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Identification of cow milk in goat milk by nonlinear chemical fingerprint technique

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ABSTRACT

The objective of this paper was to develop a nonlinear chemical fingerprint technique for identifying and detecting adulteration of goat milk with cow milk. In this study, by taking the Belousov-Zhabotinsky oscillatory chemical reaction using acetone and substrates in goat milk or cow milk as main dissipative substances, when the same dosage of goat milk and cow milk was introduced to the "$H^+ + Mn^{2+} + BrO_3^- +$ acetone" oscillating system respectively, nonlinear chemical fingerprints were obtained for goat milk and cow milk from the same origin. The results showed that inductive time value and the content of cow milk in goat milk had a linear relationship in the range of 0—100% and the corresponding regression coefficient was 0.9991. A detection limit of 0.0107 g/g was obtained, and the content of cow milk in mixed milk was calculated. The proposed method in this study was simple, economical and effective. In addition, the method did not need the pretreatment and separation of samples for identifying and evaluating cow milk adulteration in goat milk.

1. Introduction

Goat milk is a kind of highly nutritious food because it possesses unique properties which distinguish it from cow milk. Recently, goat milk plays an increasingly important role in the human diet, and it suits not only for infants but also for adults, especially for nursing mothers [1]. Furthermore, goat milk has become popular as an infant diet because the fat globules of goat milk are smaller than those of cow milk and goat milk has higher percentage of short-and medium-chain (C_6-C_{14}) fatty acids in comparison with cow milk, which is probably main reason for the easy digestion of goat milk [2—4]. However, for higher profit, increasing demand for goat milk might result in the adulteration by cheaper cow milk, which has become a serious quality problem. Due to the similarity of goat milk and cow milk in appearance and composition, it is difficult to

differentiate goat milk and cow milk [5]. This situation also presents a risk for people afflicted with cow milk allergies, when they consume adulterated goat milk [6,7]. Thus, analytical methods with high selectivity to identify and evaluate adulteration in goat milk are required.

Currently, chemical fingerprint analysis has been applied as an effective method for milk and dairy products quality control. These fingerprinting methods include HPLC [5], capillary electrophoresis [8], isoelectric focusing [9,10], enzyme-linked immunosorbent assay [11], PCR technology [6,12], pulse polarography method [13], liquid chromatography tandem mass spectrometry [14] and so on. Most of these methods exhibit high sensitivity and can meet the requirements for identifying and evaluating adulteration of milk and dairy products. However, these techniques described above are mainly used to identify and distinguish adulteration of milk and dairy products by detecting various chemical compositions of milk, such as the detection of specific protein components by HPLC [15]. Moreover, these methods usually require complex preprocessing, such as separation and purification. So the research on the effective method for directly showing the throng character of chemical components in cow milk and goat milk has become one of the primary tasks which the analysts are facing. In addition, it would be beneficial to develop simple and cost effective methods based on different principles from different aspects for distinguishing and evaluating samples, and the techniques may be complementary with each other.

The discovery of Belousov–Zhabotinskii (B–Z) oscillatory reaction (Belousov and Zhabotinsky were the names of the two Russian scientists, who were the first ones to study the reaction) began the times to investigate nonlinear chemical reaction systems [16], and the research on the application of nonlinear chemical reaction had aroused increasing attention. Chemical oscillation based on Belousov–Zhabotinskii (B–Z) reaction was a common phenomenon in nonlinear chemistry. The phenomena of the reaction are complex, involving chemical oscillation, chemical turbulence, chemical patterns and chemical waves [16]. The reaction mechanism and applications of chemical oscillation in single component detection had been investigated extensively and thoroughly by domestic and foreign scholars [16–19]. While the research to apply it in fingerprint analysis for characterizing throng characteristic of the components in foods was rare abroad, and also started rather late in China. Such as authenticity identification and quality evaluation of soya sauce had been reported by nonlinear electrochemical fingerprint and system similarity [20]. According to the literatures [21,22], because of the differences in the types and the content of their components, different samples had different influences on an identical nonlinear chemical reaction, which caused that the shapes of the relevant potential-time (E–t) curves were different from each other. The E–t curve is very helpful for the rapid identification and evaluation of cow milk in goat milk because it contains abundant qualitative and quantitative information. In this work, we introduced goat milk with or without artificially added cow milk to "H$^+$ + Mn^{2+} + BrO$_3^-$ + acetone" oscillating system, and cow milk adulteration in goat milk was identified and evaluated by a nonlinear chemical fingerprint technique.

Thus, the objective of this study was to evaluate the feasibility to apply nonlinear chemical fingerprint technique to detect and identify cow milk adulteration in goat milk. The content of cow milk in mixed milk was calculated by the least square method, and the mechanism of the method was introduced using H$^+$, Mn^{2+}, BrO$_3^-$, acetone and glucose as the reaction substrates. The method developed in this study has the advantages of low operational cost and no pretreatment for identifying and evaluating cow milk adulteration in goat milk.

2. Materials and methods

2.1. Materials

All chemicals used were of analytical grade. Sulfuric acid (1.0 mol L^{-1}), acetone (1.0 mol L^{-1}), sodium bromate (0.8 mol L^{-1}) and manganese sulfate (0.08 mol L^{-1}) were used. Solutions were kept at a constant temperature (50.0 °C) until used. Double distilled water was used throughout the experiments. In addition, acetic acid (5%), dichloromethane (200 μL) and formic acid (0.2%) were used.

2.2. Milk samples

Raw goat milk and cow milk were collected from local farms. Then, milk samples were freeze dried milk powder. A series of adulteration samples were made of raw cow milk and raw goat milk in volume ratios 0, 5%, 10%, 20%, 40%, 60%, 80%, and 100% v/v.

2.3. Main apparatus

A nonlinear chemical fingerprint instrument (Model MZ-1B) developed by Central South University and Xiangtan Ltd. (Hunan, China) was used. A Type 217 calomel electrode was used as reference electrode and a Type 213 platinum electrode was used as working electrode (both were purchased from Shanghai Precision & Scientific Instrument Co., China). All studies were conducted using the experimental set-up which was illustrated in the literature [21]. In addition, an ion trap mass spectrometer (Thermo Finnigan, San Jose, USA) was used.

2.4. Procedure

The following procedure was used in all experiments. The nonlinear chemical reaction mixture was prepared by mixing 25.00 mL of sulfuric acid, 10.00 mL of acetone, 12.00 mL of manganese sulfate, 10.00 ml of double distilled water and appropriate dosage of cow milk or goat milk sample. All components of reaction mixture were added into the reactor. The reactor cover with two injection holes, the electrodes and a thermometer was closed. The instrument was then turned on, with temperature and stirring rate adjusting to 50.0 °C and 800 r/min, respectively. After stirring for 5.0 min, 5.00 mL of sodium bromate solution was injected into the reactor. Electric potential-time (E–t) curve was immediately obtained and finished as soon as the potential oscillation disappeared.

3.   Results and discussion

3.1.   Reaction condition and basic process of nonlinear chemical fingerprint

Kinetic and thermodynamic conditions on nonlinear chemical fingerprint had been discussed, and the entropy change laws and expression suitable for describing the entropy change rates of any thermodynamic system had been interpreted on the basis of the relevant literature [22]. It was demonstrated that an open system without complementarity of dissipative substances and a close system far from the equilibrium were suitable for studying nonlinear chemical fingerprint since the chemical reaction was able to be accomplished in a properly short period of time in these systems.
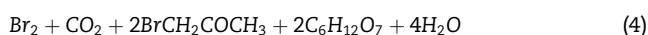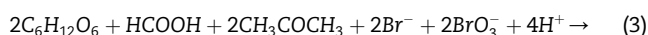
Process of a nonlinear chemical reaction is very complicated and involves oxidation–reduction reaction, precipitation reaction, neutralization reaction and free radical reaction [23]. In this study, the mechanism of B–Z oscillatory reaction to use Mn^{2+}, BrO_3^-, H^+, acetone and glucose as the reaction substrates was discussed. Although B–Z oscillatory reaction includes many kinetic steps like dozens of elementary reactions [24,25], its mechanism can be summed into two main processes, namely, inductive process and oscillatory process [25], which are explained by Process I and Process II, respectively.

Process I:

$$3BrO_3^- + 6H^+ + 6Mn^{2+} + 2CH_3COCH_3 \rightarrow \tag{1}$$

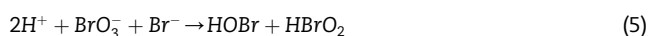$$3Br^- + 6Mn^{3+} + 3H_2O + 2HCOOH + 2CH_3COOH \tag{2}$$
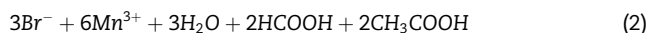
It can be seen from process I that bromine ion is generated in the inductive reaction.

Process II:

$$2C_6H_{12}O_6 + HCOOH + 2CH_3COCH_3 + 2Br^- + 2BrO_3^- + 4H^+ \rightarrow \tag{3}$$

$$Br_2 + CO_2 + 2BrCH_2COCH_3 + 2C_6H_{12}O_7 + 4H_2O \tag{4}$$
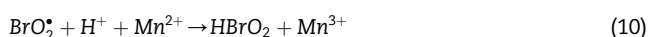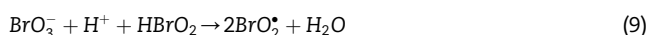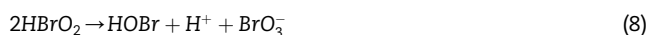
Process II is the total reaction of the oscillating reaction in the reaction system. It involves three processes, namely, process A, process B and process C.
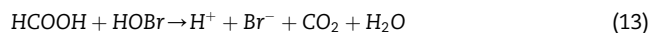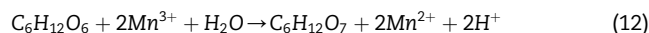
Process A:

$$2H^+ + BrO_3^- + Br^- \rightarrow HOBr + HBrO_2 \tag{5}$$

$$Br^- + H^+ + HBrO_2 \rightarrow 2HOBr \tag{6}$$

$$Br^- + H^+ + HOBr \rightarrow Br_2 + H_2O \tag{7}$$

Process B:

$$2HBrO_2 \rightarrow HOBr + H^+ + BrO_3^- \tag{8}$$

$$BrO_3^- + H^+ + HBrO_2 \rightarrow 2BrO_2^{\bullet} + H_2O \tag{9}$$

$$BrO_2^{\bullet} + H^+ + Mn^{2+} \rightarrow HBrO_2 + Mn^{3+} \tag{10}$$

Process C:

$$CH_3COCH_3 + Br_2 \rightarrow Br^- + H^+ + BrCH_2COCH_3 \tag{11}$$

$$C_6H_{12}O_6 + 2Mn^{3+} + H_2O \rightarrow C_6H_{12}O_7 + 2Mn^{2+} + 2H^+ \tag{12}$$

$$HCOOH + HOBr \rightarrow H^+ + Br^- + CO_2 + H_2O \tag{13}$$

B–Z oscillating reaction is initiated by Br^- when the concentration of Br^- in the reaction system is higher than $[Br^-]_{crit}$, namely, the critical concentration [24,25]. In process A, Br^- is consumed and Br_2 is accumulated. With the reactions prolonging, the concentration of Br^- gradually decreases in system [26]. When the concentration of Br^- is lower than $[Br^-]_{crit}$, the whole oscillating reaction is dominated by process B, during which Mn^{3+} and HOBr are accumulated. $HBrO_2$ is an important intermediate that operates the switch from process B to process C. Accumulation of Br_2, Mn^{3+} and HOBr initiates process C that regenerates Br^-. Then the next new cycle will start as the concentration of Br^- being accumulated. In this way, process A, B and C move in cycles and form oscillating reaction.

According to the above described, it can be observed that nonlinear chemical reaction can not take place in the reaction system without reducing agent such as glucose can reduce Mn^{3+} into Mn^{2+}. In fact, there are many reducing substances in different samples, such as vitamin C in milk, and these reducing substances may reduce Mn^{3+} into Mn^{2+}. In other words, the condition of taking place nonlinear chemical reaction is that reducing agent is required in the reaction system. The differences of the reducing substances in different samples result in the differences of reaction mechanisms for inductive process and oscillatory process. Thus, the shape of nonlinear chemical fingerprint is changed with reaction condition, reactants, products and coexisting substances in the reaction system.

3.2.   Optimization of detecting dosage of milk

Nonlinear chemical fingerprint is very sensitive to the change of determining condition, therefore, it must be determined under the constant condition. In the experiment, the universal optimum conditions recommended in the literature [22] were utilized, and the optimization experiment of the determining dosage of milk was carried out. On selecting the determining dosage in order to determine the fingerprint of milk, both of the characteristic of the fingerprint and the determining time must be considered simultaneously. Under the optimal experimental conditions, namely, 25 mL of 1.0 mol L^{-1} sulfuric acid, 15 mL of 1.0 mol L^{-1} acetone, 5 mL of 0.8 mol L^{-1} sodium bromate, 12 mL of 0.08 mol L^{-1} manganese sulfate, 10 mL of double distilled water and temperature of 50.0 °C in the reactor, the detecting dosage of milk could be evaluated by employing oscillatory end time as the measured parameter. Therefore, the applied dosage of adulterated milk samples to the experiment was 1.00 g, and the detecting dosage was 0.90 g for the reproducibility of milk samples. Under the condition of the determining dosage, not only the characteristic differences of the fingerprints were conspicuous, but also the determining time was not too long.

3.3. Essential information in nonlinear chemical fingerprint of milk

Nonlinear chemical fingerprint contained abundant quantitative information and intuitionistic information due to its dynamic property. In this study, nonlinear chemical fingerprint was obtained by adding 0.90 g of goat milk into the reactor. The fingerprint was shown in Fig. 1.

It was obvious from Fig. 1 that the essential characteristic information of nonlinear chemical fingerprint mainly included inductive time (t_{ind}), undulatory period (τ_{und}), undulatory life (t_{und}), canyon potential (E_{can}), canyon time (t_{can}), peak top potential (E_{pet}), peak top time (t_{pet}), oscillatory start potential (E_{uns}), oscillatory end potential (E_{une}), oscillatory end time (t_{uns}), maximum amplitude (ΔE_{max}), which were defined as quantitative information. All of them were described in detail in the literature [22,23]. The researchers [23] pointed that oscillation wave shape of fingerprint could reflect the characteristics of complex samples, and inductive time, oscillatory life, oscillatory period and oscillatory curve were holistic quantitative information on chemistry components of samples. Furthermore, the fingerprint information was very important and helpful for distinguishing and evaluating of goat milk and cow milk. Thus, inductive curve, oscillatory curve, oscillatory-end curve and a part of equilibrium curve constituted the whole nonlinear chemical fingerprint [23,25].

3.4. Reproducibility and precision of nonlinear chemical fingerprint of milk

Goat milk and cow milk were analyzed to evaluate the repeatability of the method. Under the same determination conditions, according to "Procedure", 0.90 g of goat milk or cow milk with the same origin was added into the reactor, and their nonlinear chemical fingerprints were obtained. The experiments were carried out in quadruplicate. The reproducibilities of nonlinear chemical fingerprints of goat milk and cow milk were shown in Fig. 2.

The precision was assessed by determining the relative standard deviation (RSD). It can be seen from Table 1 that the

Fig. 1 – Basic characteristic information of nonlinear chemical fingerprint of goat milk (e–f: inductive curve; f–g: undulatory curve; g–h: wave-end curve; e and h are the start and end points of nonlinear chemical reaction, respectively).

RSDs were less than or equal to 1.93%. Thus, these values confirmed that nonlinear chemical fingerprint had very good reproducibility and precision.

3.5. Identification for goat milk and cow milk

In this study, by taking B–Z nonlinear chemical reaction to use acetone and substrates in goat milk or cow milk as main dissipative substances, when the same dosage of goat milk and cow milk was introduced to the "$H^+ + Mn^{2+} + BrO_3^- + $ acetone" oscillating system respectively, the fingerprints of goat milk and cow milk were obtained. As shown in Fig. 3.

The profiles of inductive curves and oscillating curves had obvious characteristic differences between the fingerprint of goat milk and cow milk. Inductive curve of goat milk was smoother. On the contrary, inductive curve of cow milk had slight fluctuation. Furthermore, compared with cow milk, the time taken to reach the maximum potential was longer for goat milk. In addition, it can be seen from Fig. 3 that inductive time of cow milk and goat milk was 1720.56 s and 2865.03 s, respectively. This indicated that goat milk and cow milk could be distinguished by comparison of the fingerprints.

At present, chromatogram and spectrum fingerprints are the most widely used methods in milk analysis. However, characteristic differences of the chromatogram and spectrum fingerprints are not obvious and intuitive. Moreover, their features must be interpreted by chemometric methods or mathematics [27]. In contrast, the shapes of nonlinear chemical fingerprints of different types of milk are obviously different. At the same time, compared with other methods such as chromatogram and spectrum, the proposed method avoids the laborious process of separation and purification of samples. Since a large number of samples contained different chemical components and contents, which leaded to be different for quantifiable parameters and intuitionistic shapes in nonlinear chemical fingerprints of goat milk and cow milk. Moreover, the holistic content of chemical constituents was higher, while it is the content of active components was also higher in terms of the same dosage of goat milk or cow milk. Thus, based on the above explanation, it was important to estimate the quality of milk by the throng character of chemical components in cow milk or goat milk. However, the shapes and quantifiable parameters of nonlinear chemical fingerprint of milk were decided by the types of milk and the content of chemical components in milk. Firstly, with regard to the content of chemical components in goat milk and cow milk, some researchers had reported that cow milk had lower percentage of short-and medium-chain (C_6-C_{14}) fatty acids in comparison with goat milk [28]. Secondly, goat milk was richer in monounsaturated (MUFA), polyunsaturated fatty acids (PUFA) and medium chain triglycerides (MCT) [7,29]. In addition, goat milk contained relatively higher levels of beta-casein and alpha-s2 casein, whereas alpha-s1 casein was the most abundant in cow milk [2,30,31]. The factors described above were probably one of the reasons for influencing on the shape of E–t curves between goat milk and cow milk. At the same time, season, climatic condition [32], feeding schemes [33] and so on were also mainly factors. This indicated that
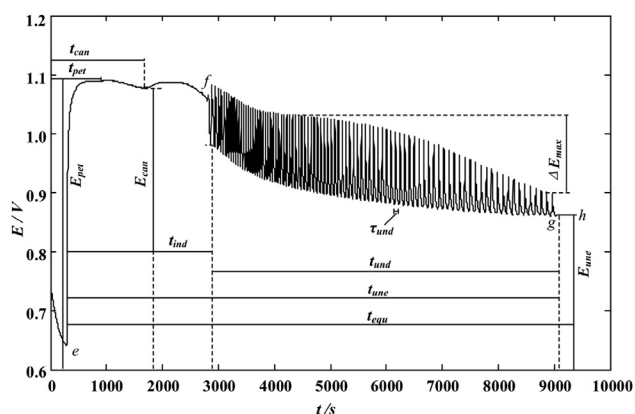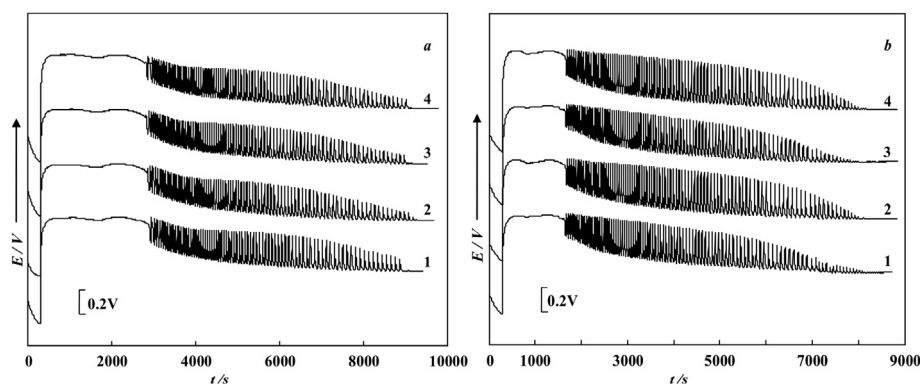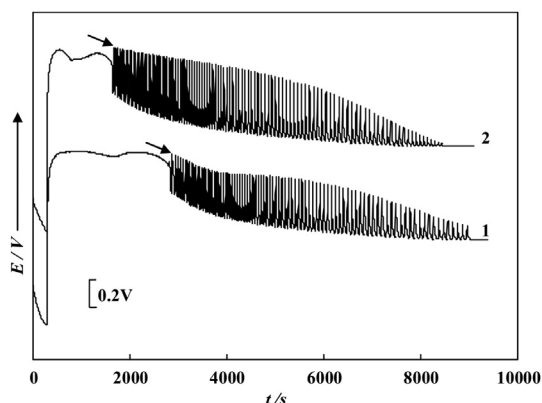
Fig. 2 – Reproducibility of nonlinear chemical fingerprint of goat milk (a) and cow milk (b) (Nonlinear chemical fingerprints of each type of milk were obtained in quadruplicate).

Table 1 – The reproducibility of nonlinear chemical fingerprint of cow milk and goat milk (n = 4).

Name of milk	E_{uns} (V)	t_{ind} (S)	t_{une} (S)	E_{une} (V)	ΔE_{max} (V)	τ_{und} (S)	E_{pet} (V)
Cow milk	1.128	1720.56	8251.03	0.9174	0.1750	43.4304	1.1543
RSD (%)	0.31	0.43	1.13	1.45	1.93	0.68	0.16
Goat milk	1.130	2865.03	9159.78	0.9151	0.1325	46.835	1.141
RSD (%)	0.96	0.19	0.38	0.80	0.62	1.72	0.47

Fig. 3 – Nonlinear chemical fingerprint of goat milk (1) and cow milk (2).

experiments with the same dosage could provide different fingerprints for different types of milk.

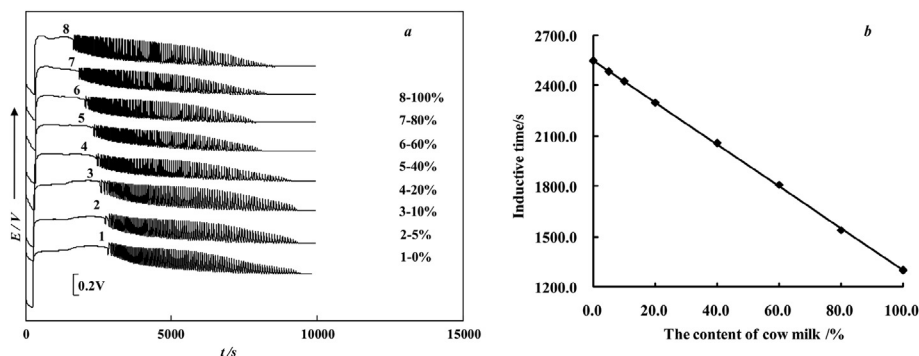### 3.6. Feasibility of nonlinear chemical fingerprint for adulterated milk samples

In order to evaluate feasibility of the method for detecting and evaluating adulterated milk samples, raw goat milk was spiked with different volume percentages of raw cow milk (0, 5%, 10%, 20%, 40%, 60%, 80%, and 100% v/v), which were also used to determine the detection limit for milk adulteration. Then adulterated samples were analyzed by nonlinear chemical fingerprint. The results were shown in Fig. 4. In order to find a strategy to evaluate the percentage of cow milk present in adulterated goat milk, the attention was focused on

inductive time. It could be seen that inductive time was decreasing with increasing of the volume percentage of cow milk added in goat milk. Moreover, when adding different proportion of cow milk into goat milk, the shapes of nonlinear chemical fingerprint had obvious changes. From the fingerprints in Fig. 4(a), there were obvious characteristic differences between the fingerprints of mixed milk. The proposed method was validated using high-performance liquid chromatography with electrospray ionization mass spectrometry method (HPLC/ESI/MS) [5]. Thus, the method of this study was feasible for identifying and evaluating cow milk adulteration in goat milk.

The content of cow milk in mixed milk had greatly influenced on the quantitative information of nonlinear chemical fingerprint. As shown in Fig. 4(b). The results showed that inductive time value and the content of cow milk in goat milk had a linear relationship in the range of 0–100%, and the regression equation was $t_{ind} = -1249.9C + 2550.2$ with a linear correlation coefficient (R^2) of 0.9991, which provided a theoretical basis for identification of samples and determination of the content of adulterated milk. In this study, the least square method was adopted as a quantitative assessment model for identification and evaluation of cow milk adulteration in goat milk. The principle of the method was that inductive time (t_{ind}) and the content of cow milk (C) in mixed milk had a good linear relation, namely, $t_{ind} = KC + B$. For the convenience of calculation, all inductive time (t_{ind}) was denoted by Y, namely

$$Y = KC + B \tag{14}$$

To calculate the content of cow milk in mixed milk by using quantitative information of nonlinear chemical fingerprint, K and B must be obtained firstly. Let C_i and Y_i be the content of cow milk of the i th standard sample in mixed milk and the

Fig. 4 – Effects of different volumes percentages of cow milk added in goat milk on nonlinear chemical fingerprints (a) and the linear relationship between volume percentage of cow milk in goat milk and inductive time of nonlinear chemical fingerprint (b).

Table 2 – The determination results of the content of cow milk in goat milk by nonlinear chemical fingerprint method (n = 3).

Sample no.	Original content/%	Measured value/%	HPLC/ESI/MS value/%	True value/%	RSD/%	Recovery/%
1	–	2.12	2.63	2.00	0.46	106.12
2	–	14.95	15.11	15.00	1.87	99.67
3	–	30.43	31.25	32.00	0.84	95.09
4	–	78.07	77.91	78.00	0.65	100.09
5	–	100.33	99.92	100.0	0.51	100.33

corresponding inductive time of nonlinear chemical fingerprint, respectively. Substituting n known data of C_i and Y_i into Equation (14), a linear calibration model was obtained, namely

$$\widehat{Y} = kC + b \qquad (15)$$

where k and b were the estimated values of K and B, $\widehat{Y}$ was the estimated value of Y. The essence of the least square method was that the parameter estimation value was optimal when the sum of square of deviations for the real value Y_i and the estimated value $\widehat{Y}_i$ was minimal.

Denote

$$Q(k,b) = \sum_{i=1}^{n}\left(Y_i - \widehat{Y}_i\right)^2 = \sum_{i=1}^{n}(Y_i - kC_i - b)^2 \to \min$$

Namely

$$\frac{\partial Q}{\partial b} = -2\sum_{i=1}^{n}(Y_i - kC_i - b) = 0 \qquad (16)$$

$$\frac{\partial Q}{\partial k} = -2\sum_{i=1}^{n}(Y_i - kC_i - b)C_i = 0 \qquad (17)$$

After some manipulations, we obtained

$$\begin{cases} nb + \sum\limits_{i=1}^{n} kC_i = \sum\limits_{i=1}^{n} Y_i \\ \sum\limits_{i=1}^{n} bC_i + \sum\limits_{i=1}^{n} kC_i^2 = \sum\limits_{i=1}^{n} C_i \cdot Y_i \end{cases} \qquad (18)$$

where k and b were calculated by using n known data of C_i and Y_i of n standard samples. Then, the values of k and b were obtained by Equation (18). When the content of cow milk in unknown mixed milk sample was predicted under the same

condition of the correction model, inductive time of unknown mixed milk sample was determined. According to Equation (14), we obtained,

$$C = k^{-1}(Y - b) \qquad (19)$$

The content of cow milk in unknown mixed milk could be calculated in terms of Equation (19). Thus, Equation (19) may be as the general model of mixed milk of goat milk and other milk for quantitative identification and evaluation.

According to the general model, the content of cow milk in mixed milk wasobtained, and determination results, precision and accuracy were shown in Table 2. It can be seen from Table 2 that the RSDs of determination results of the content of cow milk were less than or equal to 1.87%, and the recovery rates were between 95.09 and 106.12%. These experimental data interpreted that nonlinear chemical fingerprint method could be used to estimate the amount of adulterated cow milk. In addition, the limit of detection (LOD) at signal-to-noise ratio of 3.0(S/N = 3.0) was 0.0107 g/g. The proposed method could meet the requirements for the maximum amount of cow milk added in goat milk. Therefore, it suggests that the proposed method has good precision and accuracy for calculation of the content of cow milk in goat milk by nonlinear chemical fingerprint and the least square method.

4. Conclusion

Compared with other analytical instruments, nonlinear chemical fingerprint method was rather economic in price. Nonlinear chemical fingerprint was a kinetic fingerprint based on potential change with time, which was determined by all

components in sample. Therefore, the reproducibility of the proposed method was very well, and it not only had the advantages of simple operation and no pretreatment, but also contained abundant quantitative information and intuitionistic information. Meanwhile, the method may be easy received by routine analysis, and it can be used to calculate the content of cow milk adulteration in goat milk. The adulteration can be quantified in a range from 0 to 100%, with a detecting limit of 0.0107 g/g. When an assay was being considered for use in large scale analysis of milk and dairy products, nonlinear chemical fingerprint method was very helpful for the identification and evaluation of milk. More importantly, the proposed method can reflect the throng characters of chemical substances by adding a sample into the reactor, so it was more suitable for discriminating complicated adulterated milk samples. Accordingly, as a nonlinear chemical analysis of throng components, nonlinear chemical fingerprint technique could be a promising method for routine dairy product inspection in future. In addition, the proposed method in this study may also identify and evaluate other foodstuffs unable to be treated directly by chromatography, such as honey, vinegar, jam, syrup and so on.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgements

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