Effects of storage methods on the quality of *Isatis indigotica* radix based on active constituents and NIR spectra

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RESEARCH ARTICLE

Abstract

Isatis indigotica radix is a common traditional Chinese medicine and veterinary medicine widely cultivated in China, and inappropriate storage conditions may damage its quality. The objective of this work was to investigate the changes in epigoitrin and nucleosides compounds and Near Infrared Spectra (NIR) of *Isatis indigotica* radix packaging under vacuum (VP), nitrogen (NP), and sealing (SP), stored at different temperatures—room temperature (SPt), refrigerator (SPr), and frozen (SPf) during 12 months of storage. After 12 months of storage, the epigoitrin concentration of *I. indigotica* radix samples in VP, NP, and SPt was much higher than in SPr and SPf, while samples in SPf had more contents of nucleosides than the other. Moreover, a significant difference in principal component analysis revealed a superior storage method based on active compounds and NIR spectra. It suggested that NP was considered a better storage method for retaining the chemical and physical properties and quality of *I. indigotica* radix after 12 months of storage.

Keywords: epigoitrin and nucleosides compounds, *Isatidis radix*, near infrared spectra, principal component analysis, various storage conditions

Introduction

The root of *Isatis indigotica* (Isatidis Radix), named "Ban-Lan-Gen" in China, has a long medical history in China as traditional Chinese medicine and veterinary medicine (Tong *et al.,* 2020). It was one of the drugs recommended by the Chinese government for the prevention and control of severe acute respiratory syndrome (SARS) (Lin *et al.,* 2005). It inhibited the coronavirus disease 2019 (COVID-19) (Yu *et al.,* 2021). Given the high market demand, *I. indigotica* has grown increasingly in agricultural fields in the last two decades, and its output reached 50,000 tons in 2020 (Chen *et al.,* 2015; Fa *et al. et al.* 2021; Xu *et al.,* 2023). Due to the imbalance of production and marketing and the difference in demand, *I. indigotica* radix must be stored for a relatively long time—two or three years, after harvest and before market. However, dried *I. indigotica* radix is usually transported, stored under ambient temperature, packed in woven bags or cartons, or not packaged. *I. indigotica* radix undergoes numerous physical, chemical, and microbiological changes due to UV light, moisture vapor, oxygen, and temperature changes (King, 2006), which will damage the quality of *I. indigotica* radix products.

Proper storage methods avoid possible damages and maximally retain the product quality, hence maintaining the product market value (Nasiri *et al.,* 2017; Wang *et al.,* 2018). In recent years a wide variety of packages and storage methods have been employed to reduce the level of quality deterioration of food and herbal medicine materials, which eventually retains product quality and economic value (Chaliha *et al.,* 2013; Choe *et al.,* 2001; Liu *et al.,* 2014; Sharma *et al.,* 2001; Usai *et al.,* 2011; Di Wang *et al.,* 2021; Wang *et al.,* 2018). However, to our knowledge, studies on storage methods that influence *I. indigotica* radix quality during storage remain unexplored. The present study attempts to study changes in the main active constituents—epigoitrin, adenosine, guanosine, and uridine (Chen *et al.,* 2021) and Near Infrared Spectra (NIR) of *I. indigotica* radix by packaging under sealing, vacuum, and nitrogen and using a packaging material (polyethylene/vacuum metalized (aluminum) polyethylene terephthalate [PE/VMPET]), which is known to be effective in blocking air and moisture, to obtain a better storage method for *I. indigotica* radix, ultimately retaining the quality and economic value of the product.

Materials and Methods

Samples

Cultivar *I. indigotica* was planted at the medicinal plant garden of the School of Pharmacy, Gansu University of Traditional Chinese Medicine, Hezheng, (Gansu Province, China, 35°16' N, 103°24' E) for 1 year. The roots of 100 *I. indigotica* plants were carefully sampled, washed with tap water, and dried in the shade for 2 months. The dried roots were stored at room temperature away from direct sunlight until treated with packing. These samples are referred to as month = 0 samples throughout the paper.

Conditions for storage trial

The collected roots of *I. indigotica* were separately wrapped in different PE/VMPET envelopes (23 cm × 30 cm × 0.06 mm). The envelopes containing samples were treated with vacuum packaging (VP), nitrogen packaging (NP), and sealing packaging (SP). The envelopes in sealing packaging were separately stored at room temperature (SPt), refrigerator (SPr), and frozen (SPf) conditions before being instrumentally analyzed. The procedure was performed in triplicate. The properties of these storage methods are described in Table S1. Sampling for analysis occurred at time zero (before packaging, hereafter referred to as month = 0) and once every 2 months for 12 months, labeled months 2, 4, 6, 8, 10, and 12.

Chemicals

Epigoitrin (EP), adenosine (Ad), guanosine (Gu), and uridine (Ur) were used as standards (Shanghai Yuanye Bio-Technology Co., Shanghai, China) in our study, and the purities were above 98% according to HPLC/UV. The methanol (Shandong Yuwang Industrial Co., Shandong, China) used in our study was of HPLC grade. All other reagents and chemicals were analytical (Shandong Yuwang Industrial Co., Shandong, China).

Extraction of epigoitrin and nucleosides compounds

Epigoitrin and nucleosides compounds in radix isatidis were determined by HPLC, using an Agilent 1200 HPLC system (Agilent, California, USA) with a diode array detector (DAD) and the analytical column, Phenomenex Luna C18 reversed-phase column (250 mm × 4.6 mm i.d. 5μm particle diameter). The samples of isatidis radix (1.0 g) were added to 50 mL purified water, extracted by ultrasonic for 60 min, and then filtered (Commission 2020). Each sample was repeated four times. Samples of isatidis radix at 20 mg/mL concentration were separated with a 0.02% phosphoric acid solution mixed with methanol (7:93, v:v) (Commission 2020). The flow rate was 0.9 mL/min, and the column temperature was maintained at 30°C. The detection wavelengths were 245 nm (epigoitrin) and 254 nm (nucleosides). The total run time was 30 min. These compounds were identified by comparing retention times and UV spectra with those of the standards of compounds—epigoitrin, adenosine, guanosine, and uridine. The standard curves were performed using the linear regression method. The equations formulated relating peak areas (y in arbitrary units) and concentrations (x in mg) were $y = 2\times10^7x+175.05$ for epigoitrin; $y = 2 \times 10^6x - 5.4178$ for adenosine, $y = 272704x+1.7638$ for guanosine, and $y = 2\times10^6x+55.551$ for uridine. The correlation coefficient (r) was 0.99 or above in all cases.

Spectral acquisition

NIR spectra of the isatidis radix samples were scanned three times in the 12,000–4000 cm^{-1} range by Bruker Tango-R (Bruker, Karlsruhe, Germany), and the obtained mean spectrum was applied for further analysis.

Statistical analysis

One-way analysis of variance (ANOVA) was used to analyze the effects of storage methods on the samples. A Tukey's Honestly Significant Difference (Tukey HSD) test was conducted when the samples exhibited a significant difference between samples, with the significance level

set at $p < 0.05$. One-way ANOVA and Tukey HSD were performed with SPSS Statistics 18 (IBM, Armonk, NY, USA). The NIR data pre-processing and principal component analysis (PCA) were performed using Unscrambler X version 10.3 (Camo Software, Oslo, Norway).

Results and Discussion

Analysis of epigoitrin and nucleosides in Isatis indigotica Radix

According to Figure 1, the epigoitrin concentration in *I. indigotica* radix is subject to change during storage. There was a statistically significant interaction between the effects of storage time and storage methods on epigoitrin concentration in *I. indigotica* radix, F (24, 105) = 68.450, $p < 0.0005$. According to Table S2, after 12 months of storage, the epigoitrin concentration of *I. indigotica* radix in SPt was 0.64 ± 0.00 mg/g dry weight, which was not significantly different ($p > 0.05$) from the epigoitrin concentration of 0.59 ± 0.05 at the beginning of the storage trial (month = 0). The epigoitrin concentration of *I. indigotica* radix in NP (0.61 ± 0.01) was similar to that of *I. indigotica* radix in SPt, and the epigoitrin concentration of *I. indigotica* radix in VP (0.76 ± 0.01) was significantly greater ($p < 0.05$), which could be mainly caused by water loss during storage. However, the epigoitrin concentration of *I. indigotica* radix in SPr and SPf (0.35 ± 0.05, 0.30 ± 0.03) was significantly fewer ($p < 0.05$). This result showed that refrigerating or freezing significantly influenced the medicinal quality of *I. indigotica* radix, probably due to freezing usually resulting in mechanical damage to the cell wall and increased solute concentration (Sun and Li 2003). Increasing solute concentration means increased solubility of polysaccharide and starch grains of *I. indigotica* radix in water. Adding more polysaccharides and starch grains would increase the system's viscosity and decrease mass transfer in the system and the solubility of epigoitrin in water decreased. The sample water extract solution at the SPr and SPf storage trial (month = 12) became more cloudy while epigoitrin concentration decreased, which demonstrated this.

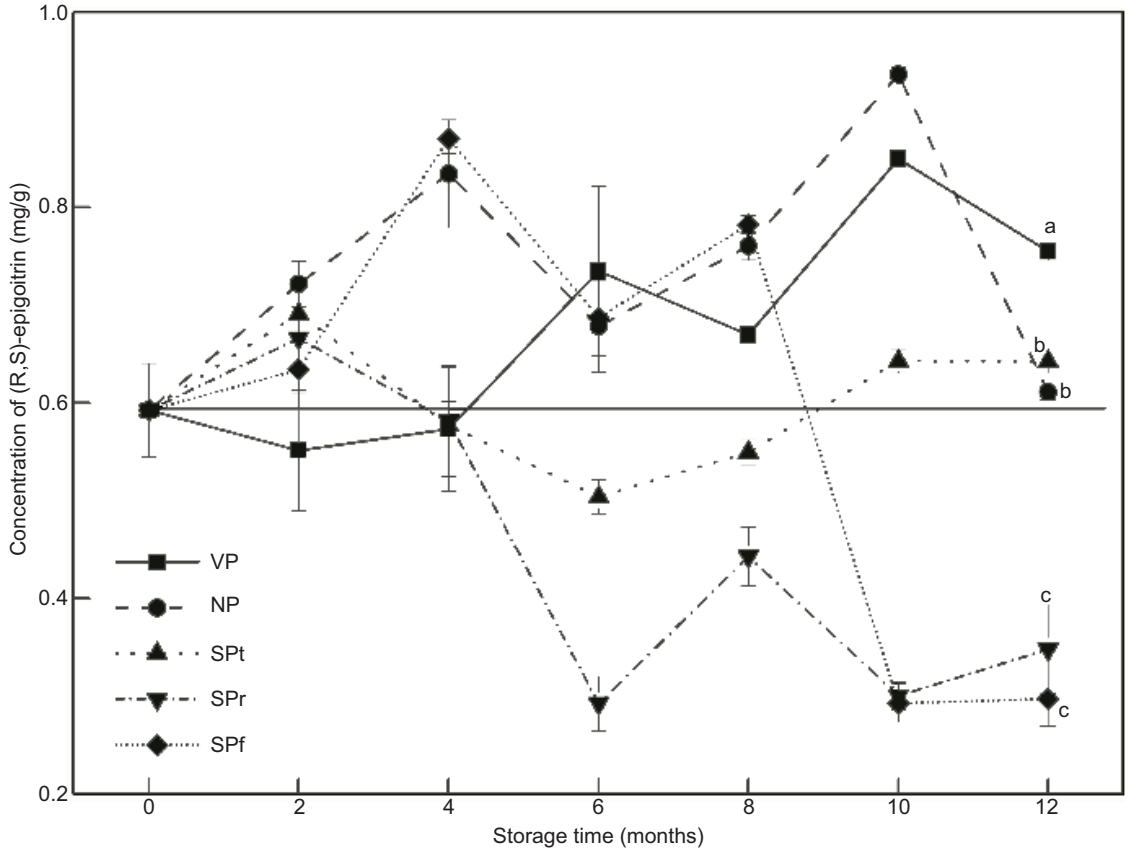

Figure 1. Changes in the concentration of epigoitrin during 12 months of storage for *Isatis indigotica* radix in storage methods VP, NP, SPt, SPr, and SPf. Results are expressed as mean ± standard deviation (n = 4). Different letters (i.e., a, b, c) denote significant differences between means according to a Tukey HSD.

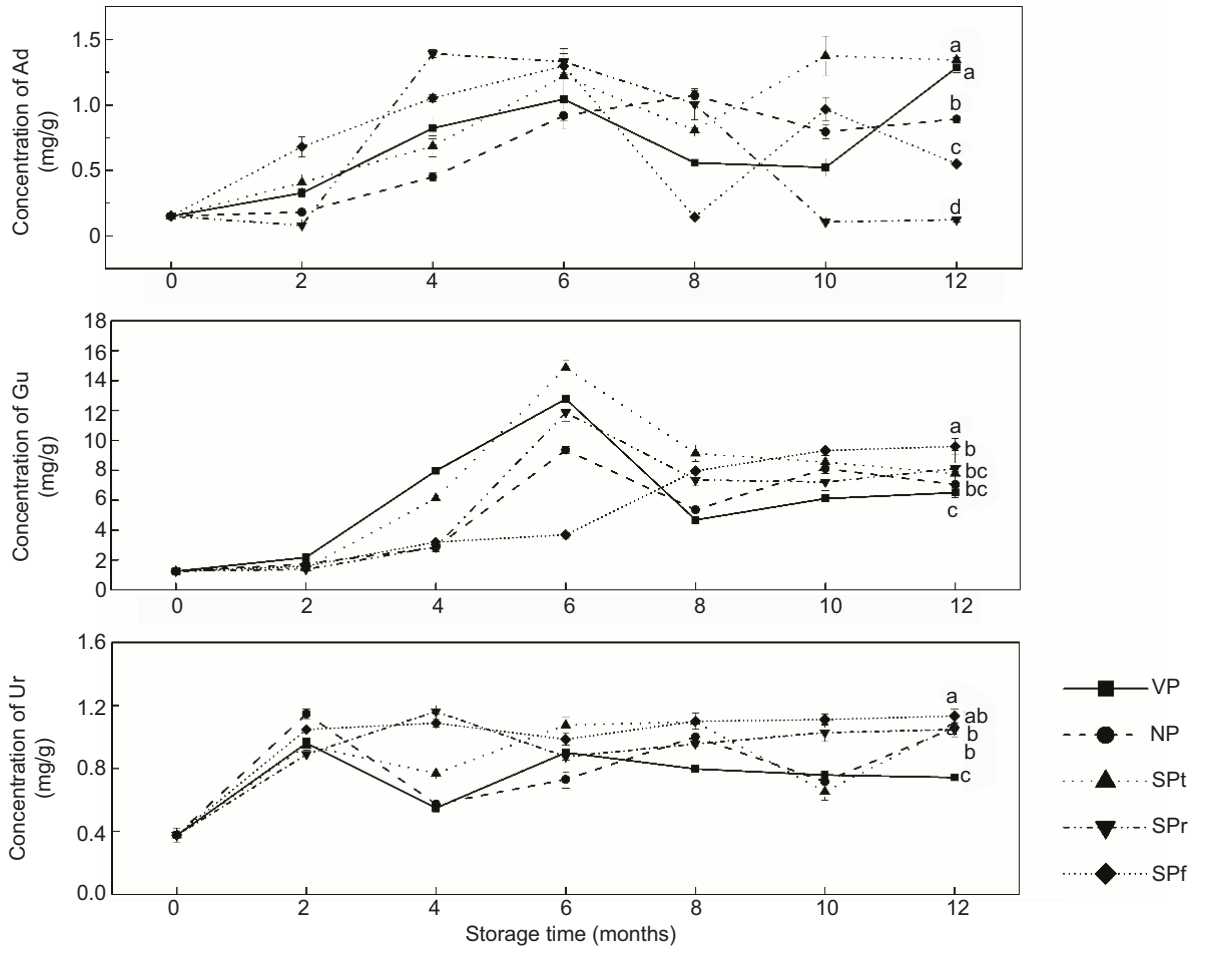

Figure 2. Changes in the concentration of adenosine (Ad), guanosine (Gu), and uridine (Ur) during 12 months of storage for *Isatis indigotica* radix in VP, NP, SPt, SPr, and SPf. Results are expressed as mean ± standard deviation (n = 4). Different letters (i.e., a, b, c) denote significant differences between means according to a Tukey HSD.

The amounts of nucleosides—adenosine, guanosine, and uridine in *I. indigotica* radix in storage methods VP, NP, SPt, SPr, and SPf are shown in Table S2. After 12 months of storage, the adenosine concentration of *I. indigotica* radix in SPt and VP were 1.34 ± 0.02 mg/g dry weight and 1.29 ± 0.04 mg/g dry weight, respectively, which were significantly higher than in the other three storage methods NP, SPr, and SPf ($p < 0.05$). The guanosine and uridine concentration in SPf was significantly higher than in the other four storage methods: VP, NP, SPt, and SPr ($p < 0.05$). According to Figure 2, an increase in the amounts of nucleosides—adenosine, guanosine, and uridine was observed after 12 months of storage. The levels of nucleosides of *I. indigotica* radix changed with the increase in storage time.

The contents of 3 nucleosides, i.e., adenosine, guanosine, and uridine peaked at storage (month = 6), then decreased gradually and came to stability after 12 months of storage in VP, NP, SPt, and SPr (Figure 3). However, the contents of 3 nucleosides of *I. indigotica* radix in SPf increased correspondingly as that of month 0 (1.77±0.13) to month 12 (11.28±0.50) (Figure 3). It may be that enzyme from frozen *I. indigotica* radix degraded authentic ATP and formed nucleosides (Pei *et al.,* 2014). Our results indicated the increase of nucleosides appearing in *I. indigotica* radix, which had been frozen and stored at −20°C for 12 months.

Spectral analysis

The average NIR spectra of *I. indigotica* radix from five different storage methods with maxima at 8323, 6762, 6340, 5675, 5160, 4762, 4374, and 4308 cm^{-1} are shown in Figure 4A. The broad band at 4764 cm^{-1} is commonly called the "carbohydrate band" (Li *et al.,* 2012). The chosen absorptions, near 7000 cm^{-1} and 6000 cm^{-1}, were due to the structure of the epigoitrin containing a secondary amine group that can form hydrogen bonds (Ma *et al.,* 2020).

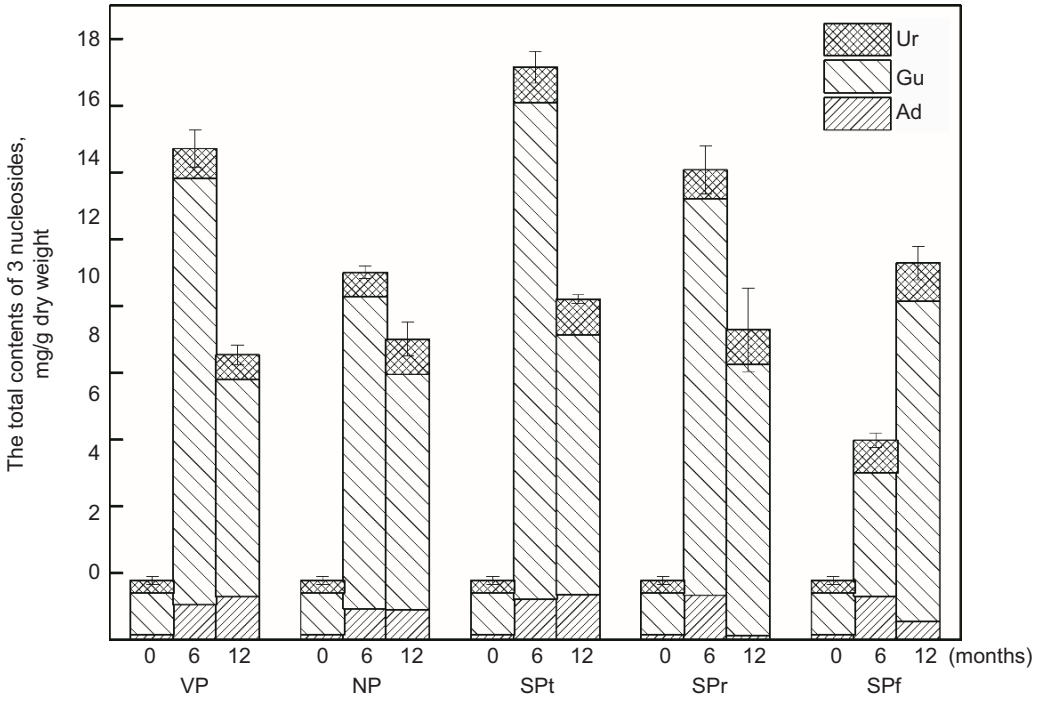

Figure 3. The total contents of 3 nucleosides—adenosine (Ad), guanosine (Gu), and uridine (Ur) during storage (month = 0, 6, 12) for *Isatis indigotica* radix in VP, NP, SPt, SPr, and SPf. Results are expressed as the means ± standard deviation (n = 4).

The NIR spectra of *I. indigotica* radix show a similar spectrum of starch whose major peaks occur at 8306, 6831, 5176, 4762, 4367, and 4303 cm^{-1} (McClure & Stanfield, 2006) because plant tissue may contain as much as 60% starch by weight (dry basis) (Li *et al.,* 2012).

It is difficult to find specific bands in the raw NIR spectra of *I. indigotica* radix based on storage methods. On the other hand, baselines of sample spectra vary widely due to particle size effect, moisture effect, noise, and so on. The NIR spectral data were pretreated with the use of the multiplicative scatter correction (MSC) method (Ni *et al.,* 2012) to minimize additive and multiplicative spectral effects (Ni *et al.,* 2012). MSC spectra of *I. indigotica* radix from five different storage methods after 12 months of storage are shown in Figure 4B. As a result, the unique spectral features associated with different samples became apparent, which were around 7700–4300 cm^{-1} were chosen for modeling.

Principal component analysis

Principal component analysis (PCA) is an unsupervised pattern recognition and data display method (Ni *et al.,* 2012). Each sample object and variable is represented by a score and loading values on each PC. A PCA was applied because it is convenient to reduce the data dimension and visualize the similarities among samples of *I. indigotica* radix. This analysis provides the locations of the samples in the principal component and the relationships between the analyzed variables (Rodríguez-Flores *et al.,* 2019). The MSC spectra and the contents of epigoitrin and 3 nucleosides were evaluated by PCA for optimum separation of samples stored with different storage methods in this study. The first three components, which describe 66%, 29%, and 5%, clarify differentiation. The distribution of *I. indigotica* radix samples and the projection of each variable in the two first components are shown in Figure 5. In the PCA model, it could be seen that samples from NP and SPt are placed in the upper right corner of the scores plot, respectively, and samples from VP in the bottom right corner. In contrast, samples from SPf and SPr are effectively clustered on the left (Figure 5). These observations suggested that the *I. indigotica* radix samples stored in NP, VP, and SPf differed. This analysis highlighted the active constituents and spectra characteristics that better differentiated the studied *I. indigotica* radix samples from five different storage methods, especially for packing under frozen (SPf and SPr) and non-frozen (SPt, NP, VP) samples, which further indicated that changes in chemical and physical properties after 12 months of refrigerated or frozen storage of *I. indigotica* radix.

The information about the variables analyzed was displayed in correlation loadings (Table 1), which provide a scale-independent assessment of the variables and may

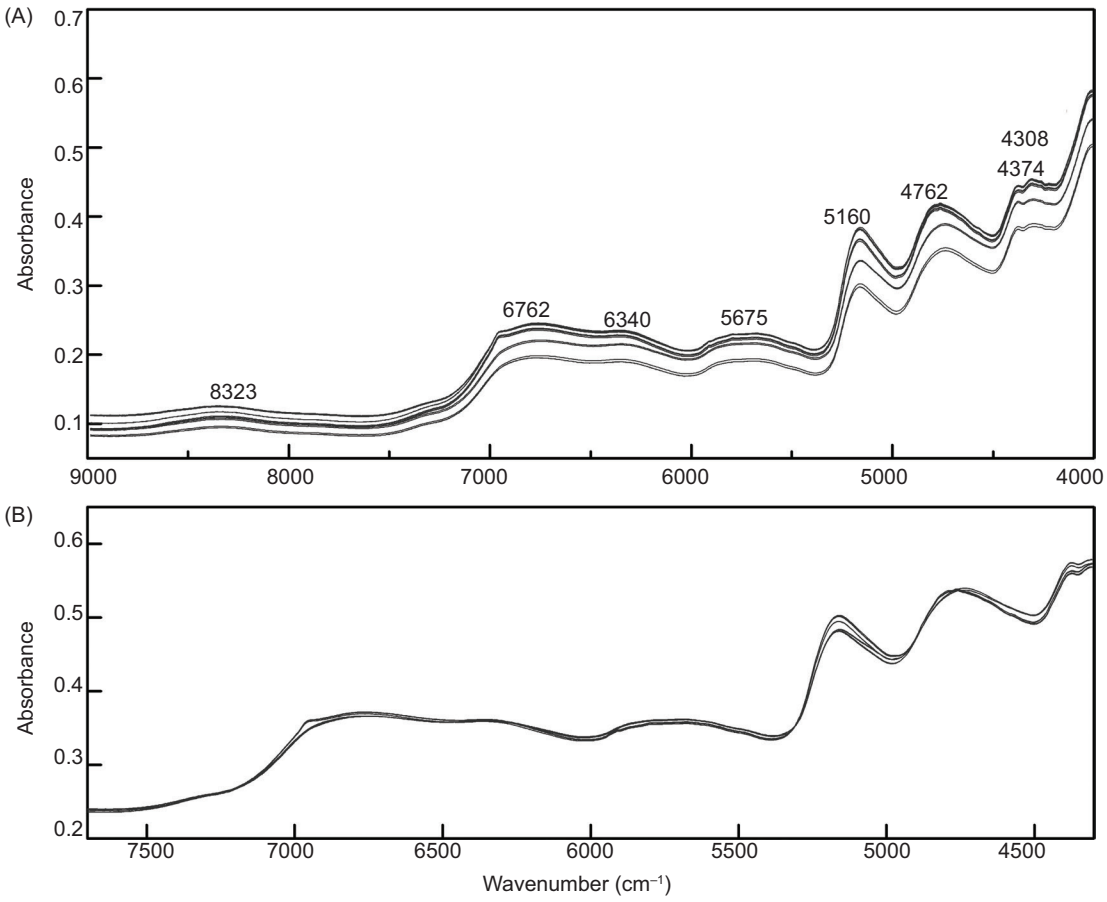

Figure 4. Raw spectra of *Isatis indigotica* radix samples from five different storage methods after 12 months of storage (A); MSC spectra of *I. indigotica* radix samples from five different storage methods after 12 months of storage (B).

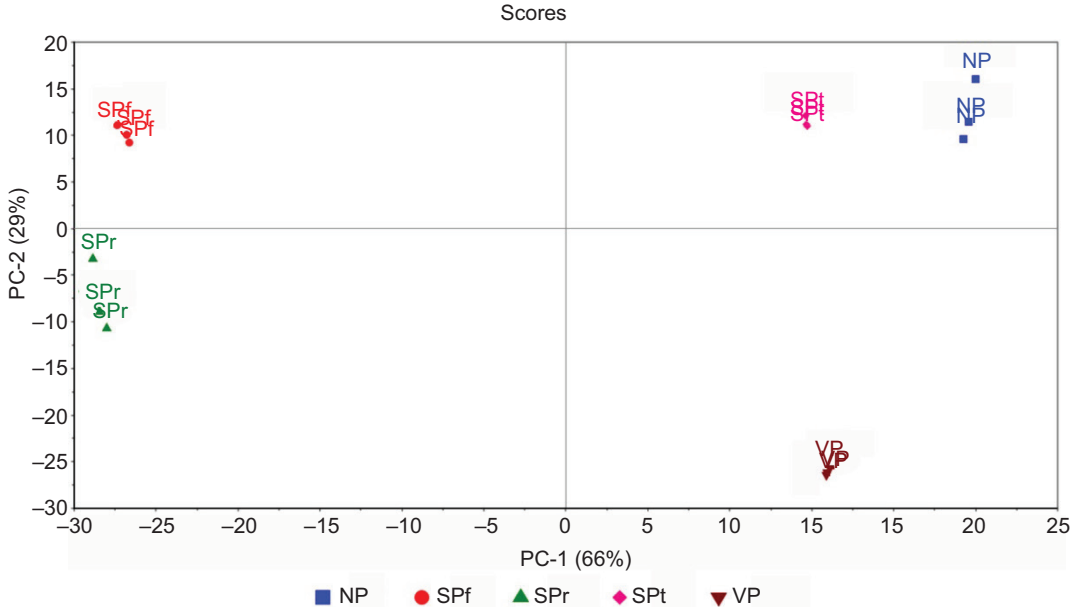

Figure 5. Score plot of the first two components obtained from the PCA. (scatter plot of samples of *I. indigotica* radix in storage methods VP, NP, SPt, SPr, and SPf after 12 months of storage).

Table 1. Components extracted and variable correlation used in the PCA.

Number of components	Component correlation loadings	
	PC-1	PC-2
epigoitrin	0.96	−0.14
adenosine	0.37	0.64
guanosine	−0.29	0.78
uridine	−0.94	−0.01
7096–6933 cm^{-1}	0.91 to 0.98	−0.36 to 0.02
6929–6627 cm^{-1}	0.97 to 0.91	0.03 to 0.39
6419–6272 cm^{-1}	0.34 to −0.18	0.91 to 0.98
6268–6252 cm^{-1}	−0.23 to −0.40	0.96 to 0.91

provide a clearer indication of variable correlations. For the correlation loadings, the variables were epigoitrin, and 7096–6627 cm^{-1} spectra characteristics along PC1, and the variables, adenosine, guanosine, and 6419–6252 cm^{-1} spectra characteristics along PC2 (Table 1). Composite the scores plot and the correlation loadings, these results indicated that samples stored in VP lying to the lower right of the score plot have higher values along PC1, while samples stored in SPf lying to the upper left of the score plot have higher values along PC2, and *I. indigotica* radix samples stored in NP lying to the upper right of the score plot have higher values for those variables (PC1 and PC2). The above results approximate the previous analysis of epigoitrin and nucleosides in *I. indigotica* radix samples stored using different storage methods.

Conclusion

The contents of epigoitrin and 3 nucleosides, i.e., adenosine, guanosine, and uridine, can be a preliminary differentiation among *I. indigotica* radix samples stored in VP, NP, and SPf by ANOVA analysis. However, the combination of the contents of epigoitrin and nucleosides, and NIR spectra characteristics variables with PCA, characterized *I. indigotica* radix samples from different storage methods having different properties with greater precision. In summary, a significant difference in PCA revealed superior storage conditions based on active compounds and NIR spectra. It revealed that NP was considered a better storage method for retaining the chemical and physical properties and quality of *I. indigotica* radix after 12 months of storage.

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Conflict of interest

The authors declare no conflict of interest.

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Supplementary

Table S1. Storage methods and temperature-humidity.

Storage methods	Temperature (°C)	Relative humidity (%)
VP	15 to 25	15 to 40
NP	15 to 25	15 to 40
SPt	15 to 25	15 to 40
SPr	2 to 8	40 to 55
SPf	−18 to −20	25 to 35

Table S2. Concentrations of bioactive components in *Isatis indigotica* Radix after storage in different storage methods (n = 4).

Bioactive components	Preservation methods	Concentrations of bioactive components[a] after storage for						
		0 month	2 months	4 months	6 months	8 months	10 months	12 months
Epigoitrin	VP[b]	0.59±0.05	0.55±0.06	0.57±0.06	0.73±0.09	0.67±0.01	0.85±0.01	0.76±0.01[a]
	NP	0.59±0.05	0.72±0.02	0.83±0.06	0.68±0.05	0.76±0.01	0.94±0.01	0.61±0.01[b]
	SPt	0.59±0.05	0.69±0.03	0.58±0.02	0.50±0.02	0.55±0.01	0.64±0.01	0.64±0.00[b]
	SPr	0.59±0.05	0.67±0.06	0.58±0.06	0.29±0.03	0.44±0.03	0.30±0.01	0.35±0.05[c]
	SPf	0.59±0.05	0.63±0.00	0.87±0.02	0.69±0.00	0.78±0.01	0.29±0.02	0.30±0.03[c]
Adenosine	VP	0.15±0.01	0.33±0.03	0.82±0.08	1.04±0.16	0.56±0.02	0.52±0.07	1.29±0.04[a]
	NP	0.15±0.01	0.18±0.01	0.45±0.03	0.92±0.11	1.07±0.04	0.80±0.05	0.89±0.03[b]
	SPt	0.15±0.01	0.41±0.06	0.69±0.08	1.22±0.17	0.80±0.02	1.38±0.15	1.34±0.02[a]
	SPr	0.15±0.01	0.08±0.01	1.39±0.03	1.33±0.10	1.01±0.12	0.11±0.01	0.12±0.02[d]
	SPf	0.15±0.01	0.68±0.08	1.05±0.03	1.30±0.05	0.14±0.01	0.97±0.09	0.55±0.03[c]
Guanosine	VP	1.25±0.09	2.18±0.11	7.96±0.18	12.77±0.40	4.68±0.19	6.11±0.20	6.50±0.32[c]
	NP	1.25±0.09	1.75±0.22	2.84±0.12	9.36±0.22	5.36±0.18	8.11±0.42	7.05±0.50[bc]
	SPt	1.25±0.09	1.47±0.11	6.12±0.16	14.86±0.48	9.14±0.55	8.56±0.43	7.79±0.12[bc]
	SPr	1.25±0.09	1.37±0.19	2.88±0.32	11.87±0.57	7.35±0.40	7.21±0.57	8.12±1.22[b]
	SPf	1.25±0.09	1.59±0.22	3.19±0.10	3.69±0.24	7.95±0.13	9.33±1.36	9.60±0.52[a]
Uridine	VP	0.38±0.04	0.96±0.05	0.55±0.03	0.90±0.05	0.80±0.00	0.76±0.01	0.74±0.02[c]
	NP	0.38±0.04	1.15±0.03	0.57±0.00	0.73±0.05	1.00±0.01	0.72±0.06	1.06±0.01[b]
	SPt	0.38±0.04	0.94±0.05	0.77±0.02	1.07±0.05	1.10±0.01	0.65±0.06	1.07±0.02[ab]
	SPr	0.38±0.04	0.89±0.07	1.16±0.02	0.87±0.10	0.96±0.01	1.03±0.06	1.05±0.05[b]
	SPf	0.38±0.04	1.05±0.12	1.09±0.03	0.98±0.04	1.10±0.05	1.11±0.03	1.13±0.05[a]

Different letters within a row (i.e., a, b, c) denote significant differences between means according to a Tukey HSD.
VP - vacuum packaging at room temperature, NP - nitrogen packaging at room temperature, SPt - sealing packaging and stored at room temperature, SPr - sealing packaging and stored at the refrigerator, SPf - sealing packaging and stored at the frozen condition.