

Short communication

## LC-APCI-MS method for detection and analysis of tryptanthrin, indigo, and indirubin in Daqingye and Banlangen

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### Abstract

A rapid, selective, and sensitive LC-APCI-MS method is developed in this study for detecting and analyzing tryptanthrin, indigo, and indirubin in Daqingye and Banlangen, which are, respectively, the leaves and roots of *Isatis indigotica* and *Strobilanthes cusia* in traditional Chinese medicine. The detection of the three active components is linear in concentrations ranging from 100 to 1500 ng/mL, the squared correlation coefficient is higher than 0.996, the precision as measured by the relative standard deviation is no larger than 9.5%, and the recovery is greater than 86.6%. The analysis of the 21 Banlangen samples led to considerably different conclusions on the contents of tryptanthrin, indigo, and indirubin in fresh leaves versus those in dried leaves. These results should shed some light on future plant selection and breeding. Compared with the traditional TLC and HPLC-UV methods, the new LC-APCI-MS approach has proven to be an optimal tool for detecting and analyzing the three marker compounds in the Chinese herbal medicines of Daqingye and Banlangen.

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**Keywords:** Tryptanthrin; Indigo; Indirubin; LC-APCI-MS; Banlangen; Daqingye; *Isatis indigotica*; *Strobilanthes cusia*

### 1. Introduction

*Isatis indigotica* (Cruciferae) and *Strobilanthes cusia* (Acanthaceae) are both biennial herbaceous plants widely distributed in Asia. Their leaves and roots known as Daqingye and Banlangen, respectively, have been used in traditional Chinese medicine (TCM) for hundreds of years as antipyretic, antiviral, anti-inflammatory, and anti-influenza agents [1,2]. During the outbreak of severe acute respiratory syndrome (SARS) in 2003, both Daqingye and Banlangen were prescribed to treat patients in China although there was no medical evidence suggesting their applicability. Since then, they have become two of the most popular TCMs.

Over the last decade, more than 50 chemical constituents have been identified in Daqingye and Banlangen including alkaloids [3–9], sucrose [5,6], organic acids [3,10], and glycosides

[7,9]. Among them, three alkaloids are selected as the markers for quality control in this paper due to their unique pharmaceutical activities: tryptanthrin, indigo, and indirubin (Fig. 1). Tryptanthrin, or indolo-[2,1-b]-quinoxaline-6,12-dione, is an indoloquinazoline alkaloid that strongly inhibits cyclooxygenase-2 (COX-2) in cellular assays (IC<sub>50</sub> 64 nm in Mono Mac 6 cells) [11]. It was reported that tryptanthrin is also a 5-lipoxygenase (5-LOX) inhibitor in vitro-pharmacological investigations [12]. Indigo and indirubin are structural isomers. Indigo is one of the oldest natural blue dye stuffs and had been used by the Asians to color artificial crafts until synthetic indigo was marketed at the end of last century. However, indigo is more than just a natural pigment since Sugihara et al. demonstrated that both indigo and indirubin have physiological effects on liver microsomes in mice [13]. They also possess the property of inducing ethoxyresorufin-*O*-dealkylase (EROD) as well as methoxyresorufin-*O*-dealkylase (MROD) activities and the influence is mediated by the hydrocarbon receptor. In addition, indigo derivatives and indirubin have been proven to have curative effects on anti-cancer drugs in humans and chronic leukemia inhibitors in mice [14,15]. Most recently, indirubin has been shown to inhibit the chemokine regulated on

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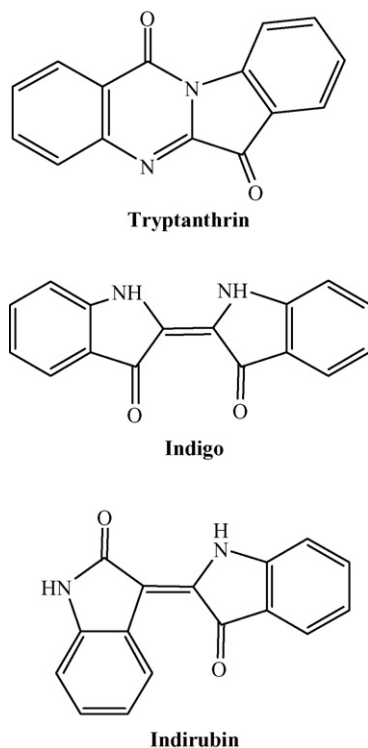


Fig. 1. The structures of tryptanthrin, indigo, and indirubin.

activation, normal T cell expressed and secreted (RANTES) [16].

The majority of methods for detecting tryptanthrin in *Isatis* plants are based on the scan mode of thin layer chromatography (TLC) and high performance liquid chromatography with an ultraviolet detector (HPLC-UV) [12]. Currently, liquid chromatography (LC) coupled with electrospray ionization mass spectrometry (ESI-MS) has been successfully employed to analyze another *Isatis* species plant *I. tinctoria* [17]. For the detection of indigo and indirubin, the most common methodologies are TLC, HPLC-UV [18–20], and fast atom bombardment mass spectrometry (FAB-MS) [21]. Szostek et al. and Puchalska et al. recently used LC-MS to investigate the natural dyes (including indigo and indirubin) in Coptic textiles and historical art objects [22,23], but the matrix effect is quite different from that in TCMs.

In sum, a careful review of the existing literature reveals that LC-MS has attracted much attention from researchers due to the fact that it does not require sample pretreatment and allows for the detection of tryptanthrin, indigo, and indirubin at the same time. By integrating it with the technique of atmospheric pressure chemical ionization (APCI), we propose the use of LC-APCI-MS to quantify the amounts of the three active components contained in Daqingye and Balangen. In other words, the main objective of this paper is to employ the new method in the SIM scan mode to simultaneously evaluate tryptanthrin, indigo, and indirubin as the marker standards for quality control of related herbs. To validate its effectiveness, our approach will be applied to fresh and dried leaves of some dye plants.

## 2. Experimental

### 2.1. Reagents and chemicals

Standards of tryptanthrin and indigo were purchased from Extrasynthese (France) and Acros Organics (Geel, Belgium), respectively. Indirubin was isolated from the leave extract of *I. indigotica* in our laboratory, and its purity and structure were confirmed by HPLC, and ESI-mass spectrometry. Tanshinone-I was purchased from Pharmaceutical Industry Technology and Development Center (Taipei, Taiwan) and used as the internal standards (IS).

### 2.2. Plant materials

Daqingye and Banlangen: The roots and leaves of *I. indigotica* were obtained from the Herbal Source Biotechnology Co. Ltd. and Agronomy department of National Chung-Hsing University (NCHU), Taichung, Taiwan. Samples of *S. cusia* were purchased from the farmer, Nantou, Taiwan. Identification of these crude drugs was confirmed by macroscopic and microscopic analysis according to the Chinese pharmacopoeia. The dried samples were finely pulverized and then stored at room temperature for 3 days before extracting. The fresh Daqingye was extracted with methanol immediately after collecting in the morning.

### 2.3. Preparation of standard solutions

The standards were individually dissolved in an appropriate volume of methanol to prepare the 10  $\mu\text{g}/\text{mL}$  stock solutions. A 3 mL aliquot of each stock solution was mixed and diluted with 1 mL of methanol to give a standard working solution containing each compounds at the concentration of 3  $\mu\text{g}/\text{mL}$ . The standard working solution was diluted with methanol and spiked appropriate volume of IS. Calibration solutions of standards at levels of 100–1500 ng/mL with 1000 ng/mL IS were prepared. All stock and working solutions were maintained at  $-4^\circ\text{C}$  in a refrigerator and warmed up to room temperature before using.

### 2.4. Preparation of plant samples

Each 1.0 g crude plant was sonicated in 10 mL HPLC grade methanol at  $25\text{--}30^\circ\text{C}$  for 1 h. And then, the mixture was filtered through Watman No. 40 filter paper into a 50 mL round-bottomed flask. The residue was extracted twice more in the same manner ( $2 \times 10\text{ mL}$ ). The combined methanol extract was evaporated under reduced pressure at  $40^\circ\text{C}$ , and the resulting residue were individually re-dissolved in 1 and 10 mL methanol to run the analysis by HPLC and LC-MS. The extracts were all kept in a refrigerator at  $-4^\circ\text{C}$  until analysis. The sample solutions were filtered through a 0.2  $\mu\text{m}$  Whatman membrane filter before LC-APCI-MS analysis.

### 2.5. Chromatographic conditions

HPLC analysis was carried out on a Agilent 1100 liquid chromatography system (Agilent technologies Inc., Waldbronn,

Germany) equipped with two pumps, a MWD UV detector and Rheodyne injector (20  $\mu$ L loop). For the LC/MS analysis, the LC was carried out on a Surveyor liquid chromatography system (Thermo Finnigan, San Jose, CA, USA), consisting of two solvent pumps and a Rheodyne injector (5  $\mu$ L loop). Chromatographic conditions for the above two system are the same as follows: the LC column was a Thermo Hypurity-Advance C18 (5  $\mu$ m, 250 mm  $\times$  3 mm i.d.) (USA) column and a Phenomenex Luna Security Guard Cartridge C18 (5  $\mu$ m, 4 mm  $\times$  2.0 mm i.d.) was preceded in-line. Two mobile phases A and B were used at flow rate of 0.4 mL/min. The mobile phase was filtered through a 0.45  $\mu$ m filter, and degassed by vacuum, followed by sonication. Mobile phase A consisted of water with 0.005% trifluoroacetic acid (TFA) and mobile phase B was ACN containing 0.005% TFA. Separation was carried out at room temperature. A gradient was used, starting at 60% A, changing to 50% A linearly in 25 min. After elution the column was washed with 100% B for 10 min then equilibrated with 60% A for 7 min under the initial conditions. The total analysis was less than 42 min for real sample.

## 2.6. Mass spectrometry

Mass spectra were obtained by using an atmospheric pressure chemical ionization (APCI) source on a quadrupole ion trap instrument (Thermo Finnigan LCQ, San Jose, CA, USA). The data acquisition software was Xcalibur, Version 1.2. Helium was used as the damping and collision gas. The LC-APCI-MS in positive ion mode operated under the optimum conditions. The MS parameters were optimized according to the sensitivity from flow injection. The MS parameters used were as follows: the sheath gas flow for the detection of tryptanthrin, indigo (or indirubin) and internal standard were 0.6, 0.3 and 1.2 L/min, respectively; the aux gas flow was 1.5 L/min for tryptanthrin, and 0 for the rest two and the discharge was set 3.5  $\mu$ A for tryptanthrin and indigo, and 5.5  $\mu$ A for IS. The vaporization temperature, capillary temperature, capillary voltage, tube lens offset and injection time of tryptanthrin, indigo, indirubin, and IS were set at 575  $^{\circ}$ C, 200  $^{\circ}$ C, 10 V, 0 V and 500 ms, respectively. Three different segments were individually optimized to monitor the four standards.

## 3. Results and discussion

### 3.1. The performance of HPLC-UV method

According to the existing literature and the Chinese Pharmacopoeia, the root part of *I. indigotica* is the main source of the herbal medicine. However, the results from our research indicate that the amounts of the three analytes in the roots Banlangen are much smaller than those in the leaves Daqingye. This implies that the traditional approach of HPLC-UV is not sensitive enough to uncover those marker compounds in the root samples due to low absorption and complicated matrix (see Fig. 2). Also, there is a clear problem of quantification due to interferences in the analysis.

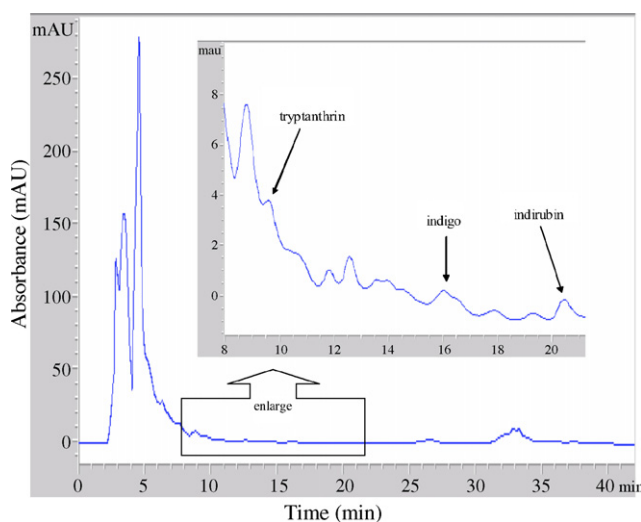


Fig. 2. HPLC chromatogram of a root extract of *I. indigotica* (sample no. 14 in Table 2), detected at UV 280 nm.

### 3.2. APCI mass spectra

APCI mass spectra were analyzed by LC-MS in the positive ion (PI) mode in a mixed mobile phase of ACN/H<sub>2</sub>O (50/50) containing 0.005% TFA at a flow rate of 0.4 mL/min. The 10  $\mu$ g/mL standards were first prepared in MeOH and then injected to the sample loop to determine the molecular ions of tryptanthrin, indigo, and indirubin. In Fig. 3, the protonated molecular ions [M + H]<sup>+</sup> of tryptanthrin and indigo (or indirubin) at *m/z* 249 and *m/z* 263, respectively, as the base ions were obtained.

### 3.3. Effect of modifier on LC-MS

The role of the amount of modifier in the mobile phase is explored to find the optimum ionization efficiency in MS. TFAs of 0.001% and 0.005% (v/v) with blank control were used and the optimum modifiers were evaluated with the high sen-

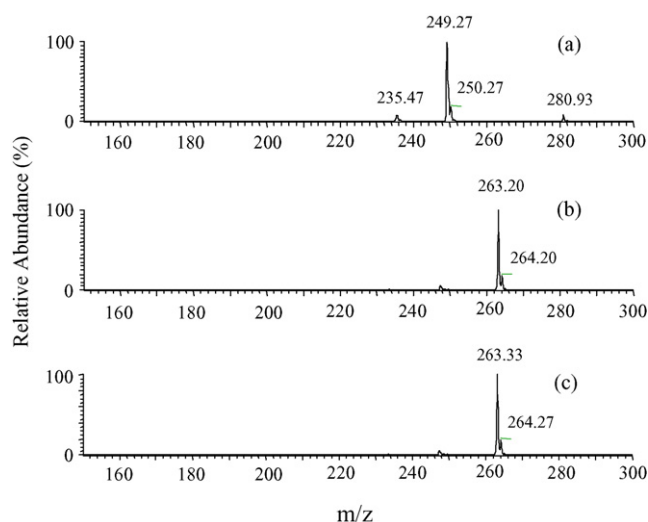


Fig. 3. The mass spectra of 10  $\mu$ g/mL (a) tryptanthrin, (b) indigo and (c) indirubin produced by (+)APCI/MS.

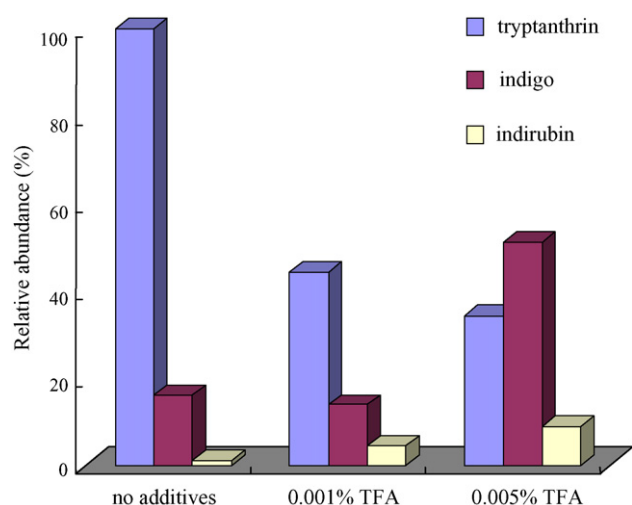


Fig. 4. The ionization efficiency of modifiers at the concentration of 0.001 and 0.005% trifluoroacetic acid, respectively, with blank control, evaluated by LC/(+)APCI/MS.

sitivity (i.e., the peak area) obtained in the SIM mode. Lower pH values (TFA > 0.005%) were not chosen for the column of concern.

For tryptanthrin, the ionization efficiency was the highest without any modifier and it decreased with the amount of TFA added (Fig. 4). For indigo and indirubin, the efficiency was enhanced by the TFA modifier and it became higher as the concentration of TFA increased. Generally, indigo and indirubin are treated as the standards for quality control in TCMs through the use of HPLC. However, TFA at the 0.005% level was selected as the modifier since indigo, indirubin and tryptanthrin were detected simultaneously in the present research. Based on our findings, this choice not only improves the ionization efficiency of each of indigo and indirubin, but also reduces the analyte retention times in the LC analysis.

#### 3.4. Selected ion monitoring (SIM) chromatogram

The mass chromatogram of analytes in the PI mode under the above mobile phase was examined. The ions in the SIM mode of the mass ion chromatogram for tryptanthrin, indigo, indirubin, and IS were at  $m/z$  249, 263, 263, and 273, respectively, and the corresponding retention times were 9.4, 16.4, 20.7, and 23.5 min. All of the peaks of analytes had a good shape and they were finely separated. The performance comparison between indigo and indirubin was made at the concentration of 250 ng/mL. The sensitivity of detection for indirubin was found to be much lower than that for indigo.

Table 1

Linear ranges, calibration curves, correlation coefficients ( $r^2$ ), and detection Limits for tryptanthrin, indigo and indirubin, as obtained by LC/(+)APCI/MS

Compounds	Linear ranges (ng/mL)	Linear equations	Squared correlation coefficient ( $r^2$ )	LODs (ng/mL)
Tryptanthrin	100–1500	$Y=0.0142X - 1.2627$	0.997	20
Indigo	100–1000	$Y=0.0116X + 0.7436$	0.996	29
Indirubin	250–1500	$Y=0.0043X - 0.5549$	0.998	67

#### 3.5. Linear detection range and limit of detection

The linearity of detection was verified by using concentrations ranging from 100 to 1500 ng/mL for the three compounds with Tanshinone-I as the IS. Triplicate injections were administered and the SIM mode was performed in quantitation. As can be seen from Table 1, the squared correlation coefficient exceeds 0.996 in each of the cases.

The linear-range experiments provided the necessary information to estimate the limit of detection (LOD), which was calculated from the slope of the calibration curve ( $b$ ) and the three-fold standard deviation ( $3S_b$ ) of the lowest detectable concentration. The LODs for tryptanthrin, indigo, and indirubin were assessed at 20, 29, and 67 ng/mL, respectively (Table 1).

#### 3.6. Precision and recovery

The precision of our proposed approach was established by five consecutive injections with the same concentration under the optimum conditions. For each of the three active components, concentrations at three different levels were assessed: 100, 750, and 1500 ng/g for tryptanthrin, 100, 500, and 1000 ng/g for indigo, and 250, 750, and 1500 ng/g for indirubin. All the signals were calculated based on the ratio of the peak area to the IS area. The precision of the analyte as measured by relative standard deviations (R.S.D.s) ranged from 1.4 to 9.5%, which was deemed acceptable.

The recovery test was carried out by using the methanol extract of *Isatis* samples as a matrix and then spiking to it each analyte at a concentration 500 ng/mL. The recoveries were computed by comparing the peak area ratio obtained from the methanol extract with the spiked *Isatis* samples with that of the standard solution. It turned out that the recoveries of the three compounds were all above 86.6% and the R.S.D.s were all below 6.47%.

#### 3.7. Application to real samples

The proposed method was tested on 21 plant samples collected from different geographical origins with an aim to demonstrate its efficacy in practical applications. As a first step towards determining the exact amount of each of tryptanthrin, indigo, and indirubin, the weight of the fresh *Isatis* leaves was converted to the dry weight by taking into account the water content. The experimental results indicated that the water contents on six fresh leaves samples varied between 81.8 and 85.6%, and their standard deviations ranged from 0.1 to 4.3%, therefore, the dry weight for each of them can be calculated based on its water content, respectively.

Table 2  
Concentrations ( $\mu\text{g/g}$ ) of the three marker components in 21 different samples from *I. indigotica*, *S. cusia* and *S. longesplicatus*

Entry	Sample	Type	Tryptanthrin ( $\mu\text{g/g}$ )	Indigo ( $\mu\text{g/g}$ )	Indirubin ( $\mu\text{g/g}$ )
1–6 <sup>a</sup>	<i>I. indigotica</i>	Fresh leaves	61.6, 119.0, 51.3, 83.4, 114.2, 49.0	74.4, 97.2, 54.1, 73.5, 63.8, 74.1,	127.2, 262.2, 93.6, 119.9, 228.1, 67.5
7–12 <sup>a</sup>	<i>I. indigotica</i>	Dry leaves	85.7, 62.6, 34.3, 109.7, 89.0, 34.2	10.0, 12.8, 12.5, 8.7, 5.1, 14.5	640.7, 689.5, 466.3, 759.3, 612.8, 617.4
13–18 <sup>a</sup>	<i>I. indigotica</i>	Dry roots	0.110, 0.614, 0.533, 0.409, 0.164, 0.153	0.016, 1.859, 1.685, 1.562, 0.693, 0.271,	0.224, 2.384, 1.148, 1.340, 0.348, 0.242,
19, 20 <sup>b</sup>	<i>S. cusia</i>	Dry leaves	32.8, 131.2	20.1, 54.0	72.0, 273.2
21 <sup>b</sup>	<i>S. longesplicatus</i>	Dry leaves	ND <sup>c</sup>	ND <sup>c</sup>	ND <sup>c</sup>

<sup>a</sup> Obtained from different provinces in China.

<sup>b</sup> Obtained from Nanto County in Taiwan.

<sup>c</sup> Not detected.

Table 2 depicted the contents of the three marker compounds in Banlangen from various sources. In particular, sample nos. 1–18 are samples of *I. indigotica* plants from different provinces in China and sample nos. 19–21 are plants of *S. cusia* and *Strobilanthes longesplicatus* from Nanto County as well as the experimental farm operated by the Agronomy Department of National Chung-Hsing University in Taiwan.

In Table 2, the first six samples pertain to fresh leaves and the real contents (ng/dry wt.) of the three active components are calculated. The *Isatis* sample nos. 7–12 are associated with the dried leaves obtained from the corresponding fresh leaves in sample nos. 1–6 after treatment with oven at 60 °C for 2 days. A close look at the amounts of the three ingredients contained in the leaves before and after drying reveals no noticeable change for tryptanthrin. However, there was a 85% decrease for indigo, which is perhaps due to the isomerization of indigo to indirubin

during drying. This is consistent with the observation of Kokubun et al. that the indigo content of *Isatis tinctoria* reduced by 67% when the fresh leaves were dried [24]. In contrast, more indirubin was found and increased four-folds in the dried leaves than in the fresh leaves. This could result from its precursor indican oxidizing to indoxyl and converting to indirubin in the drying process.

The findings from our research as reported in Table 2 indicate that the amounts of the three analytes in the roots Banlangen are much smaller than those in the leaves Daqingye. In comparison with traditional HPLC-UV, the new LC-APCI-MS method suggested in this work provides us with a more selective and sensitive tool to detect and analyze the chemical constituents, which could be quantified without interfering with each other. The mass ion chromatograms of the real *Isatis* roots were separated for these three analytes in Fig. 5.

It should be pointed out that sample nos. 19 and 20 in Table 2, which are the *Strobilanthes* species named Ma-Lan or Nan-Ban-Lan, are indigenous plants to Taiwan. They are usually used as succedaneum of Banlangen for clinical purposes. With the exception of *S. longesplicatus*, all the analytes under consideration were successfully detected in *Strobilanthes cusia*.

#### 4. Conclusions

In this paper, a rapid, selective, and sensitive LC-APCI-MS method was developed for quantitative detection and analysis of three active components in Daqingye and Banlangen. Our findings demonstrate that nearly all of the tryptanthrin contained in fresh *Isatis* leaves was retained after they had been dried. However, the amount of indigo decreased by 85% and that of indirubin increased four-folds. In view of these observations, the *Strobilanthes cusia* (sample no. 20) can be a good substitute for *Isatis indigotica* in traditional Chinese medicine. Since the contents of the *Strobilanthes* species vary with the source, it is far more important to control the quality of Taiwan's indigenous plant Nan-Ban-Lan for clinical treatment.

The analysis of the 21 Banlangen samples led to considerably different conclusions on the contents of tryptanthrin, indigo, and indirubin in fresh leaves versus those in dried leaves. These results should shed some light on future plant selection and breeding. Compared with the traditional TLC and HPLC-UV

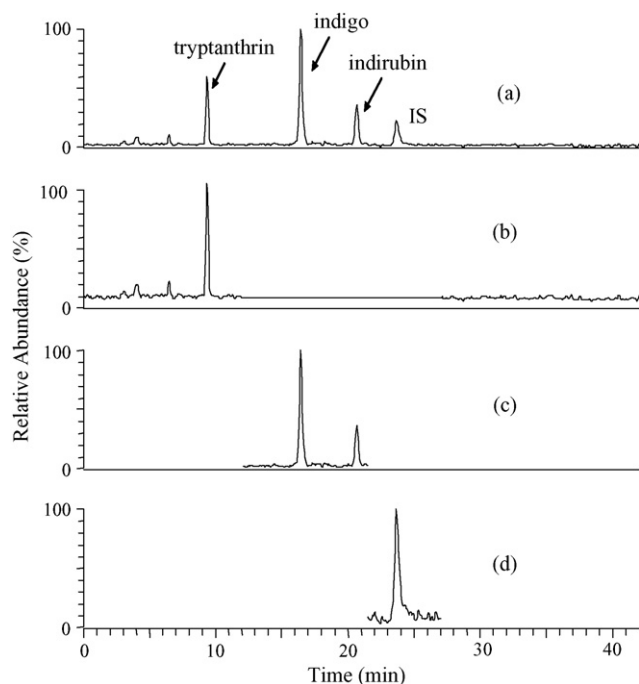


Fig. 5. Mass ion chromatograms of (a) TIC, (b) tryptanthrin ( $m/z$  249, RT=9.4 min), (c) indigo ( $m/z$  263, RT=16.4 min) and indirubin ( $m/z$  263, RT=20.7 min) and (d) Tanshinone-I (IS) ( $m/z$  273, RT=23.5 min), produced by LC/(+)APCI/MS from an extract of sample no. 14 in Table 2.

methods, the new LC-APCI-MS approach has proven to be an optimal tool for detecting and analyzing the three marker compounds in the Chinese herbal medicines of Daqingye and Banlangen.

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