ANALYSIS OF THE EXTRACTS OF *ISATIS TINCTOR*IA BY NEW ANALYTICAL APPROACHES OF HPLC, MS AND NMR

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Abstract

The methods of extraction, separation and analysis of alkaloids and indole glucosinolates (GLs) of *Isatis tinctoria* were reviewed. Different analytical approaches such as High-pressure Liquid Chromatography (HPLC), Liquid Chromatography with Electrospray Ionization Mass Spectrometry (LC/ESI/MS), Electrospray Ionization Time-Of-Flight Mass Spectrometry (ESI-TOF-MS), and Nuclear Magnetic Resonance (NMR) were used to validate and identity of these constituents. These methods provide rapid separation, identification and quantitative measurements of alkaloids and GLs of *Isatis tinctoria*. By connection with different detectors to HPLC such as PDA, ELSD, ESI- and APCI-MS in positive and negative ion modes, complicated compounds could be detected with at least two independent detection modes. The molecular formula can be derived in a second step of ESI-TOF-MS data. But for some constituents, UV and MS cannot provide sufficient structure identification. After peak purification, NMR by semi-preparative HPLC can be used as a complementary method.

Keywords: *Isatis tinctoria*; HPLC; MS; NMR; indole glucosinolates (GLs); alkaloids.

Introduction

Isatis tinctoria is widely used as anti-inflammatory and dye medicinal plant in Europe and China. It has been used and cultivated in Europe and China since antiquity. Over the last decade, more than 65 compounds of *Isatis tinctoria* including glycosides, alkaloids (Wu et al, 1997; Liu et al, 2001a,b; Liu et al, 2003), organic acids (Wang et al, 2009), and sucrose (Liu et al, 2002a,b) were identified. *Isatis tinctoria* contains indolic compounds. Among these compounds, glucobrassicin and its derivatives have antitumoral effect, especially against mammary cancer (Hoessel et al, 1999; Xu et al, 1991; Lin et al, 2002; Thornalley 2002; Galletti et al, 2006).

Different analytical approaches such as High-Pressure Liquid Chromatography (HPLC) (Qin et al, 2001; Tu et al, 2009), Liquid Chromatography with Electrospray Ionization Mass Spectrometry (LC/ESI/MS), Electrospray Ionization Time-Of-Flight Mass Spectrometry (ESI-TOF-MS), and Nuclear Magnetic Resonance (NMR) were used for analysis of the compounds in *Isatis tinctoria*. These methods provide rapid separation, identification and quantitative measurements of glucosinolates and alkaloids from *Isatis tinctoria*. The methods of extraction, preparation and analysis of qlucosinolates and

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alkaloids of *Isatis tinctoria* were reviewed in this paper.

Alkaloids

Indigo is one of the oldest natural blue dye (Uzal et al, 2010). Indole is a product of tryptophan catabolism by gut bacteria and is absorbed into the human body in substantial amounts (Gillam et al, 2000). Indole is absorbed and metabolized within the liver to indican (indol-3-ylsulfate) (Fordtran et al, 1964; Levy 1995). Indigo and indirubin have been found in human urine. Indigo and indirubin are structural isomers, which have physiological effects on liver microsomes in mice (Sugihara et al, 2004). Tryptanthrin, is an indoloquinazoline alkaloid that strongly inhibits cycloxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) in cellular assays (Henning et al, 2002a; Henning et al, 2002b; Meragelman et al, 2002; Chan et al, 2009).

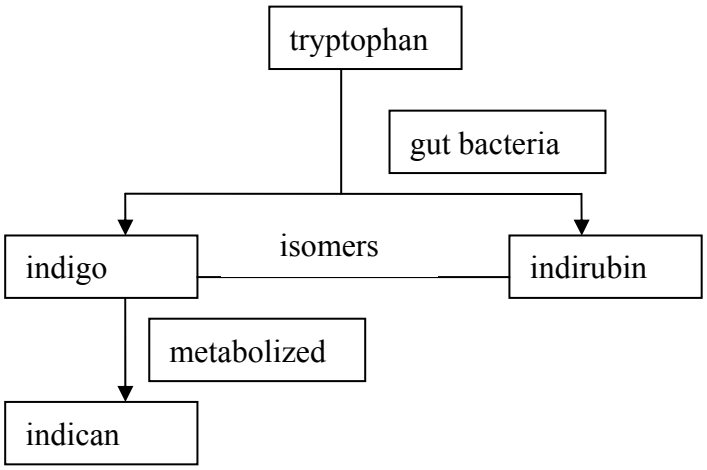

Figure 1: Relationship of tryptophan, indigo, indirubin and indican

Extraction and separation

Freshly harvested materials of *Isatis indigotica* were cut into small pieces of 2–3 cm length and immediately shock frozen with liquid nitrogen. Prior to extraction, the leaves were dried at 40 °C. Temperature, relative humidity and weight loss was monitored during the drying process. Constant weight was achieved after 3–4 days. The dried leaf samples were stored at room temperature for a few days in brown glass bottles in the dark. Immediately before extraction, dried leaf material was grounded. 1.0 g frozen and powdered samples was extracted by Pressurised Liquid Extraction (PLE) with an ASE 200 instrument (Liau et al, 2007). Bagci and Özçelik (2009) investigated fatty acid in the seeds of *Isatis tinctoria* using GC and HPLC.

Sample preparation of Mass Spectrometry

Standard solutions (0.1 mg/ml) of indigo, isoindigo, indirubin, isatin, indican, indoxyl acetate and 2-indolinone were prepared by dissolving 5 mg of each powder in 50 ml of DMSO. The solutions were filtered over a 0.45 μm syringe filter. The solution of the mixture of active agents was prepared daily by dilution of appropriate volumes of the standard solutions with acetonitrile.

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New analytical approaches

The methods used to identify indigos and its isomers include spectrophotometer in the UV, visible and IR regions (Cordy and Yeh, 1984; Miliani et al, 1998;Vautier et al, 2001), micro-Raman spectroscopy (Vandenabeele and Moens, 2003), electron spin resonance (Russell and Kaupp,, 1969), thin layer chromatography (Kokubun et al, 1998) and liquid chromatography with UV/visible detection (Wouters and Rosario-Chirinos, 1992; Cooksey and Tyrian, 2001; Maugard et al, 2001; Orska-Gawry´s et al, 2003). The alkaloids 5-hydroxyoxindole (5), 3-(20-carboxyphenyl)-quinazolin-4-on (8) and bisindigotin (21) have been described for *Isatis tinctoria* or *Isatis indigotica* before (Wu et al, 1997; Li et al, 2000; Wei et al, 2005). The available reference compounds were identified by NMR spectra. To identify indigos and its isomers, compounds were identified on the basis of their UV data in combination with MS, high resolution-MS, and literature data on their chromatographic retention (Canjura and Schwartz, 1991; van Breemen et al, 1991; Terasaki et al, 2002; de Rosso and Mercadante, 2007a, b). HPLC profiles obtained with PDA, ELSD, MS and MS/MS detectors are given as supplementary data (Van Breemen et al, 1991). APCI-MS and MS/MS data were compared with the published literature (de Rosso and Mercadante, 2007a, b).The parameters of selected indigoid compounds and their precursors are presented in Table 1.

Table 1: The parameters of selected indigoid compounds and their precursors

Class	Compounds	λmax (nm) (DMSO)	$[M + H]^+$	Reference
Indigoid coloring agents	Indigo, indigotin(blue)	617	263	Puchalska et al, 2004
	Indirubin (red)	540	263	
	Isoindirubin (red)	552	263	
	Isoindigo (brown)	365, 490	263	
Indigoid precursors	Indican	218, 280	296	
	Isatan	218, 280	310	
	Indoxyl acetate	280	176	
	2-Indolinone	-	134	
	Isatin	416	148	

HPLC method was widely used to analyze alkaloids in medicinal plants. The parameters of tryptanthrin, indigo, and indirubin analysis by HPLC are presented in Table 2.

Based on thin layer chromatography (TLC) and HPLC with an ultraviolet detector (HPLC-UV) (Ding et al, 2001; Henning et al, 2002a), LC/ESI/MS and fast atom bombardment mass spectrometry (FAB-MS) has been used to analyze *Isatis tinctoria* (Wouters et al, 1991; Ozeki et al, 1993; Sun et al, 2000). Recently, scientists have used LC/MS to investigate indirubin and indigo in textiles and historical art objects (Szostek et al, 2003; Puchualska et al, 2004). Liau et al used LC/APCI/MS to quantify the amounts of tryptanthrin, indigo, and indirubin in *Isatis tinctoria* (Zhang 1983; Liau et al, 2007).

Mass spectrometry (MS) has been applied to the identification of indigoid compounds using direct inlet into different ion sources: electron ionization (EI) (McGovern et al, 1991; Clark and Cooksey,1997) laser desorption/ionization (LDI) and APCI (Szostek et al, 2003; Bagci and Özçelik, 2009).The previous study reported LC-APCI-MS method is developed for detecting and analyzing tryptanthrin, indigo, and indirubin in the leaves and roots of *Isatis indigotica*

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(Oberthür et al, 2003; Liau et al, 2007).

Table 2: The parameters of tryptanthrin, indigo, and indirubin by HPLC

Compounds	Alkaloids : tryptanthrin, indigo, and indirubin
HPLC	[1]1100 liquid chromatography system equipped with two pumps, a MWD UV detector and Rheodyne injector. [2] L-4250 UV-VIS detector and an L-6200A pump
Column	[1]Thermo Hypurity-Advence C18 (5μm, 250mm×3mm i.d.) (USA) column and a Phenomenex Luna Security Guard Cartridge C18 (5μm, 4mm×2.0mmi.d.) [2]ZORBAX ODS
Mobile phases	A : water 0.005% trifluoroacetic acid (TFA); B: ACN containing 0.005% TFA
Conditions	[1]Gradient for the separation of fraction 2 was: 5% B for 2 min, 5–65% B in 98 min, 65–70% B in 10 min; gradient for the separation of fraction 3 was: 34% B for 13 min, 34–85% B in 87 min. 210 nm [2]B: 0-5-30 min; 20-50-100% at a flow rate of 2 ml/min;552nm
Reference	[1]Liau et al, 2007 [2] Aobchey et al, 2007

Reversed-phase LC/ESI/MS and UV/visible spectrophotometic methods were elaborated for the identification of indigoid (indigo, indirubin, isoindigo, isoindirubin) color components of natural dye stuffs and their natural or synthetic precursors (indican, isatin, indoxyl, 2-indolinone) (Puchalska et al, 2004).

Gillam et al (2000) reported the definitive identification of indigo and indirubin as products of human cytochrome P450 (P450)-catalyzed metabolism of indole by visible, ^{1}H NMR and MS. Mohn et al (2009) discovered a new indolic alkaloid from *Isatis tinctoria* by combined several detectors such as photodiode array detector (PDA), ESI/MS, and APCI-MS (positive and negative ion modes). Structural information was obtained by unspecific evaporative light scattering detector (ELSD)/MS/MS (Xiao et al, 2007) experiments and by high-resolution mass spectra recorded by ESI-TOF-MS.

Different analytic methods have been used to identity and determine the compounds in *Isatis tinctoria*. Previous reports reveal that LC/MS does not require sample pretreatment and allows for the detection of tryptanthrin, indigo, and indirubin at the same time. ESI/MS with high sensitivity, offers detection limits in the range 0.03–5.00 μg/ml for these compounds examined. Some selected parameters of these methods used to analyze alkaloid in *Isatis tinctoria* are presented in Table 3.

Previous reports show the concentrations (μg/g) of tryptanthrin, indigo, and indirubin in different samples from *Isatis indigotica* as follows: tryptanthrin 0.110, 0.614, 0.533, 0.409, 0.164, 0.153; Indigo 0.016, 1.859, 1.685, 1.562, 0.693, 0.271; Indirubin 0.224, 2.384, 1.148 (You et al, 1998; Liang et al, 2000; Liau et al, 2007). Puchalska (2004) reported the results of indigoids by HPLC/ESI/MS as presented in Table 4.

Indole glucosinolates (GLs)

Extraction and separation

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Indole glucosinolates (GLs) are highly reactive compounds, with a varying aglycon side chain. According to the type of this chain they are classified into aliphatic, aromatic and indole GLs. Among the latter, glucobrassicin (GBS), together with other indol-type GLs, was isolated from *Isatis tinctoria* (Elliott and Stowe, 1970; Elliott and Stowe 1971;

Table 3: The parameters of tryptanthrin, indigo, and indirubin analyzed by MS

Products	Alkaloids : tryptanthrin, indigo, and indirubin
Column	Zorbax SB-C18 (150 d 4.6 mm, 3.5 μm)
Instrument	HPLC: [1] LC: Surveyor liquid chromatography system. MS: APCI, quadrupole ion trap instrument. [2] 1100 LC MS: High-Resolution ESI/TOF /MS (BioTOF III; Bruker Daltonics, Inc. Billerica, MA, USA)
Eluent	A, ACN; B, 0.15% formic acid
Gradient program	A: 3 min 20% 8 min 50% 10 min 55% 22 min 55% 35 min 100% 45 min 100% Flow rate:0.6 ml/min; Injection volume: 5μl; wavelength: 280, 610 nm
MS parameters	Sheath gas for tryptanthrin, indigo or indirubin. aux gas 1.5 L/min for tryptanthrin, Nebulizing gas flow and temperature: 10 ml/min and 300 °C Vaporization temperature: 575 °C; capillary temperature, 200 °C
Voltage	Discharge: 3.5 μA for tryptanthrin indigo, and 5.5μA for IS. capillary voltage, 10V. Ionization voltage: 4000 V (positive ion mode); Orifice voltage: 90 V
Instrument	LC-MSD 1100 (Agilent Technologies)
m/z	Observed mass range (TIC): 50–500 Observed selected ions (SIM): 134, 148, 176, 205, 263, 296, 338, 340, 418, 420, 422
References	Liau et al, 2007 Chanayath et al, 2002; Puchalska et al, 2004

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Table 4: The results of indigoids by HPLC/ESI/MS

Parameter	Indigotin	Isoindigo	Indirubin	Isatin	Reference
Selected ion, *m*/*z*	263	263	263	148	Puchalska et al, 2004
Calibration curve $y = ax + b$					
a	598367	341891	319965	80950	
b	16205	430	47560	27036	
Linearity range (μg/ml)	0.03–4.20	0.03–4.00	0.03–5.00	0.45–18.20	
Detection limit(μg/ml)	0.01	0.01	0.01	0.15	
Quantification limit(μg/ml)	0.03	0.03	0.03	0.45	

Frechard et al, 2001). Some reports demonstrated that qlucosinolates is very sensitive to temperature. 60–70 °C have been used in validated PLE methods for many classes of natural products such as isatan A and B (Benthin et al., 1999; Oberth¨ur et al, 2003; Oberth¨ur et al, 2004a, b; Basalo et al, 2006). For the extraction and isolation of GLs of *Isatis tinctoria*, existing methods (Kushad et al, 1999; Kiddle et al, 2001; Verkerk et al, 2001; Bennett et al, 2007) has a problem with the extent of thermal degradation at temperature above 50 °C with. Extractions were typically carried out at temperatures between 70 °C and 100 °C. Thermal degradation of GLs during cooking has been reported (Slominski and Campbell, 1989; Oerlemans et al, 2005; Bones and Rossiter,, 2006).

Mohn et al (2007a) isolated GLs from *Isatis tinctoria* seeds by Soxhlet-extraction for 8 h with 400mL petrol ether (boiling range 40–60 °C). After evaporation to dryness, the residue was extracted three times with water (room temperature, 3×150 mL), centrifuged (5 min, 1600×*g*, room temperature) and filtered. The aqueous solutions were concentrated to 45mL in vacuo, and 5mL acetonitrile were added. After centrifugation, the supernatant was introduced into a column packed with DEAE-Sephadex A-25 (50 g). The column was eluted with water/acetonitrile 80:20 until the eluate was colorless. GLs were eluted with a mixture of 0.1M K_2SO_4/acetonitrile 80:20 at 3.2 mL/min and monitored with UV detection at 229 nm. Fractions were analyzed by HPLC–MS. Finally, isolated GLs were freeze dried. Purity and structures of isolated compounds were confirmed by NMR and LC–MS experiments.

New analytical approaches

In the recent years, new analytical approaches such as LC-based on-line spectroscopy, MS, NMR opened new avenues for increasingly comprehensive analysis of plant extracts and secondary metabolites. Researches by LC-based on-line spectroscopy has been widely taken on metabolite profiling studies (Burns et al, 2003; Yamazaki et al, 2003; Le Gall et al, 2005; Long et al, 2006; Dan et al, 2008; Ding et al, 2008; Iijima et al, 2008; Qiao et al, 2008; Schliemann et al, 2008). Previous reports on the indigo precursors and GLs showed harvest regimen affected the chemical composition of *Isatis tinctoria* leaves (Oberthür et al, 2004b; Mohn and Hamburger, 2008). Mohn et al (2008) carried out a quantitative assay for the direct analysis of intact GLs in *Isatis tinctoria* leaves. In their research, pressurized liquid extraction (PLE) was used. Detection was carried out by ESI/MS in the negative ion mode. Mohn and Hamburger (2008) used atmospheric pressure chemical ionization (APCI) and ESI/MS, and ESI-TOF-MS detectors to detect the extracts of *Isatis tinctoria*.

The dichloromethane extracts of *Isatis tinctoria* are lipophilic with active pharmacological effect (Oberthür, 2003). Mohn et al (2009) used dichloromethane with PLE method to extract lipophilic compounds. The parameters of the

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dichloromethane extracts by HPLC are presented in table 5.

Table 5: The parameters of the dichloromethane extracts by HPLC

Extracts	The dichloromethane extracts
HPLC	Agilent series 1100 system equipped with degasser, binary high pressure mixing pump, column thermostat and photodiode array (PDA) detector
Column	SunFire C18 column, 5 μm, 150× 10.0 mm I.D., equipped with a guard column (10.0 × 10.0 mm I.D)
Mobile phases	A: water with formic acid 0.1%, B: acetonitrile, flow rate: 0.5 ml/min,
Conditions	5% B isocratic for 2 min, 5%–93% B in 30 min, 93% B isocratic for 17 min, 93–100% B in 6 min. column temperature:25.0 °C,
Reference	Mohn et al, 2009

In previous reports, trifluoroacetic acid and formic acid are often used as mobile phase for chromatography of intact GLs (Mellon et al., 2002; Bringmann et al., 2005; Song et al., 2005). The parameters in Table 6 present GLs extracts analysed by HPLC-PDA-MS and HPLC-TOF-MS. The parameters of LC/TOF/ MS methods for GLs are presented in Table 7.

Table 6: The parameters of the dichloromethane extracts analyzed by LC/ESI /MS

Extracts	The dichloromethane extracts
Instrument	[1] The second splitter outlet: evaporative light scattering detector (ELSD series 2000, Alltech, Deerfield Il, USA) APCI (Bruker Daltonics; Bremen, Germany) Esquire 3000 plus ion trap ESI /MS
Conditions	[1] Ion charge conditions (positive mode: ICC 30000; negative mode: ICC 20000), scan speed:13000 m/z/s, gauss filter: 0.2 m/z, trap drive: 58.8 (negative mode: 61.4), isolation width: 4.0 m/z, fragmentation amplitude: 1.00 V. ESI: A:B= 1:3 Nitrogen pressure =2.0 bar Reference solution of sodium formate 0.1% in isopropanol/water (1:1) containing 5 mM sodium hydroxide.
Voltage	Capillary voltage:-4500 V (negative mode: +4500 V), endplate offset: -500 V, capillary exit voltage: 128.5 V (negative mode: -128.5 V), skimmer voltage: 40 V (negative mode:-40 V) hexapole at 250.0 Vpp (negative mode:230 Vpp) skimmer 1 at 40 V (negative mode: -50 V) skimmer 2 at 22.5 V (negative mode: -22.5 V).
Gas	Drying gas: nitrogen, flow rate 5μl/min

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	nebulising gas (pressure: 30 psi), collision gas: helium
Temperature	55 °C, nebuliser temperature:375 °C, Drying temperature: 250 °C. Dry gas temperature 240 °C.
m/z	100–800 in positive and negative modes for the detection of carotenoids and porphyrins in the hexane/acetone extract: 100–1200
References	Mohn et al, 2009

Table 7: The parameters of GLs analyzed by LC/TOF/ MS

Products	GLs: sinigrin, epiprogoitrin, progoitrin, gluconapin,glucotropaeolin, glucobrassicin, neoglucobrassicin, sulfoglucobrassicin, 4-hydroxyglucobrassicin
Instrument	LC/TOF/ MS, Negative ion LC–MS spectra on the ion trap
Voltage	Capillary voltage was at 4500V, endplate offset at −500V, capillary end voltage at −115.0V, skimmer voltage:−40.0V and trap drive at 53.4.
Gas	drying gas: nitrogen flow rate of 10 L/min, nebulizing gas: pressure of 30 psi
Temperature	nebulizer temperature : 300 °C
m/z	100–800 negative mode:100–600
Instrument calibration	a reference solution of sodium formate 0.1% in isopropanol/water (1:1) containing 5mM sodium hydroxide.
References	Mohn et al, 2009

Some minor constituents purified by semi-preparative HPLC were analyzed by off-line microprobe NMR spectroscopy. The analysis of GLs by NMR are presented in Table 8.

Table 8: The parameters of GLs analyzed by NMR

Products	NMR	References
GLs	NMR spectra were obtained with a 500 MHz Avance III system equipped with a 1 mm TXI probe. Data processing was with Topspin 2.1	Mohn et al, 2009
	Bruker DRX500 NMR spectrometer equipped with a 5mm SEI probe I.S.: 1,3,5-trimethoxybenzene	Mohn et al, 2007a,b,c

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Isatis tinctoria has been cultivated throughout Europe, especially in Western and southern Europe, since ancient times. In China, *Isatis tinctoria* has been used thousands of years as an important and popular herbal medicine in Traditional Chinese Medicine (TCM) for the treatment of inflammatory diseases. This paper reviewed the extraction methods of hydrophilic compounds (alkaloids) and lipophilic compounds (indole glucosinolates) of *Isatis tinctoria*. Multiple analytical approaches including HPLC, LC/ESI/MS, ESI-TOF-MS, and NMR have been reported as validation and quality control

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tools of this herbal medicine. HPLC separates complicated compounds. By connection with different detectors to HPLC such as PDA, ELSD, ESI- and APCI-MS in positive and negative ion modes, complicated compounds could be detected with at least two independent detection modes. The molecular formula can be derived in a second step of ESI-TOF-MS data. But for some constituents, UV and MS cannot provide sufficient structure identification. After peak purification, NMR by semi-preparative HPLC can be used as a complementary method. Selected parameters of these analytical methods are summarized in this article. This review may be useful to guide the analysis of the extracts of *Isatis tinctoria* in the future.

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