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Investigation on the spectrum-effect relationships of EtOAc extract from *Radix Isatidis* based on HPLC fingerprints and microcalorimetry

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ABSTRACT

This work investigated the spectrum-effect relationships between HPLC fingerprints and the anti-bacterial activities of EtOAc extracts from *Radix Isatidis*. Fingerprints of EtOAc extracts of *Radix Isatidis* from various sources were established by a High-Performance Liquid Chromatography. The process of *Escherichia coli* (*E. coli*) growth affected by EtOAc extracts was monitored using a Thermal Activity Monitor (TAM) Air Isothermal Calorimeter by microcalorimetry. By analyzing the power–time curves, quantitative parameters, such as growth rate constant k, maximum heat-production rate P_m, appearance time t and total heat-production Q were obtained to characterize the interactions of *E. coli* and the EtOAc extracts from *Radix Isatidis*. The HPLC fingerprints were investigated using hierarchical clustering analysis. The main thermo-kinetic parameters from the power–time curves were analyzed using principal component analysis. The spectrum-effect relationships between the HPLC fingerprints and anti-bacterial activities were analyzed with multivariant correlation analysis. Close correlation existed between the spectrum-effect relationships of the EtOAc extracts. Salicylic acid in the HPLC fingerprints might be one of the anti-bacterial components. This work provides a general model of the combination of HPLC and microcalorimetry to study the spectrum-effect relationships of EtOAc extracts from *Radix Isatidis*, which can be used to search for principal components of *Radix Isatidis* on bioactivity.

1. Introduction

Radix Isatidis (Banlangen in Chinese), as the Traditional Chinese Medicine (TCM), is a kind of dried root of the plant *Isatidis indigotica* Fort (family Cruciferae), and is officially listed in the Chinese Pharmacopoeia [1]. It is mainly distributed in Hebei, Jiangsu, Zhejiang, Fujian, Anhui and Gansu provinces in China. It has been used as a medicinal plant for more than 2000 years from *ShenNongBenCao-Jing*, a famous ancient Chinese medicinal literary and has a widely useful activities including anti-virus, anti-bacterial, anti-endotoxic, anti-inflammatory, and immune regulatory effects [2–6]. Because the chemical constituents with anti-bacterial effect of *Radix Isatidis* remain uncertain, the reasons for its actions have remained a puzzle for a long time. Some researchers considered that the ethyl acetate (EtOAc) extract of *Radix Isatidis* had high anti-virus effects [7]. However, whether the EtOAc extract possessed anti-bacterial activities was not clear.

High-Performance Liquid Chromatography (HPLC), as an important analytical method, has got quick development in many areas. It can be used to determine the contents, analyze the constituents of many compounds and get the fingerprints of the drug, food and other materials [8–11]. Chromatographic fingerprinting is a useful method in the identification and quality control of botanical medicines [12–14] and HPLC fingerprinting analysis of the roots and leaves of *Isatis* species has been reported [15,16]. Microcalorimetry is a quantitative and versatile method of thermo-kinetics for measuring the heat-production in many fields, such as life sciences [17], pharmacological analysis [18], biotechnology [19], and environmental sciences [20]. This is because of its high sensitivity, high accuracy and possibilities for automation [21]. *Escherichia coli* is one of the most common pathogenic bacteria. Its clinical course is very extensive, ranging from a transient uncomplicated bacterium to a complicated course of septic embolization [22]. It has been selected as the experimental target to screen the bioactive part of the folium of *I. indigotica* by microcalorimetry [23].

So, in this present paper, microcalorimetry was selected to investigate the anti-bacterial activity of EtOAc extracts from *Radix Isatidis* on *E. coli* growth. HPLC was applied to establish the fingerprints of EtOAc extracts of *Radix Isatidis* from various sources to search for and analyze the components which had the anti-bacterial

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Table 1
The sources of *Radix Isatidis*

Sample no.	Source	Sample no.	Source
A	Fiiyang city, Anhui	F	Longnan city, Gansu
B	Qixin city, Hebei	G	Anguo city, Hebei
C	Bozhou city, Anhui	H	Longxi city, Gansu
D	Qixin city, Hebei	I	Zhangye city, Gansu
E	Wudu city, Gansu	J	Changchun city, Jilin

activity. The aim of this work is to provide a model of combining HPLC fingerprint and anti-bacterial activity of EtOAc extracts to search for bioactive components of *Radix Isatidis*.

2. Materials and methods

2.1. Instruments

HPLC fingerprints were measured with an Agilent 1100 series HPLC (Agilent company, USA) equipped with a diode array detector (DAD), a quaternary pump and degasser and HP Chemstation color spectrum workstation. A Thermal Activity Monitor (TAM) Air Isothermal Calorimeter, manufactured by Thermometric AB Company of Sweden, was used to measure heat output of the metabolism of *E. coli* strain. This isothermal microcalorimeter is an eight-channel twin instrument and thermostated at the range of 5–60 °C, with a limit of detectibility of 2 μW. For more details of the instrument, see the report of Wadso [24].

2.2. Materials

E. coli strain (*E. coli* CMCC B44103) was purchased from the Chinese Center for Type Culture Collections, National Institute for the Control of Pharmaceutical and Biological Products, Beijing 100051, China. It was grown in a Lactose Broth (LB) culture medium containing 10 g peptone, 5 g yeast extract and 10 g NaCl and medium pH was adjusted to 7.0–7.2 with 1 mM NaOH before autoclaving. HPLC grade and AR grade methanol (MeOH) were bought from Fisher Scientific (USA). The water was double distilled water. Authentic reference standard salicylic acid was supplied by National Institute for the Control of Pharmaceutical and Biological Products in China. *Radix Isatidis* was derived from the dried root of *I. indigotica* Fort, which was accredited by Professor Xiao-He Xiao, of the Institute of Chinese Materia Medica in 302 Hospital of PLA, Beijing, China. The various sources of *Radix Isatidis* were showed in Table 1. The samples from the same city or province had different harvest season or elaboration methods of medical material.

2.3. HPLC fingerprints

2.3.1. HPLC condition

The samples were injected into the HPLC system. Chromatography was carried out on a Kromasil C_{18} column (250 mm × 4.6 mm, 5 μm) and kept at 30 °C. The mobile phase was 0.2% H_3PO_4 water solution (A) and methanol (B) system. The gradient elution profile was: 0–10 min A:B (81:19, v/v) to A:B (65:35, v/v), 10–35 min A:B (65:35, v/v) to A: B (65:35, v/v), 35–55 min A:B (65:35, v/v) to A:B (50:50, v/v), 55–70 min A:B (50:50, v/v) to A:B (15:85, v/v). The flow rate was 1.0 mL/min and the effluent was monitored at 230 nm. Integration was performed in the horizontal baseline mode.

2.3.2. Preparation of reference standard solution

The standard solutions were prepared by adding an accurately weighed amount of salicylic acid to a volumetric flask and dissolved with 10 mL MeOH to make the final concentration 0.30 mg/mL.

2.3.3. Preparation of sample solution

Radix Isatidis was crushed into a powder, and 2 g of the powder was accurately weighed and extracted with 80 mL of EtOAc in an ultrasonic bath for 1.5 h. After extraction, the solution was filtered, 40 mL of the filtrate was concentrated to dryness by evaporation. The residue was dissolved with 5 mL MeOH, and then filtered through 0.45 μm micropore film to yield the sample solution for HPLC to get the HPLC fingerprints.

2.3.4. Analysis of HPLC fingerprints

2.3.4.1. Definition of run time. Sample B solution (Table 1), was chosen randomly to test the feasibility and suitability of the experimental method and condition. 10 μL of sample B solution was injected into the HPLC system, and the chromatogram was run for 100 min. The results indicated that there were no significant peaks after 70 min, so 70 min was selected as the experimental run time.

2.3.4.2. Precision. To assess the repeatability of the method, samples B solution was injected three times for HPLC analysis. The relative standard deviation (R.S.D.) of the retention time and peak area of the characteristic peak were 0.09% and 3.2%, respectively. The intermediate precision was assessed by replicate injections (three times) of samples B solution after one week and one month. The day-to-day variations of the peak retention time and peak area of the reference peak were acceptable (R.S.D. < 0.6% and 5.4%, respectively).

2.3.4.3. Establishment of fingerprints. Every sample solution in Table 1 was injected into the HPLC system three times to get the fingerprints. Then, samples B solution dissolved with MeOH was dried and dissolved with double distilled water to get the water solution. The samples B solution dissolved with water was also injected into the HPLC system three times to get the fingerprint.

2.3.4.4. Evaluation of fingerprints. The HPLC fingerprints were matched automatically by a "similarity evaluation" system for chromatographic fingerprint of Traditional Chinese Medicine (TCM) (Version 2004A). Then the reference atlas, a fitting curve, was formed by this system using the Median method from the general comparison of the chromatograms of 10 batches of EtOAc extracts from Radix Isatidis and the similarities between the reference atlas and the chromatogram of EtOAc extracts were calculated using this software.

2.3.4.5. Hierarchical clustering analysis. The hierarchical clustering analysis (HCA) of samples A–J was performed using SPSS statistics software (SPSS for Windows 13.0, SPSS Inc., USA). A method called Between-Groups Linkage was applied.

2.4. Anti-bacterial experiment

2.4.1. Sample preparation

Sample preparation for anti-bacterial experiment is the same as that for HPLC analysis, except for the last step that the residue was dissolved in 5 mL of water instead of MeOH before the start of anti-bacterial assay. The final concentration of the sample solution was 4.0 mg/mL.

2.4.2. Experimental procedure

The microcalorimeter was thermostated at 37 °C, and the measurement was made using the ampoule method. At the beginning of the experiment, *E .coli* was inoculated into the LB peptone medium at a concentration of 2×10^6 cells/mL. For the experiment, 5 mL of the medium with *E. coli* was exactly added to every ampoule. The

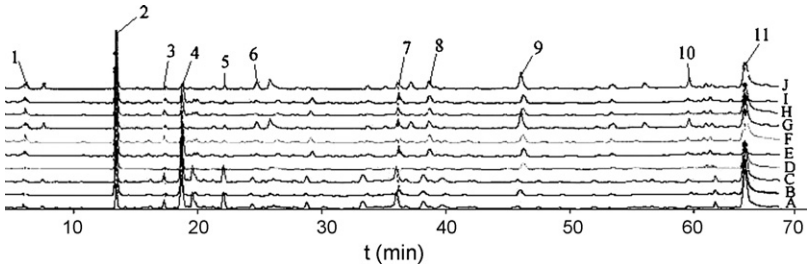

Fig. 1. The HPLC fingerprints of EtOAc extracts of *Radix Isatidis* from various sources The HPLC fingerprints of EtOAc extracts of 10 batches of *Radix Isatidis* from various sources were obtained by a High-Performance Liquid Chromatography (HPLC) method based on a Kromasil C_{18} column (250 mm × 4.6 mm, 5 μm) and kept at 30 °C, The mobile phase was 0.2% H_3PO_4 water solution (A) and methanol (B) system. The flow rate was 1.0 mL/min and the effluent was monitored at 230 nm.

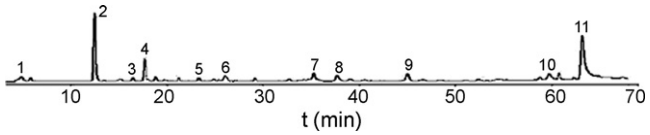

Fig. 2. The reference atlas from the ten chromatograms of EtOAc extracts. The reference atlas was formed by a "similarity evaluation" system for chromatographic fingerprint of Traditional Chinese Medicine (TCM) (Version 2004A) using the Median method from the general comparison of the chromatograms of 10 batches of EtOAc extracts from *Radix Isatidis*.

first ampoule with no EtOAc extracts solution was regarded as the negative control, while 100 μL of EtOAc extracts solution of *Radix Isatidis* from various sources were added to the other ampoules, respectively. All the ampoules were then sealed up with corks and put into the Bioactivity Monitor. After about 30 min (the temperature of ampoules reached 37 °C), the power–time curves were recorded. Since the bacterial metabolic process was monitored under the isothermal and isochoric conditions, the nutrient and oxygen consumed by cells was surely limited. When the recorder returned to the baseline and the experiment was finished. All data were collected continuously using the dedicated software package.

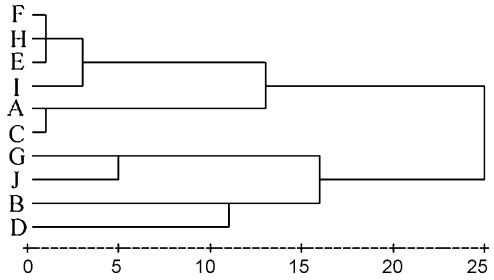

Fig. 3. Hierarchical clustering analysis of *Radix Isatidis* samples. The hierarchical clustering analysis (HCA) of samples A–J was performed using SPSS statistics software (SPSS for Windows 13.0, SPSS Inc., USA). A method called Between-Groups Linkage was applied.

2.4.3. Principal component analysis

In the study of many indexes, the phenomenon of information overlap of observational data exists because of too many variables and possible correlations between them. So we used principal component analysis to search for several factors which have no correlation to represent all the variables. In this study, the values of the thermo-kinetics parameters from the power–time curves were selected for principal component analysis by SAS statistical software (SAS for Windows 8.0, SAS Inc., USA).

2.5. Multivariant correlation analysis

Multivariant correlation analysis was used for the spectrum-effect relationships between the values of peak area in HPLC fingerprints and the thermodynamics parameters from the Principal component analysis of the power–time curves by SPSS statistics software (SPSS for Windows 13.0, SPSS Inc., USA).

3. Results and discussion

3.1. Results of HPLC experiment

3.1.1. HPLC fingerprints

Fig. 1 showed the typical HPLC fingerprints of EtOAc extracts of *Radix Isatidis* from various sources. The reference atlas was generated in Fig. 2. Eleven peaks, with large areas and good segregation from consecutive peaks, were possessed collectively by the ten chromatograms, and also the total peak areas of them were 90% more than all the peak areas in every chromatogram.

Table 2
The average relative retention time and peak area of every common characteristic peak

Peak no.	Relative retention time[a]	Average peak area of every characteristic absorption band									
		A[a]	B[a]	C[a]	D[a]	E[a]	F[a]	G[a]	H[a]	I[a]	J[a]
1	0.116	33.4	27.8	19.6	10.1	41.5	12.6	46.6	43.2	41.5	44.9
2	0.210	433.4	478.4	426.2	469.2	425.7	418.7	424.3	423.7	425.6	438.7
3	0.270	124.01	94.7	115.3	44.9	94.1	108.7	59.9	118.3	84.1	61.9
4	0.292	1424.2	1014.4	1403.1	1011.8	1585.2	1650.8	1128.6	1548.8	1568.4	1140.6
5	0.344	198.6	59.8	160.3	41.3	46.5	35.4	85.6	45.4	46.5	64.6
6	0.381	159.1	136.5	34.8	135.2	72.1	38.8	433.2	63.4	38.8	233.2
7	0.562	503.1	431.2	408.6	433.6	425.4	443.2	423.9	424.3	427.2	475.1
8	0.596	3594	320.9	357.6	82.5	370.9	351.6	342.6	349.1	374.3	326.5
9	0.714	77.5	131.9	65.6	232.4	264.8	259.5	531.9	267.2	264.8	552.3
10	0.929	76.3	64.1	68.3	63.6	60.3	52.3	101.6	68.8	52.7	151.6
11	1.000	1735.1	1463.3	1728.6	1093.1	1494.9	1402.8	1423.7	1362.8	1194.9	1223.8

[a] Average of three times experiments.

So, the eleven peaks were selected as the common characteristic peaks. Peak 11, which was salicylic acid at retention time 65.0 min was selected as the reference peak. Table 2 showed the average peak area and relative retention time of eleven common characteristic peaks. The similarities between the atlas of 10 batches of *Radix Isatidis* samples and the reference atlas were 0.968, 0.911, 0.981, 0.953, 0.921, 0.883, 0.891, 0.921, 0.932, 0.832, respectively.

3.1.2. Results of hierarchical clustering analysis

The results of the HCA were showed in Fig. 3. It was clear that the samples could be divided into two clusters: samples F, H, E, I, A, C in cluster one and samples G, J, B, D in cluster two. The samples in cluster one come from Gansu and Anhui provinces, while in cluster two come from Hebei, Jilin provinces. *Radix Isatidis* samples from the same source and similar longitude and latitude consisted of the same specie, and the differences between them were small, indicating that the reproducibility of the method was good. HCA provided a qualitative comparison of the samples. These differences between *Radix Isatidis* samples from different sources were large; for example, the differences among F, H, E, I and A, C were large, among J, G and B, D were larger, but differences between the two clusters were much larger.

3.2. Results of anti-bacterial experiment

3.2.1. The power–time curves of E. coli

The power–time curves obtained when a culture of *E. coli* was inoculated at 37 °C with EtOAc extracts of *Radix Isatidis* were showed Fig. 4. Similarity was the shape of the power–time curves of *E. coli* under the action of the extracts. The growth curve of *E. coli* can be divided into four phases, i.e., the first exponential phase, the lag phase, the second exponential phase and the decline phase. But, with the extracts from various sources, the values of P_m^1, P_m^2 and t_m^1, t_m^2 had large differences. P_m^1 and P_m^2 were the maximum power output of phases I and II. t_m^1 and t_m^2 were the maximum appearance time of phases I and II.

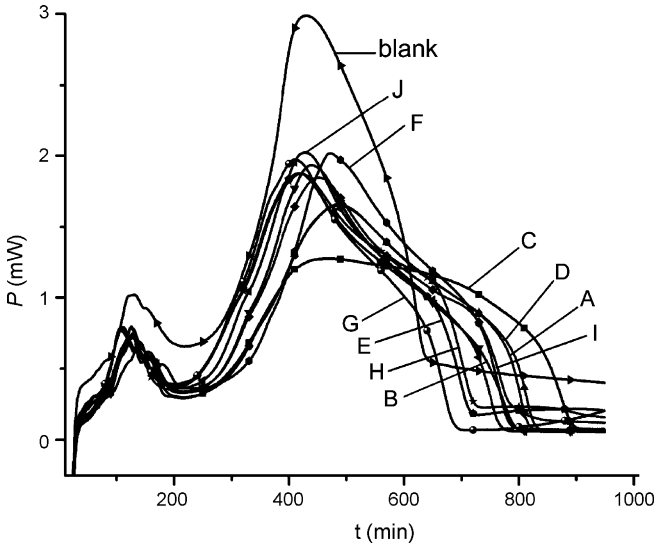

Fig. 4. The power–time curves of *E. coli* growth at 37 °C affected by EtOAc extract of *Radix Isatidis* from various sources (4.0 mg/mL). *E. coli* was cultured in LB culture medium supplemented with the same concentration of EtOAc extracts of *Radix Isatidis* from various sources, respectively, and monitored using a TAM Air Isothermal Calorimeter by the ampoule method at 37 °C.

3.2.2. Calculation of the growth rate constant (k) of E. coli

The exponential model of metabolism of *E. coli* could be used in the two growth processes.

$$P_t = P_0 \exp(kt) \quad \text{or} \quad \ln P_t = \ln P_0 + kt \tag{1}$$

P_0 represents the heat-output power at the beginning of baseline and P_t represents that at time t. The power–time curves of the exponential phase of growth corresponded to Eq. (1). Using this equation, the growth rate constants k could be calculated by fitting $\ln P_t$ and t to a linear equation. The values of k_1 and k_2 of *E. coli* growth in the eight channels which contained eight ampoules with the suspension in the absence of EtOAc extracts were showed in Table 3. k_1 and k_2 were the growth rate constant

Table 3
Growth rate constant (k) for the growth of *E. coli* at 37 °C in the absence of the extracts

	Channel							
	1	2	3	4	5	6	7	8
k_1/min^{-1}	0.1201	0.1195	0.1203	0.1208	0.1210	0.1206	0.1199	0.1204
R	0.9970	0.9968	0.9981	0.9962	0.9967	0.9975	0.9992	0.9987
k_2/min^{-1}	0.0920	0.0925	0.0929	0.0932	0.0928	0.0929	0.0931	0.0930
R	0.9984	0.9988	0.9979	0.9962	0.9967	0.9971	0.9989	0.9992

R, correlation coefficient.

Table 4
The thermo-kinetics parameters of *E. coli* growth at 37 °C affected by EtOAc extracts of *Radix Isatidis* from various sources

Samples	k_1 (min^{-1})	k_2 (min^{-1})	t_m^1 (min)	t_m^2 (min)	P_m^1 (mW)	P_m^2 (mW)	Q (mJ)	R
Control[a]	0.1208	0.0929	125	406	1.026	2.958	589,765	0.9981
A[a]	0.0539	0.0436	114	498	0.637	1.594	456,897	0.9983
B[a]	0.1106	0.0621	122	414	0.694	1.794	507,317	0.9976
C[a]	0.0528	0.0420	118	506	0.658	1.272	447,652	0.9992
D[a]	0.0565	0.0462	116	488	0.667	1.642	471,107	0.9986
E[a]	0.0713	0.0545	119	434	0.708	1.780	496,532	0.9986
F[a]	0.1021	0.0702	141	410	0.710	1.992	516,233	0.9965
G[a]	0.089	0.0690	131	412	0.602	1.972	508,496	0.9977
H[a]	0.1032	0.0562	118	418	0.699	1.789	506,454	0.9959
I[a]	0.0636	0.0540	119	458	0.639	1.760	475,065	0.9994
J[a]	0.1042	0.0852	121	414	0.936	1.966	555,608	0.9968

R, correlation coefficient.
[a] Average of three times experiments.

Table 5
Comparison between the two fingerprints got from MeOH and water reconstituted extract of sample B

Peak no.	Relative retention time[a]		R.E. (%)	Average peak area[a]		R.E. (%)
	B	B[b]		B	B[b]	
1	0.116	0.114	1.72	27.8	27.6	0.72
2	0.210	0.209	0.48	478.4	478.0	0.08
3	0.270	0.268	0.74	94.7	94.5	0.21
4	0.292	0.290	0.68	1014.4	1014.0	0.04
5	0.344	0.343	0.29	59.8	59.7	0.17
6	0.381	0.379	0.52	136.5	136.1	0.29
7	0.562	0.561	0.18	431.2	431.1	0.02
8	0.596	0.595	0.17	320.9	320.4	0.16
9	0.714	0.711	0.42	131.9	131.6	0.23
10	0.929	0.927	0.22	64.1	63.8	0.47
11	1.000	1.000	0.00	1463.3	1462.7	0.04

B, sample B solution dissolved with MeOH.
[a] Average of three times experiments.
[b] B, sample B solution dissolved with water.

of phases I and II. The results showed the mean growth rate constants in the absence of the extracts $k_1 = (0.1203 \pm 0.0015)\text{min}^{-1}$ and $k_2 = (0.0928 \pm 0.0012)\text{min}^{-1}$ and all correlation coefficients were exceeded 0.9960, indicating a good reproducibility and correlation. The second exponential phase can be regarded as a part of the stationary phase because the value of k_2 was much less than that of k_1. The smaller the value of k is, the stronger the anti-bacterial activity the drug possesses [25].

3.2.3. Analysis of anti-bacterial activity

The thermo-kinetic parameters (k_1, k_2, P_m^1, t_m^1, P_m^2, t_m^2, Q) of *E. coli* growth affected by EtOAc extracts of *Radix Isatidis* from various sources were showed Table 4. Q was the total heat-production.

The power–time curves (Fig. 4) of *E. coli* growth affected by EtOAc extracts of *Radix Isatidis* from various sources showed that the lag phase was prolonged comparing with the control. The values of k_1, k_2, P_m^1, P_m^2 reduced and the values of t_m^1, t_m^2 increased. These indicated that EtOAc extracts all had the capacity to inhibit the growth metabolism of *E. coli* to different extents and the inhibitory extent varied with various sources of extracts. The time of the lag phase of *E. coli* growth was prolonged with various sources of EtOAc extracts, indicating that the bacterial culture might took longer time to produce a sufficient number of cells for a detectable signal and that excess extracts inhibited *E. coli* growth. This probably resulted from the fact that EtOAc extracts combined with the cell to inhibit the duplication of DNA resulting in damage of the membrane structure and functions of cells. The thermo-kinetic parameters of *E. coli* growth in Table 4 were different among the samples. The smaller the values of k_1, k_2, P_m^1, P_m^2 and the bigger the values of t_m^1, t_m^2 are, the stronger anti-bacterial activities the drug possesses [25]. The general results from Fig. 4 and Table 4 showed that the magnitude of anti-bacterial activity of EtOAc extracts of *Radix Isatidis* from various sources on *E. coli* growth was: C > A > D > I > E > H > B > G > F > J. Sample C in Table 1 with the smallest $k_1, k_2, P_m^1, P_m^2, Q$ and biggest t_m^1, t_m^2 values had the strongest anti-bacterial activity, but sample J, had the adverse results. As we knew, sample C came from Fuyang city, Anhui province, the southeast area in China, while sample J was produced in Changchun city, Jilin province, in the northeast area of China. These indicated that the anti-bacterial activity of EtOAc extracts was related with the production place and the latitude and longitude of the place of *Radix Isatidis*.

3.2.4. Results of principal component analysis

The result of the principal component analysis of the thermo-kinetics parameters showed that the first two principal components contained 86.80% of the information of the original seven indexes from the results of "Eigenvalue of the Correlation Matrix", the equations of which were:

$$\begin{aligned} Z1 = {} & 0.3279(X1 - 0.7442) + 0.4337(X2 - 0.6924) \\ & + 0.2909(X3 - 220.5) - 0.3627(X4 - 741.5) \\ & + 0.3350(X5 - 0.695) + 0.3971(X6 - 1.8761) \\ & - 0.4236(X7 - 494136.5) \end{aligned}$$

$$\begin{aligned} Z2 = {} & -0.0206(X1 - 0.7442) - 0.6494(X2 - 0.6924) \\ & + 0.0568(X3 - 220.5) + 0.2249(X4 - 741.5) \\ & + 0.3712(X5 - 0.695) + 0.1926(X6 - 1.8761) \\ & + 0.5909(X7 - 494136.5) \end{aligned}$$

The values of $Z1$ and $Z2$ (the first and second principal component) were mainly decided by $X2$ (k_2) and $X7$ (Q), showing that k_2 and Q had the main influence on the anti-bacterial activity of EtOAc extract of *Radix Isatidis*.

3.3. Results of multivariant correlation analysis

The fingerprints got from sample B solutions dissolved with MeOH and water for HPLC (in Section 2.3.4.3) were established and the average peak area and relative retention time of eleven common characteristic peaks were got (Table 5). Table 5 showed that the relative error (R.E.) between the two fingerprints by comparing the average peak area and relative retention time of eleven com-

Table 6
The coefficient of partial correlation between thermo-kinetics parameters (k_2, Q) and common characteristic peaks

Parameter	Peak no.										
	1	2	3	4	5	6	7	8	9	10	11
k_2	0.2799	0.0064	−0.4990	−0.2847	−0.4887	0.4690	0.1898	0.0320	0.8350	0.7022	−0.4897
Q	−0.2291	−0.0840	0.3692	0.2272	0.5396	−0.4424	−0.2727	−0.0333	−0.7032	−0.4799	0.4585

mon characteristic peaks obtained from the water reconstituted extract and MeOH extract were all <2.00% for the relative retention time and <1.00% for the average peak area. All these illustrated that there were no significant differences between the two kinds of fingerprints and the amounts of the constituents presented in the two samples did not differ when dissolved in water and MeOH. So, the multivariant correlation analysis between thermo-kinetics parameters (k_2 and Q) of anti-bacterial activity and the area values of 11 common characteristic peaks in the HPLC fingerprints were applied (Table 6).

Table 6 showed that the anti-bacterial activity of EtOAc extracts from *Radix Isatidis* on *E. coli* had a close correlation with peaks 3, 5, 6, 9, 10 and 11 of the HPLC fingerprints. These peaks, including salicylic acid at peak 11, may be the anti-bacterial components, but what they are needs further study. The areas of peaks 3, 5, 6, 9, 10 were low but with a large influence on the anti-bacterial activity. Conversely, peaks 2, 4, 8 had little influence on the anti-bacterial activity even with big peak areas. Sample C with the largest peak area for peak 11 has the strongest anti-bacterial activity, illustrating that salicylic acid has a conspicuous effect on the anti-bacterial activity in *Radix Isatidis*. Salicylic acid, as one of the main components of *Radix Isatidis*, has strong anti-bacterial activity on *E. coli* growth [26,27]. These results were helpful for the study on *Radix Isatidis* and searching for other effective components of it.

4. Conclusions

HPLC method has got great development in many areas. It was used to analyze the chemical component of many drugs and compounds with high precision and sensitivity. Microcalorimetry, as the main mean of thermo-kinetics, takes the state parameters of the system as the study target, and can determine the energy transferring and calorific change during the organism's metabolic process when the TCM interacts with the organism. This method is appropriate to determine the heat-production curve of microbes, such as *E. coli* at a constant temperature of 37 °C and to obtain thermodynamic parameters. Additional advantages are that it generates thermodynamic information of the microbe-drug reaction when drugs are added to the culture medium. Compared with the control, the curves are usually different with each other when different drug is added to the culture medium. Though HPLC and microcalorimetry have developed rapidly, the combination of them was not reported. So, in this study, we combined HPLC and microcalorimetry to investigate the spectrum-effect relationships of EtOAc extracts from *Radix Isatidis*.

Radix Isatidis has been studied for many years, a certain number of compounds have been discovered and some bioactive functions have been reported, however, the bioactive components, especially the anti-bacterial constituents remain unclear. The HPLC method, together with microcalorimetry has been used to try to provide answers. HPLC fingerprint was used to analyze the compositions of *Radix Isatidis*. Anti-bacterial activity was applied to evaluate the efficacy of *Radix Isatidis* on *E. coli* growth. The internal quality could be evaluated by the combination of HPLC fingerprint and anti-bacterial activity and the combination could fix the possible active components of *Radix Isatidis*. The samples which had good chromatograms and great similarity with the reference atlas had strong anti-bacterial activity, for example samples C, A and D in Table 1. However, sample B was an exception. It had poor anti-bacterial activity although the similarity was large. Compared with sample D, the internal quality of sample B which was harvested at different season had large difference although both of them were from the same source. Samples A and C both come from Anhui province but with different harvest season, samples B and D both come from Hebei province with different harvest season. This illustrated that the harvest season of *Radix Isatidis* has a considerable influence on the anti-bacterial activity. The samples which had small similarity had poor anti-bacterial activity such as samples F, G and J. Sample J was distributed from Changchun city, Jilin province, China, illustrating that the place of production also had a great influence on anti-bacterial activity. All these could be seen from the HPLC fingerprints. Our study on the spectrum-effect relationships of EtOAc extracts of *Radix Isatidis* from various sources based on HPLC fingerprints and microcalorimetry can measure the internal quality and anti-bacterial activity of *Radix Isatidis*, so provides a sound experimental foundation for the study of *Radix Isatidis*.

The quality of TCM was determined mainly by the composition and content of active substances. The compositions and contents of active substances in *Radix Isatidis* related with species (internal factor), places of production (environmental factor), longitude and latitude of place, harvest season, elaboration methods of medical material, etc. This study showed that the differences of the spectrum (HPLC fingerprints) and the effect (anti-bacterial activity) were big among the samples from Anhui, Hebei, Gansu and Jilin provinces. The results of this study showed that the contents of main chemical composition of EtOAc extracts from *Radix Isatidis* were different and the differences resulted in the discrepancy of anti-bacterial activities. So, in order to control the good quality of TCM, the source of medical material must be under control.

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