Determination of Total Flavonoids in Milk Powder by Spectrophotometry

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Abstract [**Objectives**] Based on spectrophotometry, a method for determining the total flavonoid content in milk powder was established by optimizing sample pretreatment conditions, and method validation was performed. [**Methods**] Total flavonoids in milk powder were extracted with ethanol using 45 ℃ water bath ultrasonication for 60 min. Flavonoids contain a benzopyran ring structure and react with aluminum nitrate in a potassium acetate environment to form a yellow complex. The absorbance of this complex was measured at 420 nm for quantitative determination. [**Results**] The method exhibited a good linear relationship within the range of 0.2 – 1.0 mg. The limit of detection (LOD) was 0.05 g/100 g, and the limit of quantification (LOQ) was 0.3 g/100 g. When the spiked amount of total flavonoids was in the range of 0.3% to 1.0%, the spike recovery rates were 99.3% to 103.4%, and the relative standard deviations (*RSD*s) were less than 3.0%. [**Conclusions**] This method offers advantages such as rapidness, accuracy, good stability, and high sensitivity, and can be used for the detection of total flavonoid content in milk powder.

Key words Spectrophotometry, Total flavonoids, Yellow complex, Benzopyran ring, Rutin

0 Introduction

Flavonoids are derivatives of chromone or chromane. They generally refer to a series of compounds formed by two benzene rings (A-ring and B-ring) connected through a central three-carbon atom, with the basic skeleton being C_6-C_3-C_6. The C_3 part can be an aliphatic chain or form six-membered and five-membered rings with the C_6 part. Many flavonoids possess medicinal value. These compounds can exert anti-inflammatory, anti-allergic, and antibacterial effects. They can also lower blood pressure, reduce the deposition of cholesterol and blood lipids on blood vessel walls, scavenge free radicals in the body, and show significant efficacy in improving blood circulation[1-3].

Due to their multiple effects and the maturation of their identification and extraction technologies, flavonoids are currently used as food additives in the production of beverages, alcoholic drinks, baked goods, and dairy products. They impart no off-flavors and can preserve the original food aroma. Milk powder contains various nutrients such as protein, fat, lactose, vitamins, and trace elements. Therefore, it is anticipated that milk powder supplemented with flavonoids will become a new favorite in the natural health market. Developing a method for detecting total flavonoids in milk powder provides crucial technical support for product quality control[4-6].

The main methods for detecting total flavonoids include spectrophotometry, thin-layer chromatography-fluorospectrophotometry, high-performance liquid chromatography (HPLC), and high-performance capillary electrophoresis (HPCE). The principles of these methods differ, leading to inconsistent results in measured flavonoid content and a lack of unified standards. Among them, thin-layer chromatography-fluorospectrophotometry is only suitable for trace analysis and has limited application scope. When there are many interfering components, the determination results can be affected. Therefore, separation conditions must be strictly controlled, and clear color spots must be selected under a fluorescent lamp for measurement[7-9]. Although HPLC and HPCE offer good reproducibility and high recovery rates, they have higher equipment requirements, and standard reference materials are not readily available, which limits their application to some extent. Spectrophotometry has the advantages of good repeatability, accuracy, simplicity, ease of mastery, and the requirement for inexpensive and readily available reagents. Ultraviolet-visible spectroscopy (UV-Vis) is electronic spectroscopy and possesses comprehensive and holistic chemical information characteristics. Simultaneously, the instruments used for this method are inexpensive, the analysis cost is low, the analysis speed is fast, sample pretreatment is simple, and detection sensitivity is high. Therefore, it has applications in multiple fields such as quality control of traditional Chinese medicine, food origin identification, food quality control, and identification of counterfeit liquor[10-13].

This study applied a spectrophotometer to detect the total flavonoid content in milk powder. It primarily investigated extraction solvent, extraction temperature and time, extract aspiration volume, and detection wavelength. A systematic method validation was also performed, including the study of limit of detection (LOD) and limit of quantification (LOQ). By adding different concentrations of rutin standard solution to milk powder, the recovery rate and repeatability under different spiking concentrations were studied. The selectivity and robustness of the method were

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also studied. Ultimately, an analytical method for determining total flavonoids in milk powder was established. This method features simple sample pretreatment, high sensitivity, good accuracy, excellent reproducibility, ease of operation, and convenience for promotion.

1 Materials and methods

1.1 Materials and reagents Ethanol, Aluminum Nitrate, Potassium Acetate (analytical grade, Tianjin Damao Chemical Reagent Factory); Rutin (standard substance, purity ≥95%, 100 mg, Shanghai Anpel Laboratory Technologies Inc.).

1.2 Instruments and equipment Spectrophotometer TU-1810 (Beijing Purkinje General Instrument Co., Ltd.); Analytical Balance AB265-S (METTLER-TOLEDO, Switzerland); Ultrasonic Cleaner KQ-300 (Kunshan Ultrasonic Instrument Co., Ltd.); Vortex Mixer MS3 (IKA Works, Inc.).

1.3 Methods

1.3.1 Rutin standard stock solution (1.0 mg/mL). 50 mg of rutin standard substance, dried to constant weight (dried under reduced pressure at 120 ℃), was accurately weighed, dissolved with anhydrous ethanol, and diluted to volume in a 50 mL volumetric flask.

1.3.2 Standard curve construction. 0.2, 0.4, 0.6, 0.8, and 1.0 mL of rutin standard stock solution were accurately pipetted into separate 50 mL volumetric flasks. Anhydrous ethanol was added to bring the total volume to 15 mL. Then, 1 mL of aluminum nitrate solution and 1 mL of potassium acetate solution were added sequentially, and the mixture was shaken well. Water was added to the mark, the flask was shaken well, and the solution was allowed to stand for 1 h. The absorbance was measured at 420 nm using a 2 cm cuvette, with a 30% ethanol solution as the blank. A standard curve was plotted with the mass of rutin in 50 mL (mg) as the abscissa and absorbance as the ordinate, or calculated using a direct regression equation.

1.3.3 Sample preparation. Whole milk powder was selected as the experimental sample. Whole milk powder has comprehensive components without special processing and is a typical representative sample of milk powder. 2 g of milk powder sample (weighed accurately to 0.1 mg) was placed into a 50 mL volumetric flask. 10 mL of water was added and dissolved thoroughly. 35 mL of ethanol was added and mixed well. The mixture was ultrasonicated in a 45 ℃ water bath for 60 min to ensure complete extraction of total flavonoids from the sample. After ultrasonication, the mixture was cooled to room temperature. The volume was made up to the mark with ethanol and mixed well. The mixture was centrifuged at 4 ℃ and 5 000 r/min for 10 min. The supernatant (sample extract solution) after centrifugation was reserved for use[14-17].

2.0 mL of the sample extract solution to be tested (the volume can be adjusted according to the sample content) was accurately pipetted into a 50 mL volumetric flask. The procedure described in Section **1.3.2** was followed. The absorbance of the test solution was measured at a wavelength of 420 nm using a 2 cm cuvette, with the sample blank (Section **1.3.2**) as the reference. The content of flavonoid compounds in the test solution (mg) was determined by consulting the standard curve or calculated using the regression equation. The concentration of the test solution was obtained from the standard curve, and its absorbance value should be within the linear range of the standard curve.

1.3.4 Blank test. The procedure described in Section **1.3.2** was followed, except that no sample was added.

1.3.5 Sample content calculation. The total flavonoid content in the sample was calculated using Equation (1).

$$X = \frac{C \times V \times n}{m \times 1\,000} \times 100 \quad (1)$$

where X is the total content of flavonoid compounds, in g/100 g; C is the mass of rutin in the sample colorimetric solution obtained from the standard curve or calculated by the linear regression equation, in milligrams (mg); V is the constant volume, in milliliters (mL); m is the mass of the sample, in grams (g); n is the dilution factor. The calculation result was retained to two decimal places.

1.4 Method validation Precision, Recovery, and Repeatability Test: Blank milk powder samples were spiked with low, medium, and high concentration levels. Six parallel determinations were performed for each level. The detection results and recovery rates of the spiked samples were analyzed, and the relative standard deviation (*RSD*) was calculated.

LOD and LOQ Experiment: The limit of detection (LOD) was calculated as the average value of 10 blank sample detections plus 3 times the standard deviation. Total flavonoids were added to blank milk powder samples such that the detected absorbance of the sample was 0.2, which meets the minimum absorbance value requirement for quantitative analysis according to the Lambert-Beer law. The amount added at this point was taken as the limit of quantification (LOQ). The LOD and LOQ for this method were calculated[18-20].

2 Results and analysis

2.1 Selection of extraction solvent and sample weight Total flavonoids are generally insoluble or poorly soluble in water but soluble in organic solvents such as methanol, ethanol, ethyl acetate, and ether. However, considering that directly adding organic solvents to milk powder samples will easily cause protein coagulation and precipitation, encapsulating the total flavonoids and increasing extraction difficulty, water should first be added to fully dissolve the milk powder sample before extraction. After dissolving 2 g of milk powder in water, ethanol was added for extraction. The effects of the amounts of water and ethanol added on the extract were investigated[21-25], and the results are shown in Table 1.

As shown in Table 1, when the water added increased from 10 mL to 15 mL and the ethanol added decreased from 40 mL to 35 mL, the extract solution remained clear. When the water added

increased to 20 mL and the ethanol added decreased to 30 mL, the extract solution became turbid, indicating that the added ethanol was insufficient to precipitate the protein. Since total flavonoids are insoluble in water but soluble in ethanol, the optimal extraction solvent was chosen as 10 mL of water and 40 mL of ethanol[32-35].

Table 1 Amounts of water and ethanol added to the extract using different pretreatment methods for whole milk powder

No.	Sample weight//g	Water added//mL	Ethanol added//mL	Constant volume//mL	Extract solution	Reaction solution
1#	2	10	40	50	Clear	Clear
2#	2	15	35	50	Clear	Clear
3#	2	20	30	50	Turbid	Turbid
4#	3	10	40	50	Clear	Turbid
5#	4	10	40	50	Clear	Turbid

When the sample weight was increased to more than 2 g, although the extract solution was clear, the solution became turbid after adding aluminum nitrate and potassium acetate for the derivatization reaction, making subsequent absorbance measurement impossible. Therefore, the optimal sample weight was 2 g.

2.2 Selection of extraction temperature Flavonoids have moderate heat resistance, and heating can improve dissolution efficiency. The recovery rate for milk powder samples spiked with a theoretical amount of 0.5% was investigated at extraction temperatures of 35, 45, and 60 ℃ to evaluate the extraction efficiency at different temperatures[22-26].

Table 2 Detection results of total flavonoids in milk powder at different extraction temperatures

Extraction temperature//℃	Average detection result//%	Recovery rate//%	*RSD*//%
30	0.43	86	4.5
45	0.48	96	3.3
60	0.41	82	3.6

As shown in Table 2, when the extraction temperature increased from 30 ℃ to 45 ℃, the recovery rate increased from 86% to 96%. When the temperature further increased to 60 ℃, the recovery rate significantly decreased compared to 45 ℃. The increase in temperature may cause loss of total flavonoid substances. The extraction efficiency was optimal at 45 ℃.

2.3 Selection of extraction time Extraction was performed in a 45 ℃ water bath. The effect of different extraction times (30 min, 45 min, 60 min, 75 min)[27-34] on the recovery rate was investigated. Recovery experiments were conducted by adding 0.5% total flavonoids to blank milk powder.

Table 3 Detection results of total flavonoids in milk powder at different extraction times

Extraction time//min	Average detection result//%	Recovery rate// %	*RSD*//%
30	0.24	48	4.5
45	0.38	75	3.1
60	0.49	98	3.3
75	0.48	96	3.2

As shown in Table 3, when the extraction time increased from 30 min to 60 min, the recovery rate increased from 48% to 98%. Prolonging the extraction time significantly improved the extraction efficiency. When the extraction time was further extended from 60 min to 75 min, the recovery rate no longer increased. Therefore, the optimal extraction time was 60 min.

2.4 Selection of extract aspiration volume After determining the sample weight, extraction solvent, extraction temperature, and time, a certain volume of the extract needed to be aspirated for the derivatization reaction before measuring absorbance. The aspiration volume of the extract was investigated[34-39], and the results are shown in Table 4.

Table 4 Aspiration volume of extract

Test sample	Extract volume aspirated//mL	Detection deviation	Reaction phenomenon
Milk powder spiked at 0.05%	1	5.8	Clear
	2	2.1	Clear
	3	10.2	Turbid
	4	63.3	Turbid
	5	218.4	Turbid

As can be seen from Table 4, when the aspiration volume of the extract was 1 mL or 2 mL, the derivatization reaction solution appeared clear. However, when the aspiration volume was increased to 3 mL, the reaction solution became turbid, making absorbance measurement impossible. Considering that the detection deviation was smallest when the aspiration volume was 2 mL, the optimal aspiration volume was determined to be 2 mL.

2.5 Standard curve, LOD, and LOQ The standard solutions were measured according to the method described in Section **1.3.2**. The absorbance of each standard solution was measured at 420 nm. The standard curve was plotted with the total flavonoid content in the standard solution as the abscissa and absorbance as the ordinate, as shown in Fig. 1.

Regression equation: $y = 1.057\ 6x - 0.004\ 8$, with a linear range of 0.2 – 1.0 mg, correlation coefficient (r) of 0.999, LOD of 0.05%, and LOQ of 0.3%.

As shown in Fig. 1, this method exhibited a good linear relationship within the range of 0.2 – 1.0 mg, with a correlation coefficient r greater than 0.999 9. The average value of 10 blank sample detections plus 3 times the standard deviation resulted in 0.05%. Therefore, 0.05% was set as the LOD of this method. For the milk powder sample with a weight of 2 g, 0.3% standard substance was added. At this level, the absorbance was 0.2, meeting the minimum absorbance value requirement for quantita-

tive analysis according to the Lambert-Beer law. Moreover, the spike recovery rate at this level ranged from 100.4% to 103.9%. Therefore, 0.3% was set as the LOQ of this method.

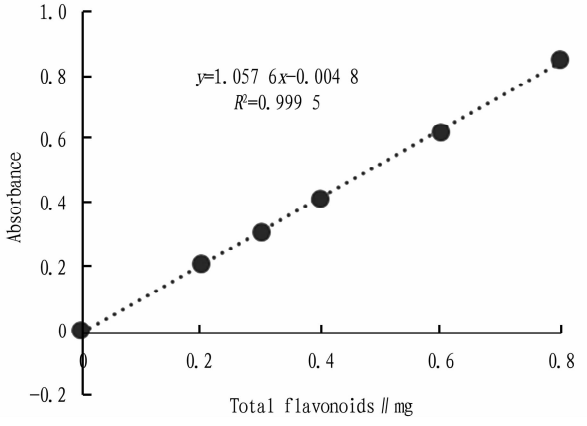

Fig.1 Standard curve

2.6 Recovery rate Recovery experiments were conducted using milk powder as the matrix. Standard solutions at three concentration levels were added to investigate the recovery rate[39-44]. The detection results are shown in Table 5.

Table 5 Recovery experiment %

Test items	Background value	Theoretical spiked amount	Detection result	Recovery rate	Recovery rate CV
Total flavonoids	Not detected	0.3	0.30	102.7	1.28
			0.31	103.9	
			0.30	100.4	
			0.30	101.6	
			0.31	103.5	
			0.30	103.1	
		0.6	0.59	101.4	0.96
			0.61	103.4	
			0.60	101.4	
			0.60	101.4	
			0.62	103.4	
			0.60	102.4	
		1.0	0.98	99.3	0.55
			0.99	100.1	
			1.00	100.4	
			1.00	101.0	
			1.00	100.4	
			0.98	100.2	

Different mass concentrations of total flavonoid standard solution were added for spike recovery experiments, with six parallel determinations performed for each level. As shown in Table 5, when total flavonoids were added at 0.3%, 0.6%, and 1.0% per 100 g of sample, the recovery rates were 99.3% to 103.9%, indicating good recovery results.

2.7 Precision Sample solutions spiked with different mass concentrations were selected. Six parallel repeatability experiments were performed for each spiking concentration. The detection results and relative standard deviations are shown in Table 6.

Table 6 Precision of spiked samples

Spiked concentration	Result	Average result	*RSD*
0.30	0.30 – 0.31	0.30	1.70
0.60	0.59 – 0.62	0.60	1.71
1.00	0.98 – 1.00	0.99	0.99

Repeatability experiments were conducted six times for milk powder samples spiked at 0.3%, 0.6%, and 1.0%. The detection result ranges and relative standard deviations are shown in Table 6. The relative standard deviations were all less than 3.0%, indicating that the accuracy of this method meets quantitative requirements and the method precision is good.

3 Conclusion

This study established a method for determining total flavonoids in milk powder based on spectrophotometry. Total flavonoids in milk powder were extracted with ethanol using 45 ℃ water bath ultrasonication for 60 min. Flavonoids contain a benzopyran ring structure and react with aluminum nitrate in a potassium acetate environment to form a yellow complex. The absorbance of this complex was measured at a wavelength of 420 nm for quantitative determination.

The method exhibited a good linear relationship within the range of 0.2 – 1.0 mg. The limit of detection was 0.05 g/100 g, and the limit of quantification was 0.3 g/100 g. When the spiked amount of total flavonoids was in the range of 0.3% to 1.0%, the spike recovery rates were 99.3% to 103.4%, and the relative standard deviations were less than 3.0%. The method has the advantages of being fast, accurate, stable, and highly sensitive, and can be used for the detection of total flavonoid content in milk powder.

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