CHARACTERIZATION OF FRESH MILK PRODUCTS BASED ON MULTIDIMENSIONAL RAMAN SPECTROSCOPY **

Zheng-Yong Zhang [1,2*], Si-Wei Li [1], Min Sha [1], Jun Liu [1]

[1] *School of Management Science and Engineering at Nanjing University of Finance and Economics, Nanjing 210023, Jiangsu, China; e-mail: zyzhang@nufe.edu.cn*
[2] *State Key Laboratory of Chemo/Biosensing and Chemometrics, Hunan University, Changsha Hunan 410082, China*

The effect of integration time on Raman spectral data collected from fresh milk products was investigated and analyzed. The collected spectral data were denoised by wavelet transform to remove signal interference. Two-dimensional correlation Raman spectra of the fresh milk products were then constructed using two-dimensional correlation analysis and laser integrating time as an external perturbation. Finally, feature extraction was carried out, and the Euclidean distance was calculated. Thus, the similarities between fresh milk products of the same brand and different brands were quantitatively analyzed and calculated from Raman spectroscopy, two-dimensional correlation Raman spectroscopy, and feature extraction. The results showed that the quality of fresh milk products of the same brand fluctuated but maintained a high consistency. The differences between different brands of fresh milk products under the same detection conditions were much greater than within the same brand samples.

Keywords: *dairy products, measurement analysis, Raman spectroscopy, two-dimensional correlation spectroscopy, quality control.*

ХАРАКТЕРИСТИКА СВЕЖИХ МОЛОЧНЫХ ПРОДУКТОВ НА ОСНОВЕ МНОГОМЕРНОЙ СПЕКТРОСКОПИИ КОМБИНАЦИОННОГО РАССЕЯНИЯ

Z.-Y. Zhang [1,2*], S.-W. Li [1], M. Sha [1], J. Liu [1]

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[1] *Школа менеджмента и инженерии при Нанкинском финансово-экономическом университете, Нанкин 210023, Цзянсу, Китай; e-mail: zyzhang@nufe.edu.cn*
[2] *Хунаньский университет, Чанша, Хунань 410082, Китай*

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Исследовано и проанализировано влияние времени интегрирования на данные комбинационного рассеяния свежих молочных продуктов. Собранные спектральные данные подверглись шумоподавлению с помощью вейвлет-преобразования для удаления помех сигнала. С использованием двухмерного корреляционного анализа и времени интегрирования лазера в качестве внешнего возмущения построены двухмерные корреляционные спектры комбинационного рассеяния свежих молочных продуктов. Выполнено извлечение признаков и вычислено евклидово расстояние. Сходства между свежими молочными продуктами одной и той же марки и разных марок проанализированы количественно и рассчитаны на основе КР-спектроскопии, двухмерной корреляционной КР-спектроскопии и выделения признаков. Результаты показали, что качество свежих молочных продуктов одной и той же марки колебалось, но сохранялось на высоком уровне. Различия между брендами свежих молочных продуктов при одинаковых условиях обнаружения намного больше, чем в образцах одной и той же марки.

Ключевые слова: *молочные продукты, измерительный анализ, спектроскопия комбинационного рассеяния, двумерная корреляционная спектроскопия, контроль качества.*

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Introduction. The quality and safety of dairy products are important issues in food science and analytical chemistry. The risks to the quality of dairy products often come from two sources: the presence of illegal additives or harmful substances; the manufacture of counterfeit products. Traditional identification methods rely mainly on sensory evaluation and instrumental analysis [1–4], but these methods are often time-consuming and laborious and require expensive instruments. In 2016, a large number of counterfeit dairy products, discovered by law enforcement officers in Shanghai, China, met the national standards and fortunately were harmless to consumers. The main reason for making counterfeit products was to use cheap ingredients to imitate high-end brand products and increase profits, so it was difficult to identify these types of counterfeit product by analyzing their components [5]. Therefore, to enforce the law on-site and identify brands quickly, analytical methods providing a rapid characterization based on spectroscopy have been developed recently.

Raman spectroscopy has attracted extensive research attention because of its superior performance based on its portability, suitability for directly testing aqueous samples, rapid acquisition of spectral signals, and ability to characterize rich structural information from samples [6]. However, its use has mainly been limited to detecting illegal additives, such as melamine and sodium thiocyanate [7, 8]. These current characterization analysis methods only make use of two-dimensional Raman spectroscopy, so the characterization information is limited. Recently, two-dimensional correlation spectroscopy has been proposed [9, 10]. This method can improve the spectral resolution and provide more information from a new analytical perspective, but its current application in research on dairy products is relatively rare. The present study has taken fresh branded milk products as an example for illustrating a quantitative method for discriminating between milk products based on the application of multidimensional Raman spectroscopy.

Materials and methods. *Materials and Raman measurements.* Guangming (GM) fresh milk products were purchased from Bright Dairy & Food Co., Ltd. (Shanghai, China), and Weigang (WG) yoghurt products were purchased from the Nanjing Weigang Dairy Co., Ltd. (Nanjing, China).

The fresh milk samples (360 μL) were placed in separate wells of a 96-well plate (Corning Inc., Corning, NY, USA). The Raman spectra of the samples were then recorded using a portable laser Raman spectrometer (Prott-ezRaman-D3, Enwave Optronics, Irvine, CA, USA). The excitation wavelength of the laser was 785 nm, and the laser power was 450 mW. The integration time could be varied from 20 to 180 s in increments of 40 s. The spectrometer operated from 250 to 2000 cm^{-1} with a resolution of 1 cm^{-1}, and the charge coupled device temperature was –85°C. The Raman spectra of the samples were obtained without any physical or chemical pretreatments.

Data processing. The baseline of the collected Raman spectra was calibrated using SLSR Reader software (version 8.3.9, Enwave Optronics). The wavelet denoising and Euclidean distance calculations were carried out using MATLAB software (MathWorks, Natick, MA, USA). The calculation of the two-dimensional correlation Raman spectra was performed using 2D-shige software (written by Shigeaki Morita, Kwansei-Gakuin University, 2004–2005).

Results and discussion. *Analysis of characteristics of fresh milk products using Raman spectra.* The Raman spectra of the fresh milk products based on different integration times are shown in Fig. 1, with the main vibrational bands listed in Table 1 with their tentative assignments according to previous reports [11–15]. This provides a wealth of molecular vibration and compositional information on the sample: for example, the Raman band at 1762 cm^{-1} could be assigned to the C=O stretching ester with the presence of fatty acids; the band at 1668 cm^{-1} could be the C=O stretching of proteins (CONH group of amide I) and C=C stretching mode of unsaturated fatty acids; and the strong Raman band at 1455 cm^{-1} (CH_2 deformation) could be mainly caused by fats and carbohydrates. Another strong and significant Raman band at 1015 cm^{-1} could be associated with the ring-breathing stretching (C-C stretching vibration in ring) from phenylalanine in the proteins [16]. From previous reports on the Raman spectra of milk powder, the following peaks were only found in fresh milk: those at 1212, 1195, and 1168 cm^{-1} may be mainly related to amino acids, which shows that the Raman characteristics of milk powder after processing are different from those of fresh milk products [17, 18]. Figure 1 shows how the Raman spectra of the samples changed with different integration times. For an integration time of 20 s, only a few main peaks at 1015 and 1455 cm^{-1} appeared. As the integration time increased, more peaks appeared and became clearer, and their intensity also increased, but the ratio between the peak areas remained basically unchanged.

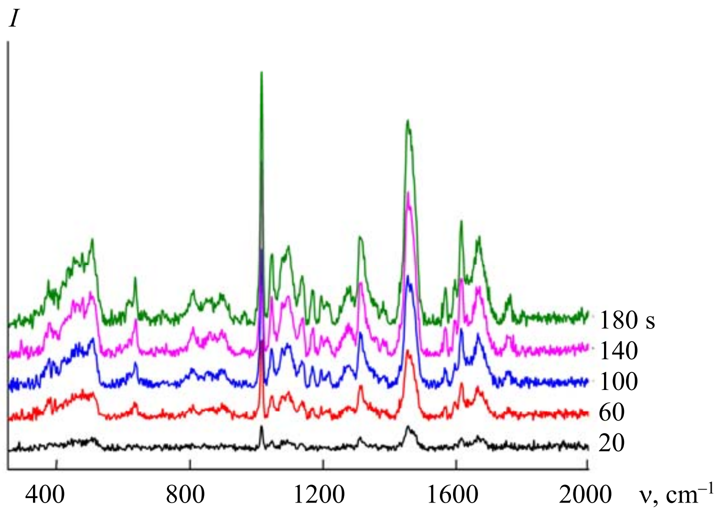

Fig. 1. Raman spectra of one fresh milk product (GM brand).

TABLE 1. The Main Peak Assignments of Raman Spectrum of One Fresh Milk Product

Wavenumber, cm^{-1}	Assignment
1762	ν(C=O)$_{ester}$
1668	ν(C=O) Amide I; ν(C=C)
1596	ν(C-C)$_{ring}$
1568	δ(N-H); ν(C-N) Amide II
1455	δ(CH_2)
1313	τ(CH_2)
1275	γ(CH_2)
1212	ν(C-N), Amide III
1195	τ(NH_2)
1168	ν(C-N)
1135	ν(C-O)+ν(C-C)+δ(C-O-H)
1096	ν(C-O)+ν(C-C)+δ(C-O-H)
1045	ν(C-O)+ν(C-C)+δ(C-O-H)
1015	Ring-breathing (phenylalanine); ν(C-C) $_{ring}$
964	δ(C-O-C)+δ(C-O-H)+ ν(C-O)
896	δ(C-C-H)+δ(C-O-C)
809	δ(C-C-O)
633	δ(C-C-O)
506	glucose
369	lactose

N o t e: ν – stretching vibration, δ – deformation vibration, τ – twisting vibration, γ – out of plane bending vibration.

Analysis of characteristics of fresh milk products using two-dimensional correlation Raman spectra. The Raman spectra of the fresh milk samples collected in the experiment exhibited some noise (Fig. 2). Noise is caused by random signals generated during signal collection, which may interfere with the subsequent measurement analysis. Therefore, the present study chose wavelet denoising as the pretreatment for denoising the spectral data [19, 20]. Briefly, the first step is decomposing the spectral signal by wavelet, followed by selecting a wavelet basis and determining a decomposition level *N*, then finally reconstructing the smoothed denoised spectral data. Figure S1 shows the Raman spectral decomposition data based on the bior 2.4 wavelet. This showed that the low frequency signal retained the signal information, with the noise information mainly concentrated at the high frequency. In the present study, the bior 2.4 wavelet with three decomposition layers ($N = 3$) was selected to provide Raman spectral data denoising, whose smoothing effect is shown in Fig. 2.

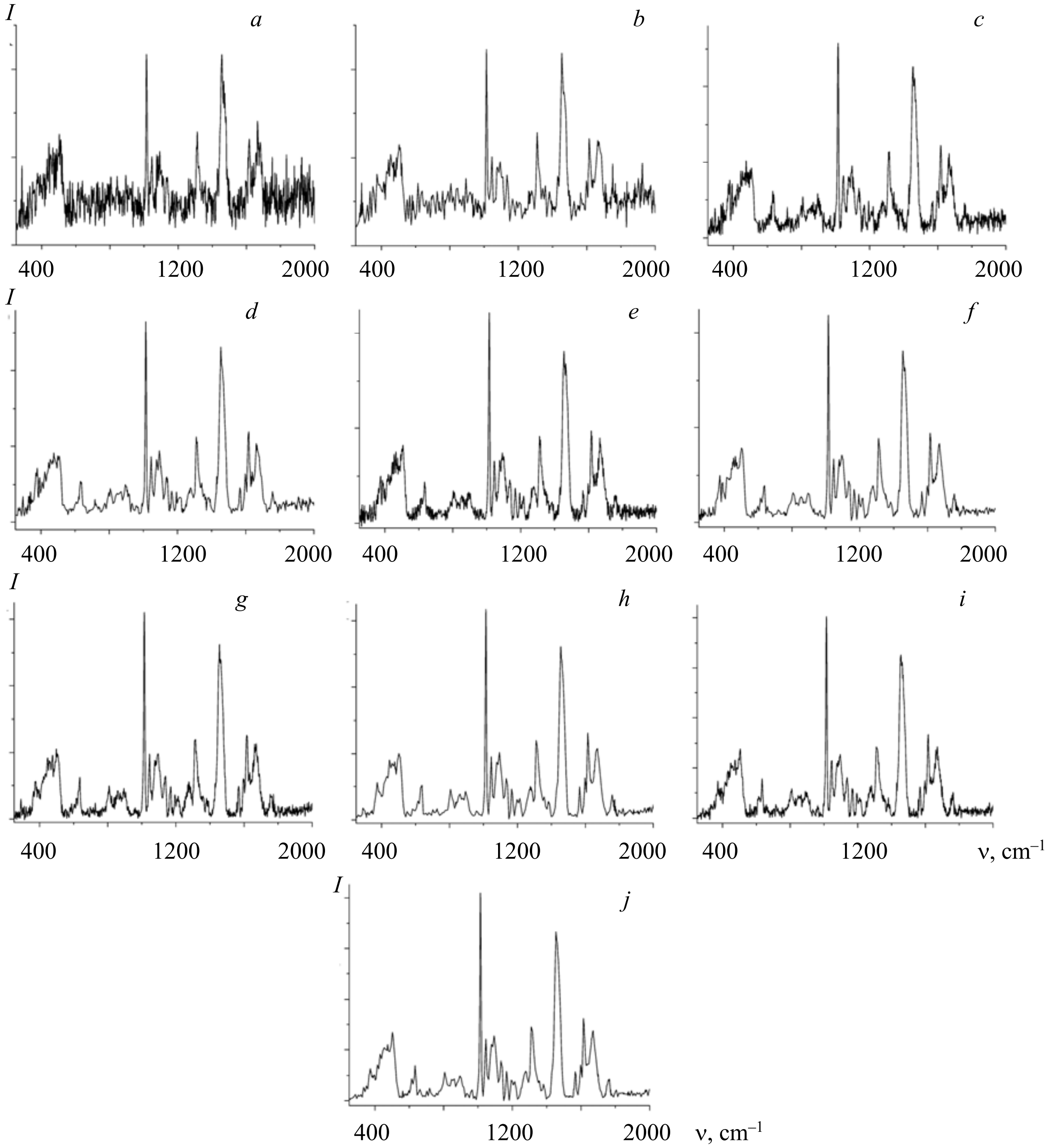

Fig. 2. Original Raman spectra with integration time of (a) 20, (c) 60, (e) 100, (g) 140, and (i) 180 s of one fresh milk product, and Raman spectra after wavelet denoising with integration time of (b) 20, (d) 60, (f) 100, (h) 140, and (j) 180 s of one fresh milk product, respectively (GM brand).

Two-dimensional correlation spectroscopy can use several two-dimensional spectra synthetically to obtain three-dimensional information on samples through a two-dimensional correlation analysis operation. This can effectively improve the resolution of spectra and provide much more sample information [21, 22]. In the present study, two-dimensional Raman correlation spectra of samples were constructed using Raman spectra signals collected at different integration times with a laser as an external perturbation, as shown in Fig. 3 (three-dimensional stereogram), and in Fig. S2a as a planar graph. The positive and negative correlation intensities are indicated by the unshaded regions and shaded regions, respectively. As the laser integration time increased, the Raman signals of the fresh milk products were enhanced, so the correlation signals are mainly positive. It can be seen that there are seven auto peaks at around (1668, 1668), (1612, 1612), (1455, 1455), (1313, 1313), (1096, 1096), (1015, 1015), and (506, 506) with positive intensities, as well as about 60 cross peaks at around (1612, 1455), (1455, 1313), (1455, 1015), (1455, 1612), (1313, 1455), (1015, 1455), and more in addition, with clear positive intensities in the fresh milk samples. There was also a high correlation between the two peaks (1015, 1015) and (1455, 1455), which became the characteristic peaks of the fresh milk samples.

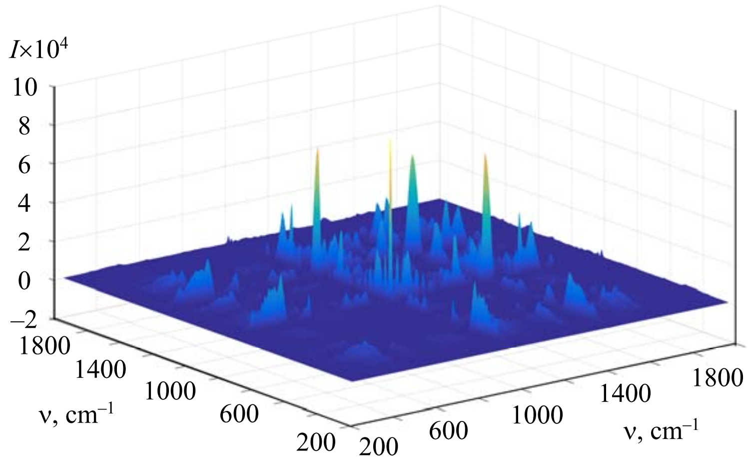

Fig. 3. Two-dimensional correlation Raman spectrum of one fresh milk product (GM brand).

Analysis of measurements of different brands of fresh milk products. The GM brand fresh milk product was used as the experimental group, with the WG brand fresh milk product as the control group. They were both a milky white color, so they could not be effectively distinguished by the naked eye. The Raman spectra and two-dimensional correlation Raman spectra of WG fresh milk products are shown in Fig. S3 (two-dimensional), Fig. 4 (three-dimensional stereogram), and Fig. S2b (planar graph). The main peaks of the Raman spectra for the WG fresh milk products were similar to those of the GM fresh milk products, indicating that their compositions were similar, but it could also be seen that the ratios of Raman spectra peaks were different. This indicated that there were differences in composition between the two brands of fresh milk products, also consistent with the actual situation. Table S1 shows that the nutrient contents of the two brands of fresh milk product were indeed different. At the two-dimensional correlation spectral level, the peaks at (1015, 1015) and (1455, 1455) and their ratios for WG fresh milk products were significantly different from those of GM fresh milk products, indicating that these Raman characteristic peaks were suitable for identifying similar products.

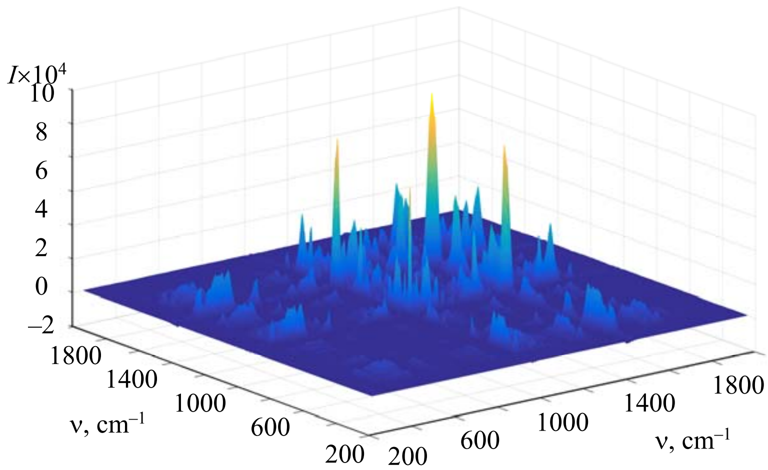

Fig. 4. Two-dimensional correlation Raman spectrum of one fresh milk product (WG brand).

Therefore, it can be seen that Raman spectroscopy and two-dimensional correlation Raman spectroscopy could provide abundant information on the components and molecular characteristics of the fresh milk samples, which could then be quantified. The Euclidean distances were calculated to quantitatively evaluate the similarity between samples [23, 24]. First, the similarity between samples of the same brand (GM) was studied. Six fresh milk products were randomly sampled to reflect the normal quality fluctuation of fresh milk products. The spectral mean value was applied to estimate their theoretical value, and then the Euclidean distance between the samples and the mean was calculated (Tables S2, S3). Using Raman spectral integration times of 20, 60, 100, 140, and 180 s, the range of Euclidean distances between the mean and each sample was 117.9387–138.9414, 172.1779–301.7697, 222.6504–423.1605, 231.6415–674.0575, and 335.9841–596.3056, respectively (Table S2). The results showed that there was a certain quality fluctuation between the samples of the same brand and the mean value, while maintaining a high consistency. As the integration time changed, there were some fluctuations in characterizing the similarity between samples. The Euclidean distances between each sample and their mean of GM brand fresh milk product based on two-dimensional correlation Raman spectra were in the range of 4.0708×10^5–6.9727×10^5 (Table S2). The results showed that the greater Euclidean distance may be due to the higher spectral resolution and larger dimension of the two-dimensional correlation spectroscopy. Feature extraction was expected to improve the discriminant efficiency. The analysis showed that the ratio between the peaks at 1015 and 1455 cm^{-1} represented a major feature of fresh milk products. Therefore, the 1015/1455 ratio was further evaluated as the input value by calculating the Euclidean distance between samples [25–27]. The range of calculated Euclidean distances between each sample and their mean of GM brand fresh milk product based on Raman spectra of the ratio of Raman peaks (at 1015/1455 cm^{-1}) for integration times of 20, 60, 100, 140, and 180 s were 0.0456–0.1085, 0.0171–0.1895, 0.0433–0.1437, 0.0011–0.2917, and 0.0674–0.1293 (Table S3), respectively. The Euclidean distances between each sample and their mean of GM brand fresh milk product based on two-dimensional correlation Raman spectra of the ratio of Raman peaks at (1015,1015)/(1455, 1455) were in the range of 0.2285–0.4522 (Table S3). It was clear that the calculation dimension decreased greatly from the Raman spectrum (1751 dimension) to the two-dimensional correlation Raman spectrum (1751×1751 dimension) to the one-dimensional. These results were consistent with those calculated previously, in that there were some fluctuations among the samples, but a high consistency was maintained.

The WG brand fresh milk products were also randomly sampled as a control group. The Euclidean distances between each sample and the mean of GM brand fresh milk products were calculated under Raman spectra, two-dimensional correlation Raman spectra, and feature extraction conditions (Tables S4, S5). The Euclidean distances calculated between each WG brand fresh milk product and its mean value based on Raman spectra for integration times of 20, 60, 100, 140, and 180 s were 147.6653–221.0413, 360.9165–506.2506, 580.9595–869.7124, 857.7067–1174.5, and 902.8853–1664.5 (Table S4), respectively. The Euclidean distances calculated between each WG brand fresh milk product and the mean of the GM brand fresh milk product based on two-dimensional correlation Raman spectra were in the range of 1.0315×10^6–2.0872×10^6 (Table S4). The Euclidean distances between each WG brand fresh milk product and the mean of the GM brand fresh milk product based on the Raman spectra of the ratio of Raman peaks (at 1015/1455 cm^{-1}) for integration times of 20, 60, 100, 140, and 180 s were 0.1293–0.4511, 0.5815–0.9210, 0.3932–0.7133, 0.3874–0.6859, and 0.3834–0.7332 (Table S5), respectively. The Euclidean distances calculated between each WG brand fresh milk product and the mean of the GM brand fresh milk product based on two-dimensional correlation Raman spectra of the ratio of Raman peaks at (1015,1015)/(1455, 1455) were in the range of 1.0807–1.4870 (Table S5). These results showed that, under the same test conditions, the Euclidean distance between the control group (WG brand fresh milk products) and the experimental group (the mean of GM brand fresh milk products) was larger than the distance between the corresponding samples in the experimental group (Fig. S4). Overall, the results of the present study have shown that the Euclidean distance combined with Raman spectroscopy, two-dimensional correlation Raman spectroscopy, and feature extraction could be used to quantify the differences between different brands of fresh milk product.

Conclusions. This study has shown that Raman spectroscopy, two-dimensional correlation Raman spectroscopy, and feature extraction have the potential for characterizing and analyzing measurements from fresh milk products. The advantage of this method is that multidimensional Raman spectroscopy can provide a great deal of molecular information on the components of the samples. The liquid samples of fresh milk products can be directly tested with no sample pretreatment, with a complete test taking only a few minutes. By adjusting the sampling integration time and two-dimensional correlation analysis, two-dimensional correlation Raman spectra of fresh milk products can be constructed to further highlight the characteristics of samples from a higher dimensional level. Based on the Raman spectroscopic characteristics of fresh milk products, the ratio of characteristic peaks can be extracted, to be used for identifying fresh milk products from several perspectives.

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SUPPLEMENT

TABLE S1. Nutrient composition and content Information of fresh milk products

Nutritional components	GM fresh milk products	WG fresh milk products
protein	3.0 g/100 g	3.0 g/100 g
fat	3.2 g/100 g	3.1 g/100 g
carbohydrate	4.8 g/100 g	4.5 g/100 g

TABLE S2. Euclidean distance results between each sample and their mean of GM brand fresh milk products based on Raman spectra (integration time: 20, 60, 100, 140, and 180 s) and two-dimensional correlation Raman spectra

Mean	GM-1	GM-2	GM-3	GM-4	GM-5	GM-6
GM-20s	117.9387	125.3345	132.9045	132.4569	118.6993	138.9414
GM-60s	172.1779	301.7697	193.3186	189.4008	189.7838	203.1871
GM-100s	228.5607	423.1605	222.6504	304.7443	316.9314	331.0362
GM-140s	231.6415	523.0716	291.2780	424.6735	470.3647	674.0575
GM-180s	492.1679	382.6337	335.9841	596.3056	369.1198	328.9778
GM-2D	6.1633×10^5	4.7285×10^5	4.0708×10^5	6.9727×10^5	4.6368×10^5	4.4449×10^5

N o t e. GM means GM brand fresh milk products. GM-1, GM-2, GM-3, GM-4, GM-5, GM-6 represent a GM fresh milk product respectively; 2-D indicates two-dimensional correlation Raman spectroscopy.

TABLE S3. Euclidean distance results between each sample and their mean of GM brand fresh milk products based on Raman spectra of the ratio of Raman peaks at 1015/1455 cm^{-1} (integration time: 20, 60, 100, 140, and 180 s) and two-dimensional correlation Raman spectra of the ratio of Raman peaks at (1015,1015)/(1455, 1455) cm^{-1}

Mean	GM-1	GM-2	GM-3	GM-4	GM-5	GM-6
GM-20s	0.0544	0.0624	0.1064	0.0456	0.0627	0.1085
GM-60s	0.0877	0.1895	0.0628	0.0972	0.0171	0.0768
GM-100s	0.0627	0.1437	0.0701	0.1045	0.1071	0.0433
GM-140s	0.0481	0.1419	0.0206	0.0011	0.1845	0.2917
GM-180s	0.0966	0.0952	0.0934	0.0674	0.1293	0.1069
GM-2D	0.2628	0.2694	0.3355	0.2285	0.4522	0.3774

N o t e. GM means GM brand fresh milk products. GM-1, GM-2, GM-3, GM-4, GM-5, GM-6 represent a GM fresh milk product, respectively; 2-D indicates two-dimensional correlation Raman spectroscopy.

TABLE S4. Euclidean distance results between each WG brand fresh milk product and the mean of GM brand fresh milk products based on Raman spectra (integration time: 20, 60, 100, 140, and 180 s) and two-dimensional correlation Raman spectra

Mean	WG-1	WG-2	WG-3	WG-4	WG-5	WG-6
GM-20s	221.0413	200.3739	181.9037	184.9411	187.9323	147.6653
GM-60s	506.2506	483.2457	435.9169	388.1332	444.3183	360.9165
GM-100s	869.7124	844.3089	704.4634	782.8796	619.2770	580.9595
GM-140s	1.1537×10^3	1.1640×10^3	860.2030	857.7067	1.1745×10^3	1.0192×10^3
GM-180s	1.5334×10^3	1.6645×10^3	1.2569×10^3	1.3293×10^3	1.4007×10^3	902.8853
GM-2D	1.5820×10^6	1.7106×10^6	1.3120×10^6	1.5382×10^6	2.0872×10^6	1.0315×10^6

N o t e. GM means GM brand fresh milk products. WG means WG brand fresh milk products. WG-1, WG-2, WG-3, WG-4, WG-5, WG-6 represent a WG fresh milk product, respectively; 2-D indicates two-dimensional correlation Raman spectroscopy.

TABLE S5. Euclidean distance results between each WG brand fresh milk product and the mean of GM brand fresh milk products based on Raman spectra of the ratio of Raman peaks at 1015/1455 cm^{-1} (integration time: 20, 60, 100, 140, and 180 s) and two-dimensional correlation Raman spectra of the ratio of Raman peaks at (1015,1015)/(1455, 1455) cm^{-1}

Mean	WG-1	WG-2	WG-3	WG-4	WG-5	WG-6
GM-20s	0.4312	0.4511	0.3961	0.3734	0.3686	0.1293
GM-60s	0.5982	0.5815	0.6052	0.7077	0.6671	0.9210
GM-100s	0.6470	0.7133	0.6102	0.4893	0.5075	0.3932
GM-140s	0.5948	0.6859	0.5564	0.3874	0.4307	0.5695
GM-180s	0.7032	0.7332	0.5533	0.5420	0.4840	0.3834
GM-2D	1.4237	1.4870	1.2070	1.1439	1.0807	1.2296

N o t e. GM means GM brand fresh milk products. WG means WG brand fresh milk products. WG-1, WG-2, WG-3, WG-4, WG-5, WG-6 represent a WG fresh milk product, respectively; 2-D indicates two-dimensional correlation Raman spectroscopy.

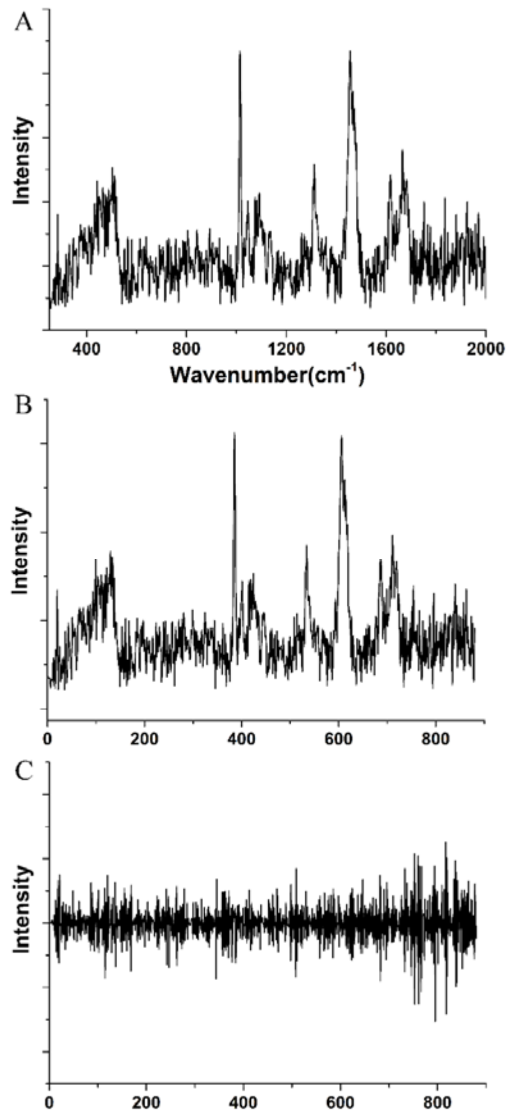

Fig. S1. Original Raman spectrum with integration time 20 s of fresh milk product (A), wavelet denoising, first layer low frequency coefficients of bior 2. 4 decomposition (B), first layer high frequency coefficients of bior 2. 4 decomposition (C).

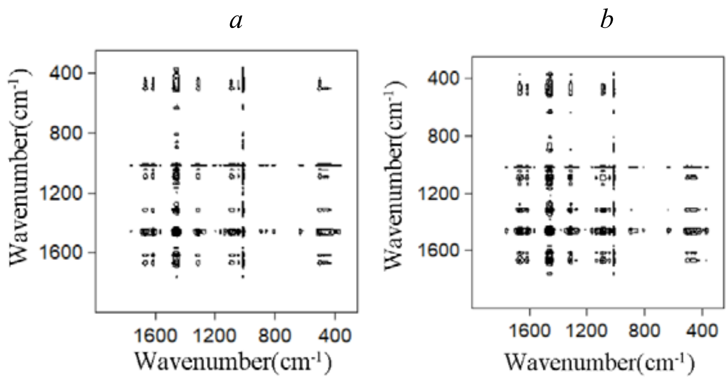

Fig. S2. Two-dimensional correlation Raman spectrum of fresh milk products (GM brand, planar graph) (a) and one fresh milk product (WG brand, planar graph) (b).

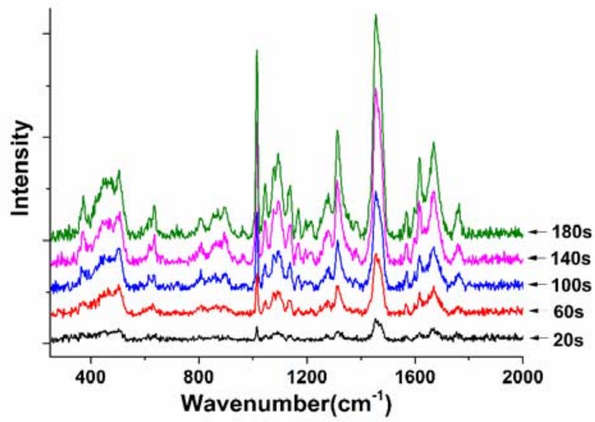

Fig. S3. Raman spectra of one fresh milk product (WG brand).

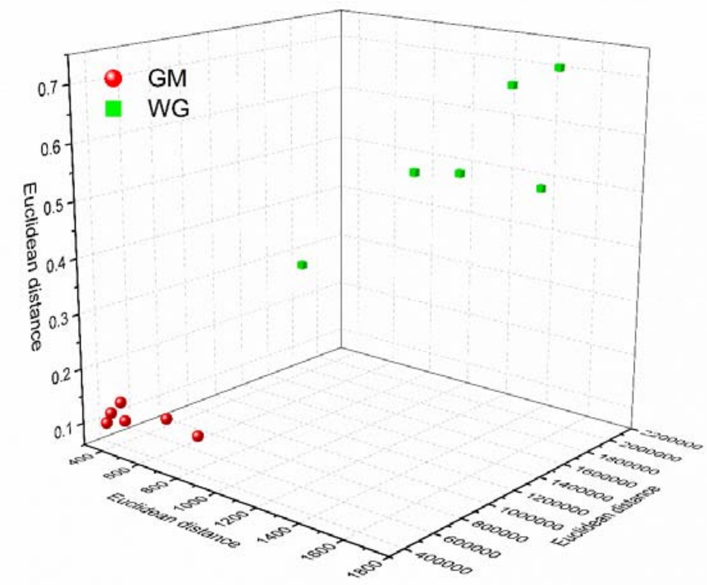

Fig. S4. A Euclidean distance 3-dimensional map of fresh milk products (The data for GM brand fresh milk products, *x*: Table S2 (row GM-180s); *y*: Table S2 (row GM-2D); *z*: Table S3 (row GM-180s); The data for WG brand fresh milk products, *x*: Table S4 (row GM-180s); *y*: Table S4 (row GM-2D); *z*: Table S5 (row GM-180s)).