A COMPREHENSIVE METABOLITE PROFILING OF *ISATIS TINCTORIA* LEAF EXTRACTS

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Dekan

Meinen Eltern und Birgit

in Liebe und Dankbarkeit

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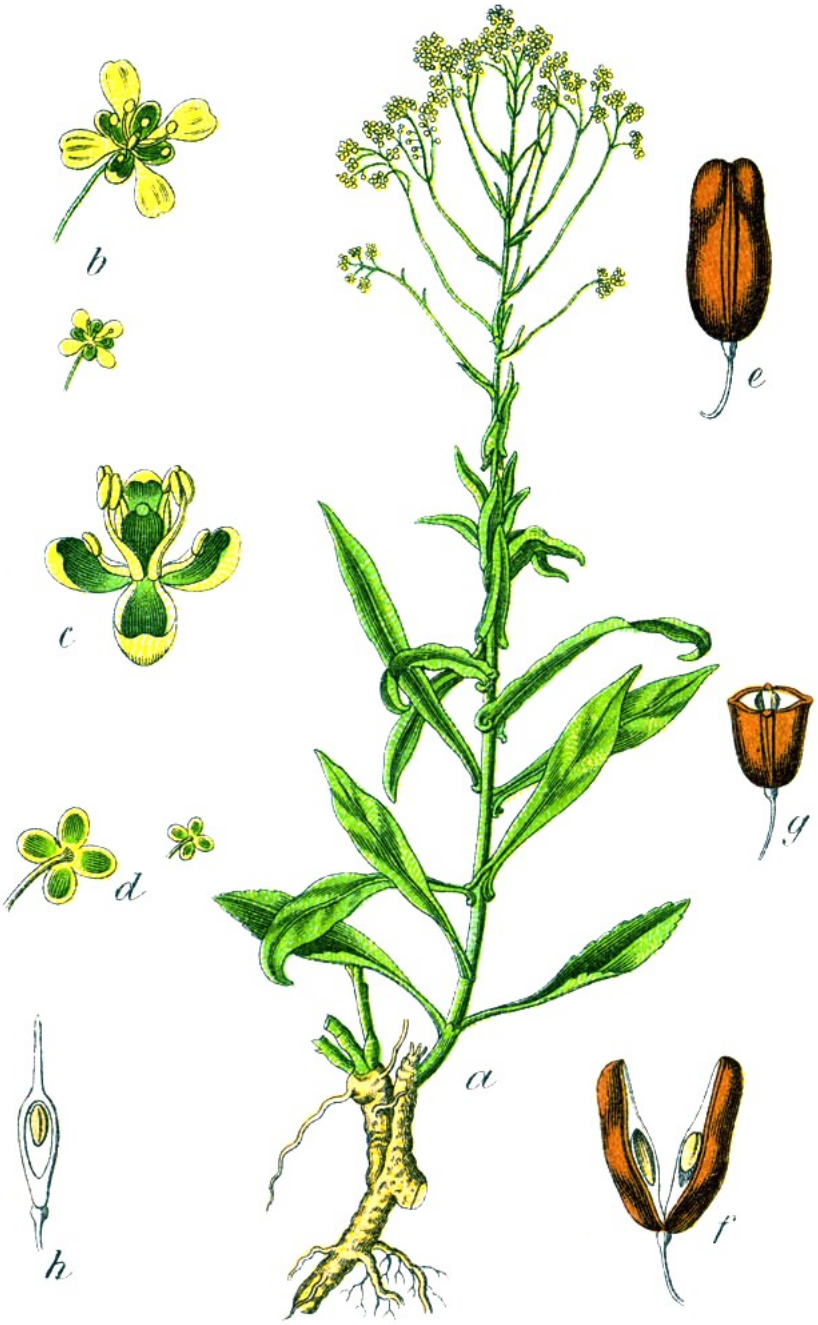

Isatis tinctoria L., Brassicaceae

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Summary

Woad (*Isatis tinctoria* L., Brassicaceae) is an ancient indigo dye and anti-inflammatory medicinal plant, which has been used and cultivated in Europe since antiquity. The anti-inflammatory potential of lipophilic leaf extracts was recently confirmed in a broad-based pharmacological profiling, in various animal models and in a clinical pilot study. Tryptanthrin, an indolin-2-one derivative, and γ-linoleic acid were identified as pharmacologically active compounds inhibiting cyclooxygenase-2 (COX-2), 5-lipoxygenase (5-LOX), the expression of the inducible nitric oxide synthase (iNOS), human neutrophil elastase, and the release of histamine from mast cells. Indirubin inhibits inflammatory reactions in delayed-type hypersensitivity and is a potent inhibitor of cyclin-dependent kinase 5 (CDK5) and glycogen synthase kinase 3β (GSK3B).

In view of a development of this lipophilic woad extract towards a new anti-inflammatory herbal medicine, analytical tools were developed to address various issues related to quality control. This study was conducted as part of the development process and had the purpose to characterise the pharmacologically active leaf extracts of *I. tinctoria* and the closely related species *I. indigotica* Fort. As a first step, a gradient LC-PDA-MS method for the quantitative determination of pharmacologically relevant constituents and the dye indigo was developed and validated. The assay enabled quality assessment in the development of phytomedicinal preparations based on woad extracts.

A comprehensive phytochemical profiling of the pharmacologically active dichloromethane extract from *I. tinctoria* leaves was carried out. The polar constituents of *I. tinctoria* (MeOH extract) and the taxonomically closely related TCM plant *I. indigotica* were also profiled for comparative purposes. PDA, ELSD, APCI- and ESI-MS, and ESI-TOF-MS detectors were used in parallel to ensure a wide coverage of secondary metabolites with highly diverging structural properties. Off-line microprobe NMR after peak purification by semi-preparative HPLC enabled the structure elucidation of some minor constituents. More than 75 compounds belonging to various structural

classes such as alkaloids, flavonoids, fatty acids, porphyrins, lignans, carotenoids, glucosinolates and cyclohexenones were unambiguously identified, and tentative structures were proposed for additional compounds. Numerous compounds were identified for the first time in the genus *Isatis*, and a new indole alkaloid was discovered.

The content of indole glucosinolates in woad, and their possible role as precursors of the alkaloid tryptanthrin was investigated. At first, a robust HPLC-MS method for the quantitative determination of glucosinolates was developed and validated. Direct analysis of glucosinolates was rendered possible by the use of a volatile ion-pairing reagent. Extraction via pressurised liquid extraction was optimized, and purity assessment of reference compounds was carried out with quantitative ^{1}H-NMR.

Seasonal fluctuations of glucosinolates in the leaf material of five defined *I. tinctoria* and one *I. indigotica* accessions were assessed. Significant qualitative and quantitative differences in the glucosinolate patterns were observed when comparing the two species, while differences among the various *I. tinctoria* accessions were much smaller. Repeated harvesting during the growth season did not have a major effect on glucosinolate concentrations of newly grown leaves.

We investigated the influence of the drying procedure on the glucosinolate content. In contrast to shock frozen samples, none of the glucosinolates could be detected in plant material that had been dried at ambient temperature or at 40 °C. However, none of the known breakdown products of indole glucosinolates could be detected.

The glucosinolate patterns in seeds of the five *I. tinctoria* and two *I. indigotica* accessions were also investigated. The glucosinolate patterns of the seeds showed characteristic differences compared to leaves. Qualitative and quantitative differences in glucosinolate patterns between both *Isatis* species were found, enabling a differentiation of the two species at the level of seed material.

Zusammenfassung

Färberwaid (*Isatis tinctoria* L.) ist eine historische Indigofärberpflanze mit enzündungshemmenden Eigenschaften und wird bereits seit der Antike in Europa kultiviert und angewendet. Die entzündungshemmende Wirkung der lipophilen Blattextrakte wurde kürzlich in einem breit angelegten, pharmakologischen Screeningprogramm sowie in zahlreichen Tierversuchen und einer klinischen Pilotstudie bestätigt.

Tryptanthrin, ein Indolin-2-on-derivat und γ-Linolensäure wurden als pharmakologisch wirksame Verbindungen identifiziert und es konnte gezeigt werden, dass diese Stoffe in der Lage sind Targets wie Cyclooxygenase-2 (COX-2), 5-Lipoxygenase (5-LOX), die humane, neutrophile Elastase, die Expression der induzierbaren NO Synthase (iNOS) sowie die Freisetzung von Histamin aus Mastzellen zu hemmen. Indirubin stoppt die Entzündung bei Überempfindlichkeitsreaktionen vom Spättyp und ist ein wirksamer Inhibitor der cyclin-abhängigen Kinase 5 (CDK5) und der Glycogensynthase Kinase 3β (GSK3B).

Für die Weiterentwicklung dieses entzündungshemmenden, lipophilen Waidextraktes zu einem Phytopharmakon wurden analytische Methoden für verschiedene Fragestellungen in der Qualitätskontrolle erarbeitet. Diese Arbeit ist als Teil dieses Entwicklungsprozesses einzuordnen und hat den Zweck die pharmakologisch aktiven Blattextrakte von *I. tinctoria* und der nahe verwandten Art *I. indigotica* Fort. zu charakterisieren. In einem ersten Schritt wurde eine LC-PDA-MS Methode entwickelt und validiert, die eine quantitative Bestimmung der pharmakologisch relevanten Inhaltsstoffe sowie des Indigofarbstoffs ermöglicht. Diese Methode kann zur Qualitätsprüfung bei der Entwicklung eines pflanzlichen Arzneimittels aus Färberwaidextrakten eingesetzt werden.

Ein umfangreiches, phytochemisches Profiling des pharmakologisch aktiven *Isatis tinctoria* Dichlormethanextraktes wurde durchgeführt. Die polaren Verbindungen in

I. tinctoria (MeOH Extrakt) und die taxonomisch nah verwandte TCM Pflanze *I. indigotica* wurden für Vergleichszwecke ebenfalls untersucht. Verschiedene Detektoren wie PDA, ELSD, APCI- and ESI-MS und ESI-TOF-MS wurden dabei parallel eingesetzt um eine möglichst groβe Auswahl von strukturell unterschiedlichen Sekundärmetaboliten abzudecken. Ausserdem kam eine neuartige NMR Technologie mit Mikroprobenkopf zum Einsatz, die die Strukturaufklärung einiger Minorverbindungen nach semi-preparativer Auftrennung ermöglichte. Die Strukturen von mehr als 75 Verbindungen aus zahlreichen Strukturklassen wie bespielsweise Alkaloiden, Flavonoiden, Fettsäuren, Porphyrinen, Lignanen, Carotinoiden, Glucosinolaten und Cyclohexenonen wurden zweifelsfrei aufgeklärt und für einige weitere Verbindungen konnten Strukturvorschläge getroffen werden. Neben zahlreichen in der Gattung *Isatis* bisher unbekannten Stoffen wurde dabei auch ein neues Indolalkaloid entdeckt.

Der Gehalt an Indolglucosinolaten in Waid und deren mögliche Rolle als Vorstufe für das Alkaloid Tryptanthrin wurde untersucht. Zuerst wurde eine HPLC-MS Methode für die quantitative Bestimmung der Glucosinolate entwickelt und validiert. Der Einsatz eines flüchtigen Ionenpaarreagenzes ermöglichte die Direktbestimmung der Glucosinolate. Die Extraktion mittels Hochdruckflüssigextraktion wurde optimiert und die Reinheit der Referenzsubstanzen wurde mit Hilfe einer quantitativen ^{1}H-NMR Methode bestimmt.

Saisonale Schwankungen des Glucosinolatgehaltes in Blättern von fünf definierten *I. tinctoria* Kulturen sowie einer *I. indigotica* Varietät wurden untersucht. Beim direkten Vergleich beider Arten waren signifikante qualitative und quantitative Veränderungen der Glucosinolatmuster festzustellen, während die Unterschiede innerhalb der verschiedenen *I. tinctoria* Stämme deutlich kleiner waren. Die Wiederholung der Ernte während der Wachstumsperiode hatte keine deutliche Auswirkung auf den Gehalt der Glucosinolate in nachwachsenden Blättern.

Wir untersuchten den Einfluss des Trocknungsverfahrens auf den Glucosinolatgehalt. Im Gegensatz zu den schockgefrorenen Proben konnten wir keine Glucosinolate in Pflanzenmaterial finden, das zuvor bei Raumtemperatur oder bei 40 °C getrocknet wurde. Allerdings konnten auch keine bekannten Abbauprodukte der Indolglucosinolate identifiziert werden.

Die Glucosinolatmuster in Samen von fünf definierten *I. tinctoria* und zwei *I. indigotica* Kulturen wurden ebenfalls untersucht. Dabei wurden in den Samen deutliche Unterschiede im Vergleich zu den Blättern festgestellt. Qualitative und quantitative Unterschiede zwischen beiden *Isatis* Arten wurden entdeckt, was eine Unterscheidung beider Arten bereits auf Ebene des Saatgutes ermöglicht.

1. AIM OF THE WORK

The potential of woad (*Isatis tinctoria* L., Brassicaceae) extracts as new active ingredient for anti-inflammatory phytopharmaceuticals was explored during the last years and is still ongoing. Tryptanthrin, γ-linolenic acid and an indolin-2-one derivative were shown to be active principles inhibiting cyclooxygenase-2 (COX-2), 5-lipoxygenase (5-LOX), the expression of inducible nitric oxide synthase (iNOS), human neurophil elastase, and the release of histamine from mast cells. Indirubin inhibits inflammatory reactions in delayed-type hypersensitivity and is a potent inhibitor of cyclin-dependent kinase 5 (CDK5) and glycogen synthase kinase 3β (GSK3B), whereas indigo is a side product of indirubin formation from indigo precursors during the drying of woad.

In contrast to the numerous pharmacological studies on woad and its constituents, comparably few analytical work had been performed. A range of suitable analytical tools are, however, needed for the quality control and optimization of breeding, post-harvest treatment etc. As a first step, a suitable HPLC method for the quantification of the pharmacologically active constituents and indigo should be developed and validated, to be used in quality control for herbal extract preparations based on woad.

Besides the quantity of active compounds, the overall composition (fingerprint) of an extract is important in quality control of phytopharmaceuticals, since legal authorities consider the extract in its entirety as active ingredient even if specific activities can be attributed to single compounds. A second aim was, therefore, to analyse the spectrum of extracted metabolites as comprehensively as reasonably possible, for the pharmacologically active lipophilic leaf extract of. *I. tinctoria*. This profiling study should be complemented with an analysis of polar metabolites, and a profiling of the closely related *I. indigotica* Fort. A detailed knowledge of the chemical composition of the active extract should provide some clues on the nature of the compounds responsible for the solubility enhancing effects observed in a previous microdialysis study.

Oberthür et al. (2004) observed that the phytochemical profile of woad underwent profound changes during post-harvest treatment. Indigo precursors largely disappeared, whereas the pharmacologically active tryptanthrin and indirubin were formed only during the drying process. The precursor molecules of tryptanthrin and indirubin remained, however, unknown. A systematic investigation of possible candidates is crucial to optimise cultivation and harvesting conditions in the development of a new herbal drug. Indole glucosinolates occur in rather high concentrations in fresh woad leaves and are thus possible precursors of these indole alkaloids. The second part of the metabolite profiling study should, therefore, focus on the indole glucosinolates in *Isatis,* and their role as putative precursors of tryptanthrin and other indole alkaloids.

First, a robust method should be developed for the quantitative determination of glucosinolates. Relevant parameters for sample preparation, extraction and analysis should be optimised and validated. Required reference compounds, which are not commercially available, should be isolated and their purity should be determined with a suitable procedure.

The developed method should then be used to investigate seasonal fluctuations of glucosinolate patterns in leaves of defined *Isatis* accessions. Furthermore, the effects of repeated harvesting during the growth season and the influence of the drying procedure on the glucosinolate content should be examined. The glucosinolate patterns in seeds of defined *I. tinctoria* and *I. indigotica* strains should be analysed for comparative purposes.

2. INTRODUCTION

2.1 Woad (*Isatis tinctoria* L.)

Historical background of woad as source of indigo

Woad (*Isatis tinctoria* L., Brassicaceae) has a long and well documented history as an indigo dye plant and as a medicinal herb. This biennial herbaceous plant has been known in Central Europe for centuries. From the Middle Ages up to the 18th century, the cultivation of woad played an important economical role in certain parts of Europe. Centres of indigo production were located in Germany (especially in Thuringia) as well as in France (Normandy, Languedoc, Somme), England (Somerset, Lincolnshire) and Italy (Piedmont, Tuscany). The import of cheaper indigo from *Indigofera suffruticosa* initiated the decline of the woad industry in the late 17th century and woad cultures disappeared completely with the production of synthetic indigo in the late 1890's[1,2].

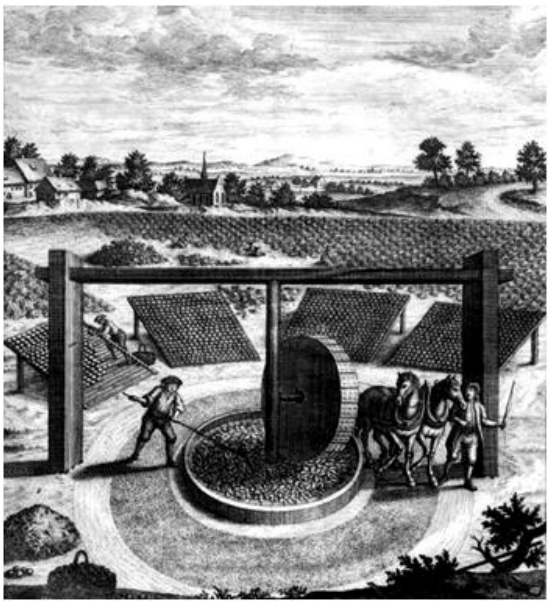


Figure 1. Traditional woad mill in Thuringia.
(Illustration: 17th century) Source: Hurry[2]

Medicinal uses, phytochemistry and biological activities of woad

The medicinal properties of the plant were esteemed in Europe and in Traditional Chinese Medicine (TCM) for centuries. The application of woad for the treatment of wounds, ulcers and haemorrhoids was described by Hippokrates (460 B.C.), Galen (129-216 A.D.) and Pliny (23-79 A.D.). During the late Middle Ages *Isatis* was used to

treat snake bites, wounds and other inflammatory ailments. Its curative properties fell into oblivion after woad lost its importance as source of indigo for dying in Europe[2].

In China, Banlangen (*Isatis* root) and Daqingye (*Isatis* leaf) from the taxonomically closely related *Isatis indigotica* Fort. are still important and popular herbal drugs in Traditional Chinese Medicine (TCM) used for the treatment of inflammation, fever or swelling and are official drugs of the Chinese Pharmacopoeia up to the present time. Antibacterial and antiviral properties have also been described[3].

A considerable number of phytochemical, biological and pharmacological investigations have been carried out, mostly over the last 4 decades. More than 100 secondary metabolites have been identified from *I. tinctoria* and *I. indigotica*. They include numerous indole derivatives like tryptanthrin[4] (**1**), indirubin[5] (**2**), indigo[5,6] (**3**), indolinone[7] (**4**), deoxivasicinone[8] (**5**), or the indigo precursors isatan A (**6**) and isatan B[9] (**7**), but also aliphatic and indolic glucosinolates[10-13], aromatic and aliphatic carboxylic acids[14,15], aliphatic alcohols, esters, ethers, aldehydes, hydrocarbons, nitriles and furans[15,16], lignans[17], various glycosides[18,19], amino acids[20,21], isoprenoids[15,18], flavonoids[8,22,23], sphingolipids[24] and anthranoids[8] (Figure 2).

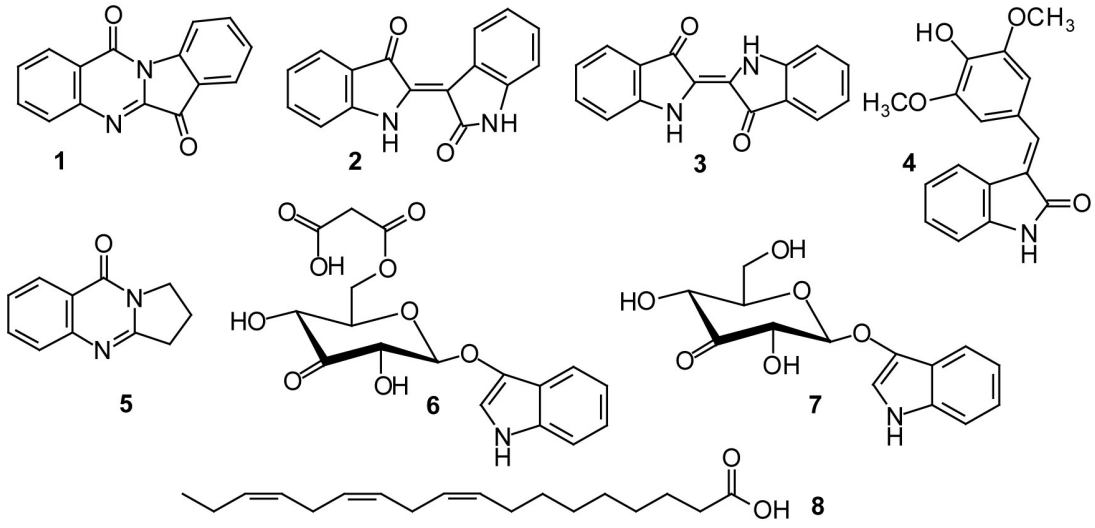


Figure 2: Structures of compounds **1**-**8**.

Tryptanthrin (**1**) was originally isolated by Honda et al.[4] as an anti-dermatophytic compound. High antimycobacterial activity of the compound and some derivatives was observed against *Mycobacterium smegmatis* in several *in vitro* studies[25-27] but a notable *in vivo* efficacy was lacking[28]. An aqueous extract of woad was tested in a chronic pulmonary infection model in rats. The observed effect was positive and was deemed promising for human studies[29]. Furthermore, its applicability was investigated in the context of the development of wood protecting varnishes. Fermented woad juice inhibited wood rotting fungi[30] and weak insecticidal activity was found for tryptanthrin[31] (**1**).

Isatis extracts and selected constituents were screened for antiviral, antifungal, antibacterial and cytoinhibitory activities. However, its potential as an anti-inflammatory was not discovered at that time. Some years ago, a broad-based pharmacological screening was initiated and tryptanthrin (**1**), γ-linoleic acid (**8**) and (*E*)-3-(3',5'-dimethoxy-4'-hydroxy-benzylidene)-2-indolinone (**4**) were shown to inhibit cyclooxygenase-2 (COX-2), 5-lipoxygenase (5-LOX), expression of inducible nitric oxide synthase (iNOS), human neutrophil elastase and the release of histamine from mast cells[32-36].

Leclerc et al.[37] found that indirubin (**2**) inhibits inflammatory reactions in delayed type hypersensitivity and is a potent inhibitor of cyclin-dependent kinase 5 (CDK5) and glycogen kinase 3β (GSK3B). These findings were corroborated by pharmacological studies on tryptanthrin (**1**) and indirubin (**2**) performed by other groups[4,38-40]. A clinical pilot study in experimentally induced skin erythema[41] and *in vivo* studies in models of acute and chronic inflammation, contact allergy, and rheumatoid arthritis confirmed the anti-inflammatory activity of lipophilic *Isatis tinctoria* extracts[42,43].

Parallel to these pharmacological investigations, advanced phytochemical and analytical studies of the plant have been carried out. Oberthür et al. identified the correct structures of the major indigo precursors (**6** and **7**) in woad and observed profound post-harvest changes in metabolite patterns during the drying of *Isatis* leaves[9].

Indigo precursors largely disappear, whereas pharmacologically active compounds such as tryptanthrin (**1**) were formed during the drying process[44]. However, the precursor molecules of tryptanthrin (**1**) or indirubin (**2**) are so far unknown and a systematic investigation of possible candidates is essential to optimise cultivation and harvesting conditions in order to establish a new herbal drug.

Figure 3. Experimental research plots with *Isatis* plants
at the Agricultural research station of Thuringia, Dornburg.

The clinical efficacy of *Isatis* extracts can not be explained solely by the presence of individual active compounds like tryptanthrin (**1**), indirubin (**2**) and (*E*)-3-(3',5'-dimethoxy-4'-hydroxy-benzylidene)-2-indolinone (**4**) because these compounds occur in too low amounts. Synergistic effects between extract constituents seem to play a central role and/or compounds in the complex extract matrix presumably increase the solubility of these poorly soluble alkaloids.

2.2 Interaction and synergy in phytomedicines

Commonly, plant extracts are very complex mixtures, whose individual constituents are known only to a limited extent. The extracted compounds can be divided into main active substances and concomitant compounds. Pharmacologically active main compounds are responsible for the therapeutic effect of the extract, while concomitant compounds can change physico-chemical properties and therefore influence biopharmaceutical parameters like solubility and bioavailability of the main active substances.

Several factors like conditions of cultivation, harvest, drying or storage, but also the selection of an appropriate extraction method, particle size, solvent and extraction duration have a major impact on composition and concentration of active compounds in an extract. As a general rule, the extract yield increases with the polarity of the solvent or solvent mixture used. This results in a lower drug-to-extract ratio[45-47].

The pharmacological activity of plant extracts is often not attributable to a single compound, and interactions between the extracts constituents have to be expected. In fact, proponents of phytotherapy consider the occurrence of synergistic effects in herbal extracts as a major advantage of this type of medicines. However, surprisingly few studies have been carried out to understand synergistic effects and interactions between plant constituents.

Synergistic interactions between plant constituents

Synergistic interactions have been described for constituents from an extract of a single plant, as well as for those from different plants in a multi-herbal formulation. The general understanding of synergy is that an effect of a combination of substances is greater than what can be expected from the sum of their individual contributions[48,49]. For

example, Wagner et al. found that isolated ginkgolides A and B from *Ginkgo biloba* L. were pharmacologically less effective as anti-inflammatory agents *in vitro* than a mixture of the two compounds[50]. Another case of synergistic interactions was described for *Piper methysticum* G. Forst (Kava-Kava), where the anticonvulsant activity of the kava lactones yangonin and desmethoxyyangonin was found to be much higher in combination with other kava constituents[51]. Keledjian et al. investigated the absorption of the kava lactones dihydrokavain, kavain, desmethoxyyangonin and yangonin in mice. The authors found higher concentrations of yangonin and kavain in the brain after administration of an extract compared to the equivalent amount of pure compounds[52]. Similar observations were described by Nahrstedt for kavain. After oral administration of pure kavain to mice the plasma concentration was more than 50% lower compared than that found after administration of the same amount of kavain in a refined *Piper methysticum* extract[53].

Flavonoids are present in many phytomedicines but their role as synergistic compounds is still not clear. Philippson observed that the activity of artemisinin in an *in vitro* antimalarial test was enhanced in the presence of the flavonoids artemetin and casticin[54]. Flavonoids were also involved in synergistic effects observed in a clinical trial with willow bark (*Salix alba* L.) for the treatment of ostheo-arthritis. According to the work of Schmidt et al., the applied amount of salicin (240 mg daily) was too low to explain the observed activity. Interestingly, gastrointestinal side effects associated with non-steroidal anti-inflammatory drugs such as acetylsalicylic acid were not observed, although it is known that the salicylalcohol derivative salicin is transformed in the liver into salicylic acid[55].

Butterweck et al. investigated the mode of action of St. Johns Wort (*Hypericum perforatum* L.) which is used for the treatment of mild forms of depression. Several compounds were isolated from the plant and used for an *in vitro* screening on several receptors, transporters and ion channels. Synergistic interactions between different

compounds were observed, and the authors concluded that this effect is responsible for the antidepressant activity in St. Johns wort[56].

Interaction by solubility enhancement

Solubility enhancing properties have been described for saponins. It is known, for example, that saponins from *Ginseng* lead to a pronounced enhancement of the solubility of saikosaponins in water[57]. Kimata et al. found a considerable solubility enhancement of saikosaponin A, (a triterpene saponin of Bupleuri radix) by the *Ginseng* saponin chikusetsusaponin V (a bidesmoside of the oleanolic acid type)[58]. The solubility of saikosaponin A in water can also be enhanced by glycyrrhizin and glycyrrhizin 30-β-glucoside ester and glycyrrhizin-30-β-glucuronide ester[59]. Zhou et al. showed that the significant solubility enhancement of saicosaponin B in water in the presence of a ginseng-saponin mixture is caused by malonyl-ginsenosides. No solubility enhancing effects were observed with neutral ginseng-saponins of the dammarane type. In contrast, solubility enhancing effects of malonyl-saponins were increased in the presence of dammarane saponins[60].

Schöpke and Bartlakowski studied solubility enhancing effects that single saponins or mixtures of saponins had on quercetin, which was used as a model compound with poor solubility in water. The authors found that the saponin mixture enhanced the solubility of quercetin far below the critical micelle concentration (CMC), while this effect was not observed with a single saponin[61]. This result indicates that solubility enhancement can not be explained completely by micellar solubilisation.

Liquorice (*Glycyrrhiza glabra* L.) contains a large variety of saponins and is used in many mixtures in Chinese medicine as an agent with synergistic and solubility enhancing properties. For example, it potentiates the effect of the neuromuscular blocking agent paeoniflorin[62]. Schindler found that adding 10% glycyrrhizinic acid, the

major saponin in *Glycyrrhiza glabra*, to am aqueous medium can enhance the solubility of the sapogenin isoliquiritigenin by a factor of 570[63].
However, a further systematic investigation of solubility enhancing effects of saponins has not been carried out so far.

Solubility enhancing effects observed in *Isatis*

Solubility enhancing effects were also found for *Isatis tinctoria.* The skin penetration of pharmacologically active tryptanthrin (**1**) from solutions of pure compound and lipophilic *Isatis* leaf extracts was investigated with a microdialysis study using an *ex vivo* pig foreleg model[64]. Tryptanthrin concentrations in the dialysates increased faster when the alkaloid was applied in the extract matrix. When applied in pure solutions the alkaloid crystallised on the skin surface. One explanation for this was that extract constituents act as solubility enhancers and increase the cutaneous penetration of poorly soluble compounds like tryptanthrin. Also synergistic pharmacological effects from other constituents in the extract have to be taken into account when considering the in vivo activities reported.

Negative aspects of compound interaction in plant extracts

In general, synergistic effects are considered to be positive because lower doses have to be applied but also less desirable interactions have been reported[48]. The presence of tannins in an herbal drug, for example, can lead to lower absorption of proteins or alkaloids due to the formation of poorly soluble complexes[65]. In case of St. John's wort (*Hypericum perforatum*), the induction of the cytochrome P450 enzyme systems may accelerate the metabolism of other drugs which finally results in blood levels that are too low for a therapeutic effect[48].

Franz[66] observed that polysaccharides can generate a hydrocolloid film in the gastrointestinal tract and hereby decrease the absorption of other compounds. An absorption lowering effect has also been described for resins, gum and pektins[67].

Due to the complex composition of most plant extracts, it was possible only in rare cases to unequivocally assign factors which are responsible for the solubility enhancement and higher dissolution rate of poorly soluble compounds. Several factors that influence synergistic effects may interfere with each other. So far, only a small number of examples have been investigated[45,46,48], and a systematic investigation of this issue is still lacking.

These selected examples of influences of concomitant compounds on the dissolution and absorption of active compounds show, that concomitant compounds should not be regarded as worthless. It is therefore right that legal authorities consider the extract in its entirety as active ingredient, even if the specific activity can only be attributed to single compounds. Besides the quantity of active compounds also the composition of an extract is important in quality control of phytopharmaceuticals.

2.3 The role of plant extracts in the pharmacopoeia, and aspects of quality control

Medicinal plants and plant-derived phytopharmaceuticals account for approx. 25% of prescribed medicines and approx. 50% of the market share in over-the-counter products in industrial countries[68,69]. Safety and efficacy of these products are a central issue for regulatory authorities like BfArM (Germany) or Swissmedic (Switzerland) and for manufacturers. Compulsory guidelines to guarantee the quality of medicinal products including all phytopharmaceuticals are specified in pharmacopoeias such as the European Pharmacopoeia, the British Pharmacopoeia or the US Pharmacopeia.

Definitions by the European Pharmacopoeia

If the active constituents of herbal extracts are not known, marker compounds are used for standardisation to guarantee a constant quality. The European Pharmacopoeia (PhEur 6.0) distinguishes between different types of extracts according to their type of marker compounds[70].

"Standardised extracts" are adjusted to a given content of constituents with known therapeutic activity within an acceptable tolerance. The amount of active ingredient is defined in the final product but the amount of native extract varies. "*Quantified extracts"* are adjusted to a defined range of pharmacologically active constituents, or else, to a limited concentration of toxicologically critical compounds. Such adjustments are made by blending batches of extracts.

Production of quantified extracts is only possible in cases where toxicological or pharmacological data of marker compounds exist. Examples are Devils Claw dry

extracts (Harpagophyti extractum siccum), standardised Horse Chestnut dry extract (Hippocastani extractum siccum normatum) or St. John's Wort dry extract (Hyperici herbae extractum siccum).

"*Other extracts*" are essentially defined by their production process. Factors like herbal material, extraction conditions or extraction solvents are specified. Quantification of analytically relevant markers is carried out for each individual batch and the amount of extract per dose is constant.

Often, marker compounds of pharmacological, toxicological or analytical relevance are used for quality control purposes. Examples for the selection of compounds with pharmacological activity as markers are oligomeric procyanidines in Hawthorn dry extract (Crataegi folii extractum siccum), hyperforin in St. John's Wort dry extract (Hyperici herbae extractum siccum) or ginkgolides in standardised *Ginko biloba* dry extracts (Ginkgo extractum siccum normatum). Aristolochic acid in Traditional Chinese Medicine (TCM) plants or pyrrolizidine alkaloids in butterbur (*Petasites hybridus* L. Gaertn.) dry extracts are examples of toxicologically relevant markers. Increase and decrease of marker compounds with analytical relevance give information on the identity and purity of extracts or herbal drugs.

A special kind of extract as defined by the European Pharmacopoeia is the so called "*refined extract*". If the active compounds are known, it is possible to obtain such extracts by several enrichment steps to remove unwanted compounds. Techniques for the separation of complex mixtures are selective extraction and partitioning methods, for example liquid-liquid-extraction, adsorption techniques and precipitation procedures in combination with filtration. Especially precipitation steps are widely used in industrial scale to remove unwanted constituents like proteins, polyphenols or waxy compounds.

Only for very few phytopharmaceuticals the global composition of the active ingredient (extract) has been analysed in a more comprehensive manner and data published.

Notable examples include the special extracts EGb 761 (derived from *Ginkgo biloba* leaves) and ZE 331 (obtained from *Petasites hybridus* leaves). Both examples are discussed in the following sections.

Example: the refined *Ginkgo biloba* extract EGb 761

One example for a refined extract is the *Ginkgo biloba* extract EGb 761, where some compound classes are enriched and others are depleted compared to their original proportions in the plant. This is achieved in a sophisticated multi step extraction procedure (Figure 4).

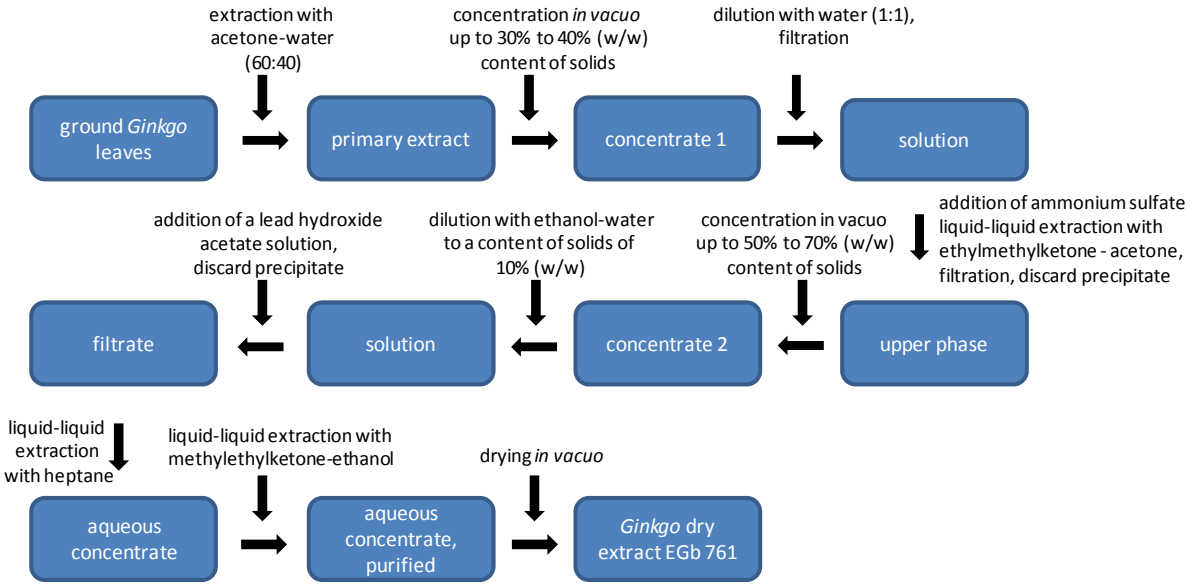


Figure 4. Simplified production scheme of the *Ginkgo biloba* extract EGb 761. Adapted from Hänsel and Spieβ[71] with minor modifications.

Compounds which are responsible for allergic reactions[72-74] or serious mucosal disturbances[75] such as ginkgolic acid derivatives are removed. An overview of compounds present in *Ginkgo biloba* leaves but not in EGb 761 is shown in Table 1.

Compounds, which are regarded as pharmacologically active like flavonol glycosides, ginkgolides and bilobalide can be enriched by this extraction procedure. According to DeFeudis[76] about 2.5 kg of extract is obtained from 100 kg of *Ginkgo* leaves. The extract contains approximately 24% (w/w) flavonoid glycosides, 3.6% (w/w) ginkgolides, 2.9% (w/w) bilobalide, 6.5% (w/w) proanthocyanidins and less than 1 ppm alkyl phenol compounds (ginkgolic acid derivatives). The final EGb 761 product is standardised to contain 24% flavonol glycosides and 6% terpene lactones (3.1% ginkgolides, 2.9% bilobalide) which is controlled analytically to ensure the consistency of its composition.

Compound class	Content [%]
Flavonol glycosides	24
Terpene lactones, thereof	6
Bilobalide	2.9
Ginkgolides A, B and C	3.1
Oligomeric proanthocyanidines	5-10
Carboxylic acids (among others 6-hydroxy-kynurenic acid, vanllic acid, protocatechuic acid, p-hydroxybenzoic acid)	approx. 9
Ginkgoles and ginkgolic acids	0.0005
Biflavones	0.1

Table 1. Composition of the *Ginkgo biloba* extract (50:1) EGb 761.
Source: Hänsel and Spieβ[71]

Example: the *Petasites hybidus* extract Ze 339

An other example for a well characterised extract is the butterbur (*Petasites hybridus*) extract Ze 339 used for the treatment of allergic rhinitis. Careful selection of plant material for cultivation, study of harvest conditions and choice of an appropriate extraction method (the manufacturer used an extraction technique with subcritical CO_2) enable an enrichment of the pharmacologically active petasins (petasin, neo-petasin, iso-petasin, S-petasin, neo-S-petasin and iso-S-petasin), while toxic pyrrolizidine

alkaloids (such as senecionine, integerrimine and their N-oxides) are absent from Ze 339[77,78]. The composition of the respective compound classes is shown in Table 2.

Compound class	Content [%]
Sum of petasins including	20.3
petasin, neo-petasin, iso-petasin, S-petasin, neo-S-petasin, iso-S-petasin	
Fatty acids (total)	40.2
unsaturated cis-fatty acids	1.1
poly-unsaturated cis-fatty acids	33.8
unsaturated trans-fatty acids	0.0
poly-unsaturated trans-fatty acids	5.3
Aroma components	7.0
Steroids/Phytosterols	1.2
Pyrrolizidine-alkaloids	n.d.
Remaining substances	30
apolar constituents without chromophors	6.9
water	

Table 2. Composition of the refined *Petasites hybridus* extract Ze 339. n.d.: not detectable; Source: Brattsröm (2003)[78]

Drawbacks of current methods

Special care has to be taken for the quality control of raw and final formulated products due to their complex composition. Current methods such as TLC, HPLC-UV or GC-MS provide only a limited insight into the complexity of the samples and often show limited separation and detection capabilities owing to the physicochemical properties of the analytes under investigation (e.g. poor volatility, lack of chromophores etc.). These methods are adapted to fulfil control needs of regulatory authorities and pharmaceutical manufacturers to track known adulterations but unknown adulterations can be easily overlooked. As a consequence of the lack of efficient tools in quality control, large variations of phytopharmaceuticals can be found on the market. The analysis of 14 commercially available feverfew samples showed that each batch had a characteristic

spectra profile and two of the batches were distinctly different from the other twelve[79]. Since the chemical composition of a phytopharmaceutical critically depends on the plant growth environment, harvesting conditions and used preparation methods, it is essential that quality control of phytomedicines takes these factors into account. It is therefore desirable to establish analytical methods, which are able to profile plant extracts in a holistic manner and to provide a means for standardisation and quality control of phytopharmaceuticals based on their entire composition without reference to “active” molecules.

2.4 Metabolite profiling and quality control of medicinal plants

Terminology and definitions

Several approaches like metabonomics, metabolomics or metabolomic profiling have been developed during the last years. All these techniques try to give detailed qualitative and quantitative metabolite overviews in organisms or extracts. Metabonomics is a systems oriented approach describing "the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification" as defined in 1999 by Nicholson et al.[80] Metabolomics is defined as "a comprehensive analysis in which all the metabolites of a biological system are identified and quantified"[81] while, in contrast, metabolic profiling is defined as an approach using predefined metabolites which are biogenetically closely related and limited in number [82]. Hence metabolic profiling has to be considered as a biased approach. An overview on strategies for metabolic analysis has been given by Dunn and Ellis[82] and is shown in Table 3.

Strategy	Description
Metabolomics	Non-biased identification and quantification of all metabolites in a biological system. Sample preparation must not exclude metabolites, and selectivity and sensitivity of the analytical technique must be high
Metabolite profiling	Identification and quantification of a selected number of pre-defined metabolites, generally related to a specific metabolic pathway(s). Sample preparation and instrumentation are employed so to isolate those compounds of interest from possible matrix effects prior to detection, normally with chromatographic separation prior to detection with MS. In the pharmaceutical industry, this is widely used to study drug candidates, drug metabolic products and the effects of therapeutic treatments
Metabolic fingerprinting	High-throughput, rapid, global analysis of samples to provide sample classification. Quantification and metabolic identification are generally not employed. A screening tool to discriminate between samples of different biological status or origin. Sample preparation is simple and, as chromatographic separation is absent, rapid analysis times are small (normally 1 min or less)
Metabolite target analysis	Qualitative and quantitative analysis of one or a few metabolites related to a specific metabolic reaction. Extensive sample preparation and separation from other metabolites is required and this approach is especially employed when low limits of detection are required. Generally, chromatographic separation is used followed by sensitive MS or UV detection
Metabonomics	Evaluation of tissues and biological fluids for changes in endogenous metabolite levels that result from disease or therapeutic treatments

Table 3. Overview on strategies for metabolic analysis.
adapted from Dunn and Ellis[82] with minor modifications.

Analytical platforms utilized for metabolic profiling - general considerations

These modern metabolic strategies have to cope with the enormous number of unknown compound structures. Therefore analytical techniques as HPLC-MS using MS^n and high accuracy mass spectrometric detection, offline NMR and HPLC-NMR couplings are required for structure elucidation and identification of complex metabolite structures[83].

However, some issues need to be considered when working with these hyphenated techniques. In metabolomic analysis factors such as age, type of tissues, developmental stage, environmental conditions and harvesting time greatly affect the

metabolome obtained even from the same genotype. Therefore, the herbal material should be frozen directly after harvesting to avoid any change. The subsequent sample preparation procedure has to be carried out most carefully to avoid artefact formation.
As metabolomics aims at comprehensive fingerprinting of all metabolites, the used extraction methods should cover all possible plant metabolites. Unfortunately, there is no single extraction method to achieve this goal because the polarity of the extraction solvent limits the range of compounds that can be extracted. Thus, it is indispensible to extract the plant material under investigation with different polar and non-polar solvents to cover a wide range of metabolites and to optimise the extraction procedure to achieve an exhaustive but non-destructive extraction. In this context, a denaturation of enzymes involved in metabolite alteration should be considered, which can be achieved by drying of the plant material before extraction (for example by freeze drying), storing, grinding and extraction at low temperatures, or by the choice of a denaturating extraction solvent. A sample transformation for example by pH shifts has to be excluded before analysis[82].

For HPLC-MS techniques, modifiers are added to the mobile phase to increase ionisation efficiency and care must be taken to keep the physicochemical characteristics of the final samples (as pH, ion strength) constant over the whole sample set. Ion suppression effects are another well known problem in mass spectrometry of complex biological matrices. This phenomenon leads to a decreased ion yield and is not necessarily constant over a chromatographic run. Therefore, care must be taken that analyte responses are not unpredictably influenced by this effect, if compounds from different samples should be quantified and compared. Ion suppression can be minimised with a suitable sample preparation procedure, together with optimisation of chromatographic separation conditions[84,85]. An enhanced chromatographic resolution can be achieved for example with recent technologies as ultra performance chromatographic systems (for example the Waters UPLC™ system). The method is

based on the application of very small particles (1.7 µm) at elevated pressure to achieve superior theoretical plate numbers and resolution. When applied to natural product analysis, UPLC showed great advantages compared to standard HPLC[86,87]

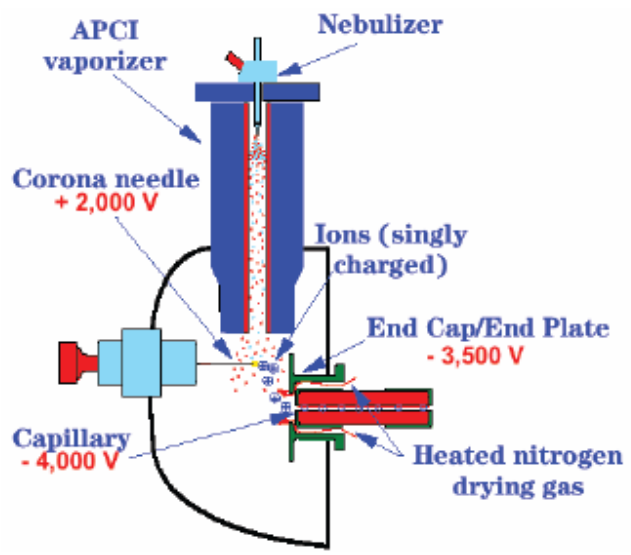
For NMR based approaches, factors as the intrinsic pH of the extract, NMR probe temperature stability, and quality of the residual solvent suppression affect the robustness of the applied method. Imperfect sample preparation techniques might introduce inhomogeneities to NMR spectra as described by Defernez and Colquhoun[88]

Mass spectrometric approaches for metabolite profiling

Ionisation techniques

Gas chromatographic and liquid chromatographic techniques have gained a considerable importance for metabolic analysis. Within the past years, different methods for ion formation and ion selection have been developed. The oldest and most frequently used ionisation technique is electron impact ionisation (EI). The analytes of interest have to be vaporized prior to ionization, and this technique is therefore commonly used in GC-MS analysis. Due to the high reproducibility of EI spectra, they are amenable to database searches. Extensive databases such as the NIST database are available. Over the past two decades, ionisation techniques such as electrospray ionisation (ESI), atmospheric pressure chemical ionisation (APCI), or matrix-assisted laser desorption ionisation (MALDI) have been developed for the analysis of non-volatile and thermally labile compounds. In ESI, ions are generated from a liquid by the formation of highly charged droplets and their subsequent evaporation. ESI is probably the most used ionisation technique for a wide range of biological analytes. It can be used in combination with separation techniques as HPLC or CE, and is considered to be a soft ionisation technique which does not result in extensive fragmentation of molecular ions as observed in electron impact mass spectrometers. This is the reason

why direct metabolite identification is not possible by comparing ESI mass spectra. Also, ESI mass spectral libraries are not available as in the case for GC-MS.



A common alternative to ESI is atmospheric pressure chemical ionisation (APCI). With APCI, the HPLC eluent is heated to temperatures of about 400 °C and sprayed with nitrogen into the source. The ionisation is initiated by a corona discharge of a needle followed by a complex reaction cascade leading to an efficient ionisation of analytes (Figure 5).

Figure 5. Interface design of an APCI source.
Illustration: Bruker Daltonics

Typically, the gas phase ionisation from APCI generates more fragment ions relative to the parent ion than the liquid phase ionisation technique ESI. With APCI it is also possible to ionise weakly polar analytes not existing as preformed ions in solution with ESI. Thus, the two ionisation techniques are complementary.

APCI is known for its sensitivity, robustness and reliability but is less useful for thermally labile compounds and requires some compound volatility. In contrast to ESI, APCI has the advantage of being less susceptible to matrix interferences from salts[85,89-92].

Unlike ESI or APCI in which analyte ions are produced continuously, ions in matrix-assisted laser desorption ionisation (MALDI) are produced by pulsed-laser irradiation of a sample. The sample is co-crystallised with a solid matrix that can absorb a wavelength of light emitted by the laser. MALDI is the method of choice for high throughput analysis, since target plates loaded with hundreds of samples can be used. The major drawback of MALDI lies in the high degree of chemical noise as a result of

the required matrices, especially if low molecular weight compounds have to be analysed. Therefore, a complete and reproducible MALDI of a metabolic profiling sample still remains a challenge[93].

Mass analysers

For metabolic profiling strategies, several low resolution (single or triple quadrupole and ion trap) and high resolution (time of flight, FT-ICR-MS, orbitrap) mass analysers are utilised (Figures 6 to 8). In low resolution instruments, quadrupoles were the most commonly used mass analysers in the past, particularly in combination with EI ionisation in GC-MS platforms. Their major drawback is their relatively slow scanning rate (<4000 u/s) in full scan mode, not suitable for modern high-speed separations with narrow peaks. Triple quadrupole mass spectrometers give more structural information on the target analyte by arrangement of three quadrupoles, whereas the second quadrupole is used as collision cell for a controlled ion fragmentation. This setup allows the combination of ion selection, ion reaction and ion scan processes. Another way to obtain structural information by controlled ion fragmentation (collision induced dissociation (CID)) is the application of an ion trap as mass analyser. Ions are “trapped” with these devices by electrodynamic focusing and can be forced to leave the trap by putting them in unstable orbits by increasing the voltage. Ion traps enable the possibility to perform MS^n experiments, are robust and are relatively inexpensive compared to other mass spectrometric devices and are therefore widely used. Quantitative performance and dynamic range could be improved with the recently developed linear ion traps.

Time-of-flight mass analysers provide high resolution mass data by the separation of ions based on their mass-to-charge dependent velocity. All ions are formed in the ion source and accelerated through a fixed potential and consequently small mass ions

arrive to the detector earlier than large mass ions with the same charge. A combination of a TOF mass analyser with other mass spectrometric devices such as a second TOF, an ion tap or one or two quadrupoles (Q-TOF) enable exceptionally high scan rates (up to 10^6 u/s) in combination with high resolving power. These hybrid mass spectrometers are used for large scale routine metabolic profiling experiments[82,85,89-92,94].

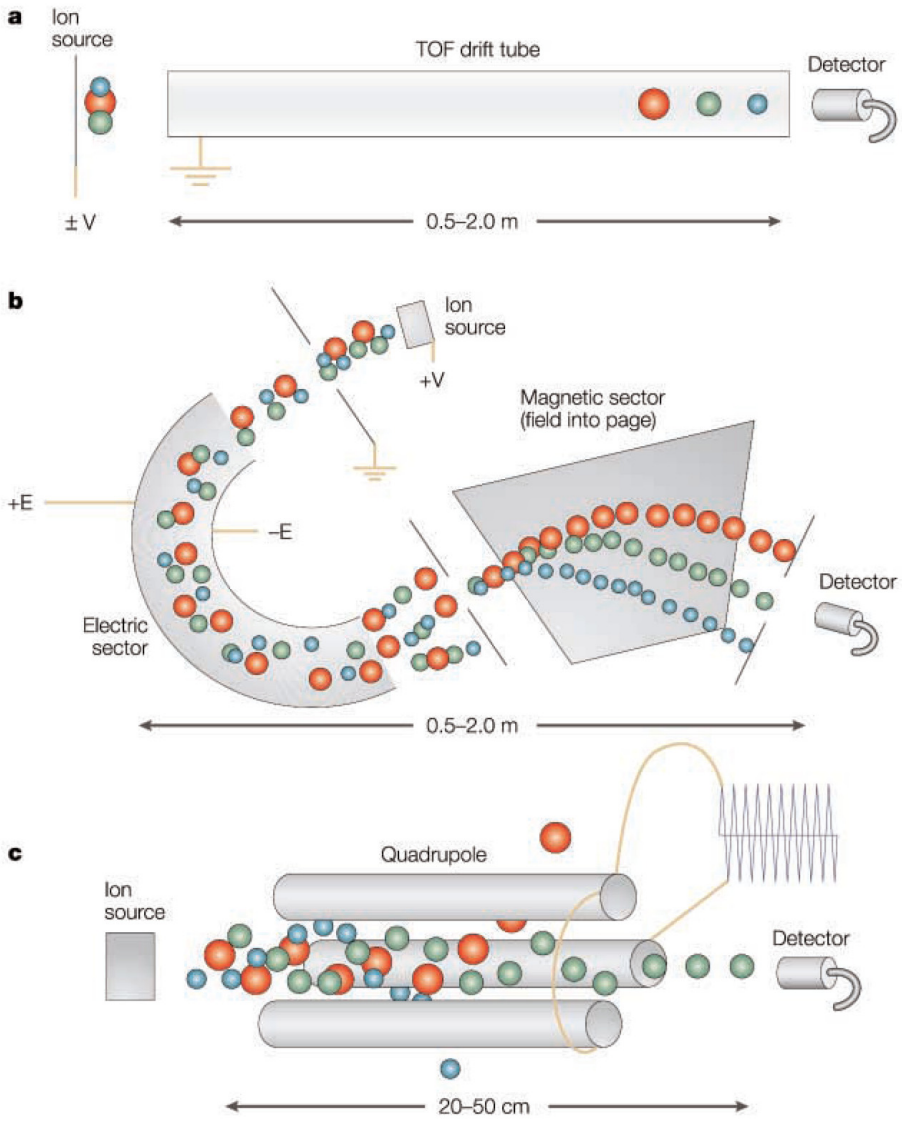


Figure 6. Pictorial diagrams of the common beam mass analysers:
a single focusing time-of-flight (TOF) mass analyser
b double-focusing time-of-flight analyser
c quadrupole mass analyser; Illustration from Glish and Vachet[90]

A different type of mass analyser is the Fourier transform ion cyclotron mass spectrometer (FT-ICR-MS) with an unmatched resolving power. The FT-ICR-MS uses a magnetic field and the ions oscillate around the magnetic field with a cyclotron frequency that is inversely related to the *m*/*z*. Very simplified, the cyclotrone frequencies of the ions trapped in the FT-ICR are measured and converted into *m*/*z*. Today's electronic equipment is able to measure frequencies with extremely high precision and this translates to a very high mass resolution. The potential of FT-ICR-MS has been discussed in articles of Wang et al.[95] or Ohta et al.[96] Although the capabilities of FT-ICR-MS are undeniable, one has to be aware of the considerable cost and effort to operate an instrument based on a high-powered multi Tesla magnet.

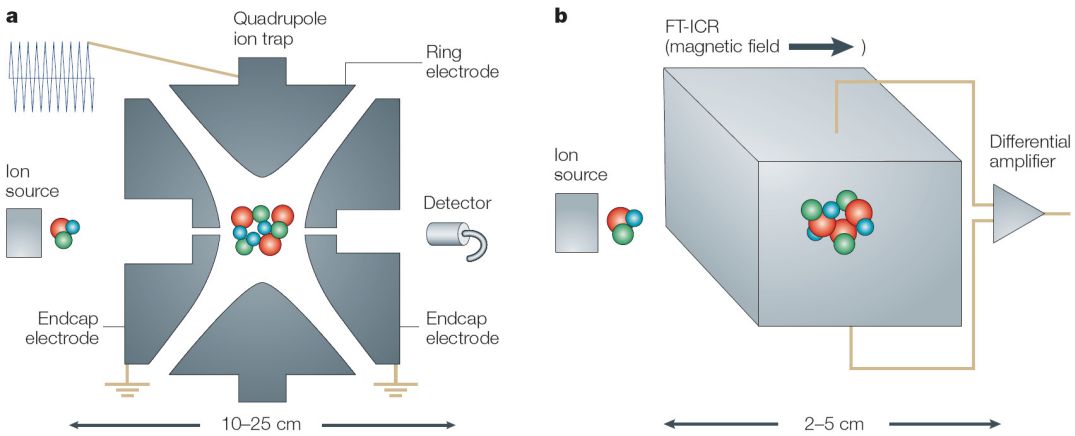


Figure 7. Pictorial diagrams of the common trapping mass analysers.
a quadrupole ion trap
b Fourier-transform ion-cyclotron resonance (FT-ICR)
Illustration from Glish and Vachet[90]

The latest development, the orbitrap, was introduced on the market in 2005 as an alternative system to the FT-ICR-MS. The hybrid instrument consists of a linear ion trap which is used to selectively fill an intermediate ion storage device (C-trap) with ions of interest which are subsequently injected into the orbitrap (Figure 8). This device uses image current detection and Fourier transform similar to the FT-ICR-MS but does not

require a superconducting magnet which significantly reduces costs and maintenance requirements[97,98].

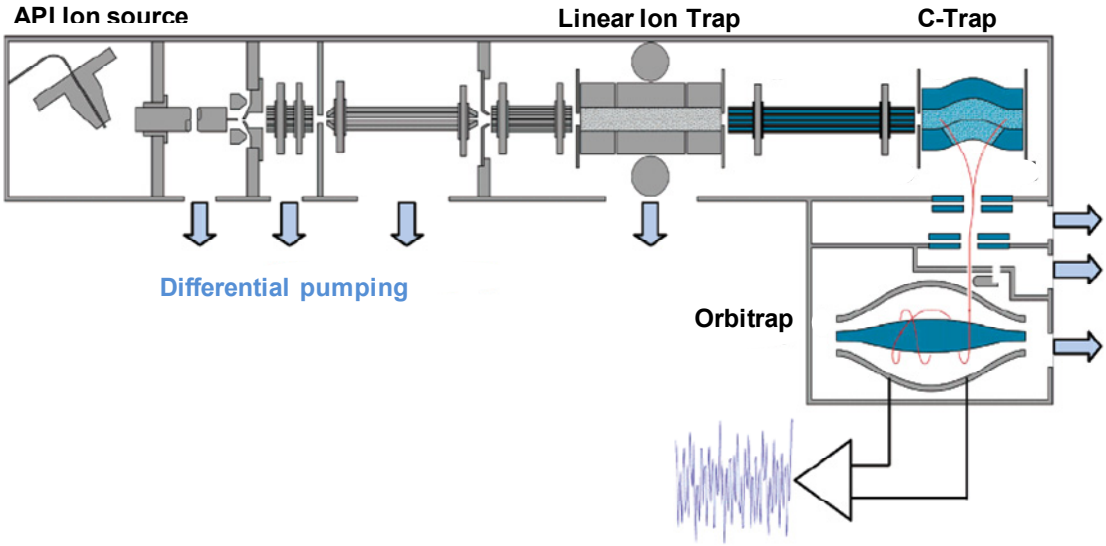


Figure 8. Pictoral diagram of a commercially available LTQ-Orbitrap instrument. Illustration from Makarov et al.[97] with minor modifications.

MS coupling to separation techniques

In theory, the optimal way to obtain a mass spectrum of all metabolites in a sample in an unbiased way would be the direct application into the MS instrument without any purification or separation. However, if working with complex biogenic matrices, this approach is associated not only with an enormous instrument maintenance as cleaning of ion source and transfer optics but also with several interferences due to unspecific matrix effects and ion suppression by competitive ionisation. Therefore, commonly a chromatographic separation (for example by HPLC, UPLC or GC) of the matrix is necessary prior mass spectrometric detection.

A complementary promising combination of a highly efficient separation technique and MS as detector is CE-MS. A wide range of analytes can be separated by capillary electrophoresis (CE) ranging from low-molecular weight compounds up to proteins[99]. However, the development of a robust CE-MS method is still challenging, since CE principally differs in theory and practice from HPLC and GC and only a few metabolic profiling studies have been carried out so far with this technique[100-102].

LC-MS in metabolite profiling and their application in quality control: some examples

In recent years, significant advances in LC-based on-line mass spectroscopy opened new avenues for increasingly comprehensive analysis of plant extracts. These possibilities have been exploited in metabolite profiling studies in which plant secondary metabolism was investigated from various perspectives. However, given the enormous difficulties of a comprehensive analysis, these studies have been usually focused on a limited range of compound classes.

The metabolite profile of the model plant *Arabidopsis thaliana* L. Heynh. has been investigated quite intensively by LC-MS[103-105].

Dan et al. investigated the metabolite profile in different plant organs of *Panax notoginseng* Wall. The authors identified numerous saponins in flowers, roots and rhizomes using UPLC coupled to a quadrupole time-of-flight mass spectrometer[106].

Several authors developed HPLC-MS methods to investigate the composition of *Ginkgo biloba* preparations. For example Jensen et al.[107] and Xie et al.[108] used LC-APCI-MS methods to evaluate the contents of bilobalides and ginkgolides of commercial *Ginkgo* preparations while a capillary LC system coupled to an ion trap spectrometer was applied by Ding et al. for a fingerprinting of *Ginkgo biloba* preparations[109].

Artemisia annua L. is a potent drug for the treatment of malaria. The activity of the plant is ascribed to the sesquiterpenelactone artemisinin, which is very effective against drug-

resistant plasmodium species[110]. In view of a possible use of an herbal preparation rather than the pure artemisinin, an HPLC-MS method was proposed for the identification and quantification of artemisinin and other constituents[111]. An HPLC-MS/MS method has been developed for a fingerprint profiling for seven different black cohosh (*Cimicifuga*) species and six different commercial products by Wang et al.[112] The validated method was utilised for a botanical identification of *Cimicifuga* species according to their triterpene glycoside patterns, and for quality control of black cohosh products.

A HPLC system connected to a hybrid ion trap and time-of-flight mass spectrometer was used for the determination of flavanolignans in the hepatoprotective plant milk thristle (*Silybum marianum* L. Gaertn.). The authors proposed the application of this method as a general method for quality control of milk thistle products[113].

Nuclear magnetic resonance spectroscopy approaches for metabolite profiling

General considerations

As a tool for metabolic profiling, NMR has some unique advantages over MS-based methods. It can provide a detailed view on the molecular composition in relatively short time while relatively simple sample preparation is required. NMR is a universal detector for all molecules with NMR-active nuclei, unlike MS where detection of analytes is dependent on ionisation conditions or UV spectrometers where only chromophore bearing compounds are detectable[84,114]. In proton NMR spectroscopy (^{1}H-NMR) the intensity of proton signals is proportional to the concentration of the metabolite. With the application of an internal standard, the real concentration of metabolites can be easy calculated[115]. Compared to modern mass spectrometry instrumentation, NMR spectroscopy is a rather insensitive analytical method but considerable effort has been conducted during the last years to overcome this issue. One major advantage of NMR

spectroscopy compared to other analytical techniques used for metabolite profiling is its matchless reproducibility and stability. A typical day to day variation often found with MS-based approaches is uncritical for NMR-based platforms[85,114].

Pattern recognition analysis in NMR fingerprinting

The information content of NMR spectra is exceeding that of mass spectrometry by far because of the fact that each proton or carbon in a molecule gives a unique signal. Therefore multivariate computer-based pattern recognition methods have been developed during the last years to reduce the complexity of ^{1}H-NMR profiles of herbal extracts. In this process spectra are analysed commonly after a data reduction step where each spectrum is digitised into integrated regions over fixed spectral intervals. After removal of solvent signals the spectra are normalised to the sum of the total spectral intensity to compensate differences in the amount of plant material analysed for each sample and the obtained data can be utilised for statistical analysis[114] (Figure 9).

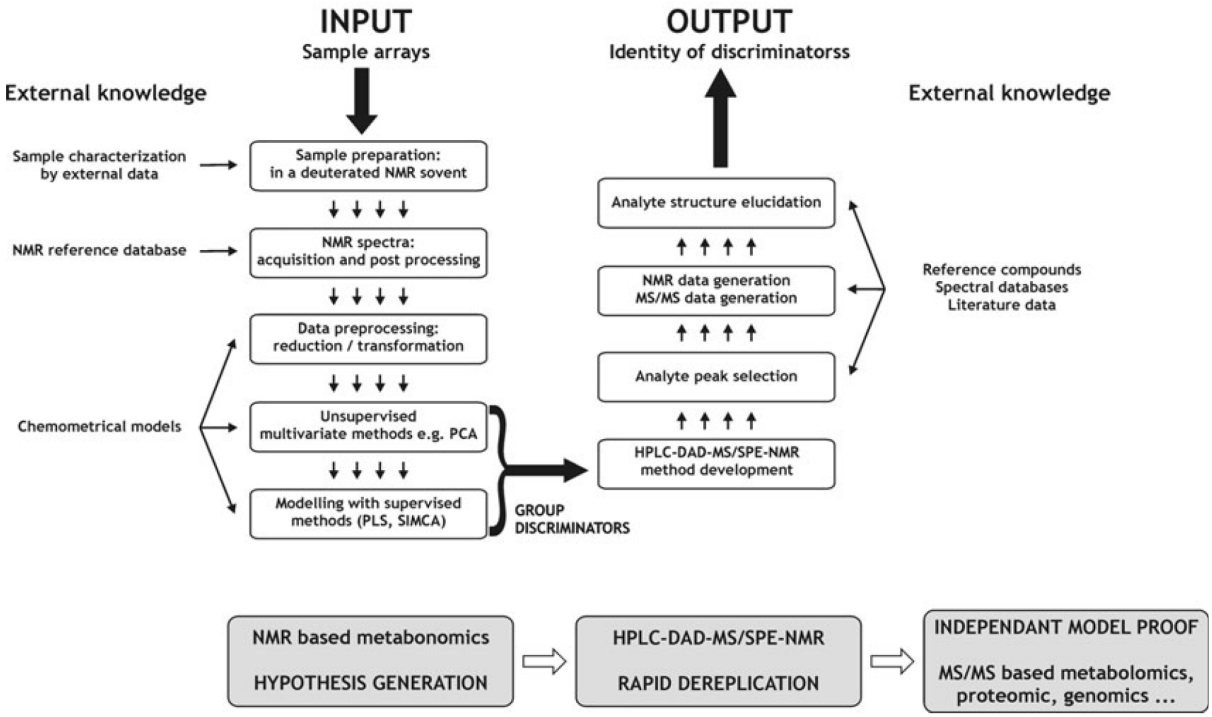


Figure 9. Schematic overview of the process of pattern recognition in complex biogenic matrices by a combination of NMR based metabonomics as discovery tool and modern hyphenated assays as analyte identification tools. Source: Holmes et al.[114]

The potential of this approach for a possible application in quality control of phytomedicines has been demonstrated in a recent study by Rasmussen et al. The authors investigated nine commercially available products of St. John's wort (*Hypericum perforatum*) by ^{1}H-NMR in combination with multivariate analysis and found major variations to their global composition[116]. A similar study has been carried out by Seger et al. with seven crude drug lots of *Hypericum perforatum.* They used ^{1}H-NMR fingerprinting with principal component analysis (PCA) and compared their results to those obtained by routinely used HPLC-DAD analysis and found that the discriminatory power between both methods was similar[117].

Bailey et al. investigated the composition of commercially available feverfew (*Tanacetum parthenium* L.) samples of several different manufacturers by high resolution NMR spectroscopy in combination with pattern recognition. A considerable

batch to batch variation was found even between products originating from the same manufacturer while a replication of experiments showed a high degree of reproducibility. They concluded that a better standardisation of these products is necessary[79].

NMR-based methods have also been applied to characterise extracts and phytopharmaceutical preparations of for example *Arnica montana* L.[118], *Artemisia annua*[111,119], *Matricaria recutita* L.[120], *Piper methysticum*[121], *Cannabis sativa* L. [122], *Panax ginseng* C. A. Mey.[123], *Angelica* sp.[124], *Ephedra* sp.[125] and *Ilex* sp.[126]

NMR Hyphenation: direct versus indirect techniques

No analytical technique is suitable for the detection, identification and quantification of all metabolites in an herbal sample[92]. The most common strategy for structural determination is to isolate and identify metabolites, but additional options for structure elucidation have been developed in the last years. These include hyphenation of the NMR spectrometer to HPLC (as HPLC-NMR or HPLC-SPE-NMR). Such technologies employ a chromatographic work-up of the analysed mixture prior to the structural characterisation without time consuming and laborious preparative-scale isolation processes. This speeds up extract dereplication and helps to avoid re-isolation of already known extract constituents.

“Continuous flow” (on-flow) experiments with direct coupling of the NMR spectrometer to a HPLC system were the first HPLC-NMR experiments to be done and are still frequently used to gain an overview of the metabolites present in an extract. The sensitivity of continuous flow methods is, however, limited by the short residence time of analytes in the NMR flow- cell, so the method is restricted to major components and only provides preliminary information.

The “stopped-flow” NMR technique addresses this problem by stopping the flow of the HPLC pump as soon as a fraction of interest is inside the NMR flow cell. Stopped-flow

experiments enable much longer spectra acquisitions because analytes can be kept for a longer time in the NMR flow-cell. Even the acquisition of 2D experiments is possible, which is necessary for definitive structural assignments. One major disadvantage of stopped-flow measurements is a diffusion-mediated band broadening of analytes on the HPLC column. This effect results in a substantial decrease of concentration within the elution bands and closely spaced peaks can merge. An analyte eluting as a large peak may further contaminate the following minor peaks because of the peak-broadening effect of the NMR flow cell.

In order to avoid these problems, a peak storage device for HPLC-NMR has been introduced that directs individual chromatographic peaks into capillary loops, where they are sealed and can be subsequently transferred into the NMR-flow probe. An advantage of this “loop storage” is that only minimal peak broadening occurs since the loops are made of capillaries and not of cavities like NMR flow-cells. Collected fractions can be measured in the NMR system as long as necessary and the flow cell can be washed between the transfer of individual peaks to avoid cross-contamination of analytes. Although the acquisition of NMR spectra is chronologically disconnected from the chromatographic separation, the NMR data are still obtained in the solvents used for HPLC separation. Therefore, the loop storage technique can be regarded as intermediate between direct and indirect hyphenated HPLC-NMR methods.

An alternative is the recently introduced HPLC-SPE-NMR technique, where HPLC peaks are collected on solid phase extraction (SPE) cartridges in order to remove the analyte from the HPLC mobile phase, followed by an elution with deuterated solvents. This indirect HPLC-NMR hyphenation offers numerous advantages compared to direct HPLC-NMR methods. The purified HPLC fractions typically elute in very small solvent volumes and are therefore highly concentrated. Moreover, the whole chromatography can be run with regular protic solvents, and deuterated solvents are only required for elution from the cartridge. Multiple trapping leads to an increase of analyte amounts

available for NMR, enabling acquisition of high-quality 2D NMR data within a short time. Working with this technology also has its difficulties. A prerequisite for a successful operation of the HPLC-SPE-NMR system is that the analyte is quantitatively retained on and eluted from the SPE cartridge. Selection of the appropriate SPE material and eluting conditions can be challenging especially for unknown compounds and needs to be optimised[127].

An overview on these HPLC-NMR transfer methods is given in Figure 10 and numerous examples of identified plant metabolites were discussed in recent reviews[128-133]

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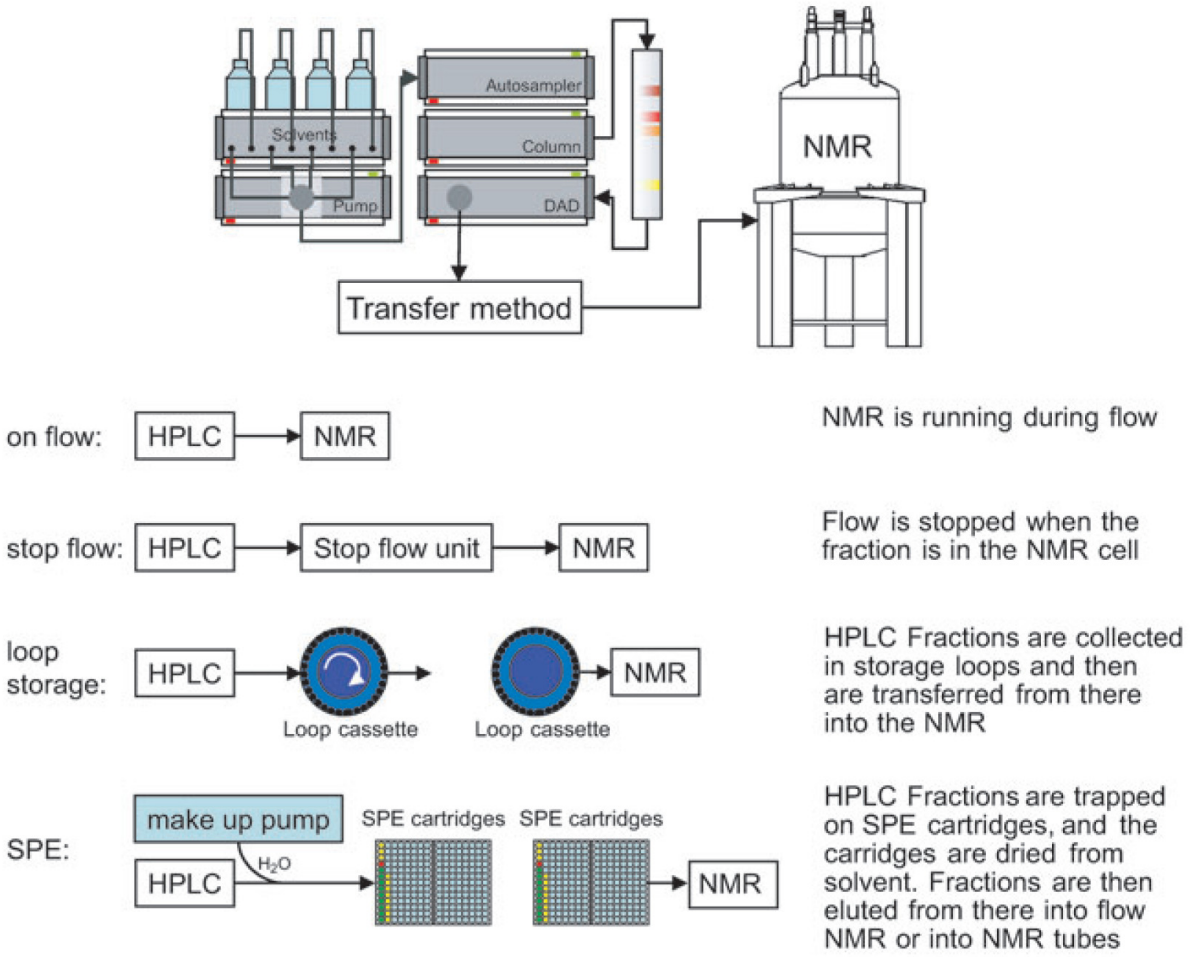

Figure 10: Various LC-NMR coupled techniques. Illustration from Bross-Walch et al.[132]

A different approach is the development of specially designed microliter NMR probes as the 1 mm triple resonance (TXI) (^{1}H,^{13}C,^{15}N) probe recently introduced into the market by Bruker. This probe has been especially developed for the structural characterisation of mass and volume limited samples such as natural products and allows the measurement of 1D ^{1}H-NMR or 2D and 3D inverse heteronuclear NMR experiments with a few micrograms of sample. Here a single glass capillary tube for each individual sample is used in contrast to previously described directly coupled HPLC-NMR systems. NMR capillaries with microfractionated LC peaks can easily be collected and efficiently measured with the TXI probe using NMR automation and sample changing routines. This off-line approach offers some additional benefits, for example that potential dead volume effects caused by long transfer capillaries are minimised, and those caused by valve switching and fluidic problems are eliminated. LC peak dilution is kept to an absolute minimum, which is crucial for obtaining an optimal signal-to-noise ratio of mass-limited samples. The size difference between a conventional 5 mm NMR tube and a 1 mm capillary tube is shown in Figure 11.

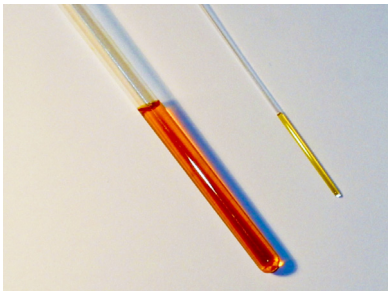

Figure 11: The 1 mm capillary tube (right) allows to identify structures from compounds in microgram range due to a higher sensitivity as result of a sample up-concentration (solvent volume approximately 5 µl). The standard NMR tube with an average sample volume of 500 µl is shown on the left side.

The enormous potential of this 1 mm microliter probe has been demonstrated recently. Schlotterbeck et al. found an increase in mass sensitivity by a factor of five, which corresponds to a 25 fold reduction in measuring time of the same sample amount compared to a conventional 5 mm probe[134]. Furthermore, the amount of expensive deuterated solvent could be reduced by a factor of 100. Griffin et al. used the 1 mm probe for a metabolic profiling of biological fluids from rats and mice. An active volume of 2 µl was used to acquire ^{1}H-NMR spectra of rat blood plasma and both rat and mouse cerebral spinal fluids - a volume which was easily removed without a required

termination of the animal. Several 1D and 2D experiments with different solvent presaturation sequences allowed the identification of numerous compounds, which finally allowed conclusions about the distribution of metabolites between blood plasma and cerebrospinal fluids[135].

HPLC coupling to mass spectrometry and NMR techniques: some examples

When considering advantages and disadvantages of NMR and MS technologies, it becomes obvious that both strategies are complementary since different analyte concentrations and analyte classes are addressed. A combination of these complementary techniques in a single setup coupled to HPLC has been described by Hostettmann et al. (Figure 12). The authors demonstrated the advantages of this powerful tool in extract screening for new antifungal agents and for the investigation of polyphenols in Gentianaceae species with monoamine-oxidase inhibitory (IMAO) properties[136,137].

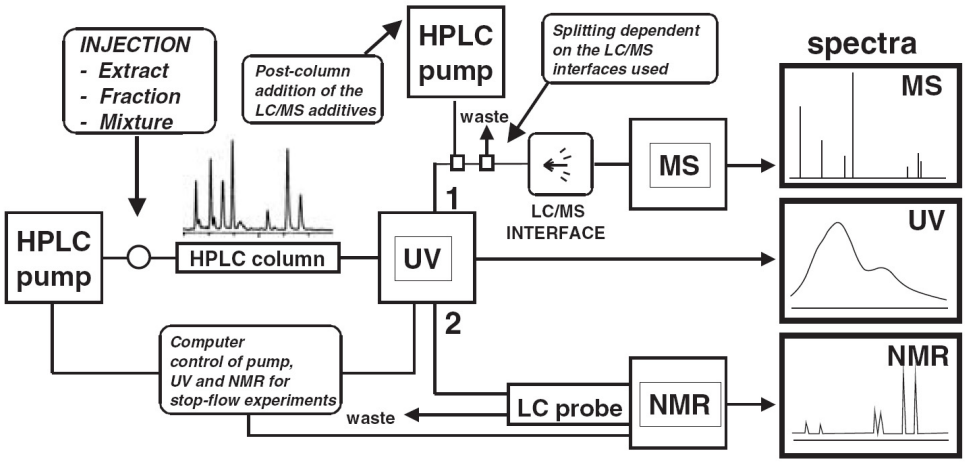

Figure 12: Schematic representation of an experimental setup used for LC-UV-MS-NMR analyses. Source: Hostettmann et al.[137]

Further studies with HPLC-MS-NMR-based methods have been carried out to identify substances from a variety of structural classes such as iridoid glycosides and phenyl propanoid glycosides from *Harpagophytum procumbens* Burch. DC ex. Meissn. (Devils claw)[138,139], pyrrolizidine alkaloids from *Senecio* sp.[140], iridoids from *Jamesbrittenia fodina* Wild O. M. Hilliard[141,142], oxylipins from *Arabidopsis thaliana*[143], carotenoids from *Spinacia oleracea L.* (spinach)[144], furanocoumarins and quinoline alkaloids from *Stauranthus perforatus* Liebm.[145]. Various structures were identified from *Hypericum perforatum*[146,147], *Matricaria recutita* (chamomile)[148], *Potamogeton* sp.[149,150], *Terminalia macroptera* Guill. & Perr. and *Jasminum subtriplinerve* Blume[151].

Bringmann et al. showed that it is possible to elucidate the complete structure (including absolute configuration) of novel secondary plant metabolites directly from the extract matrix without a single isolation step. They used a variety of on-line spectroscopic measurements (HPLC coupled to MS, NMR and CD detection) and elucidated the structures of several naphtylisoquinoline alkaloids from from the tropical lianas *Ancistrocladus griffithii* Planch.[152] and *Habropetalum dawei* (Hutch & Dalziel) Airy Shaw[153]. However, the circular dichroism (CD) detector is relatively insensitive and has so far only been applied for very few examples in natural products structure elucidation like in case of the biarylic naphtylisoqunioline alkaloids which have strong CD effects.

Other technology platforms in metabolite profiling

Some years ago, Fourier transform infrared reflectance (FT-IR) spectroscopy has been introduced as a metabolic fingerprinting technique for the analysis of plant material. This analytical technology enables a rapid, non-destructive, high-throughput analysis of a diverse range of sample types with a minimum of sample preparation. The principle of FT-IR is based on the fact that chemical bonds absorb irradiated light at a specific wavelength and vibrate in a number of ways such as stretching or bending vibrations.

Due to its holistic nature, FT-IR is able to detect a wide range of compound classes simultaneously, but it has, however, some drawbacks. IR absorption of water in samples under investigation is very intense and can lead to signal overlapping. Moreover, sensitivity and selectivity are lower compared to previously discussed methods. Gidman et al. successfully used FT-IR to investigate global metabolites changes as a result of plant-plant interferences[154] and Johnson et al. used this technology for metabolic fingerprinting of salt-stressed tomatos[155]. Fourier transform infrared spectroscopy has been applied to metabolomic questions far more than either raman or near infrared reflectance (NIR) spectroscopy. In NIR predominantly overtones and combination vibrations are measured while FT-IR spectra gave much more information in terms of chemical content[156]. NIR analysis was used by Huck et al. for a quantification of trimethoxyflavones in *Primula veris* L. extracts[157].

A new strategy in metabolomics is to focus on a cellular level and to investigate the metabolic flux between tissues and cells. Schneider and Hölscher studied the metabolite profile in specialised cells of different *Dilatris* species by using laser microsection (LMS) for tissue cryosectioning in combination with NMR and compared these obtained results to those of a whole leaf extract. By the application of this technology the authors could demonstrate that some of the secondary metabolites are located only in specific cells[158]. A similar approach was carried out on stone cells of Norway spruce bark with a combination of LMS/NMR/MS[159].

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3. RESULTS AND DISCUSSION

3.1 Quantification of active principles and pigments in leaf extracts of *Isatis tinctoria* by HPLC/UV/MS

(Mohn et al., Planta Med 2007; 73:151-156).

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Quantification of Active Principles and Pigments in Leaf Extracts of *Isatis tinctoria* by HPLC/UV/MS

Abstract

An HPLC method has been developed and validated for the quantification of the pharmacologically active principles tryptanthrin (**1**), 1,3-dihydro-3-[(4-hydroxy-3,5-dimethoxyphenyl)methylene]-2*H*-indol-2-one (indolinone) (**3**), indirubin (**4**), α-linolenic acid (**2**), and indigo (**5**), an isomer of indirubin, in extracts from the traditional anti-inflammatory plant *Isatis tinctoria* (woad). The chromatographic separation was performed on a C-18 column with a linear gradient of acetonitrile in water containing 0.1% formic acid. The method combines UV and electrospray MS detection in the positive ion mode for the detection of the alkaloids, with a switch to the negative mode for the analysis of α-linolenic acid. The method was applied to the analysis of woad extracts obtained by supercritical fluid (SFE) CO_2 extraction, and by pressurized liquid extraction (PLE) with dichloromethane and methanol, respectively. While the highest concentration of α-linolenic acid was found in the SFE extract (7.43%), the concentrations of tryptanthrin , indolinone, indirubin and indigo were the highest in the dichloromethane extract (0.30, 0.035, 2.48 and 0.84%, respectively). Compound **3** was not detected in the methanolic extract and only traces of compounds **1**, **4** and **5** and low amount of α-linolenic acid (0.39%) were present in this extract.

Key words
Isatis tinctoria · Brassicaceae · tryptanthrin · indirubin · indigo · indolin-2-one · linoleic acid · quantitative analysis · HPLC-MS

Introduction

Woad (*Isatis tinctoria* L., Brassicaceae) had been used in Central Europe since antiquity as indigo dye and as a medicinal plant for the treatment of inflammatory ailments [1] until it fell into oblivion due to the import of cheaper indigo and the disappearence of woad cultures. Woad was the main blue dye in the antique world and was reportedly used by Celtic and Teutonic warriors as a hair and skin colouring [1]. The taxonomically closely related *Isatis indigotica* remains until now one of the most important and popular herbal drugs of the Traditional Chinese Medicine (TCM) to be used in the context of inflammatory afflictions [2]. Based on this track record, we initiated some years ago a systematic investigation of the potential of *Isatis tinctoria* as an evidence-based herbal anti-inflammatory [3]. Among others, we identified several constituents responsible for the anti-inflammatory properties observed *in vitro* [3], [4], [5], [6], and confirmed the anti-inflammatory activity of lipophilic extracts and single compounds in animal models of acute and chronic inflammation, contact allergy, as well as rheumatoid arthritis [7], [8], and in a clinical pilot study of cutaneous irritation [9]. Tryptanthrin (**1**), α-linolenic acid (**2**), and 1,3-dihydro-3-[(4-hydroxy-3,5-dimethoxyphenyl)-methylene]-2*H*-indol-2-one (indolinone, **3**) were shown to be active principles inhibiting cyclooxygenase-2, 5-lipoxygenase, the expression of inducible nitric oxide synthase, human neurophil elastase, and the release of histamine from mast cells [3], [4], [5], [6], [10]. Indirubin (**4**) inhibits inflammatory reactions in delayed-type hypersensitivity [11] and is a potent inhibitor of cyclin-de-

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pendent kinase 5 and glycogen synthase kinase 3 [12], whereas indigo (**5**) is a side product of indirubin formation from indigo precursors during the drying of woad [13].

A quantitative assay for simultaneous dosage of the pharmacologically relevant constituents in woad is important in view of a development towards a new phytopharmaceutical. While quantitative assays have been reported for indigo [14] and tryptanthrin [15], [16], no method was available which would encompass all active constituents of woad. Here, we describe an HPLC procedure combining electrospray ionization (ESI) in the positive and negative modes with UV detection for the determination of **1**–**5** (Fig. **1**). We briefly report on validation and on the application of the assay to analysis of woad extracts obtained by accelerated solvent extraction (ASE) and supercritical fluid extraction (SFE).

Materials and Methods

Plant material

Leaf material was harvested in 2001 from first year plants (rosette stage) of a defined *Isatis tinctoria* L. genotype mix, "Thüringer Waid", grown on experimental plots of the Agricultural Research Station of Thuringia, Dornburg, Germany. A voucher specimen (ISAR01) is preserved in the Herbarium of the Institute of Pharmacy, University of Jena, Germany.

Solvents and chemicals

Tryptanthrin (**1**), indolinone (**3**), indirubin (**4**) and indigo (**5**) were synthesized according to established procedures [17], [18], [19]. Purities of **1** and **3**–**5** were ≥ 99% as determined by HPLC and NMR. α-Linolenic acid (**2**) (≥ 99.0%) was purchased from Sigma-Aldrich (Buchs, Switzerland). Solvents were of analytical grade (extraction) or HPLC grade (chromatography) and were from Scharlau (Barcelona, Spain). Formic acid was purchased from Sigma-Aldrich. HPLC grade water was obtained by an EASYpure II (Barnstead; Dubuque, IA, USA) water purification system.

Supercritical CO_2 extract

Fresh leaves were dried on a band drier operating at 60 °C and coarsely powdered with an SK 100 cross-beater mill (Retsch; Haan, Germany) equipped with a 2 mm sieve. The supercritical CO_2 (SFE) extract was prepared at the Adalbert-Raps-Zentrum, Technical University München-Weihenstephan, in a pilot-plant extractor by extraction with CO_2 at 800 bar and 50 °C for 3 h. The extraction yield was 0.8%. The extract corresponded to the SFE extract used in previous pharmacological and clinical studies [3], [7], [8], [9].

Dichloromethane and methanol PLE extracts

The plant material was dried in a thermostatted oven at 40 °C and powdered with an ultracentrifugal mill (Retsch ZM100) under continuous cooling with liquid nitrogen. The extracts were obtained by pressurized liquid extraction (PLE) using an ASE 200 extractor with solvent module (Dionex; Sunnyvale, CA, USA). Two cycles of extraction of 5 min each were performed. To prepare the dichloromethane extract, 5 g of powdered drug were extracted in a 22-mL cartridge at 70 °C and 120 bar. The methanol extract was obtained by extracting 1.0 g of drug in an 11-mL cartridge at 60 °C and 120 bar. The extracts corresponded to methanolic and dichloromethane extracts used in previous pharmacological and clinical studies [3], [7], [8], [9].

HPLC system

HPLC separations were carried out on an Agilent series 1100 system equipped with a degasser, a binary pump, a column oven and a DAD detector (Agilent Technologies; Waldbronn, Germany). A liquid handler 215 (Gilson; Mettmenstetten, Switzerland) was used as autosampler. The HPLC was coupled to an Esquire 3000 plus ion trap mass spectrometer (Bruker Daltonics; Bremen, Germany) fitted with an ESI source. Data acquisition and processing were performed using HyStar 3.0 software (Bruker Daltonics).

Optimization of the analytical method

Several stationary phases from different suppliers [Purosphere and Lichrospher (Merck; Darmstadt, Germany), Nucleodur (Macherey Nagel; Düren, Germany), Sunfire (Waters; Milford, MA, USA)] were tested in preliminary experiments. Due to its superior resolution power, the Sunfire column clearly showed the best results and was selected for further work. A linear gradient of acetonitrile in water containing 0.1% formic acid

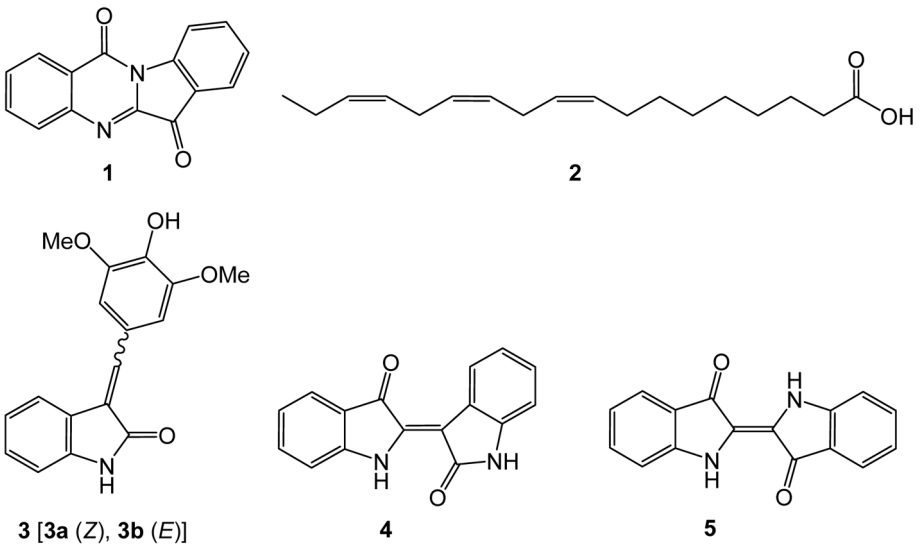

Fig. **1** Structures of compounds **1**–**5**.

proved to afford good separation and ionization of all compounds to be analyzed.

HPLC-UV-MS Analyses

Analyses were performed at 25 °C on a Sunfire C18 column (3.5 μm, 150 × 3.0 mm I.D.; Waters), equipped with a guard column (20 × 3.0 mm I.D.). The mobile phase consisted of water containing 0.1 % formic acid (eluent A) and acetonitrile (eluent B). The gradient program used was as follows: 10 % to 100 % B (30 min), 100 % B isocratic (5 min), 100 % to 10 % B (3 min). Before each separation, an equilibration time of 7 min was allowed. The flow rate was 0.5 mL/min and the injection volume was 20 μL. UV/Vis spectra were recorded in the range of 210 – 700 nm at a spectral acquisition rate of 2 scans/s. ESI-MS conditions were as follows: spectra were recorded under ion charge control conditions (ICC 30 000) at a scan speed of 13 000 *m/z*/s with a Gauss filter width of 0.2 *m/z*. Nitrogen was used as drying gas at a flow rate of 10 L/min and as nebulizing gas at a pressure of 30 psi. The nebulizer temperature was set at 300 °C. Positive ions were detected between 0 min and 25 min and negative ions between 25 min and 35 min. In the positive ion mode (0 – 25 min), spectra were recorded in the range from *m/z* 200 to 600 amu; capillary voltage was set at –4500 V, end plate offset at –500 V, capillary exit at 109.8 V, skimmer voltage at 65.0 V and trap drive at 39.8. In the negative ion mode (25 – 30 min), spectra were recorded in the range from *m/z* 180 to 650 amu; capillary voltage was set at 4500 V, end plate offset at –500, capillary end voltage at –111.8 V, skimmer voltage –40 V and trap drive at 43.7.

Method validation

Peak purity was established from the 2D-DAD (**1** and **4**) and MS data (**1** – **5**). Reproducibility was assessed with solutions of compounds **1** – **4** at 10 μg/mL (UV detection) or 1 μg/mL (ESI-MS detection). For indigo (**5**), a solution of 0.4 μg/mL was used. Analyses were performed in five replicates and the relative standard deviation was calculated (Table **1**). Retention time deviations were ≤ 0.03 min. Intra-day and inter-day repeatability was assessed in the course of the analysis with a standard mixture of **1** – **4**. No significant variation of UV signal intensity of **1** and **4** was observed (≤ 0.9 % after 18 h, ≤ 1.7 % after 65 h). MS signal sensitivity of **3** decreased over the time of analysis (–9.9 % after 18 h, –19.8 % after 68 h) while signal intensity of **2** remained less affected (+ 4.3 % after 18 h, –10.9 % after 65 h). Limit of detection (S/N ratio of 3) and limit of quantification (S/N ratio of 10) were determined by serial dilution of a standard solution containing **1** – **5** and are listed in Table **2**.

Accuracy was determined by spiking the dichloromethane extract with defined amounts of **1** – **5**. The amounts added were selected to be in the same range as the amounts of compounds originally present in the extract. Extract concentration in analysis was 1 mg/mL for **1** – **4**. For solubility reasons, indigo (**5**) was quantified in a separate experiment, at an extract concentration of 0.0025 mg/mL. Amounts added and recovery rates of **1** – **5** are listed in Table **3**.

Calibration curves

1 mg/mL stock solutions of **1** – **5** were prepared in DMSO. Calibration solutions containing a mixture of compounds **1** – **4** and separate reference solution of **5** were prepared from the stock solutions by dilution with methanol over a concentration range of 50 – 0.1 μg/mL (**1** – **4**) or 0.5 – 0.1 μg/mL (**5**). Each standard solution was measured in triplicate. For indolinone **3**, areas of MS signals corresponding to *Z* and *E* isomers were added. Calibration curves are shown in Table **4**.

Quantitative analysis of woad extracts

The dried extracts were dissolved with DMSO-MeOH (1 : 1) at a concentration of 5 mg/mL. Solutions at concentrations of 1.0 mg/mL, 0.1 mg/mL, 0.02 mg/mL and 0.005 mg/mL were prepared from the stock solution by dilution with methanol. Analyses were made in triplicate.

Table **1** RSD (%) (n = 5) for ESI-MS and UV detection of compounds **1** – **5**

	1	*2*	*3*	*4*	*5*
UV	0.3	nd	nd	0.8	10.6
ESI-MS	1.7	2.3	0.8	2.3	1.5

Amounts of compounds: UV: 200 ng (**1** and **4**) or 10 ng (**5**); ESI-MS: 20 ng (**1** – **4**) or 8 ng (**5**). nd: not determined.

Table **2** Limits of detection (LOD) and quantification (LOQ). Amount of compound injected [ng]

	LOD	*LOQ*
ESI-MS		
Tryptanthrin (**1**)	0.5	1.5
Linolenic acid (**2**)	0.5	1.5
Indolinone (**3**)	0.5	1.5
Indirubin (**4**)	0.25	0.75
Indigo (**5**)	0.5	1.5
UV		
Tryptanthrin (**1**) (254 nm)	2	5
Indirubin (**4**) (550 nm)	3	10
Indigo (**5**) (600 nm)	3	10

Table **3** Recovery rates

Compound	*Method*	*Amount added to extract*	*Recovered amount* *	*Relative recovery rates*
Tryptanthrin (**1**)	UV 254	60.0	60.5 ± 0.3	101
Tryptanthrin (**1**)	MS 249	60.0	60.8 ± 0.9	101
Linolenic acid (**2**)	MS 277	300.0	303.7 ± 2.4	101
Indolinone (**3**)	MS 298	5.00	5.31 ± 0.04	106
Indirubin (**4**)	UV 550	200.0	195.5 ± 0.8	98
Indirubin (**4**)	MS 263	200.0	211.7 ± 1.9	106
Indigo (**5**)	MS 263	4.00	3.51 ± 0.06	88

Amounts in ng on column (20 μL injection). Determinations were made in triplicate.

Table **4** Calibration curves for compounds **1** – **5** with ESI-MS or UV detection

Compound	*Method*	*Curve*	*Concentration* [mg/mL], R^2*
Tryptanthrin (**1**)	UV 254	$y = 8.6488x - 33.267$	0.001 – 0.05; $R^2 = 0.9999$
Tryptanthrin (**1**)	MS 249	$y = -609.42x^2 + 282895x + 76422$	0.0001 – 0.005; $R^2 = 0.9998$
Linolenic acid (**2**)	MS 277	$y = -63.702x^2 + 107169x - 101983$	0.0001 – 0.02; $R^2 = 0.9985$
Indolinone (**3**)	MS 298	$y = -181.53x^2 + 263424x + 339242 - 68630$	0.0001 – 0.01; $R^2 = 0.9998$
Indirubin (**4**)	UV 550	$y = 2.4893x - 17.573$	0.001 – 0.05; $R^2 = 0.9999$
Indirubin (**4**)	MS 263	$y = -826.86x^2 + 179170x + 62576$	0.0001 – 0.005; $R^2 = 0.9994$
Indigo (**5**)	MS 263	$y = -253.84x^2 + 31017x - 4739.7$	0.0001 – 0.0005; $R^2 = 0.9994$

Solutions were analysed in triplicates. For indolinone, areas of MS signals corresponding to *Z* and *E* isomers were added up.

* Ranges of concentration where the curves apply.

Results

The HPLC-MS analysis of a standard mixture of **1** – **5** under optimized conditions is shown in Fig. **2**. Separation on a reversed phase C-18 column with a linear gradient of acetonitrile in water containing 0.1 % formic acid provided a good separation of compounds **1** – **5** within 30 min. The separation was carried out at a flow rate of 0.5 mL/min without post-column eluent split. ESI-MS detection of the alkaloids **1** and **3** – **5** was performed in the positive ion mode. After 25 minutes, the polarity was switched to the negative mode for the measurement of α-linolenic acid. The positive ESI mass spectra showed strong $[M + H]^+$ quasimolecular ions together with $[M + Na]^+$ adducts. In the spectrum of α-linolenic acid, $[M - H]^-$ was the base peak. At the same time, UV detection at 254 and 550 nm could be used for the detection of tryptanthrin and indirubin, respectively. Calibration curves (see Table **4**) were found to be linear over a broad concentration range for UV detection in case of **1** and **4** ($R^2 > 0.999$), or fitted quadratic functions in the case of mass spectrometric detection ($R^2 > 0.998$). Spiking experiments demonstrated the accuracy of the method. Recovery rates between 98 and 106 % were obtained for compounds **1** – **4**. A recovery rate of 88 % was found for indigo (**5**) which had to be quantified in a highly diluted extract solution due to its poor solubility (see Table **3**).

The RSD for UV detection was lower than for MS detection (0.3 – 0.8 % *vs.* 0.8 – 2.5 %), but LOD and LOQ were approximatively 5-fold lower for MS (Table **2**). Due to better reproducibility and repeatability, UV detection was selected for the quantitative assay of tryptanthrin (**1**) and indirubin (**4**). Determination of indigo (**5**), indolinone (**3**) and α-linolenic acid (**2**) was performed with ESI-MS. Extracted ion traces corresponding to $[M + H]^+$ and $[M - H]^-$ quasimolecular ions, respectively, were used for signal integration. Due to the extremely low solubility of indigo, the calibration curve had to be measured with highly dilute solutions. Thus, the calibration range was much smaller than for other con-

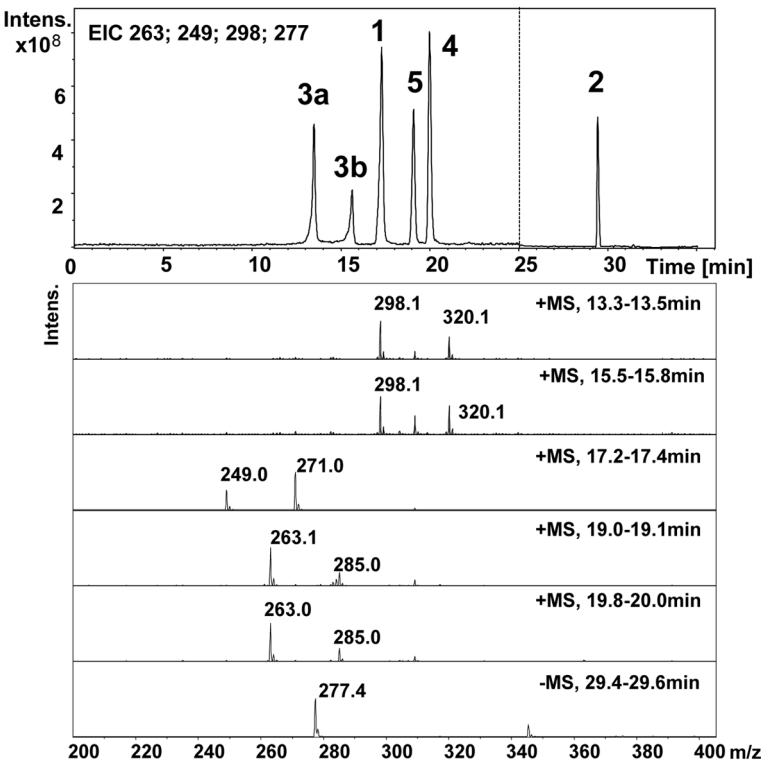

Fig. **2** HPLC/MS analysis of a mixture of **1** – **5** (10 ng each). Compound **3** exists as a mixture of *Z* (**3a**) and *E* (**3b**) isomers in equilibrium.

Table **5** Content (%) of compounds **1** – **5** in woad extracts

Compound	*Method*	*CO_2 (SFE)*	*CH_2Cl_2 (PLE)*	*MeOH (PLE)*
Tryptanthrin (**1**)	UV 254	0.224 ± 0.002[a)]	0.299 ± 0.001[a)]	nd[e)]
Linolenic acid (**2**)	MS 277	7.43 ± 0.31[b)]	3.58 ± 0.24[b)]	0.393 ± 0.014[a)]
Indolinone (**3**)	MS 298	0.0261 ± 0.0005[a)]	0.036 ± 0.001[a)]	nd[d)]
Indirubin (**4**)	UV 550	1.29 ± 0.02[a)]	2.48 ± 0.01[a)]	0.066 ± 0.004[e)]
Indigo (**5**)	MS 263	0.150 ± 0.003[c)]	0.84 ± 0.01[d)]	0.047± 0.002[c)]

Measurements were made in triplicates.

Extract concentrations used were: [a)] 1.0 mg/mL, [b)] 0.1 mg/mL, [c)] 0.02 mg/mL, [d)] 0.005 mg/mL, [e)] 5.0 mg/mL. nd: not detected.

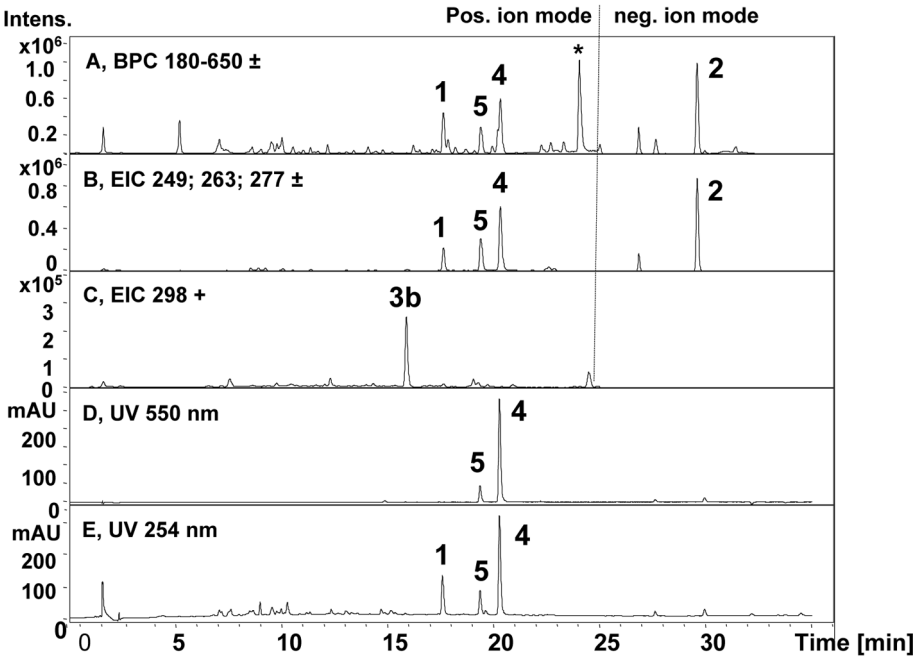

Fig. **3** HPLC/UV/MS analysis of the dichloromethane PLE extract of woad. Traces A (base peak chromatogram) and B: 2 μg of extract; traces C – E: 20 μg of extract. Only the *E* isomer of indolinone was detected. * System peak.

stituents (see Table **4**). In the case of indolinone **3**, *E* and *Z* isomers which are known to undergo slow inter-conversion at room temperature [4], [18] were both present in the reference substance. Since comparable ionization properties can be reasonably assumed for both isomers, the peak areas of both isomers were added for quantitative determination.

We then applied the method for the determination of compounds **1** – **5** in different woad extracts. The SFE and dichloromethane extracts corresponded to the preparations which had been previously found to possess *in vivo* anti-inflammatory properties in various animal models and in the clinical pilot study [7], [8], [9], while the methanolic PLE extract was supposed to contain the entire spectrum of secondary metabolites of woad. The compounds to be dosed in these extracts were thus present at different concentrations. For this reason and due to the higher sensitivity of MS detection as well, different extract concentrations were used for the quantitative determinations, depending on compound, extract and detection mode (see Table **5**). In particular, high dilutions of extracts (0.002 mg/mL for SFE and methanol extracts, 0.0005 mg/mL for the dichloromethane extract) were required for the quantification of indigo due to the poor solubility of this compound. Interestingly, while both geometric isomers of **3** were present in the reference compound, only **3b** was detected in the extracts. It has been tentatively assigned the *E* configuration since only the *E* isomer has been reported until now in the genus *Isatis* [20]. The analysis of **1** – **5** in the dichloromethane extract is shown in Fig. **3**, together with the UV and extracted ion traces used for peak integration.

The highest concentration of α-linolenic acid (**2**) was found in the SFE extract whereas the highest concentrations of the alkaloids were present in the dichloromethane extract. The quantitative differences between the two extracts were in accord with their lipophilicity and respective solubilities in the two extraction media. Compound **3** was not detected in the methanolic extract, and only traces of compounds **1**, **4** and **5** and a low amount of α-linolenic acid (0.39%) were found (Table **4**). This extract contained mainly highly polar compounds which eluted very early. *Isatis tinctoria* is known to contain high concentrations of indigo precursors [21], [22], glucosinolates [3] and other glycosides [23].

Discussion

The method presented here enables a quantitative determination of the pharmacologically relevant constituents in *Isatis tinctoria* extracts and the analysis of the dye indigo in a single run. We had previously developed assays for the determination of tryptanthrin in woad extracts [15] and in dialysates collected by cutaneous microdialysis [16]. This isocratic method, however, was not suitable for the dosage of compounds with a broad polarity range as for **1** – **5**. For tryptanthrin (**1**), the sensitivity (LOD and LOQ) in MS detection in the method developed here is comparable to that achieved in the previous assay with external standard (LOQ 1 ng) [15], and slightly lower than that of the stable isotope dilution assay (LOQ 500 pg) [16]. However, given that the tryptanthrin concentration in the lipophilic extracts is sufficiently high, UV detection was preferred here because of smaller RSD.

A major difficulty in the dosage resulted from the widely differing concentrations of compounds **1** – **5** in extracts. Compounds such as the pharmacologically highly potent alkaloids tryptanthrin (**1**) and indolinone (**3**), and indigo (**5**) were present in very low concentrations, whereas linolenic acid (**2**) reached up to 7.4 % in the SFE extract. Indigo (**5**) is *per se* not a pharmacologically relevant compound. However, it is here an unwanted side product in the formation of the bioactive indirubin (**4**) from indigo precurors [13] and, as such, should be determined.

We recently carried out extensive surveys of seasonal and post-harvest changes in the content in indigo precursors and tryptanthrin, analyzing shock-frozen and conventionally dried *Isatis tinctoria* and *I. indigotica*, and observed profound changes in the secondary metabolite profiles during the drying process [21], [22]. Pharmacologically important compounds such as tryptanthrin (**1**) and indirubin (**4**) are formed after harvest from indigo precursors in the case of **4** [13], or via unknown pathways in the case of **1** [24]. Quantitative determination of these compounds and the other anti-inflammatory compounds in herbal raw material and in extracts is thus important for quality control in the development of a phytomedicine based on woad extract. Given the phytochemical similarity and taxonomical proximity with European woad, the assay developed here will also be useful for the quality assessment of the TCM herb *Isatis indigotica* and products derived from it.

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3.2 A comprehensive metabolite profiling of *Isatis tinctoria* leaf extracts

(Mohn et al., Phytochemistry; submitted for publication).

A comprehensive metabolite profiling of *Isatis tinctoria* leaf extracts

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Abstract

A broad-based characterisation of a pharmacologically active dichloromethane extract from *Isatis tinctoria* leaves was carried out. For a comprehensive picture we also included the polar constituents of *I. tinctoria* (MeOH extract) and, for comparative purposes, the taxonomically closely related TCM plant *I. indigotica*. PDA, ELSD, APCI- and ESI-MS, and ESI-TOF-MS detectors were used in parallel to ensure a wide coverage of secondary metabolites with highly diverging analytical properties. Off-line microprobe NMR after peak purification by semi-preparative HPLC served for structure elucidation of some minor constituents.

More than 65 compounds belonging to various structural classes such as alkaloids, flavonoids, fatty acids, porphyrins, lignans, carotenoids, glucosinolates and cyclohexenones were unambiguously identified, and tentative structures proposed for additional compounds. Numerous compounds were identified for the first time in the genus *Isatis,* and *a* new indolic alkaloid was discovered.

Keywords: *Isatis tinctoria*, *Isatis indigotica*, Brassicaceae, Metabolite profile, LC-MS, Pressurised Liquid Extraction, offline LC-NMR

1. Introduction

Woad (*Isatis tinctoria* L., Brassicaceae) has been used in Central Europe since antiquity as indigo dye and as medicinal plant for the treatment of inflammatory diseases until it fell into oblivion due to import of cheaper indigo and, as a consequence, the disappearance of woad cultures (Hamburger 2003). In China, Banlangen (*Isatis* root) and Daqingye (*Isatis* leaf) from the taxonomically closely related *Isatis indigotica* Fort. remain until now important and popular herbal drugs in Traditional Chinese Medicine (TCM) for the treatment of inflammatory diseases. Over the past years, we have been exploring the potential of *Isatis tinctoria* extracts as new active ingredient for anti-inflammatory phytopharmaceuticals. As a starting point we confirmed the anti-inflammatory potential of lipophilic (CO_2 and dichloromethane) *I. tinctoria* leaf extracts in a broad-based pharmacological screening (Hamburger, 2003). With the aid of HPLC-based activity profiling (Potterat and Hamburger, 2006) we then identified several pharmacologically active metabolites in the extracts, such as tryptanthrin (**13**), α-linolenic acid (**22**) and (*E*)-3-(3',5'-dimethoxy-4'-hydroxy-benzylidene)-2-indolinone (**9**) as inhibitors of cyclooxygenase-2, 5-lipoxygenase, expression of inducible nitric oxide synthase (iNOS), human neutrophil elastase, and release of histamine from mast cells (Danz et al., 2001; Danz et al., 2002; Hamburger et al., 2006; Oberthür et al., 2005; Rüster et al., 2004). These findings were corroborated by pharmacological studies on tryptanthrin (**13**) and indirubin (**15**) performed by other groups (Hoessel et al., 1999; Honda et al., 1980; Ishihara et al., 2000; Kimoto et al., 1999). The potential of lipophilic *Isatis tinctoria* extracts was substantiated by *in vivo* studies in models of acute and chronic inflammation, contact allergy, and rheumatoid arthritis (Recio et al., 2006a, b), and by a clinical pilot study in experimentally induced skin erythema (Heinemann et al., 2004). With the aid of a skin microdialysis study, we demonstrated that in topical application the extract matrix had a solubilising and penetration enhancing effect on the poorly soluble alkaloid tryptanthrin (**13**) (Oberthür et al., 2003).

Parallel to these pharmacological investigations, we identified the correct structure of the major indigo precursors in woad (Oberthür et al. 2004) and identified new flavone

glycosides (Cheng et al., 2005). We analysed composition and seasonal variation of indigo precursors and glucosinolates in defined accessions of *Isatis tinctoria* and *I. indigotica* leaves and seeds, and investigated the influence of harvest regimen on the chemical composition of woad leaves (Oberthür et al., 2004; Mohn and Hamburger, 2008; Mohn et al., 2008). We observed profound post-harvest changes in metabolite pattern during the drying of leaves, chiefly the virtual disappearance of glucosinolates and indigo precursors, and the appearance of tryptanthrin (**13**) (Oberthür and Hamburger, 2004), besides the well known post-harvest artefacts indigo (**14**) and indirubin (**15**).

There is a difficulty with the chemical characterisation of herbal extracts used as phytomedicines. Regulatory agencies such as BfArM (Germany) or Swissmedic (Switzerland) consider the extract in its entiety as active ingredient. Manufacturers of phytomedicines often use active marker substances for quality control purposes. For very few phytopharmaceuticals, the global composition of the active ingredient (extract) has been analysed in a more comprehensive manner and data published. Notable examples include the special extracts EGb 761 (derived from *Ginkgo biloba* leaves) (Hänsel and Spieβ, 2004) and ZE 331 (obtained from *Petasites hybridus* leaves) (Brattström, 2003). However, the analyses include only a limited number of compound classes, and major portions of these extracts remain uncharacterised.

In recent years, significant advances in LC-based on-line spectroscopy opened new avenues for increasingly comprehensive analysis of plant extracts. These possibilities have been exploited in metabolite profiling studies in which plant secondary metabolism was investigated from various perspectives (Burns et al., 2003; Dan et al., 2008; Ding et al, 2008; Iijima et al., 2008; Le Gall et al., 2005; Long et al., 2006; Qiao et al., 2008; Schliemann et al., 2008; Yamazaki et al., 2003). However, given the enormous difficulties of a comprehensive analysis, these studies have been usually focused on a limited range of compound classes.

Here, we report on a broad-based characterisation of the pharmacologically active extract from *Isatis tinctoria* leaves, using multiple detection systems to ensure a wide coverage of secondary metabolites with highly diverging analytical properties. For a comprehensive picture we also included the polar constituents of *I. tinctoria* (MeOH

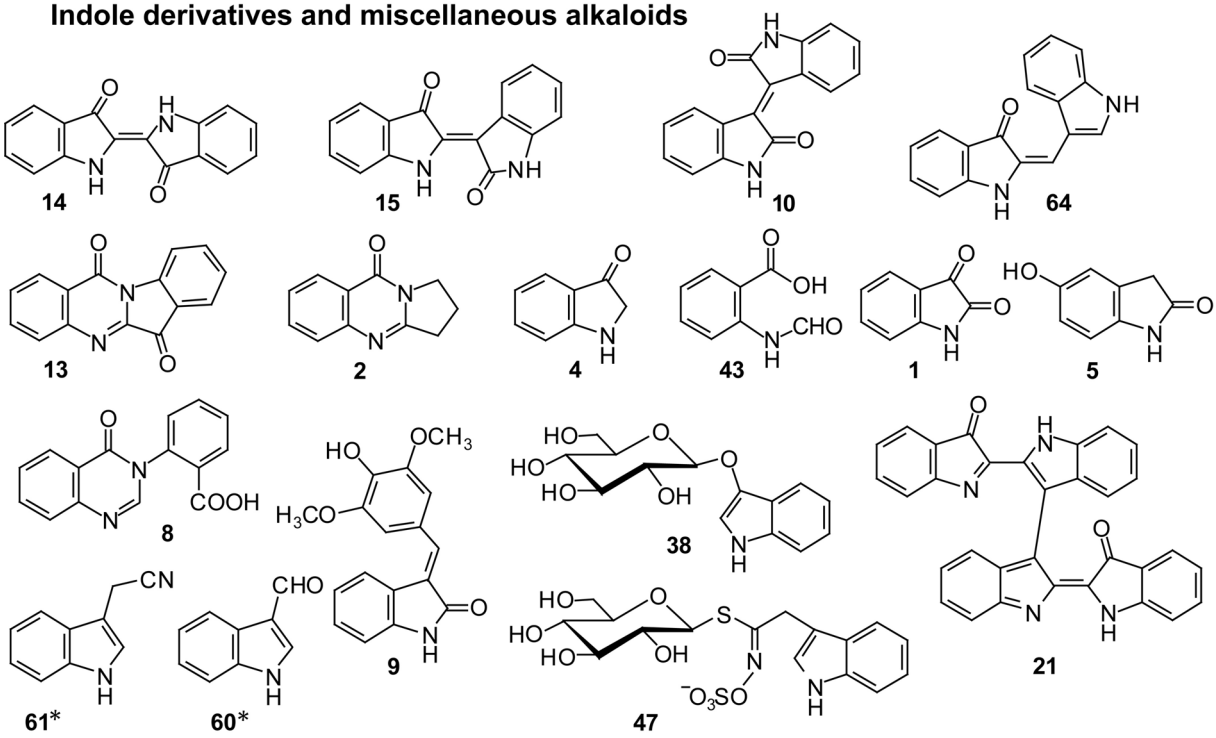
extract) and, for comparative purposes, the taxonomically closely related TCM plant *I. indigotica*.

2. Results

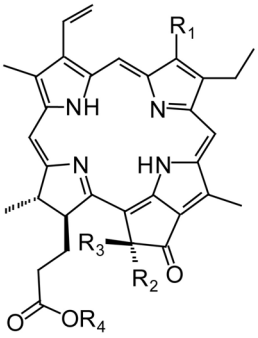
2.1 Metabolite profile of Isatis dichloromethane extracts

Isatis tinctoria and *Isatis indigotica* leaves were submitted to pressurised liquid extraction (PLE) using dichloromethane and methanol. The dichloromethane extract corresponded to the pharmacologically active lipophilic extract reported earlier (Hamburger, 2003). Extracts were analysed by HPLC-PDA-MS and HPLC-TOF-MS. An overview of identified compounds is given in Figure 1. Figure 2A shows the HPLC profile of an *I. tinctoria* dichloromethane extract on C_{18} column (HPLC system 1). For the characterisation of the pharmacologically active extract in its full complexity, we combined several detectors as PDA, ESI-MS and APCI-MS (positive and negative ion mode). The unspecific evaporative light scattering detector (ELSD) was used to ensure that all separated compounds were assigned with at least one mass spectrometric detection mode. Additional structural information was obtained by MS/MS experiments and by high-resolution mass spectra recorded by ESI-TOF-MS (traces are not shown in Figure 2A). An overview of all compounds identified in the dichloromethane extract, and molecular formulae and UV data of further compounds is given in Table 1. Compounds **1-4**, **6-7**, **9-10**, **13-15**, **18**, **22**-**25**, **29**, **30**, **33**, **34** and **37** were identified by analysis of on-line spectroscopic data and subsequent confirmation by co-chromatography with reference compounds.

Indole derivatives and miscellaneous alkaloids

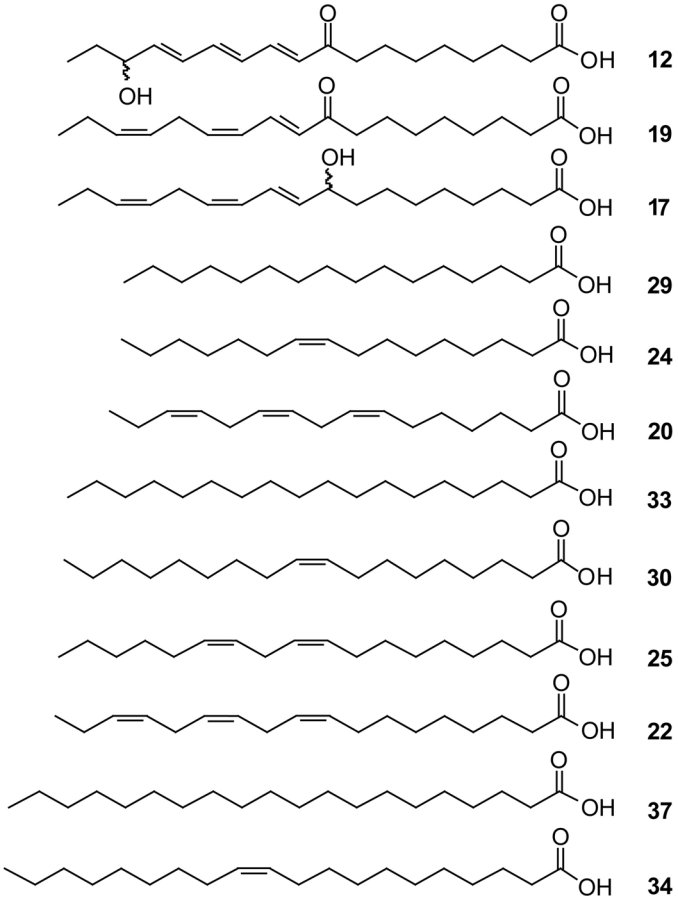


Porphyrins



R_1	R_2	R_3	R_4	
CH_3	$COOCH_3$	H	H	**27**
CH_3	H	$COOCH_3$	H	**28**
CH_3	H	H	H	**31**
CH_3	$COOCH_3$	OH	H	**26**
CHO	$COOCH_3$	H	$C_{20}H_{39}$ (phytyl-)	**56**
CH_3	$COOCH_3$	H	$C_{20}H_{39}$ (phytyl-)	**57**
CHO	H	H	$C_{20}H_{39}$ (phytyl-)	**58**
CH_3	H	H	$C_{20}H_{39}$ (phytyl-)	**59**

Fatty acids



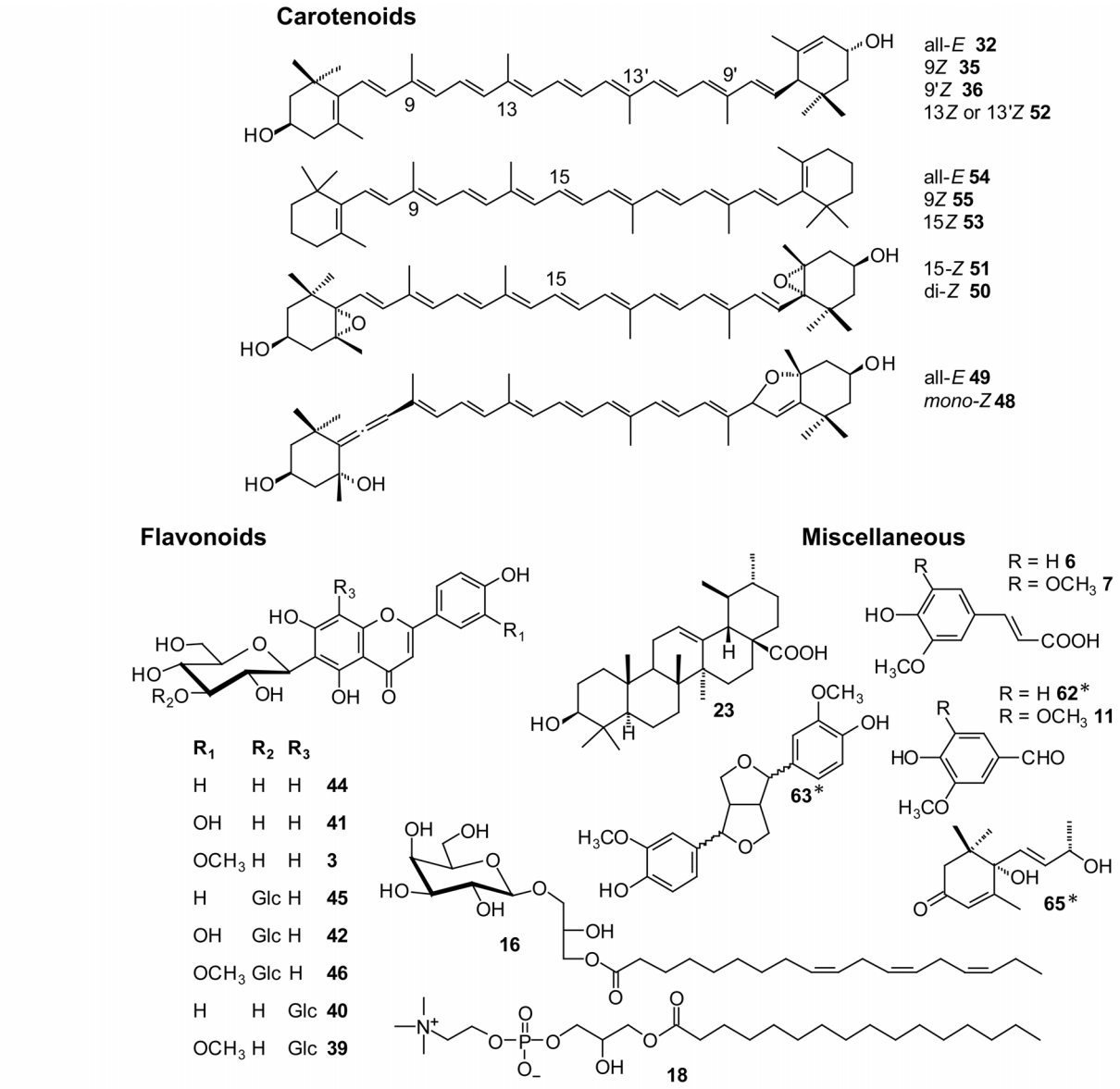


Figure 1. Structures of compounds **1** to **65**. Consecutive numbering is according to their chromatographic retention. Compounds designated with (symbol ∗) were not detectable in the crude extracts and were identified only after MPLC pre-fractionation.

Compounds **5**, **8**, **20**, **21**, **26**-**28**, **31**, **32**, **35** and **36** were identified on the basis of their UV data in combination with MS^n, high resolution-MS, and literature data on their chromatographic retention (Terasaki et al., 2002; van Breemen et al., 1991; Canjura and Schwartz, 1991; de Rosso and Mercadante, 2007a, b). The alkaloids 5-hydroxyoxindole (**5**), 3-(2'-carboxyphenyl)-quinazolin-4-on (**8**) and bisindigotin (**21**) have been described for *I. tinctoria* or *I. indigotica* before (Li et al., 2000; Wei et al., 2005; Wu et al., 1997) while the unsaturated fatty acid ((7*Z*, 10*Z*, 13*Z*)-hexadecatrienoic acid (**20**)) and the porphyrins 10-hydroxy-phaeophorbide (**26**), phaeophorbide a (**27**), its epimeric form phaeophorbide a' (**28**) and

pyrophaeophorbide a (**31**), and the lutein isomers (**32**), (**35**) und (**36**) are compounds that are widely occurring in higher plant families.

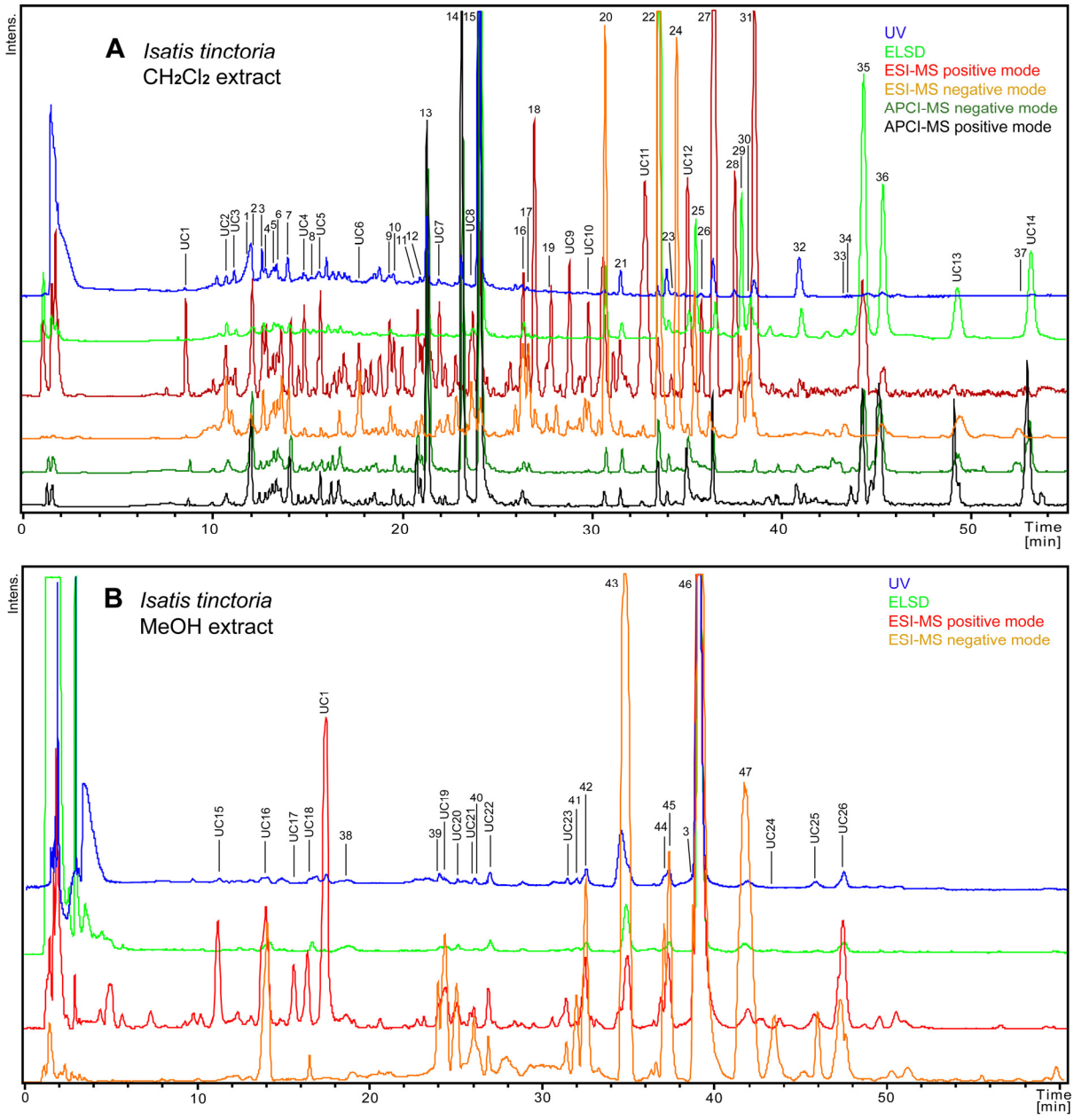


Figure 2. HPLC profiles of *Isatis tinctoria* dichloromethane (**A**) and methanol (**B**) extracts recorded with UV, ELSD, and ESI-MS and APCI-MS detectors in positive and negative ion mode. Peak labelling designates unambiguously identified compounds, and compounds for which only partial structural information was obtained (UC1 to UC26).

Additional peaks of interest which could not be identified by on-line spectroscopy and reference compounds were earmarked for small scale purification and off-line NMR analysis. The dichloromethane extract was separated by MPLC into seven fractions, and fractions 2 and 3 were separated by semi-preparative HPLC. Collected peaks were submitted to NMR and TOF-MS analysis after removal of solvents. Structures of additional 12 compounds (**11**, **12**, **16**, **17**, **19**, **43**, **60**-**65**) were elucidated with this approach. NMR spectra of **60-62** were identical with those of available reference compounds. Other compounds were identified by comparison with literature data, e.g. syringic aldehyde (**11**) (Hiltunen et al., 2006), 16-hydroxy-9-oxo-(10*E*, 12*E*, 14*E*)-octadecatrienoic acid (**12**) (Yoshikawa et al., 1998) , 1-*O*-β-D-galactopyranosyl-3-*O*-linolenylglycerol (**16**) (Hohmann et al., 1996), 9-hydroxy-(10*E*, 12*Z*, 15*Z*)-octadecatrienoic acid (**17**) (McLean and Reynolds, 1996), 9-oxo-(10*E*, 12*Z*, 15*Z*)-octadecatrienoic acid (**19**) (Koch et al., 2002), pinoresinol (**63**) (Xie et al., 2003) and 6,9-dihydroxy-4,7-megastigmadien-3-one (**65**) (Cutillo et al., 2005) (configurations of hydroxyl groups for compounds **12** and **17**, and absolute and relative configurations for compounds **63** and **65** were not determined). The separation of fraction 3 gave a peak at 48.5 min which was identified as a new compound, (2*Z*)-2-(1*H*-indol-3ylmethylidene)-1,2-dihydro-3*H*-indol-3-one (**64**) (Fig.1). Its molecular formula was established as $C_{17}H_{12}N_2O$ by HR-ESI-MS (m/z 261.1031 ($[M+H]^+$, calc. 261.1029). HSQC and HMBC spectra (Fig. 2S) revealed that the ^{13}C resonances of 16 carbons were in the range of 105 -153 ppm and indicated a highly aromatic character of **64**. A carbonyl resonance appeared at δ 185.0 ppm. Two NH groups (δ 9.28 and 11.91) and ten additional protons were observed in the ^1H-NMR spectrum. The signals at δ 6.89 (H-5), 7.17 (H-7), 7.17 (H-5'), 7.22 (H-6'), 7.48 (H-6), 7.48 (H-7'), 7.57 (H-4), 7.83 (H-4') were characteristic for two ortho-substituted benzene moieties, and two singulets at δ 7.07 (H-10) and 8.17 (H-2') indicated two additional double bonds. 1D-TOCSY experiments were used for the determination of overlapping aromatic signals, while HSQC and HMBC correlations (Figure 3) were used for further structure assignments. 1D NOESY experiments indicated spacial proximity of H-1 (9.28 ppm), H-10 (7.07 ppm) and H-2' (8.17 ppm) and, consequently, a *Z*-configuration of the linkage of the two indole moieties in **64**.

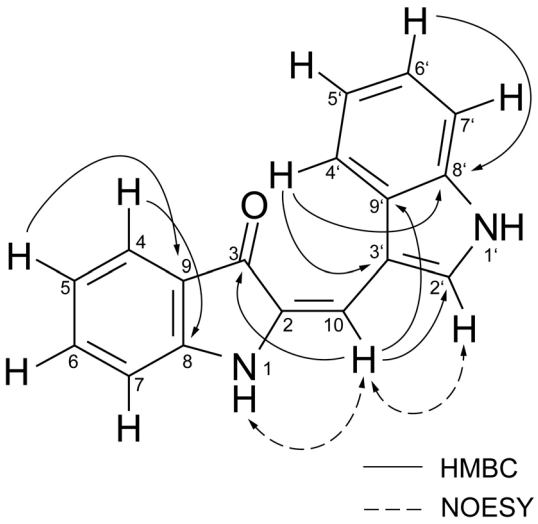


Figure 3. Structure of new indole alkaloid **64** (2*Z*)-2-(1*H*-indol-3-ylmethylidene)-1,2-dihydro-3*H*-indol-3-one with key HMBC and NOESY correlations.

Numerous compounds from the dichloromethane extract of *I. tinctoria* could be also detected in the closely related species *I. indigotica.* A complete list of identified compounds and HPLC profiles obtained with PDA, ELSD and MS detectors are given as supplementary data (Table 1S and Figure 1S).

2.2 Profiling of Isatis methanol extracts

In previous studies (Oberthür et al., 2004, Mohn et al., 2008), we had observed profound changes in the spectrum of polar metabolites in woad leaves during the drying process. Therefore, we carried out a profiling of the MeOH extracts following extraction with dichloromethane. The separation (HPLC system 2) of the *I. tinctoria* MeOH extract is shown in Figure 2B. With exception of peaks **38** and **47** (indican and glucobrassicin), the extract mainly contained flavone-*O*- and *C*-glycosides. Compounds **3**, **38**, **41**, **44**-**47** were identified with the aid of spectroscopic data (Table 2), and structures were confirmed by co-chromatography with reference substances. Characteristic MS/MS fragments in combination with UV and HRMS spectra, retention time and literature data were used for the identification of the flavone-di-*C*-glycosides **39** and **40** and for compound **42** with *O*- and *C*-linked sugar moieties (Gattuso et al., 2006; Deng et al., 2008). No fragments corresponding to a cleavage of a hexosyl moiety ($[M-162]^+$) were observed in the ESI-MS spectra of **39** and **40**

recorded in positive ion mode, but numerous small fragments resulting from two *C*-glycosidic moieties. In contrast, the spectrum of **42** showed loss of an *O*-glycosidic hexosyl moiety, followed by a partial fragmentation of *a C*-linked hexosyl moiety in MS^2 and MS^3. These results are in agreement with literature reports (Gattuso et al., 2006; Li and Claeys, 1994; Waridel et al., 2004; Wolfender et al., 2001) and own experiments with flavone *O*- and *C*-glycosides. Glucobrassicin (**47**) and indican (**38**) were the only glucosinolates and indigo precursors that could be detected.

Semi-preparative isolation and NMR analysis of a peak at 35.0 min lead to the structure of *N*-formyl anthranilic acid (**43**), a compound which has been described for *I. tinctoria* by Hartleb (1994).

The majority of compounds identified from the MeOH extract of *I. tinctoria* were also present in *I. indigotica* (Fig. 1S and Table 2S; supplementary data). Besides the absence of some unidentified minor compounds only N-formyl anthranlic acid (**43**) and glucobrassicin (**47**) were not detected in *I. indigotica*. Absence of the latter compound in *I. indigotica* leaves had already been observed (Mohn et al. 2008).

2.3 Profiling of carotenoids

Several carotenoids (**32, 35** and **36**) were detected in the dichloromethane extract of *Isatis tinctoria* and *I. indigotica* leaves. Extraction and separation conditions, however, were not optimal for analysis of these highly lipophilic metabolites. For a more comprehensive analysis of the carotenoid pattern in dried leaves, we used dedicated extraction and separation protocols (extraction with hexane / acetone (1:1) (Britton, 1995) and HPLC on a C_{30} column (de Rosso and Mercadante, 2007b)). A HPLC chromatogram is shown in Figure 4, and an overview of identified compounds and spectroscopic data is given in Table 3. Although only (all-*E*)-β-carotene (**54**) was available as reference compound, it was possible to identify other carotenoids (**32**, **35**, **36** and **48**-**55**) by a combination of spectroscopic and chromatographic information, including UV-vis spectra (λ_{max}, relative intensities of absorption maxima, and presence/absence of an absorption band at approximately 330 nm indicative of *cis*-configured double bonds), elution order on a C_{30} column, APCI-MS and MS/MS

data, and comparison with published literature (de Rosso and Mercadante, 2007a, b). The elution order for *E*- and *Z*- isomers has been described for lutein by Updike and Schwartz (2003), for violaxanthin by Meléndez-Martínez et al. (2007), and for β-carotene by Jaime et al. (2007).

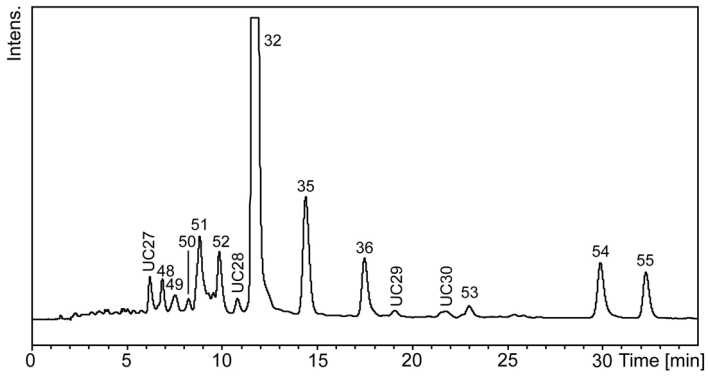


Figure 4. Carotenoid profile of *Isatis tinctoria* hexane/acetone leaf extract. Peak labelling **32**, **35**, **36**, and **48**-**55** designates characterised compounds. UC27 to UC30 are carotenoids which could not be unambiguously identified.

The lipophilic chlorophyll degradation products phaeophytin b (**56**), phaeophytin a (**57**), pyrophaeophytin b (**58**) and pyrophaeophytin a (**59**) were detected, if no saponification procedure was carried out. Spectral properties (UV, MS and MS/MS data) and elution order of these compounds were in accord with previously published data (van Breemen et al., 1991; Canjura and Schwartz, 1991).

3. Discussion and conclusion

The purpose of this study was to analyse the spectrum of extracted metabolites as comprehensively as reasonably possible, for the pharmacologically active lipophilic leaf extract of *Isatis tinctoria*, and to complement this profiling study with an analysis of polar metabolites and the closely related *I. indigotica*. In total, 65 compounds belonging to various structural classes could be unambiguously identified, including alkaloids, flavone glycosides, glucosinolates and indigo precursors, fatty acids, porphyrins, carotenoids, lignans, cyclohexenones and phenylpropanoids. Additional

compounds could be assigned to specific compound classes, or earmarked on the basis of their molecular formula derived from ESI-TOF-MS data. Numerous compounds have been identified for the first time in the genus *Isatis* (compounds **11**, **12**, **16**-**19**, **26**-**28**, **32**, **35**, **36**, **39**, **40**, **48**-**59**), and compound **64** is a new natural product. Compounds **2**, **8**, **20**, **21**, **31**, **41**, **46**, **63** and **65** were known so far only for *I. indigotica* and are reported here for *I. tinctoria.*

The example demonstrates that only the parallel use of multiple detectors and detection modes can adequately reveal the complexity of a plant extract. With a combination of PDA, ELSD, ESI- and APCI-MS in positive and negative ion modes, virtually all peaks could be detected with at least two independent detection modes, and molecular formula could be derived in a second step from ESI-TOF-MS data. For a number of peaks, UV-vis and MS data did not permit unambiguous structural assignment. This was, for example, the case with fatty acids, where location of functional groups and E/Z geometry of double bonds could not be established. Off-line microprobe NMR after peak purification by semi-preparative HPLC proved to be complementary. For truly minor constituents, a rapid pre-fractionation of the extract by MPLC was necessary to render compounds detectable by LC-PDA-MS and amenable to semi-prep. purification for NMR spectroscopy. The power of this approach could be demonstrated by the identification of a new indole alkaloid in the dichloromethane extract.

Analysis of the MeOH extract of *Isatis tinctoria* and *I. indigotica* corroborated previous observations of an nearly complete disapearance of labile indigo precursors isatan A and B, and of glucosinolates. These two compound classes together correspond to approx. 5% to 10% of dry weight of fresh *Isatis* leaves (Oberthür et al., 2004, Mohn et al. 2008). Only the rather stable indigo precursor indican (**38**) and small amounts of the major glucosinolate glucobrassicin (**47**) could be detected. Thus, the MeOH extract of dry leaves consisted mainly of flavone glycosides. In contrast to the notable differences in glucosinolate profiles (Mohn et al., 2008; Mohn and Hamburger, 2008), the flavonoid patterns of *Isatis tinctoria* and *I. indigotica* were rather similar qualitatively even though differing quantitatively. The drying of leaves has been shown to be a prerequisite for anti-inflammatory activity of woad extracts

(Hamburger, 2003), and at least two of the known active compounds, tryptanthrin (**13**) and indirubin (**15**), are formed during the drying process.

Taking the relative peak areas in the ELSD profile as a measure, one can estimate that approximately 90% of the dichloromethane extract could be assigned to particular compounds or compound classes, respectively. This is comparable or better than the state of knowledge for well-characterised special extracts, such as EGb 761 from *Gingko biloba* L. (Hänsel and Spieβ) and ZE 331 from *Petasites hybridus* L. (Brattström, 2003) which are active ingredients in well-known phytopharmaceuticals. However, in *Isatis,* pharmacologically active compounds (**9**, **13**, and **15**) known so far amount to a small portion (3.6% (Mohn et al., 2007) of the total extract only, if one excludes linolenic acid (**22**). The promising activity of *Isatis* extract can be explained, in part, by the high potency of compounds such as tryptanthrin (**13**) and indirubin (**15**). However, we assume that additional compounds contribute to the pronounced *in vivo* activity of *Isatis* extracts (Recio et al., 2006a, b). Potential candidates are likely to be found among the minor alkaloids which form a highly complex pattern in the HPLC profile (chiefly peaks between 8 and 24 min in Fig. 1a, with high signal intensity in positive ion ESI-MS). The extract matrix mainly consists of fatty acid derivatives, porphyrins and carotenoids, which account for >70% of the extract as estimated from the ELSD trace. We assume that these compounds are responsible for increasing the solubility of poorly soluble alkaloids, a fact that was previously observed, directly and indirectly, in the skin penetration study and in the clinical pilot trial comparing tryptanthrin and *Isatis* extract (Heinemann et al., 2004; Oberthür et al., 2003). Synergistic effects of constituents in a herbal extract are thought to play a central role, as the low amounts of individual compounds would not explain clinical efficacy of these products (Williamson, 2001). However, experimental evidence in support of this central paradigm of phytotherapy is relatively scarce (Eder and Mehnert, 1998). In case of the lipophilic *Isatis tinctoria* extract, a combination of pharmacological synergies (through compounds acting on different targets involved in inflammation) and bioavailability enhancement (through solubility enhancement by the extract matrix) seems to occur.

4. Experimental

4.1 Chemicals and reference compounds

Analytical grade solvents for extraction and HPLC grade solvents for chromatography were purchased from Scharlau (Barcelona, Spain). HPLC grade water was obtained by an EASY-pure II (Barnstead, Dubuque IA, USA) water purification system. Deuterated solvents were purchased from Armar Chemicals (Döttingen, Switzerland).

Isatin (**1**), sinapic acid (**6**), ferulic acid (**7**), syringic aldehyde (**11**), indigo (**14**), linolenic acid (**22**), palmitoleic acid (**24**), linoleic acid (**25**), palmitic acid (**29**), oleic acid (**30**), stearic acid (**33**), eicosenoic acid (**34**), eicosanoic acid (**37**), indican (**38**), β-carotene (**54**), indole-3-carboxaldehyde (**60**), indole-3-acetonitrile (**61**), vanillin (**62**), butylated hydroxytoluene, butylated hydroxyanisole and formic acid were purchased from Sigma-Aldrich (Buchs, Switzerland). Isoscoparin (**3**), ursolic acid (**23**), isoorientin (**41**) and isovitexin (**44**) were from Extrasynthese (Genay, France), and α-lysolecithin (**18**) was from Alexis (Lausen, Switzerland). Deoxyvasicinone (**2**), indoxyl (**4**), 3-(4-Hydroxy-3,5-dimethoxybenzylidene)-2-indolinone (**9**), isoindigo (**10**), tryptanthrin (**13**), indirubin (**15**) had been previously synthesised at the Institute of Pharmaceutical Biology, University of Jena according to published procedures (Hoessel et al., 1999; Friedländer and Roschdestwenski, 1915; Sun et al., 1998). Isovitexin-3''-*O*-β-D-glucopyranoside (**45**), isoscoparin-3''-*O*-β-D-glucopyranoside (**46**) and glucobrassicin (**47**) were isolated and characterised as previously described (Cheng et al., 2005; Mohn et al., 2007).

4.2 Plant material

Leaf material of defined strains of *I. tinctoria* (“Thüringer Waid”), and *Isatis indigotica* was harvested on 15th September 2003 from first year plants (rosette stage). Plants were cultivated on experimental plots of the Agricultural Research Station of Thuringia (TLL), Dornburg, Germany as previously described (Mohn et al., 2008). Specimens of seeds from the strains are kept at the TLL, under accession numbers 153/PG 1 (Thüringer Waid) and 153 PG 12 (*Isatis indigotica*).

4.3 Sample preparation and extraction

Freshly harvested *Isatis* leaf material was cut into small pieces of 2-3 cm length and immediately shock frozen with liquid nitrogen. Prior to extraction, the leaves were dried in a thermostatted oven at 40 °C. Temperature, relative humidity and weight loss were monitored during the drying process. Constant weight was achieved after 3-4 d. 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 ground with a ZM 1 ultracentrifugal mill (Retsch, Haan, Germany, with 0.75 mm Conidur sieve. Pressurised liquid extraction (PLE) of 1.0 g frozen and powdered samples was carried out with an ASE 200 instrument (Dionex, Sunnyvale, CA, USA) with attached solvent controller. Conditions for all extractions were as follows: extraction solvent: dichloromethane, temperature: 70 °C; 2 extraction cycles of 5 min; preheat time: 1 min; flush: 100% of cell volume; purge: 80 s with nitrogen; pressure: 120 bar; 22 ml steel cartridges. The extracts of two extraction cycles were combined, and the same plant material was extracted with methanol under same conditions.

4.4 HPLC conditions

HPLC separations were carried out on an Agilent series 1100 system equipped with degasser, binary high pressure mixing pump, column thermostat and photodiode array (PDA) detector (Agilent Technologies; Waldbronn, Germany). A liquid handler 215 (Gilson; Mettmenstetten, Switzerland) was used as autosampler. The HPLC was coupled to an Esquire 3000 plus ion trap mass spectrometer equipped with electrospray (ESI) or atmospheric pressure chemical ionisation (APCI) interface (Bruker Daltonics; Bremen, Germany). High resolution mass spectra were obtained on a micrOTOF ESI-MS system (Bruker Daltonics) connected to an Agilent 1100 series HPLC. Data acquisition and processing was performed using HyStar 3.0 software (Bruker Daltonics).

Separation conditions for the dichloromethane extracts (HPLC system 1): SunFire C_{18} column (3.5 µm, 150 x 3.0 mm I.D., Waters, Milford, MA, USA) equipped with a

guard column (20.0 x 3.0 mm I.D.); mobile phase A: water with formic acid 0.1%, mobile phase B: acetonitrile, flow rate: 0.5 ml / min, column temperature: 25.0 °C, sample injection volume: 10 µl, solvent composition: 5% B isocratic for 2 min, 5% to 93% B in 30 min), 93% B isocratic for 17 min, 93% B to 100% B in 6 min. A split ratio of 1:3 was used with the ESI interface.

An evaporative light scattering detector (ELSD series 2000, Alltech, Deerfield Il, USA) was coupled to the second splitter outlet. ELSD conditions were: nitrogen flow: 1.5 l/min, impactor: on, temperature: 55 °C, detector gain: 8.

Separation conditions for the MeOH extracts (HPLC system 2): same conditions as for HPLC system 1 but with the following solvent composition: 5% B for 2 min, 5% B to 18% B in 30 min, 18% B to 24% B in 28 min.

Sample preparation for carotenoids and chlorophyll degradation products was carried out with an *Isatis tinctoria* dichloromethane extract according to a previously reported method (Britton, 1995), with some modfications: The extract was dried *in vacuo* and 300 mg of the residue was re-dissolved in 100 ml of a mixture of acetone and hexane (1:1) containing butylated hydroxytoluene (62.5 mg/ 100 ml) and butylated hydroxyanisole (62.5 mg/ 100 ml). After filtration, the solution was transferred to a separatory funnel and 100 ml of a sodium chloride solution (10 g / 100 ml) was added. The suspension was shaken in a separatory funnel. After phase separation, the aqueous phase was discarded and the organic layer washed twice with water (2 x 100 ml), dried with anhydrous sodium sulphate and evaporated *in vacuo.* For identification of the chlorophyll degradation products **56**-**59**, the residue was dissolved in 2-propanol, filtered through a membrane filter (0.2 µm) prior to HPLC-MS analysis. For identification of carotenoids, chlorophyll had to be degraded by saponification. The residue was dissolved in 10 ml diethyl ether and mixed with 10 ml of methanolic KOH (10%). The flask was flushed with nitrogen and kept in the dark at ambient temperature for 3 h. The alkaline mixture was transferred to a separatory funnel containing 100 ml of diethyl ether. The organic solution was washed three times with water, dried with anhydrous sodium sulphate and evaporated to dryness. The residue was dissolved in mobile phase, filtered (0.2 µm) and analysed by HPLC-MS.

Separation conditions for the carotenoid separation (HPLC system 3) were adopted from de Rosso and Mercadante (2007a, b) using a YMC C_{30} column (3 µm, 150.0 x 3.0 mm I.D.; Stagroma, Reinach, Switzerland) with guard column (10.0 x 3.0 mm I.D.) and a linear gradient of methanol / methyl-*tert*-butyl ether (MTBE).

4.5 MS instrumentation and parameters

Positive and negative ion LC-MS spectra on the ion trap instrument with electrospray ionisation (ESI) were recorded after optimisation of settings, under ion charge conditions (positive mode: ICC 30000; negative mode: ICC 20000) at a scan speed of 13000 *m*/*z*/s, using a gauss filter width of 0.2 *m*/*z*. Nitrogen was used as a drying gas at a flow rate of 10 l/min and as a nebulising gas at a pressure of 30 psi. The nebuliser temperature was set at 300 °C. Spectra were recorded in the range of *m*/*z* 100 to 800. Capillary voltage was at -4500 V (negative mode: +4500 V), endplate offset at -500 V, capillary exit voltage at 111.8 V (negative mode: -111.8 V), skimmer voltage 40.0 V (negative mode: -40.0 V) and trap drive at 41.8. MS^n experiments were performed on the quasimolecular ions $[M+H]^+$ or $[M-H]^-$ using helium as collision gas. The isolation width was set to 4.0 *m*/*z* and the fragmentation amplitude was set to 1.00 V in the 'smart fragmentation' mode.

Settings for APCI were as follows: Ion charge conditions (ICC 30000 positive mode; ICC 20000 negative mode), scan speed: 13000 *m/z/s*, gauss filter: 0.2 *m*/*z*, nitrogen as drying gas (flow rate 5 l/min) and nebulising gas (pressure: 30 psi), nebuliser temperature: 375 °C, drying temperature: 250 °C, scan range *m*/*z* 100 – 800 (for detection of carotenoids and porphyrins in the hexane/acetone extract: *m*/*z* 100 – 1200), 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), trap drive: 58.8 (negative mode: 61.4). For MS^n experiments: 'smart fragmentation mode', isolation width: 4.0 m/z, fragmentation amplitude: 1.00 V, helium as collision gas.

Conditions for LC–TOF-MS were as follows: spectra were recorded in the range of *m*/*z* 100–800 in positive and negative mode. Nitrogen was used as nebulising gas at

a pressure of 2.0 bar and as drying gas at a flow rate of 9.0 l/min (dry gas temperature 240 °C). Capillary voltage was at -4500 V (+4500 V in negative mode), endplate offset at −500 V, hexapole at 250.0 Vpp (negative mode: 230 Vpp), skimmer 1 at 40 V (negative mode: −50 V) and skimmer 2 at 22.5 V (negative mode: −22.5 V). Instrument calibration was performed using a reference solution of sodium formate 0.1% in isopropanol/water (1:1) containing 5 mM sodium hydroxide. Typical mass accuracy was ± 2 ppm.

4.6 Off-line semi-preparative HPLC-NMR analysis

Powdered *Isatis tinctoria* leave material (400 g) was percolated with 600 ml petrol ether (boiling point 40-60 °C) followed by 2 l of dichloromethane to afford 14.5 g of dichloromethane extract. Pre-fractionation of the dichloromethane extract was carried out on a Sepacore MPLC system equipped with two pump modules C-605, UV photometer C-635, fraction collector C-660 and Sepacore record software (Büchi, Flawil, Switzerland). Due to poor solubility of the dichloromethane extract, 1 g of extract was adsorbed on 10 g C_{18} packing material and filled into an empty precolumn cartridge (120 x 17 mm I.D.). Adsorbed analytes were eluted onto a prepacked Sepacore C_{18} column (50 µm, 150 x 40 mm I.D) for separation. Conditions were as follows: mobile phase A: H_2O, mobile phase B: MeOH, flow rate: 15 ml/min, mobile phase composition: 15% B isocratic for 2 min, 15% B to 85% B in 68 min, 85% B to 100% B in 10 min, 100% B for 20 min. One fraction per minute was collected and appropriate fractions were combined (fractions 1-7). Fractions 2 (29.4 mg) and 3 (32.6 mg) were selected for semi-preparative HPLC on a SunFire C_{18} column, 5 µm, 150 x 10.0 mm I.D., (Waters, Milford, MA, USA) equipped with a guard column (10.0 x 10.0 mm I.D.). Separation conditions were as follows: mobile phase A: H_2O with formic acid 0.1%, mobile phase B: MeOH, flow rate: 5 ml/min, column temperature: 25 °C, injection volume: 900 µl (corresponding to 15 mg of the respective fraction) dissolved in 5% B. Gradient for the separation of fraction 2 was: 5% B for 2 min, 5% B to 65% B in 98 min, 65% B to 70% B in 10 min; gradient for the separation of fraction 3 was: 34% B for 13 min, 34% B to 85% B in 87 min.

Conditions for the semi-preparative isolation of compound **43** from the methanol extract of *Isatis tinctoria* were identical as for HPLC system 2 with exception of column dimensions (SunFire C_{18}, 5 µm, 150 x 10.0 mm I.D; Waters) and flow rate (5 ml/min). Peak based collection was monitored at 210 nm. Mobile phase was removed by parallel evaporation (Genevac EZ-2, Genevac, Gardiner, NY, USA), and samples were submitted to LC-TOF-MS and NMR analysis.

NMR spectra were obtained with a 500 MHz Avance III system (Bruker, Fällanden, Switzerland) equipped with a 1mm TXI probe. Data processing was with Topspin 2.1 (Bruker).

(2Z)-2-[1H-indol-3-ylmethylidene)-1,2-dihydro-3H-indol-3-one (**64**): orange film, UV (online, MeOH / 0.1% aq HCOOH) λ_{max} = 269, 392 and 494 nm; ESI-HRMS, *m*/*z* 261.1031 ($[M+H]^+$, $C_{17}H_{12}N_2O$, calc. 261.1029). ESI-MS, *m*/*z* (rel. int.): 261 $[M+H]^+$, 283 $[M+Na]^+$; ^{1}H-NMR (500 MHz, DMSO-d_6): δ 6.89 (1H, *dd*, H-5, $J_{4,5}$ = 7.4 Hz, $J_{5,6}$ = 7.4 Hz), 7.07 (1H, *s*, H-10), 7.17 (1H, *d*, H-7, $J_{6,7}$ = 7.7 Hz), 7.17 (1H, *dd*, H-5', $J_{4',5'}$ = 7.7 Hz, $J_{5',6'}$ = 7.7 Hz), 7.22 (1H, *dd*, H-6', $J_{5',6'}$ = 7.7 Hz, $J_{6',7'}$ = 7.7 Hz), 7.48 (1H, *dd*, H-6, $J_{5,6}$ = 7.4 Hz, $J_{6,7}$ = 7.7 Hz), 7.48 (1H, *d*, H-7', $J_{6'7'}$ = 7.7 Hz), 7.57 (1H, *d*, H-4, $J_{4,5}$ = 7.7 Hz), 7.83 (1H, *d*, H-4', $J_{4',5'}$ = 7.7 Hz), 8.17 (1H, *s*, H-2'), 9.28 (1H, *bs*, H-1), 11.91 (1H, *bs*, H-1'). ^{13}C-NMR (extracted from 2D-HMBC and HSQC spectra): δ 104.6 (C-10), 105.2 (C-3'), 112.5 (C-7'), 112.8 (C-7), 118.7 (C-4'), 119.6 (C-5), 120.8 (C-5'), 121.4 (C-9), 122.9 (C-6'), 124.0 (C-9'), 127.6 (C-9'), 127.9 (C-2'), 135.2 (C-6'), 135.6 (C-6), 136.6 (C-8'), 153.5 (C-2), 185.0 (C-3). 2D-NMR spectra of **64** are shown in Figure 2S (supplementary data).

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Supplementary Data

Figures representing HPLC profiles of *Isatis indigotica* dichloromethane and methanol extracts (Fig. 1S) and 2D-NMR spectra of **64** (Fig. 2S). Tables of identified compounds in *I. indigotica* dichloromethane (Table 1S) and methanol extracts (Table 2S).

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Tables

Table 1. Compounds identified from dichloromethane extract of *Isatis tinctoria* leaves.

Peak	R_t (min)	λ_{max} (nm)	ESI^+	ESI^-	$APCI^+$	$APCI^-$	Acc. mass found[a]	Acc. mass calc.[a]	Assignment
UC1	8.7	235; 310	337 $[M+H]^+$	335 $[M-H]^-$	337 $[M+H]^+$	335 $[M-H]^-$	337.1852	337.1838	$C_{21}H_{22}N_2O_2$
UC2	10.8	240	fragments	-	-	-	n.d.	-	unknown
UC3	11.3	207; 247; 300	297 $[M+H]^+$	-	-	-	n.d.	-	unknown
1	11.8	209; 241; 303; 416	148 $[M+H]^+$	-	148 $[M+H]^+$	-	148.0406	148.0399	Isatin
2	12.2	207; 226; 259; 302; 313	187 $[M+H]^+$	-	187 $[M+H]^+$	-	187.0868	187.0872	Deoxyvasicinone
3	12.7	216; 270; 345	463 $[M+H]^+$; 485 $[M+Na]^+$	461 $[M-H]^-$	463 $[M+H]^+$	461 $[M-H]^-$	463.1248	463.1241	Isoscoparin
4	12.9	205; 248	134 $[M+H]^+$	-	134 $[M+H]^+$	-	134.0612	134.0607	Indoxyl
5	13.2	258; 302	150 $[M+H]^+$	148 $[M-H]^-$	150 $[M+H]^+$	-	150.0552	150.0556	5-Hydroxyoxindole
6	13.3	235; 322	225 $[M+H]^+$	223 $[M-H]^-$	-	223 $[M-H]^-$	225.0758	225.0764	Sinapic acid
7	14.0	238; 322	195 $[M+H]^+$; 411 $[2M+Na]^+$	193 $[M-H]^-$	195 $[M+H]^+$	193 $[M-H]^-$	195.0653	195.0658	Ferulic acid
UC4	15.0	309	517 $[M+H]^+$	515 $[M-H]^-$	517 $[M+H]^+$	515 $[M-H]^-$	517.1963	517.1976	$C_{29}H_{28}N_2O_7$
8	15.3	220; 275; 309	267 $[M+H]^+$	265 $[M-H]^-$	267 $[M+H]^+$	265 $[M-H]^-$	267.0779	267.0770	3-(2'-Carboxyphenyl)-quinazolin-4-one
UC5	15.8	316	501 $[M+H]^+$	499 $[M-H]^-$	501 $[M+H]^+$	499 $[M-H]^-$	501.2055	501.2067	$C_{34}H_{28}O_4$
UC6	17.8	-	351 $[M+Na]^+$	327 $[M-H]^-$	-	327 $[M-H]^-$	327.2178	327.2171	$C_{18}H_{32}O_5$ probably trihydroxyoctadienoic acid
9	19.5	210; 258; 380	298 $[M+H]^+$	-	298 $[M+H]^+$	-	298.1087	298.1080	(*E*)-3-(3',5'-dimethoxy-4'-hydroxy-benzylidene)-2-indolinone

10	19.8	203; 266; 391; 478	263 $[M+H]^+$;	-	263 $[M+H]^+$	-	263.0828	263.0821	Isoindigo
11	20.9	227; 307	183 $[M+H]^+$; 205 $[M+Na]^+$	-	183 $[M+H]^+$	-	183.0657	183.0658	Syringic aldehyde
12	21.0	309	309 $[M+H]^+$; 331 $[M+Na]^+$	307 $[M-H]^-$	309 $[M+H]^+$	307 $[M+H]^-$	309.2064	309.2067	Corchorifatty acid B
13	21.4	252; 311; 392	249 $[M+H]^+$; 271 $[M+Na]^+$	-	249 $[M+H]^+$	-	249.0664	249.0665	Tryptanthrin
UC7	22.0	260	267 $[M+H]^+$	265 $[M-H]^-$	-	-	n.d.	-	unknown
14	23.2	243; 285; 606	263 $[M+H]^+$;	-	263 $[M+H]^+$	-	263.0821	263.0821	Indigo
UC8	23.8	-	699 $[M+Na]^+$	675 $[M-H]^-$	-	675 $[M-H]^-$	n.d.	-	unknown
15	24.1	240; 290; 362; 541	263 $[M+H]^+$, 285 $[M+Na]^+$	-	263 $[M+H]^+$	-	263.0829	263.0821	Indirubin
16	26.4	-	537 $[M+Na]^+$	513 $[M-H]^-$	-	513 $[M-H]^-$	513.3057	513.3063	1-*O*-β-D-galactopyranosyl-3-*O*-linolenylglycerol
17	26.7	240	317 $[M+Na]^+$	293 $[M-H]^-$	-	293 $[M-H]^-$	293.2107	293.2116	9-Hydroxy-(10*E*, 12*Z*, 15*Z*)-octadecatrienoic acid
18	27.1	-	496 $[M+H]^+$; 518 $[M+Na]^+$	-	-	-	496.3392	496.3404	α-Lysolecithin
19	27.6	279	315 $[M+Na]^+$	291 $[M-H]^-$	-	-	291.1953	291.1959	9-Oxo-(10*E*, 12*Z*, 15*Z*)-octadecatrienoic acid
UC9	28.8	250	381 $[M+H]^+$; 403 $[M+Na]^+$	-	381 $[M+H]^+$	-	381.1596	381.1604	$C_{25}H_{20}N_2O_2$
UC10	29.8	-	509 $[M+H]^+$; 531 $[M+Na]^+$	507 $[M-H]^-$	-	-	509.3025	509.3016	$C_{30}H_{40}N_2O_5$
20	30.7	-	-	249 $[M-H]^-$	-	249 $[M-H]^-$	249.1851	249.1854	(7*Z*, 10*Z*,13*Z*)-Hexadecatrienoic acid
21	31.5	261; 351; 568	491 $[M+H]^+$	489 $[M-H]^-$	491 $[M+H]^+$	489 $[M-H]^-$	491.1506	491.1508	Bisindigotin
UC11	32.8	-	672	-	-	-	n.d.	-	unknown
22	33.5	-	279 $[M+H]^+$; 301 $[M+Na]^+$	277 $[M-H]^-$	279 $[M+H]^+$	277 $[M-H]^-$	277.2161	277.2167	Linolenic acid
23	34.3	-	457 $[M+H]^+$	455 $[M-H]^-$	-	-	457.3683	457.3682	Ursolic acid
24	34.7	-	-	253 $[M-H]^-$	-	-	253.2182	253.2167	Palmitoleic acid

UC12	35.1	410; 664	563 $[M+H]^+$	561 $[M-H]^-$	282	282	n.d.	-	unknown porphyrin derivative
25	35.4	-	-	279 $[M-H]^-$	-	-	279.2321	279.2323	Linoleic acid
26	35.8	325; 407; 505; 533; 605; 664	609 $[M+H]^+$; 631 $[M+Na]^+$	607 $[M-H]^-$	-	-	609.2723	609.2714	10-Hydroxy phaeophorbide
27	36.5	320; 409; 505; 534; 606; 664	593 $[M+H]^+$; 693 $[M+Na]^+$	591 $[M-H]^-$	593 $[M+H]^+$	591 $[M-H]^-$	593.2763	593.2765	Phaeophorbide a
28	37.5	321; 408; 506; 535; 606; 666	593 $[M+H]^+$; 693 $[M+Na]^+$	591 $[M-H]^-$	593 $[M+H]^+$	591 $[M-H]^-$	593.2768	593.2765	Phaeophorbide a'
29	37.8	-	-	255 $[M-H]^-$	-	255 $[M-H]^-$	255.2316	255.2323	Palmitic acid
30	38.3	-	-	281 $[M-H]^-$	-	281 $[M-H]^-$	281.2474	281.248	Oleic acid
31	38.6	321; 410; 507; 537; 606; 666	535 $[M+H]^+$	533 $[M-H]^-$	535 $[M+H]^+$	533 $[M-H]^-$	535.2712	535.2710	Pyrophaeophorbide a
32	41.0	420; 443; 470	569 $[M+H]^+$	-	569 $[M+H]^+$	567 $[M-H]^-$	569.4362	569.4359	(all-*E*)-Lutein
33	43.3	-	-	283 $[M-H]^-$	-	-	283.2630	283.2636	Stearic acid
34	43.5	-	-	309 $[M-H]^-$	-	-	309.2809	309.2793	Eicosenoic acid
35	44.1	328; 416; 439; 465	-	-	569 $[M+H]^+$	-	569.4357	569.4359	(9*Z*)-Lutein
36	45.0	328; 416; 440; 467	-	-	569 $[M+H]^+$	-	569.4361	569.4359	(9'*Z*)-Lutein
UC13	49.0	-	-	621	397	621	n.d.	-	unknown
37	52.4	-	-	311 $[M-H]^-$	-	-	311.2970	311.2949	Eicosanoic acid
UC14	52.7	-	-	-	855; 792	819	n.d.	-	unknown

n.d. = not detected; [a] = found and calculated accurate mass for the respective $[M+H]^+$ or $[M-H]^-$ ion; UC1 to UC 14 are compounds for which only partial structure information was obtained.

Table 2. Compounds identified from methanol extract of *Isatis tinctoria* leaves.

Peak	R_t (min)	λ_{max} (nm)	ESI^+	ESI^-	Acc. mass found[a]	Acc. mass calc.[a]	Assignment
UC15	11.3	n.d.	337 $[M+H]^+$	-	337.1852	337.1838	$C_{21}H_{24}N_2O_2$
UC16	14.2	209; 253; 301	317 $[M+H]^+$; 339 $[M+Na]^+$	315 $[M-H]^-$	317.1131	317.1138	$C_{16}H_{16}N_2O_5$ unknown anthranilic acid derivative
UC17	15.8	n.d.	307 $[M+H]^+$	-	307.1745	307.1732	$C_{20}H_{22}N_2O$
UC18	16.5	n.d.	337 $[M+H]^+$	-	337.1853	337.1838	$C_{21}H_{24}N_2O_2$
UC1	17.6	236; 312	337 $[M+H]^+$	-	337.1850	337.1838	$C_{21}H_{24}N_2O_2$
38	18.3	222; 280	-	294 $[M-H]^-$	294.0971	294.0977	Indican
39	24.1	210; 269; 334	595 $[M+H]^+$; 617 $[M+Na]^+$	593 $[M-H]^-$	595.1652	595.1664	Vicenin-2
UC19	24.5	238	409 $[M+ H]^+$	407 $[M-H]^-$	409.1772	409.1764	$C_{23}H_{24}N_2O_5$
UC20	25.2	213; 268; 330	757 $[M+H]^+$; 779 $[M+Na]^+$	755 $[M-H]^-$	757.2192	757.2191	$C_{33}H_{40}O_{20}$ unknown flavone triglycoside with two *C*-linked and one *O*-linked hexosyl moieties
UC21	25.9	210; 270; 333	625 $[M+H]^+$; 647 $[M+Na]^+$	623 $[M-H]^-$	625.1765	625.1769	$C_{28}H_{32}O_{16}$ unknown flavone di-*C*-glycoside similar to Stellarin-2
40	26.2	210; 270; 343	625 $[M+H]^+$; 647 $[M+Na]^+$	623 $[M-H]^-$	625.1763	625.1769	Stellarin-2
UC22	27.0	211; 270; 338	787 $[M+H]^+$; 809 $[M+Na]^+$	785 $[M-H]^-$	787.2298	787.2298	$C_{34}H_{42}O_{21}$ unkown flavone triglycoside with two *C*-linked and one *O*-linked hexosyl moieties
UC23	31.5	213; 268; 342	625 $[M+H]^+$; 647 $[M+Na]^+$	623 [M-H]-	625.1759	625.1769	$C_{28}H_{32}O_{16}$ unknown flavone di-*C*-glycoside
41	32.1	211; 268; 348	449 $[M+H]^+$; 471 $[M+Na]^+$	447 $[M-H]^-$	449.1093	449.1085	Isoorientin
42	32.6	212; 269; 348	611 $[M+H]^+$; 633 $[M+Na]^+$	609 $[M-H]^-$	611.1617	611.1613	Isoorientin 3"-*O*-β-D-glucopyranoside
43	35.0	207; 249; 301	166 $[M+H]^+$; 148 $[M-H_2O]^+$	164 $[M-H]^-$	164.0344	164.0347	*N*-formyl anthranilic acid

44	37.1	213; 269; 336	433 $[M+H]^+$; 455 $[M+Na]^+$	431 $[M-H]^-$	433.1132	433.1135	Isovitexin
45	37.4	214; 269; 335	595 $[M+H]^+$; 617 $[M+Na]^+$	593 $[M-H]^-$	595.1659	595.1664	Isovitexin 3"-*O*-β-D-glucopyranoside
3	38.8	216; 269; 345	463 $[M+H]^+$; 485 $[M+H]^+$	461 $[M-H]^-$	463.1237	463.1241	Isoscoparin
46	39.4	213; 269; 348	625 $[M+H]^+$; 647 $[M+Na]^+$	623 $[M-H]^-$	625.1763	625.1769	Isoscoparin 3"-*O*-β-D-glucopyranoside
47	41.8	215; 274	-	447 $[M-H]^-$	447.0526	447.0531	Glucobrassicin
UC24	43.5	n.d.	-	621 $[M-H]^-$	621.1447	621.1454	$C_{28}H_{30}O_{16}$ unknown flavonoid with two *O*-linked hexosyl moieties
UC25	46.0	295; 321	-	591 $[M-H]^-$	591.1341	591.1349	$C_{27}H_{26}O_{15}$ unknown flavonoid with two *O*-linked hexosyl moieties
UC26	47.8	240; 325	-	621 $[M-H]^-$	621.1444	621.1454	$C_{28}H_{30}O_{16}$ unknown flavonoid with two *O*-linked hexosyl moieties

n.d. = not detected; [a] = found and calculated accurate mass for the respective $[M+H]^+$ or $[M-H]^-$ ion; UC1 and UC15 to UC26 are compounds for which only partial structures were obtained.

Table 3. Identified carotenoids from an acetone/hexane (1:1) extract of *Isatis tinctoria* leaves

Peak	Rt (min)	λ_{max} (nm)	% III/II[a]	$[M+H]^+$ (*m*/*z*)	MS/MS	Assignment
UC27	6.2	399, 420, 447	80	601	583, 565, 221	unknown
48	6.9	399, 419, 446	75	601	583, 565, 221	(*Z*)-Neochrome
49	7.5	400, 420, 446	80	601	n.d.	(all-*E*)-Neochrome
50	8.2	329, 407, 430, 455	10	601	583, 565, 221	(di-*Z*)-Violaxanthin
51	8.9	330, 415, 437, 464	40	601	583, 565, 509	(15*Z*)-Violaxanthin
52	9.8	330, 415, 437, 464	45	569	551, 533, 476	(13*Z*)- or (13'*Z*)-Lutein
UC28	10.8	382, 401, 425, 444, 471	n.c.	601	n.d.	mixture
32	11.9	420, 443, 470	60	569	551, 533, 459	(all-*E*)-Lutein
35	14.4	328, 416, 439, 465	65	569	551, 533, 509	(9*Z*)-Lutein
36	17.5	328, 416, 440, 467	60	569	551, 533	(9'*Z*)-Lutein
UC29	19.1	327, 418, 442, 467	15	569	551	unknown
UC30	21.7	336, 405, 428, 451	n.c.	569	551	unknown
53	23.0	338, 422, 446, 470	10	537	n.d.	(15*Z*)-β-Carotene
54	30.0	423, 451, 477	25	537	444, 347, 197	(all-*E*)-β-Carotene
55	32.3	340, 423, 443, 469	30	537	444, 209, 357	(9*Z*)-β-Carotene

n.d. = not detected; n.c. = not calculated; [a] = spectral fine structure obtained according to the method of Lee et al. (2001).
UC27 to UC30 are carotenoids which could not be unambiguously identified.

Supplementary Data

A comprehensive metabolite profiling of *Isatis tinctoria* leaf extracts

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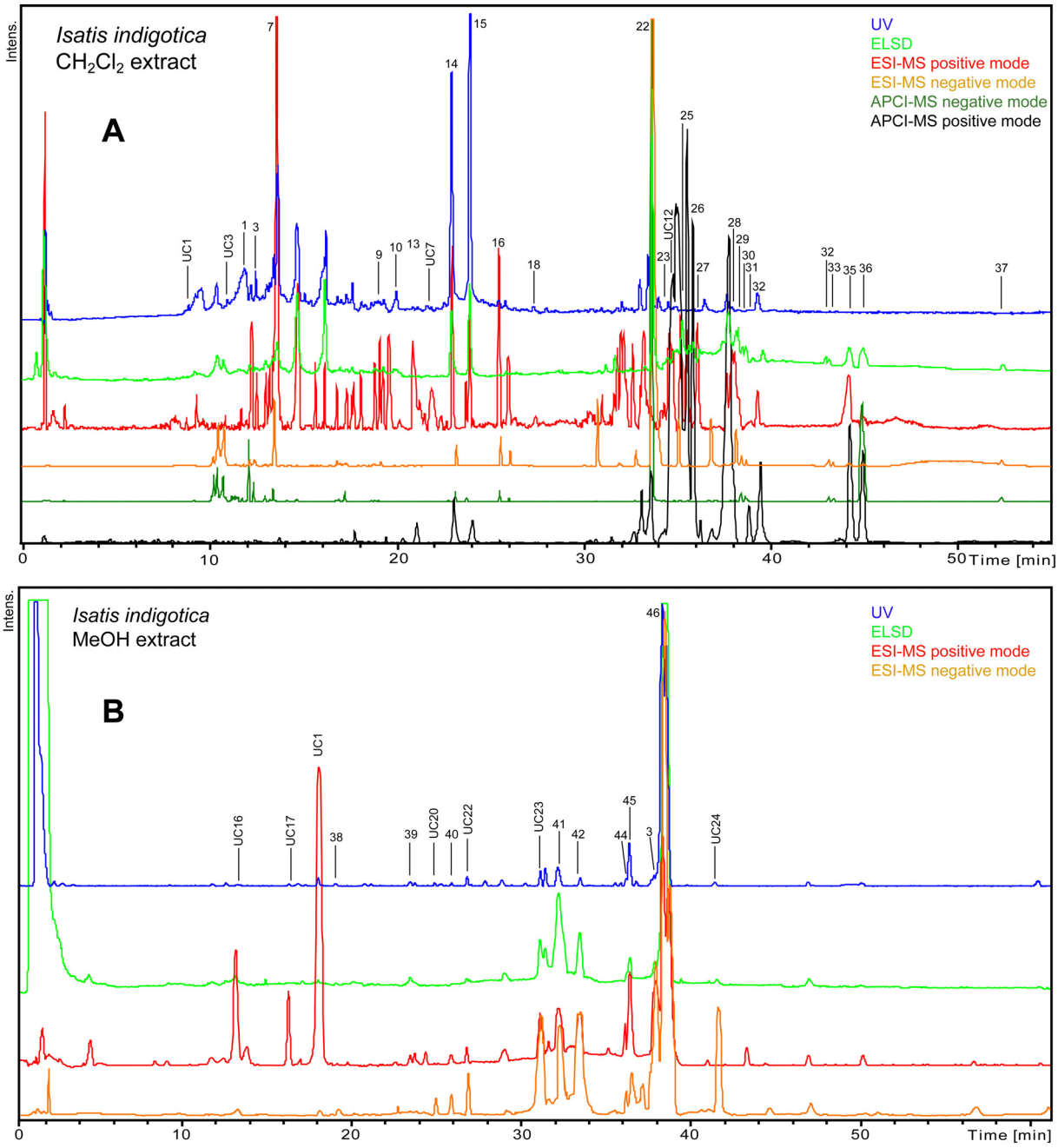

Fig.1S. Dichloromethane (**A**) and methanol (**B**) extracts of *Isatis indigotica* were separated by HPLC. Application of several detectors as PDA, ELSD, and mass spectrometric systems with electrospray (ESI) and atmospheric pressure chemical ionisation (APCI) techniques in positive and negative mode reveal the complexity of both extracts. The majority of compounds were identical with compounds observed in the closely related species *Isatis tinctoria*.

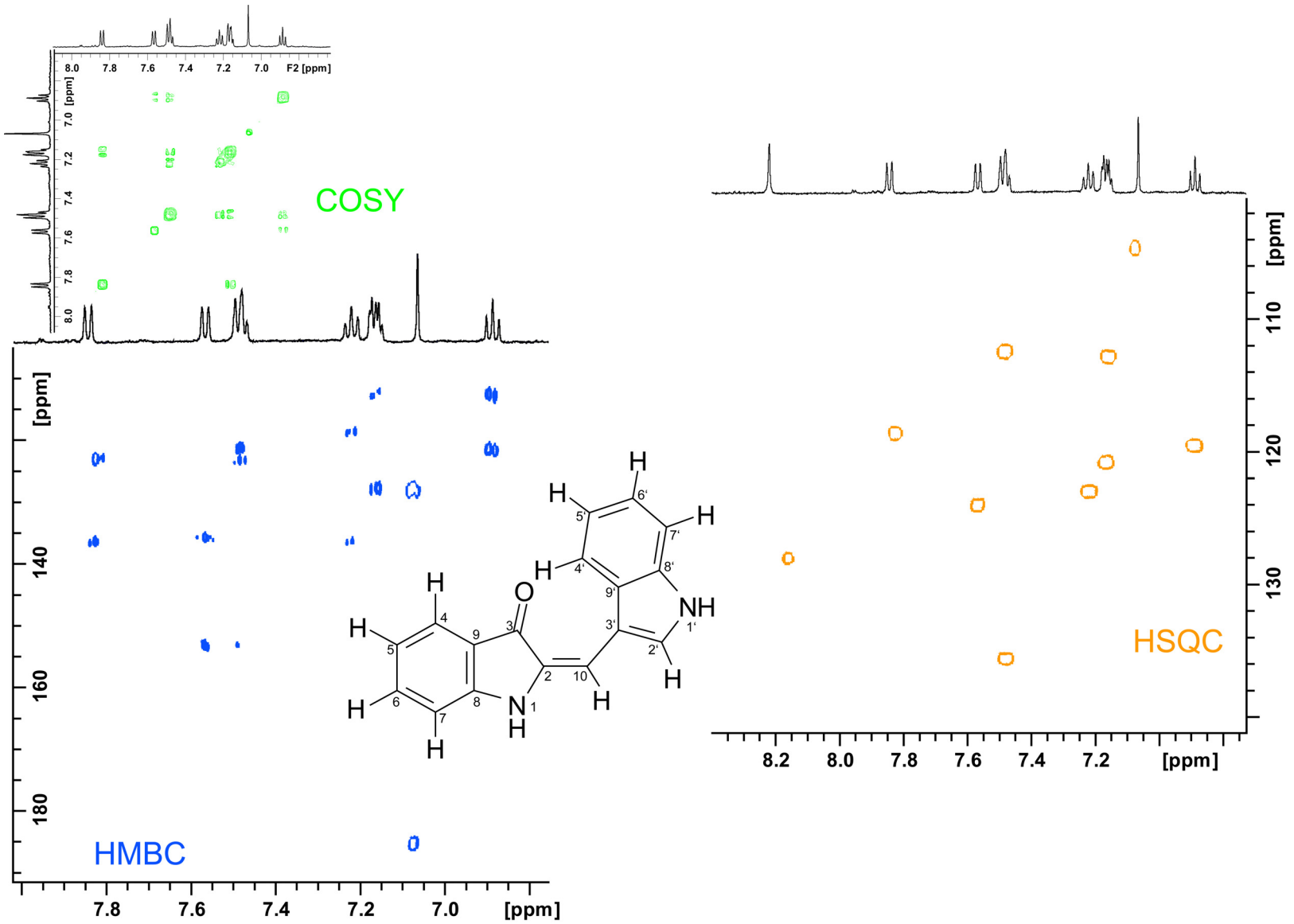

Fig. 2S. 2D-NMR spectra of compound **64**.

Table 1S. Identified compounds **1**-**37** in a dichloromethane extract of *Isatis indigotica*. All compounds were identified on the basis of mass spectrometric, high-resolution-mass spectrometric and UV data or by reference compounds. Details are available in Table 1.

Peak	R_t (min)	Assignment
UC1	9.0	$C_{21}H_{24}N_2O_2$
UC3	11.0	unknown
1	12.0	Isatin
3	12.8	Isoscoparin
7	13.8	Ferulic acid
9	19.5	(*E*)-3-(3',5'-dimethoxy-4'-hydroxy-benzylidene)-2-indolinone
10	20.3	Isoindigo
13	21.3	Tryptanthrin
UC7	22.0	unknown
14	23.0	Indigo
15	24.0	Indirubin
16	25.8	1-*O*-β-D-galactopyranosyl-3-*O*-linolenylglycerol
18	27.3	α-Lysolecithin
22	33.6	Linolenic acid
23	34.5	Ursolic acid
UC12	34.9	unknown porphyrin derivative
25	35.4	Linoleic acid
26	35.6	10-Hydroxy-phaeophorbide
27	36.4	Phaeophorbide a
28	37.8	Phaeophorbide a'
29	38.0	Palmitic acid
30	38.3	Oleic acid
31	38.6	Pyrophaeophorbide a
32	39.7	(all-*E*)-Lutein
33	43.3	Stearic acid
34	43.7	Eicosenoic acid
35	44.1	(9*Z*)-Lutein
36	45.0	(9'*Z*)-Lutein
37	52.4	Eicosanoic acid

Table 2S. Identified compounds **3**, **38**-**45** in a methanol extract of *Isatis indigotica*. All compounds were identified on the basis of mass spectrometric, high-resolution-mass spectrometric and UV data or by reference compounds. Details are available in Table 2.

Peak	R_t (min)	Assignment
UC16	13.7	$C_{16}H_{16}N_2O_5$ unknown anthranilic acid derivative
UC17	15.9	$C_{20}H_{22}N_2O$
UC1	17.8	$C_{21}H_{24}N_2O_2$
38	18.7	Indican
39	23.7	Vicenin-2
UC20	25.0	$C_{33}H_{40}O_{20}$ unknown flavones triglycoside with two *C*-linked and one *O*-linked hexosyl moieties
40	25.9	Stellarin-2
UC22	26.8	$C_{34}H_{42}O_{21}$ unknown flavone triglycoside with two *C*-linked and one *O*-linked hexosyl moieties
UC23	31.2	$C_{28}H_{32}O_{16}$ unknown flavone di-*C*-glycoside
41	32.2	Isoorientin
42	33.5	Isoorientin-*O*-glucopyranoside
44	36.3	Isovitexin
45	36.8	Isovitexin-3"-*O*-β-D-glucopyranoside
3	38.3	Isoscoparin
46	38.8	Isoscoparin-3"-*O*-β-D-glucopyranoside
UC24	41.8	$C_{28}H_{30}O_{16}$ unknown flavonoid with two *O*-linked hexosyl moieties

3.3 Extraction and analysis of intact glucosinolates – A validated pressurized liquid extraction / liquid chromatography - mass spectrometry protocol for *Isatis tinctoria*, and qualitative analysis of other cruciferous plants

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Extraction and analysis of intact glucosinolates—A validated pressurized liquid extraction/liquid chromatography–mass spectrometry protocol for *Isatis tinctoria*, and qualitative analysis of other cruciferous plants

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Abstract

Glucosinolates have attracted significant interest due to the chemopreventive properties of some of their transformation products. Numerous protocols for the extraction and analysis of glucosinolates have been published, but limited effort has been devoted to optimize and validate crucial extraction parameters and sample preparation steps. We carried out a systematic optimization and validation of a quantitative assay for the direct analysis of intact glucosinolates in *Isatis tinctoria* leaves (woad, Brassicaceae). Various parameters such as solvent composition, particle size, temperature, and number of required extraction steps were optimized using pressurized liquid extraction (PLE). We observed thermal degradation of glucosinolates at temperatures above 50 °C, and loss of >60% within 10 min at 100 °C, but no enzymatic degradation in the leaf samples at ambient temperature. Excellent peak shape and resolution was obtained by reversed-phase chromatography on a Phenomenex Aqua column using 10 mM ammonium formate as ion-pair reagent. Detection was carried out by electrospray ionisation mass spectrometry in the negative ion mode. Analysis of cruciferous vegetables and spices such as broccoli (*Brassica oleracea* L. var. *italica*), garden cress (*Lepidium sativum* L.) and black mustard (*Sinapis nigra* L.) demonstrated the general applicability of the method.

Keywords: Glucosinolates; *Isatis tinctoria*; Brassicaceae; Pressurized liquid extraction (PLE); Accelerated solvent extraction (ASE); Quantitative analysis; Quantitative NMR; LC–MS; Ion-pair chromatography; Broccoli; Mustard; Garden cress

1. Introduction

Glucosinolates are a class of nitrogen and sulfur containing secondary metabolites which are characteristic of the plant order of Capparales. More than 120 glucosinolates have been described so far, mostly from the family Brassicaceae [1]. The structure of glucosinolates consists of a β-D-glucopyranosyl moiety linked via a sulfur atom to an *N*-hydroximinosulfate ester, and of a modified amino acid side chain. The highly variable structure of the side chain includes aliphatic (straight chain, branched chain, hydroxylated, sulfur containing, keto-derivatives), alkenylic, aromatic, hydroxyalkyl benzoated or indolic moieties and can be multiply glycosylated. Glucosinolates are found in all plant parts, but occurrence and concentration vary according to species, environmental factors, age or type of tissue [2]. Following tissue damage, these nonvolatile precursors undergo enzymatic hydrolysis by a thioglucohydrolase (E.C. 3.2.1.147, myrosinase) to glucose and a variety of degradation products (isothiocyanates, nitriles, thiocyanates, epithiocyanates, epithionitriles and oxazolidines), which are responsible for the hot, pungent taste and most biological activities of glucosinolates [3].

Intact glucosinolates and their breakdown products possess chemoecological functions and serve not only as defense mechanisms against herbivores and pathogens, but also as attractant to specialized toxin tolerant insects [4–6].

Glucosinolates occur in numerous vegetables and spices such as cabbage, broccoli, cauliflower, cress, mustard, and horseradish. Interest in the role of dietary glucosinolates has been stimulated by the observation of cancer chemopreventive

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properties of certain breakdown products, such as sulforaphane derived from glucoraphanin (4-methylsulfinyl butyl glucosinolate) found in broccoli. Sulforaphane and other isothiocyanates prevent tumor growth in animal models by blocking cell cycle progression and promotion of apoptosis [2,4,7,8].

Given the importance of glucosinolates, numerous methods for extraction and analysis of these compounds have been published. In addition, official protocols have been issued, e.g. for quantitative analysis of glucosinolates in vegetable and spices [9]. Typically, extraction is carried out in water [10–12] or a mixture of methanol and water [13–15]. To prevent enzymatic degradation by myrosinase, the material to be extracted is first treated at 100 °C [16–19]. Others have performed extraction at elevated temperature, typically 70–100 °C, to inactivate myrosinase [10–15]. However, these conditions have not been optimized or validated.

The majority of methods analyze glucosinolates by reversed-phase HPLC of their desulfo-derivatives. In these cases, the extracted glucosinolates are adsorbed onto a solid support, typically Sephadex A-25, followed by enzymatic desulfation and elution of the desulfoglucosinolates [9,20]. More recently, methods for direct separation of intact glucosinolates have been proposed to circumvent this tedious sample preparation. Here, ion-pairing reagents such as tetraalkyl ammonium bromide [21,22], trifluoroacetic acid [16,18,23,24], ammonium acetate [25] or triethylamine/formic acid buffers [26] have been used in conjunction with reversed-phase chromatography on RP-18 columns. However, peaks usually suffered from inacceptable tailing, and separation was usually not optimal. The general problem with published protocols for extraction and analysis of glucosinolates was that, in most cases, insufficient information is available concerning method validation.

Due to the fact that specific biological effects of different glucosinolates vary considerably, it is critical to develop sensitive and reliable methods for efficient identification and quantification of the particular glucosinolate under investigation. We here report on the optimization and validation of a method for extraction and direct analysis of glucosinolates, and we demonstrate the applicability of the assay with the analysis of woad (*Isatis tinctoria* and *I. indigotica*), a dye and medicinal plant with a long history in Europe and in Traditional Chinese Medicine. The general applicability of the HPLC conditions for the analysis of intact glucosinolates is explored with a number of glucosinolate-containing dietary plants and spices such as broccoli (*Brassica oleracea* L. var. *italica*), garden cress (*Lepidium sativum* L.) and black mustard (*Sinapis nigra* L.).

2. Experimental

2.1. Plant material

Leaf material of woad was harvested in August 2003 from first year plants (rosette stage) of defined *I. tinctoria* L. selections "Jenaer Waid", and "Kieler Waid" and *Isatis indigotica* Fort. cultures, which were grown on experimental plots of the Agricultural Research Station of Thuringia (TLL), Dornburg, Germany. Seeds from *I. tinctoria* were also obtained from TLL, Dornburg.

Broccoli heads (*B. oleracea* L. var. *italica*) and garden cress (*L. sativum* L.) were purchased on the local market in Basel, Switzerland. Black mustard seeds (*Brassica nigra* L.) were purchased from Hänseler AG, Herisau, Switzerland.

Fresh *Isatis* leaf material was shock frozen with liquid nitrogen immediately after harvest. Prior to extraction, the leaves were lyophilized, powdered with a ZM 1 ultracentrifugal mill (Retsch, Haan, Germany) and kept frozen at below −20 °C until extraction. Broccoli and garden cress were treated the same way, black mustard seeds were shock frozen and cryomilled before extraction.

2.2. Chemicals

Analytical grade solvents for extraction and HPLC grade solvents for chromatography were purchased from Scharlau (Barcelona, Spain). HPLC grade water was obtained by an EASY-pure II (Barnstead; Dubuque IA, USA) water purification system. Ammonium formate (≥99.995%), formic acid (98.0–100%), sinigrin (**2**) (2-propenyl glucosinolate) (≥99%) and 1,3,5-trimethoxybenzene (≥99%) were purchased from Sigma–Aldrich (Buchs, Switzerland).

Potassium salts of progoitrin (**3**) (2(*R*)-2-hydroxy-3-butenyl glucosinolate), epiprogoitrin (**1**) (2(*S*)-2-hydroxy-3-butenyl glucosinolate), gluconapin (**4**) (3-butenyl glucosinolate) and glucotropaeolin (**7**) (phenylmethyl glucosinolate) were purchased from the Department of Natural Sciences, University of Copenhagen, Denmark.

2.3. Isolation of glucosinolates

Glucobrassicin (**8**) (3-indolylmethyl glucosinolate), sulfoglucobrassicin (**5**) (1-sulfo-3-indolylmethyl glucosinolate), 4-hydroxyglucobrassicin (**6**) (4-hydroxy-3-indolylmethyl glucosinolate) and neoglucobrassicin (**9**) (*N*-methoxy-3-indolylmethyl glucosinolate) were isolated from seeds of *I. tinctoria* ("Kieler Waid").

Isatis seeds (21.6 g) were defatted by Soxhlet-extraction for 8 h with 400 mL petrolether (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 (standard laboratory paper filter, 7–12 μm pore size, Schleicher & Schuell, Feldbach, Switzerland). The aqueous solutions were concentrated to 45 mL in vacuo, and 5 mL 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. Glucosinolates were eluted with a mixture of 0.1 M K_2SO_4/acetonitrile 80:20 at 3.2 mL/min and monitored with UV detection (Pharmacia Uvicord S II) at 229 nm. Fractions were analyzed by HPLC–MS and appropriate fractions combined and evaporated to dryness. For desalting, isolated sulfoglucobrassicin (**5**) was dissolved in aqua dest., applied on a Sephadex G10 column (Sephadex G10, particle size 40–120 μm in water and filled in a column of 2.5 cm diameter) and eluted with water. Finally, isolated glucosinolates were freeze dried. Purity and structures

of isolated compounds were confirmed by NMR and LC–MS experiments.

2.4. Quantitative NMR (qHNMR) for purity assessment of reference compounds

The purity of reference glucosinolates was determined by quantitative NMR with 1,3,5-trimethoxybenzene as internal standard. All NMR spectra were recorded at 303 K on a Bruker DRX500 NMR spectrometer equipped with a 5 mm SEI probe with radio-frequency coils optimized for the detection of ^{1}H.

The longitudinal relaxation constant, T1, was measured to determine the recycle delay needed for the magnetization to return to equilibrium. This was achieved with the pulse sequence "t1ir" from Bruker. Trimethoxybenzene was measured with 40 equally spaced inversion recovery delays between 0.25 and 10.0 s. Following four dummy scans, eight scans were recorded for each inversion recovery delay with a recycle delay of 100 s between successive scans. The T1 value of the trimethoxy hydrogens in trimethoxybenzene was measured to be 2.4 s.

To determine the concentration of the glucosinolates, the data was recorded using the pulse sequence "zgpr" from Bruker. The carrier frequency was set to the resonance frequency of the residual HOD solvent signal at 4.705 ppm. The presaturation pulse consisted of a rectangular pulse applied for 1 s at an intensity of 24 Hz. A spectral width of 20 ppm was monitored with 64k points, implying an FID resolution of 0.16 Hz per point. For each sample, following two dummy scans, 16 scans were recorded. To ensure the full recovery of the magnetization following each 90° pulse, a recycle delay of 20 s, a value longer than five times the T1 of any signal used to quantify the concentration.

Trimethoxybenzene (1.0 mg; Mettler Toledo AT 20 microbalance, reproducibility: 4 μg) was dissolved in 1.8 mL methanol-d_4 (adjusted to give a concentration of 3.333 mM). This solution was diluted by a factor of 10 with D_2O. Glucosinolates were separately weighed into NMR tubes (1.0 mg) and dissolved with the diluted trimethoxybenzene solution to give an exact concentration of 3 mM glucosinolate. Data were processed and analyzed using XWIN-NMR version 3.6 running on a Linux PC. Each data size for each experiment was doubled by zero-filling in the time domain. The FID was then multiplied by an exponential decay apodization function of 0.3 Hz prior to Fourier transformation. To determine the concentration of compounds **5**–**9**, the aromatic region of the spectrum between 7 and 8 ppm was integrated and divided by the appropriate number of hydrogens from which it originated. The concentration of glucosinolates **1**–**4**, which do not contain aromatic hydrogens, was determined by integration of the anomeric hydrogen at 4.5 ppm. The obtained values were compared to that of the integrated trimethoxy signal from trimethoxybenzene whereas nine protons of trimethoxybenzene (0.333 mM) corresponded to one proton glucosinolate (3 mM).

2.5. Pressurized liquid extraction (PLE) of plant samples

Pressurized liquid extraction was carried out with an ASE 200 instrument (Dionex; Sunnyvale, CA, USA) with attached solvent controller. Extraction of 1.0 g frozen, powdered plant material was carried out in 11 mL steel cartridges. Standard conditions for all extractions were as follows: preheat time: 1 min; static extraction per cycle: 5 min; flush: 100 % of cell volume; purge: 80 s with nitrogen; pressure: 120 bar.

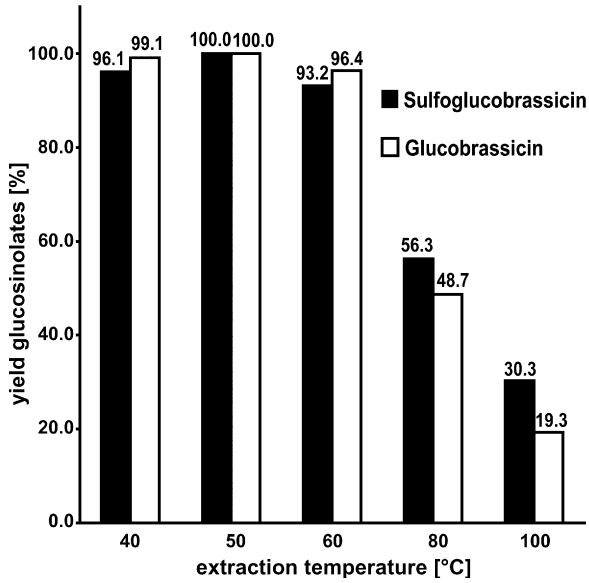


Fig. 1. Optimization of extraction parameters: influence of the extraction temperature on the yield of glucosinolates **5** and **8** from *Isatis tinctoria* leaves ("Jenaer Waid") obtained with 70% MeOH and three extraction cycles. The yields obtained at 50 °C were set as 100%.

Solvent composition (Supplementary Material, Fig. 1), temperature (40–100 °C) (Fig. 1), number of required extraction steps (Fig. 2) and particle size (sieve size 0.5, 0.75 and 1.0 mm) (Supplementary Material, Fig. 2) were optimized by repeated extractions and changing the parameter of choice. Final conditions were: particle size 0.5 mm, 50 °C, 70 % methanol in water, three extraction cycles of 5 min. Possible degradation of glucosinolates by myrosinase activity was analyzed by extraction of freshly thawed leaf material at defined time points of 0, 50, 100 and 200 min after filling the cartridge (Supplementary Material, Fig. 3).

2.6. LC–MS instrumentation and conditions

HPLC separations were carried out on an Agilent series 1100 system equipped with degasser, binary high pressure mixing pump, column thermostat and photodiode array (PDA) detector (Agilent Technologies; Waldbronn, Germany). A liquid handler 215 (Gilson; Mettmenstetten, Switzerland) was used as autosampler. The HPLC was coupled to an Esquire 3000 plus ion trap mass spectrometer equipped with an electrospray (ESI) interface (Bruker Daltonics; Bremen, Germany). High-resolution mass spectra were obtained on a micrOTOF ESI-MS system (Bruker Daltonics) connected to an Agilent 1100 series HPLC. Data acquisition and processing was performed using HyStar 3.0 software (Bruker Daltonics).

Negative ion LC–MS spectra on the ion trap instrument were recorded after optimization of settings, under ion charge control conditions (ICC 30000) at a scan speed of 13000 *m/z*/s, using a

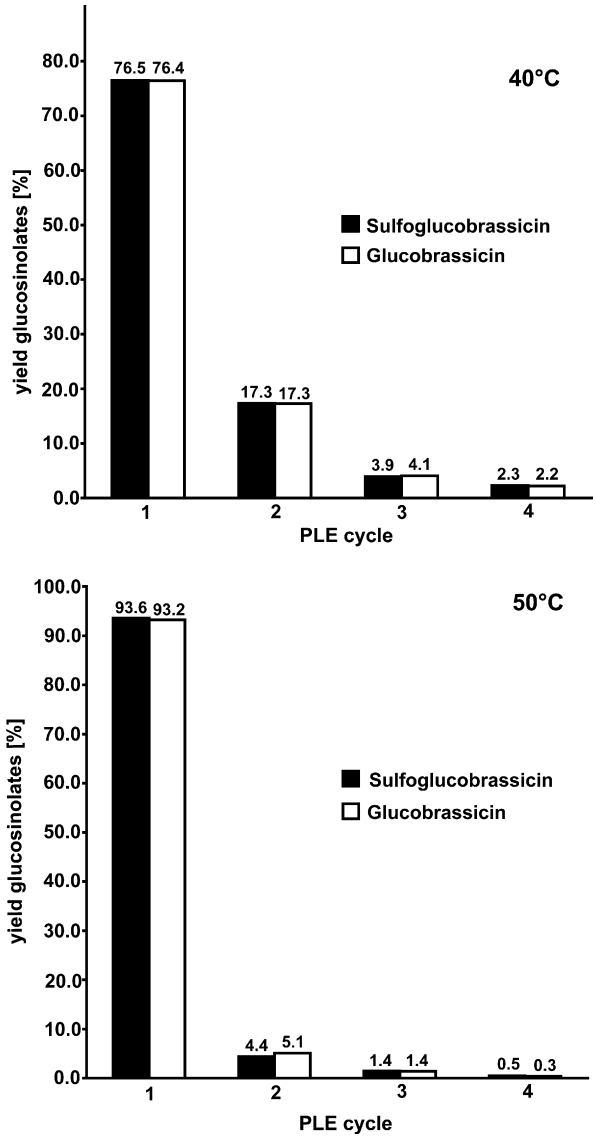


Fig. 2. Influence of the extraction temperature on yields of glucosinolates **5** and **8** in successive PLE extraction cycles. Plant material was identical with that used in Fig. 1. The cumulative yields of four extraction cycles were set as 100%.

gauss filter width of 0.2 *m/z*. Nitrogen was used as a drying gas at a flow rate of 10 L/min and as a nebulizing gas at a pressure of 30 psi. The nebulizer temperature was set at 300 °C. Spectra were recorded between 0 min and 25 min in the range of *m/z* 100–800. Capillary voltage was at 4500 V, endplate offset at −500 V, capillary end voltage at −115.0 V, skimmer voltage −40.0 V and trap drive at 53.4.

Conditions for LC–TOF MS were as follows: spectra were recorded in the range of *m/z* 100–600 in negative mode. Nitrogen was used as a nebulizing gas at a pressure of 2.0 bar and as a drying gas at a flow rate of 9.0 L/min (dry gas temperature 240 °C). Capillary voltage was at 4500 V, endplate offset at −500 V, hexapole at 250.0 V_{pp}, skimmer 1 at −50 V and skimmer 2 at −22.5 V. Instrument calibration was performed using a reference solution of sodium formate 0.1% in isopropanol/water (1:1) containing 5 mM sodium hydroxide. Typical mass accuracy was ±1 ppm.

2.7. HPLC–MS method development and validation

RP-18 columns from different manufacturers, including Atlantis and Sunfire (Waters; Milford, MA, USA), Luna and Aqua (Phenomenex; Aschaffenburg, Germany)], OmniPac pax 500 (Dionex; Sunnyvale, CA, USA) and LiChrospher 60 rp-select b (Merck; Darmstadt, Germany) columns, were tested for suitability in glucosinolate analysis. Finally separations were carried out on an Aqua C18 125 Å column (5 μm, 250 × 4.6 mm I.D.; Phenomenex) equipped with a guard column (4.0 × 3.0 mm I.D.). Mobile Phase A consisted of a solution of 10 mM aqueous ammonium formate, adjusted to pH 6.4 with formic acid, and mobile phase B was acetonitrile. A linear gradient starting at 1% B to 3% B (7 min), 3% B to 15% B (5 min), 15% B to 30% B (13 min) was shown to give best separation of all compounds to be analyzed. Flow rate was 1.0 mL/min. A split ratio of 1:4 was used with the ESI interface. Column temperature was 20.0 °C. The sample injection volume was 20 μL.

Reproducibility was assessed in five replicates with a standard solution of all glucosinolates at a concentration of 1 μg/mL and the relative standard deviation was calculated. Intra-day and inter-day repeatability was verified in the course of the analysis. Limit of detection (S/N ratio of 3) and limit of quantification (S/N ratio of 10) were determined by serial dilution of a standard solution containing all glucosinolates (Table 1). Accuracy was determined for the major compounds sulfoglucobrassicin and glucobrassicin, and the minor compound progoitrin. An extract of "Jenaer Waid" was spiked with defined amounts, whereby the amounts added were comparable to those of compounds originally present in the extract. Amounts added and recovery rates are listed in Supplementary Material, Table 1.

2.8. Quantitative analysis

A solution of 10 mM ammonium formate containing sinigrin (2 mg/L) as internal standard (IS solution) was used for all dilution steps. Stock solutions (1 mg/mL) of all reference compounds were prepared in IS solution, serial dilutions covered a concentration range of 20–0.1 μg/mL. Each standard solution was measured as five replicates. Calibration curves are shown in Supplementary Material, Table 2.

The dried extracts, obtained by extraction of 1.0 g frozen, powdered plant material, were dissolved in 100.0 mL IS solu-

Table 1
Limits of detection (LOD) and quantification (LOQ) for glucosinolates **1–9**

Compound	LOD	LOQ
Epiprogoitrin (**1**)	1	3
Sinigrin (**2**)	1	3
Progoitrin (**3**)	1	3
Gluconapin (**4**)	0.5	1
Sulfoglucobrassicin (**5**)	4	12
4-Hydroxyglucobrassicin (**6**)	2	5
Glucotropaeolin (**7**)	0.2	0.5
Glucobrassicin (**8**)	1	3
Neoglucobrassicin (**9**)	1	3

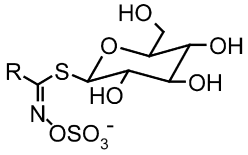
Amount of compound injected (ng).

tion. Dilutions of 1:10 and 1:100 were prepared with IS solution. All solutions were centrifuged (5 min, 1600 × *g*, 4 °C) prior to HPLC analysis. Measurements were done in triplicate.

3. Results

3.1. Isolation of reference compounds and control of purity

Most glucosinolates used in this study (Fig. 3) were not commercially available and had to be isolated as reference compounds (see Section 2). LC–MS analysis of isolated compounds showed a purity of higher than 99 %. Structures were also confirmed by NMR. The spectra did not show any additional signals of organic impurities. Given that the glucosinolates used as reference compounds are present as potassium salts and may contain an unknown amount of water of crystallization, we determined the absolute content with the aid of quantitative ^{1}H NMR (qHNMR). The purity was >99% for sinigrin (**2**) (calculated as $C_{10}H_{17}NO_9S_2$), 87% for epiprogoitrin (**1**) (as $C_{11}H_{19}NO_{10}S_2$), 90% for progoitrin (**3**) (as $C_{11}H_{19}NO_{10}S_2$), 71% for gluconapin (**4**) (as $C_{11}H_{19}NO_9S_2$) and >99% for glucotropaeolin (**7**) (as $C_{14}H_{19}NO_9S_2$), and 70% for glucobrassicin (**8**) (as $C_{16}H_{20}N_2O_9S_2$), 61% for neoglucobrassicin (**9**) (as $C_{17}H_{22}N_2O_{10}S_2$), 19% for sulfoglucobrassicin (**5**) (as $C_{16}H_{20}N_2O_{12}S_3$) and 17% for 4-hydroxyglucobrassicin (**6**) (as $C_{16}H_{20}N_2O_{10}S_2$).



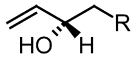
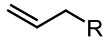
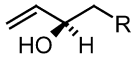
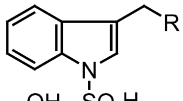
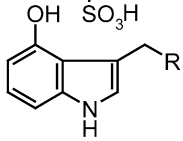
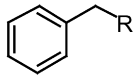
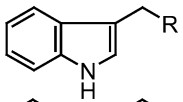
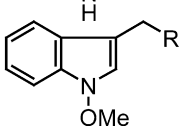
Peak number	m/z	Structure of R	Trivial name
1	388		Epiprogoitrin
2	358		Sinigrin
3	388		Progoitrin
4	372		Gluconapin
5	527		Sulfoglucobrassicin
6	463		4-Hydroxy-glucobrassicin
7	408		Glucotropaeolin
8	447		Glucobrassicin
9	477		Neoglucobrassicin

Fig. 3. Structures of glucosinolates **1–9**.

3.2. Development and validation of LC–MS conditions

LC–MS analysis of intact glucosinolates requires the use of ion-pair reagents to neutralize the negative charge of the sulfate groups. Preliminary tests with trifluoracetic acid (TFA; 0.05%, 0.1% and 0.5%) resulted in inacceptable peak tailing and separation, especially for sulfoglucobrassicin which contains two sulfate groups. Non-volatile ion-pair reagents such as tetraalkyl ammonium bromides could not be used because of MS detection. However, symmetrical peak shapes and good separation were also obtained with ammonium formate which was then used for all further separations.

We initially tested a range of RP-18 columns in conjunction with TFA and ammonium formate buffer in several concentrations. Best results were obtained on a Phenomenex Aqua column which was used for all subsequent experiments. The influence of the concentration of ammonium formate buffer (1, 5, 10 and 20 mM) on peak shape and resolution was investigated. Best results were obtained with 10 mM ammonium formate. In a range between 5 mM and 20 mM only minor shifts in retention time (<0.2 min) were observed, without change in peak shape, but a severe decline in peak symmetry and resolution occurred with a 1 mM buffer concentration. For further optimization of chromatographic separation, we investigated the influence of column temperature (15.0 °C, 20.0 °C, 25.0 °C and 30.0 °C). Best resolution and peak shape was observed at 20.0 °C, in particular for compounds **1–3**. At 30.0 °C, peaks **1–3** could not be resolved. The solvent used for dissolution of samples proved to be critical. Injecting methanolic or 70% aqueous-methanolic solutions of reference compounds and extracts resulted in peak tailing, but symmetrical and sharp peaks were obtained when samples were dissolved in mobile phase A. The HPLC–MS analysis of a standard mixture of **1–9** under optimized conditions is shown in Fig. 4.

For quantitative analysis, extracted ion traces corresponding to $[M-H]^-$ quasimolecular ions were used for signal integration, with exception of sulfoglucobrassicin (**5**). Here, the highest signal (*m/z* 447) in the spectrum, corresponding to the loss of a sulfate moiety, was used. Sinigrin (**2**), a glucosinolate which is not present in the genus *Isatis*, was used as internal standard for quantitative analysis of woad (*I. tinctoria* and *I. indigotica*) samples. Calibration curves (Supplementary Material, Table 2) were found to be quadratic functions for mass spectrometric detection ($R^2 \geq 0.9998$). Reproducibility was satisfactory (RSD 1.0–2.5%; $n = 5$). Inter-day repeatability over time of analysis for **1–9** was between 96.5% and 101.0% after 67 h. Limit of detection (S/N ratio of 3) and limit of quantification (S/N ratio of 10) were determined by serial dilution of a standard solution of all glucosinolates (Table 1).

Accuracy of the method was determined with spiking experiments. Recovery rates for the major compounds sulfoglucobrassicin (**5**) (98%) and glucobrassicin (**8**) (97%), as well

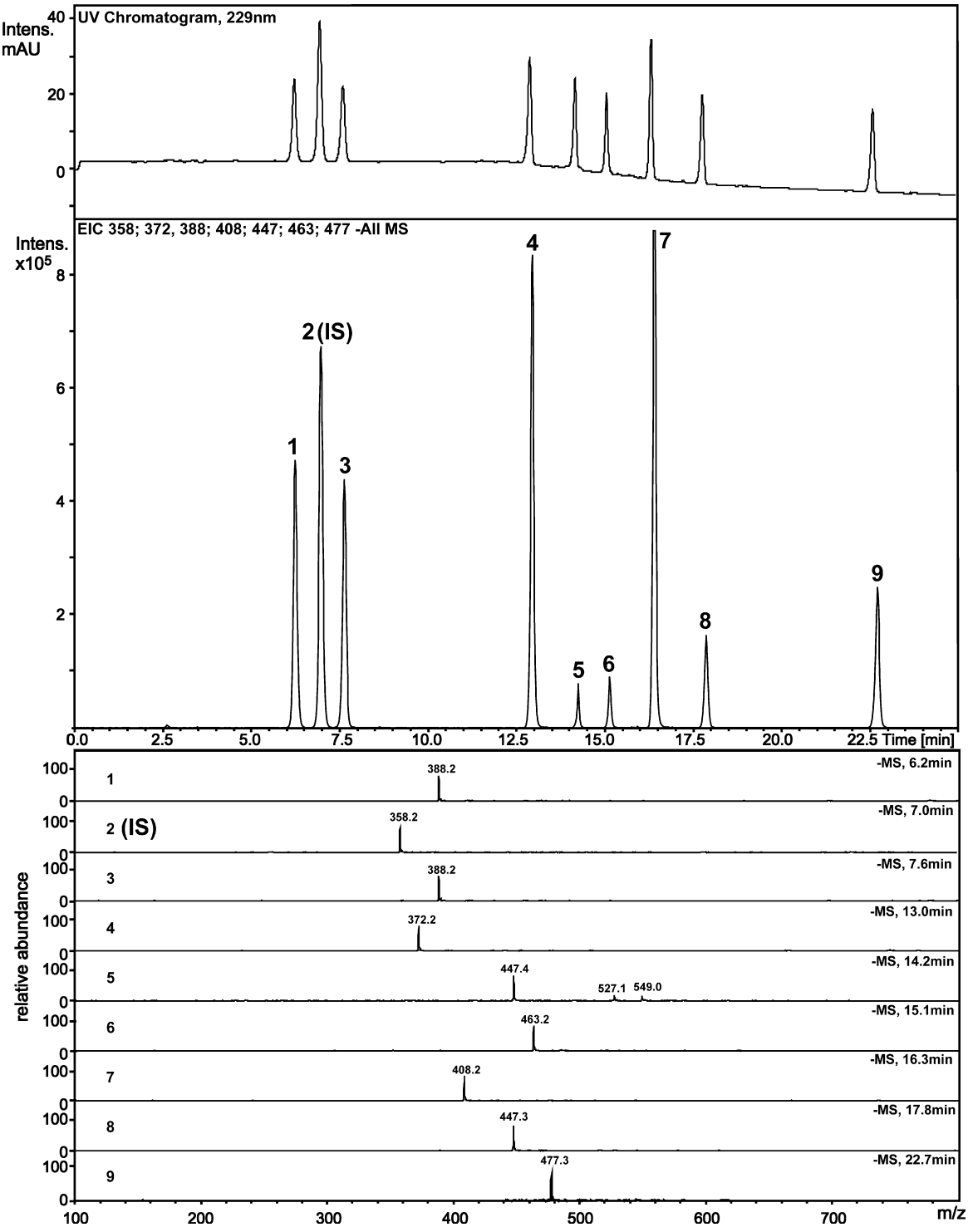


Fig. 4. HPLC separation of a mixture of reference compounds **1–9**. Sinigrin (**2**) is used as internal standard (**IS**). The chromatogram on top was recorded at 229 nm; below are the extracted ion chromatogram (EIC) obtained by ESI-MS in the negative ion mode, and the mass spectra of the individual compounds.

as for progoitrin (**3**) (102%) were obtained (Supplementary Material, Table 1).

3.3. Optimization and validation of the extraction protocol

The optimized LC–MS conditions were then employed for the optimization and validation of an extraction protocol with *Isatis* samples. We decided upon pressurized liquid extraction (PLE) as an extraction technique, given the numerous advantages over other methods of plant extraction and our own experience with the method [27–30]. We selected the major glucosinolates **5** and **8** as markers for the optimization and validation procedure.

In a preliminary experiment, solvent compositions and extraction temperatures typically used in published methods were compared. The results showed that a combination of aque-

ous MeOH (70%) and moderately elevated temperatures were likely to give the highest yields (Supplementary Material, Fig. 1). In the next step, we investigated the influence of extraction temperature on the yield of **5** and **8** in more detail. As shown in Fig. 1, thermal degradation of glucosinolates was observed at temperatures above 50 °C. A loss of >60% was found at 100 °C when using three extraction cycles of 5 min each. The total yield of glucobrassicin (**8**) and sulfoglucobrassicin (**5**) at 40 °C was almost as high as at 50 °C. Analysis of the yield of the successive extraction cycles (Fig. 2), showed that extraction efficiency was clearly better at 50 °C. Cumulative yields of 99.4% and 99.7% were achieved for **5** and **8** with three extraction cycles of 5 min, whereas only 97.7% and 97.8 % were obtained at 40 °C. An extraction temperature of 50 °C and three extraction cycles of 5 min each were used for all subsequent experiments. We also investigated the influence of particle size of the herbal material on the yields of extracted glucosinolates. *Isatis* leaves were cryomilled in an ultracentrifugal mill, using sieve sizes of 1.0, 0.75 and 0.5 mm. Differences observed in cumulative yields were small. With a sieve size of 1 mm, 98.5% of sulfoglucobrassicin (**5**) were extracted after 3 extraction cycles, compared to 99.5% with sieve sizes 0.75 and 0.5 mm. For glucobrassicin (**8**), the yields were 98.9% for sieve size of 1.0 mm, and 99.7% for 0.75 and 0.5 mm. (Supplementary Material, Fig. 2).

The enzyme thioglucohydrolase (E.C. 3.2.1.147, myrosinase), is responsible for the hydrolysis of glucosinolates to glucose and a variety of breakdown products, which are responsible for the hot and pungent taste of many cruciferous vegetable and spices. Myrosinase is known to be quite stable in dried plant material at room temperature and can be reactivated upon addition of water, e.g. during extraction. To determine whether myrosinase activity in thawed *Isatis* leaf material possibly confounded the yields of glucosinolates in extractions with our PLE protocol, we carried out an experiment in which PLE cartridges filled with identical *Isatis* leaf samples were extracted at defined times after thawing. The sample extracted immediately (0 min) served as reference (100%). No measurable decrease of sulfoglucobrassicin (**5**) and glucobrassicin (**8**) was seen in cartridges which were kept at ambient temperature up to 200 min after the start of the experiment (Supplementary Material, Fig. 3). We concluded that myrosinase activity was negligible in our assay conditions and that, consequently, larger sample series could be prepared for extraction without undue risk of loss in analytes.

3.4. Quantitative analysis of woad extracts

The optimized and validated method for sample preparation and LC–MS analysis was used to determine the content in glucosinolates **1**, and **3–9** in three different woad samples. PLE extracts of two defined cultures of *I. tinctoria* ("Jenaer Waid" and "Kieler Waid") and of the taxiconomically closely related *Isatis indigotica* were prepared. The two *Isatis* species are indigo dyes and medicinal plants with important anti-inflammatory and anti-allergic properties [31–34]. Fig. 5 shows the base peak chromatogram (BPC) of the extract and the extracted ion chromatograms (EIC) used for peak integration of the identified glucosinolates.

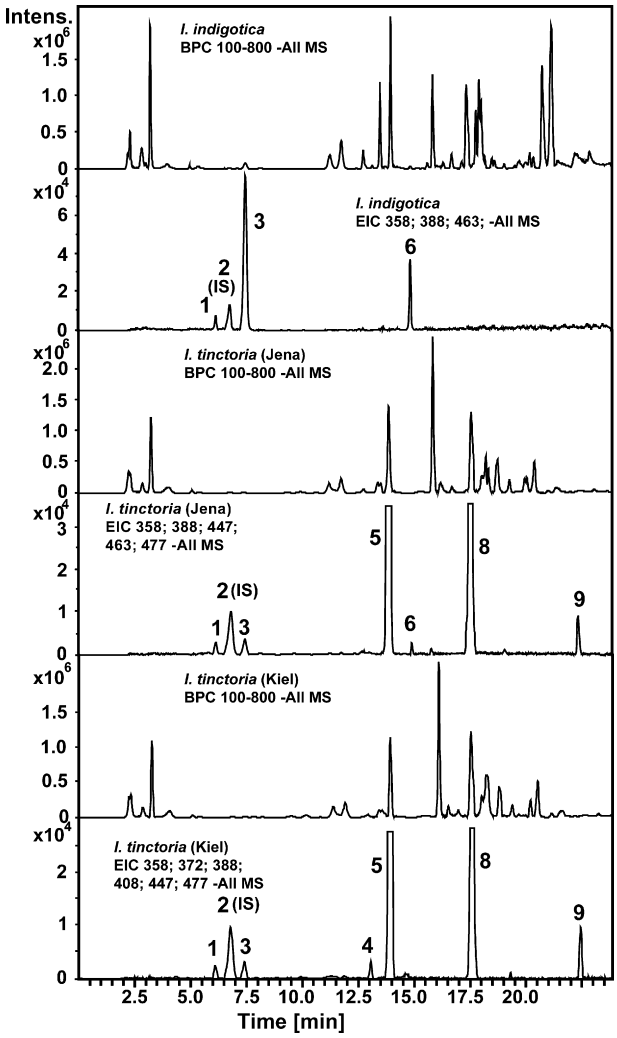


Fig. 5. Glucosinolates in *Isatis* leaf extracts. Base peak chromatograms (BPC) and extracted ion chromatograms (EIC) of major glucosinolates are shown for two *Isatis tinctoria* accessions ("Jenaer Waid" and "Kieler Waid"), and for *I. indigotica.* Sinigrin (**2**) is used as internal standard (**IS**).

Due to large differences in concentration of glucosinolates, it was necessary to analyze the extracts in different dilutions for quantification. In particular, high dilutions (1:100) of *I. tinctoria* extracts were prepared to determine sulfoglucobrassicin (**5**) and glucobrassicin (**8**), while progoitrin (**3**) and hydroxyglucobrassicin (**6**) were measured in a 1:10 extract dilution of *I. indigotica*. The remaining glucosinolates were quantified in undiluted extracts (Table 2).

The glucosinolate spectra of both *Isatis* species varied remarkably. In comparison, the extract of *I. indigotica* did not contain sulfoglucobrassicin (**5**) or glucobrassicin (**8**) which are the major glucosinolates of *I. tinctoria*. Progoitrin (**3**) and 4-hydroxyglucobrassicin (**6**) were the main glucosinolates in the *I. indigotica* extract. The aromatic glucotropaeolin (**7**) as well as other indole-type glucosinolates such as glucoisatisin and hydroxyglucoisatisin which have been reported from *I. tinctoria* L. seeds could not be found in the *Isatis* leaf samples.

Table 2
Glucosinolate content (in μmol/g dry weight) in woad extracts

Compound	*I. indigotica*	*I. tinctoria* (Kiel)	*I. tinctoria* (Jena)
Epiprogoitrin (**1**)	0.16 ± 0.01[a]	0.10 ± 0.01[a]	0.16 ± 0.01[a]
Progoitrin (**3**)	2.54 ± 0.03[b]	0.15 ± 0.01[a]	0.20 ± 0.01[a]
Gluconapin (**4**)	n.d.	0.03 ± 0.01[a]	n.d.
Sulfoglucobrassicin (**5**)	n.d.	11.38 ± 0.37[c]	13.51 ± 0.23[c]
4-Hydroxyglucobrassicin (**6**)	0.43 ± 0.01[b]	n.d.	0.04 ± 0.01[a]
Glucotropaeolin (**7**)	n.d.	n.d.	n.d.
Glucobrassicin (**8**)	n.d.	6.14 ± 0.12[c]	14.29 ± 0.25[c]
Neoglucobrassicin (**9**)	n.d.	0.19 ± 0.01[a]	0.16 ± 0.01[a]

All measurements were in triplicate.; n.d.: not detected.
[a] Undiluted extract.
[b] Dilution 1:10.
[c] Dilution 1:100.

3.5. Qualitative analysis of glucosinolates in cruciferous vegetables and spices

Cruciferous vegetables and spices such as broccoli, garden cress and black mustard were analyzed to demonstrate the general applicability of the method. LC–TOFMS in negative mode was used to confirm the molecular formula of glucosinolates. Fig. 6 shows extracted ion chromatograms of glucosinolates identified in broccoli extracts. Besides minor glucosinolates, glucoerucin ($C_{12}H_{23}NO_9S_3$, calc. 421.0535; found: 421.0535), glucoraphanin ($C_{12}H_{23}NO_{10}S_3$, calc.: 437.0484; found: 437.0483), glucobrassicin (**8**) ($C_{16}H_{20}N_2O_9S_2$, calc.: 448.0610; found 448.0608), 4-methoxyglucobrassicin ($C_{17}H_{22}N_2O_{10}S_2$, calc.: 478.0716 found: 478.0719) and neoglucobrassicin (**9**) ($C_{17}H_{22}N_2O_{10}S_2$, calc.: 478.0716 found: 478.0715) could be identified.

Glucotropaeolin (**7**) ($C_{14}H_{19}NO_9S_2$, calc.: 409.0501; found 409.0503) eluted at 16.2 min was the major glucosinolate in garden cress, followed by 4-methoxyglucobrassicin ($C_{17}H_{22}N_2O_{10}S_2$, calc.: 478.0716; found 478.0715) at 19.9 min as a minor compound (Fig. 7). Black mustard seeds contained sinigrin (**2**) ($C_{10}H_{17}NO_9S_2$, calc.: 359.0345; found 359.0346) as the dominant compound, and gluconapin (**4**) ($C_{11}H_{19}NO_9S_2$, calc.: 373.0501; found 373.0498), glucoibervirin ($C_{11}H_{21}NO_9S_3$, calc.: 407.0378; found 407.0376), gluconasturtiin ($C_{15}H_{21}NO_9S_2$, calc.: 423.0658; found 423.0656) and 4-hydroxyglucobrassicin (**6**) ($C_{16}H_{20}N_2O_{10}S_2$, calc.: 464.0559; found 464.0557) as minor glucosinolates (Fig. 8).

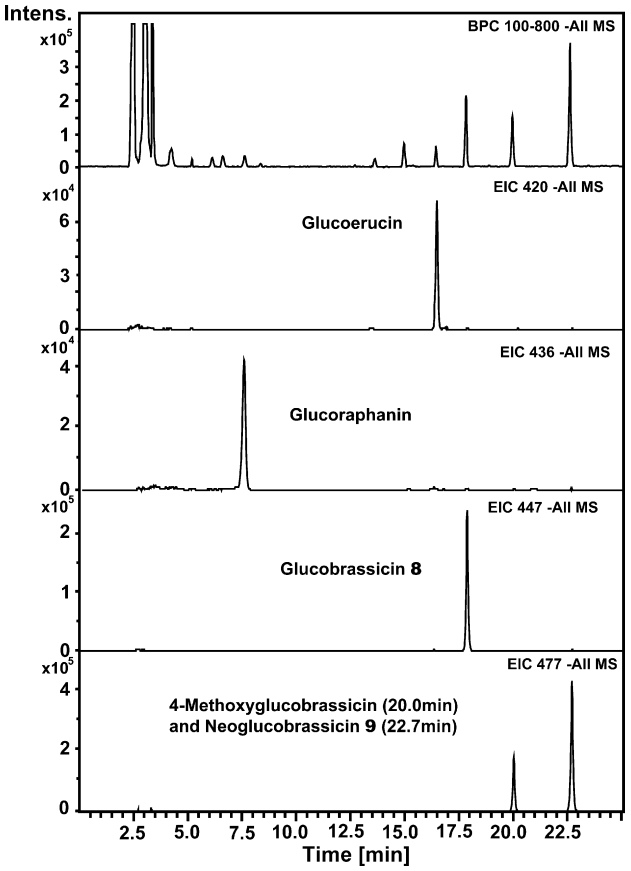


Fig. 6. Major glucosinolates identified in broccoli. Base peak chromatogram (BPC) of the extract (top) and extracted ion chromatograms (EIC) of major glucosinolates are shown.

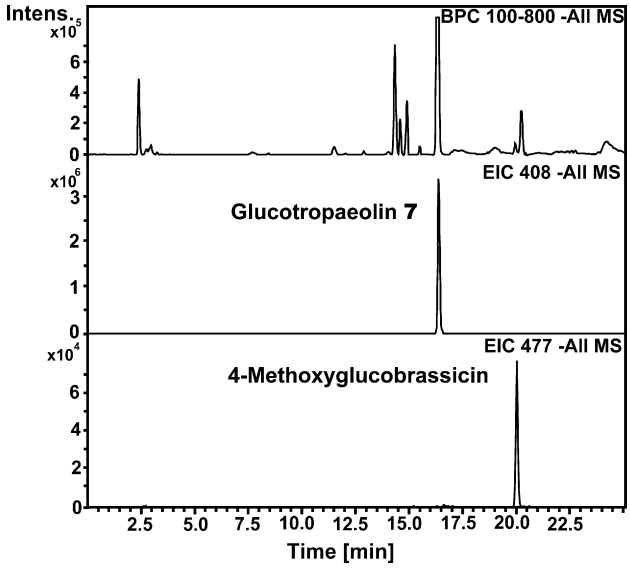


Fig. 7. Major glucosinolates identified in garden cress. Base peak chromatogram (BPC) of the extract (top) and extracted ion chromatograms (EIC) of major glucosinolates are shown.

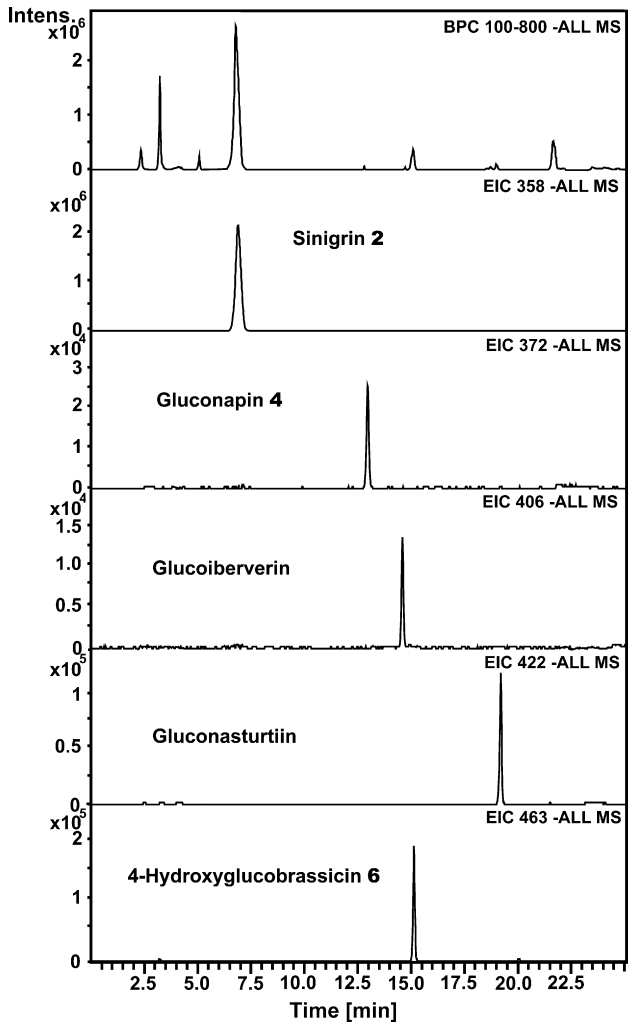


Fig. 8. Major glucosinolates identified in black mustard seeds. Base peak chromatogram (BPC) of the extract (top) and extracted ion chromatograms (EIC) of major glucosinolates are shown.

4. Discussion

In 1982, Minchinton et al. [20] published a desulfatation procedure for the quantification of glucosinolates, which served as the basis for the official method of the European Community [9], and for the majority of publications reporting quantitative data on glucosinolates. Comparatively few publications have recently attempted a direct analysis of glucosinolates, and limited information is available concerning validation of extraction, sample workup and chromatographic analysis.

For natural product analysis, purity assessment of reference compounds is often incomplete or even lacking. This is also the case for glucosinolates [10,15,24]. Due to their ionic nature, assessment of the true content is particularly critical, as reference samples may contain unknown amounts of salts and water of crystallization. Quantitative ^{1}H NMR (qHNMR) is the currently most appropriate method to determine the absolute amount of compound in a reference sample, and has been successfully applied in natural product analysis [35,36]. Using 1,3,5-trimethoxybenzene as reference, we determined the content of all purchased and isolated glucosinolates references. For compounds **1–9**, previous analysis by HPLC and by qualitative NMR gave >99% purity. However, qHNMR data revealed significant differences of true content, ranging from >99% for sinigrin (**2**) to 17% for 4-hydroxyglucobrassicin (**6**). The majority of references were in the range of 60–90% purity.

The result of any quantitative assay of a herbal drug critically depends on the extraction step which precedes the dosage. In all published methods for glucosinolate analysis, limited effort was devoted to optimization and validation of crucial extraction parameters and sample preparation steps. In our study, we used pressurized liquid extraction (PLE), a technique that is well suited for plant extraction [30,37]. Based on own experience in development of validated PLE procedures [27–29,38–40], we optimized critical parameters such as solvent composition, particle size of herbal drug, temperature, and number of required extraction steps. An unexpected finding was the extent of thermal degradation at temperature above 50 °C. This observation revealed a problem with existing methods [9–15], where extractions were typically carried out at temperatures between 70 °C and 100 °C to inactivate myrosinase, even though thermal degradation of glucosinolates during cooking of vegetables has been reported [3,41,42]. Compared to other classes of plant metabolites, glucosinolates appeared particularly sensitive to heat. Temperatures of 60–70 °C have been, typically used in validated PLE methods for various classes of natural products [27–29,38] including labile compounds such as the indigo precursors isatan A and B in woad [39], and temperatures as high as 120 °C were optimal for the extraction of triterpenoidal esters in evening primrose seeds [40].

Enzymatic hydrolysis of glucosinolates by myrosinase has been considered a critical issue in extraction, and published quantitative assays therefore aimed at inactivating the enzyme, by using denaturing solvents [13–15,25,26] or extraction at elevated temperatures [9–15,20,22]. We investigated possible loss of **5** and **8** due to myrosinase. In *I. tinctoria* leaves which had been freeze-dried, stored at −20 °C and thawed prior to extraction, we found no enzymatic degradation over 200 min at ambient temperature (Supplementary Material, Fig. 3). These data, however, cannot be extrapolated to fresh plant material, where myrosinase activity is expected due to the high water content.

Chromatography of intact glucosinolates requires suitable additives in the mobile phase. Trifluoracetic acid has been used in earlier studies [16,18,23] but did not result in acceptable peak shape and chromatographic resolution in our preliminary tests. Better results were obtained with ammonium formate, but extensive testing of several stationary phases from different manufacturers revealed major differences in performance. Sample preparation for HPLC analysis turned out to be critical. Removal of the extraction solvent (70% methanol) and redissolving in mobile phase A substantially improved peak shape.

Thanks to the use of the internal standard sinigrin (**2**), the LC–MS performance was sufficiently stable over extended periods. Inter-day repeatability was in the range of 96.5% to 101.0% over 67 h. Accuracy was satisfactory, as determined by spiking experiments with the major glucosinolates sulfoglucobrassicin

(**5**) (98%) and glucobrassicin (**8**) (97%), and with the minor compound progoitrin (**3**) (102%) (Supplementary Material, Table 1). Limited information is available concerning method validation of published procedures. Song et al. [18] and Tian et al. [24] determined validation parameters such as limit of detection, accuracy and intra-assay precision with intact glucosinolates. Reported LODs (0.4–0.6 pmol [24]) were comparable to our findings. However, analyte recoveries determined by spiking experiments were lower (≥85%) than in our experiments (≥97%).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2007.08.028.

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Supplementary Material

Extraction and Quantitative Analysis of non-derivatized Glucosinolates in Plant Extracts – a Validated PLE/LC-MS Protocol

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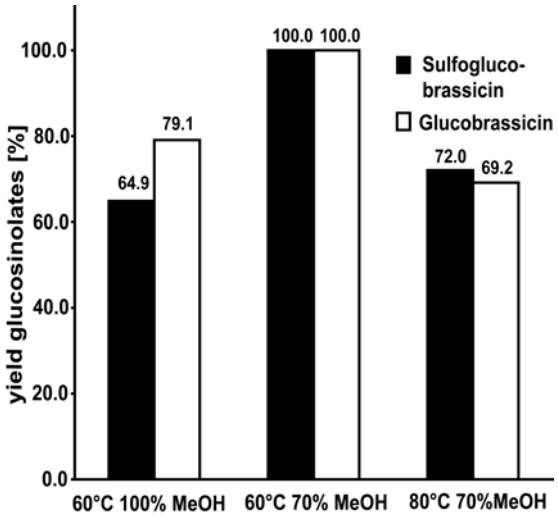

Figure 1: Optimization of extraction parameters. Influence of the solvent composition on the yield of glucosinolates **5** and **8** from *Isatis tinctoria* leaves ("Jenaer Waid"). The yields obtained at 60°C with 70% MeOH were set as 100%.

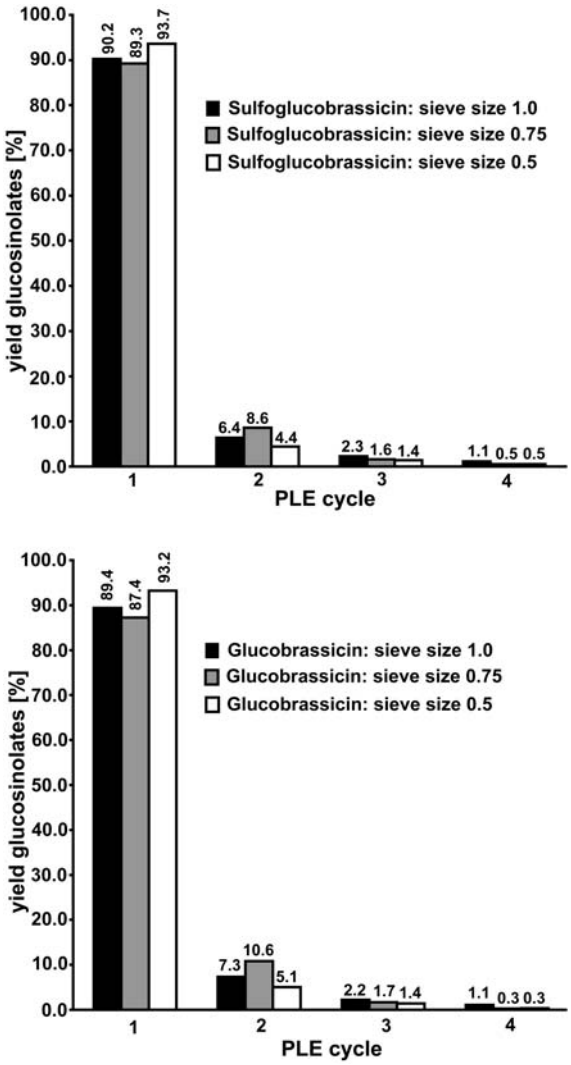

Figure 2: Optimization of extraction parameters. Influence of the particle size on the yield of glucosinolates **5** and **8** extracted from *Isatis tinctoria* leaves ("Jenaer Waid"). The cumulative yields of four extraction cycles were set as 100%.

Table 1: Recovery rates for compounds **3**, **5** and **8**.

Compound	Amount added to extract	Recovered amount*	Relative recovery rates
Progoitrin (**3**)	10.0	10.2 ± 0.6	102%
Sulfoglucobrassicin (**5**)	70.0	68.5 ± 0.3	98%
Glucobrassicin (**8**)	20.0	19.4 ± 0.3	97%

*Amounts in ng on column (20µl injection). Determinations were made in triplicate.

Table 2: Calibration curves for compounds **1,** and **3**-**9**

Compound	Curve	Concentration* [mg/ml], R^2
Epiprogoitrin (**1**)	$y = 85.907x^2 - 3115.1x + 71372$	0.0001 - 0.02; $R^2 = 0.9999$
Progoitrin (**3**)	$y = 97.22x^2 - 3818.4x + 88592$	0.0002 - 0.02; $R^2 = 0.9999$
Gluconapin (**4**)	$y = 192.58x^2 + 1023.1x + 28740$	0.0001 - 0.02; $R^2 = 1$
Sulfoglucobrassicin (**5**)	$y = 7.7837x^2 + 596.33x - 7186.8$	0.001 - 0.02; $R^2 = 0.9999$
4-Hydroxyglucobrassicin (**6**)	$y = 18.002x^2 - 34.08x + 14458$	0.0005 - 0.02; $R^2 = 1$
Glucotropaeolin (**7**)	$y = 189.91x^2 + 9274.3x - 58217$	0.0001 - 0.02; $R^2 = 0.9998$
Glucobrassicin (**8**)	$y = 48.575x^2 - 1221.7x + 46037$	0.0002 - 0.02; $R^2 = 0.9999$
Neoglucobrassicin (**9**)	$y = 69.715x^2 + 856.11x + 27276$	0.0002 - 0.02; $R^2 = 1$

* Concentration range for which the curves apply. Solutions were analyzed in five replicates.

3.4 Seasonal changes and effect of harvest on glucosinolates in *Isatis* leaves

(Mohn et al., Planta Med 2008; 74:582-587).

Seasonal Changes and Effect of Harvest on Glucosinolates in *Isatis* leaves

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Key words
- *Isatis tinctoria*
- *Isatis indigotica*
- Brassicaceae
- seasonal variation
- harvest
- glucosinolates

Abstract

The seasonal fluctuation of glucosinolates in five defined *Isatis tinctoria* and one *Isatis indigotica* accessions (first year, rosette stage), grown on field plots under identical conditions, was investigated. Analysis of the intact glucosinolates was carried out with shock frozen, freeze dried leaf samples using a recently developed and validated PLE (pressurized liquid extraction) protocol and ion-pair HPLC coupled with ESI-MS in the negative mode. When comparing the two *Isatis* species, significant qualitative and quantitative differences in the glucosinolate patterns were observed. Differences among the various *Isatis tinctoria* accessions were much smaller. We studied the effects of repeated harvesting during the growth season on glucosinolate concentrations and found that repeated harvest did not have a major effect on glucosinolate concentrations of newly grown leaves. Glucosinolates could not be detected in woad leaves submitted to conventional drying.

Abbreviations

AFLP: amplified fragment length polymorphism
BPC: base peak chromatogram
COX2: cyclooxygenase 2
EIC: extracted ion chromatogram
FW: "French woad"
IS: internal standard
JW: "Jenaer Waid"
KW: "Kieler Waid"
5-LOX: 5-lipoxygenase
PLE: pressurized liquid extraction
SW: "Swiss woad"
TW: "Thüringer Waid"
TCM: Traditional Chinese Medicine
TLL: Agricultural Research Station of Thuringia

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

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Introduction

Woad (*Isatis tinctoria* L., family Brassicaceae) had been used as an indigo dye in Central Europe since antiquity and as a medicinal plant for the treatment of inflammatory diseases [1]. In China, Banlangen (*Isatis* root) and Daqingye (*Isatis* leaf) from the taxonomically closely related *Isatis indigotica* Fort. remain until now important herbal drugs in Traditional Chinese Medicine (TCM) for the treatment of inflammatory ailments. We investigated the anti-inflammatory potential of *I. tinctoria* leaf extracts in a broad-based pharmacological screening, and tryptanthrin, γ-linolenic acid and 1,3-dihydro-3-[(4-hydroxy-3,5-dimethoxyphenyl)methylene]-2*H*-indol-2-one were identified as active principles inhibiting cyclooxygenase-2, 5-lipoxygenase, the expression of the inducible nitric oxide synthase, human neutrophil elastase, and the release of histamine from mast cells [2], [3], [4], [5], [6]. Animal pharmacological studies [7], [8] and a clinical pilot study [9] corroborated the potential of lipophilic *Isatis* extracts as an anti-inflammatory.

Parallel to these pharmacological investigations, phytochemical and analytical studies of the plant were carried out. We previously reported seasonal variations of indigo precursors in woad leaves and observed that the phytochemical profiles of *Isatis tinctoria* L. (woad) and *I. indigotica* Fort. underwent profound changes during post-harvest treatment. Indigo precursors largely dissappeared whereas pharmacologically active compounds such as tryptanthrin were formed during

the drying process [10], [11]. In fresh woad leaves, two classes of secondary metabolites occur in rather high concentrations, namely the indigo precursors, such as indican, and isatans A and B [12], and glucosinolates [13], [14], [15], [16].

As part of a comprehensive metabolite profiling, we here report on the seasonal fluctuation of glucosinolates in five *I. tinctoria* accessions grown under identical conditions. For comparative purposes, we also analyzed the glucosinolate pattern of the closely related *I. indigotica*. The effects of repeated harvesting during the growth season on glucosinolate concentrations, and the influence of post-harvest processing were studied.

Materials and Methods

Chemicals and reference compounds

Analytical grade solvents for extraction and HPLC grade solvents for chromatography were purchased from Scharlau. HPLC grade water was obtained by an EASY-pure II (Barnstead) water purification system. Ammonium formate (≥ 99.995 %) and sinigrin (**2**) (propenyl glucosinolate) (> 99 %) were purchased from Sigma-Aldrich.

Potassium salts of progoitrin (**3**) [2(*R*)-2-hydroxy-3-butenyl glucosinolate], epiprogoitrin (**1**) [2(*S*)-2-hydroxy-3-butenyl glucosinolate], gluconapin (**4**) (3-butenyl glucosinolate) and glucotropaeolin (**7**) (phenylmethyl glucosinolate) were purchased from the Department of Natural Sciences, University of Copenhagen, Denmark. Glucobrassicin (**8**) (3-indolmethyl glucosinolate), sulfoglucobrassicin (**5**) (1-sulfo-3-indolylmethyl glucosinolate), 4-hydroxyglucobrassicin (**6**) (4-hydroxy-3-indolylmethyl glucosinolate) and neoglucobrassicin (**9**) (*N*-methoxy-3-indolylmethyl glucosinolate) were isolated from seeds of *I. tinctoria*. Purity and structures of isolated compounds were confirmed by NMR and LC-MS experiments as previously described [17].

Plant material

Leaf material of five defined strains of *I. tinctoria* ("Thüringer Waid", "Jenaer Waid", "Kieler Waid", "French Woad" and "Swiss Woad") was harvested in 2003 on June 11 and 23, on July 16 and 29, on August 21, and on September 15, from one-year-old plants (rosette stage). Furthermore, leaf material of *I. indigotica* Fort. was collected on the same days as *I. tinctoria*. Plants were grown under identical conditions on experimental plots of the Agricultural Research Station of Thuringia (TLL), Dornburg, Germany, in uniform rows without randomization. "Thüringer Waid is a mix of genotypes growing in the state of Thuringia which has been cultured for many years. The strains "Jenaer Waid", "Kieler Waid" and "Swiss woad" originated from plants grown at the botanical gardens of the University of Jena, Germany, the University of Kiel, Germany, and the University of Lausanne, Switzerland, respectively. "French woad" is from a collection of wild plants growing at Château de Magrin, near Toulouse, France. *I. indigotica* was from the botanical garden of the University of Bristol, England. The strains were obtained in 1991/1992 by selection breeding of plants with positive traits grown and multiplied under isolation conditions. Annual elimination of plants which did not correspond to the desired phenotype led to a high degree of phenotypical homogeneity of the strains by the year 2003. Specimens of seeds from the strains are kept at the TLL, under accession numbers 153/PG 1 (Thüringer Waid), 153/PG 4 (Swiss woad, 153 PG 5 (French woad), 153 PG 9 (Kieler Waid), 153 PG 10 (Jenaer Waid) and 153 PG 12 (*Isatis indigotica*). Sowing was in early spring at 5 kg seeds/ha, nitrogen fertilization was at 120 kg N/ha at the time of sowing, and 50 kg N/ha each after first and second cuts. A defined treatment of herbicides was applied for weed control. In addition to plants that were left to grow over the entire growth period without being cut, further plants from the strains "Jenaer Waid" and "Thüringer Waid" were cultivated under identical conditions but leaf rosettes were cut with a mowing machine on June 24 and July 31, and the newly grown leaves were analyzed to study the effects of repeated harvesting. Samples were taken on the same harvest dates as described above, with the exception of June 11.

Sample preparation and extraction

Freshly harvested *Isatis* leaf material was cut into small pieces of 2 – 3 cm length and immediately shock frozen with liquid nitrogen. Prior to extraction, the leaves were lyophilized for 48 h (Freezemobile 12XL, Virtis), powdered frozen in liquid nitrogen with a ZM 1 ultracentrifugal mill (Retsch, with 0.75 mm Conidur sieve) and kept frozen at below –20 °C until extraction. In addition, entire leaves were dried at room temperature or at 40 °C in a thermostatted oven as described previously [11] to investigate the influence of different post-harvest treatments. Pressurized liquid extraction (PLE) of 1.0 g frozen and powdered samples was carried out with an ASE 200 instrument (Dionex) with attached solvent controller. Conditions for all extractions were as follows: extraction solvent: 70 % methanol in water; temperature: 50 °C; 3 extraction cycles of 5 min; preheat time: 1 min; flush: 100 % of cell volume; purge: 80 s with nitrogen; pressure: 120 bar; 11 mL steel cartridges. The extracts of 3 extraction cycles were combined, and the solvent was evaporated under reduced pressure. Dried extracts were stored at below -20 °C until analysis.

LC-MS instrumentation and conditions

HPLC separations were carried out on an Agilent series 1100 system equipped with degasser, binary high pressure mixing pump, column thermostat and photodiode array (PDA) detector (Agilent Technologies). A liquid handler 215 (Gilson) was used as autosampler. The HPLC was coupled to an Esquire 3000 plus ion trap mass spectrometer equipped with an electrospray (ESI) interface (Bruker Daltonics). Data acquisition and processing was performed using HyStar 3.0 software (Bruker Daltonics).

Separations were carried out on an Aqua C18 125 Å column (5 μm, 250 × 4.5 mm I.D.; Phenomenex) equipped with a guard column (4.0 × 3.0 mm I.D.). Mobile Phase A consisted of a solution of 10 mM aqueous ammonium formate, adjusted to pH 6.4. Mobile phase B was acetonitrile. A linear gradient starting at 1 % B to 3 % B (7 min), 3 % B to 15 % B (5 min), 15 % B to 30 % B (13 min) was used to separate all compounds of interest. Flow rate was 1.0 mL/min. A split ratio of 1 : 4 was used with the ESI interface. Column temperature was 20.0 °C. The sample injection volume was 20 μL.

Negative ion LC-MS on the ion trap instrument were recorded after optimization of settings, under ion charge conditions (ICC 30 000) at a scan speed of 13 000 *m/z*/s, using a Gauss filter width of 0.2 *m/z*. Nitrogen was used as a drying gas at a flow rate of 10 L/min and as a nebulizing gas at a pressure of 30 psi. The nebulizer temperature was set at 300 °C. Spectra were recorded between 0 min and 25 min in the range of *m/z* 100 to 800. Capillary voltage was at 4500 V, endplate offset at –500 V, capillary end voltage at –115.0 V, skimmer voltage –40.0 V and trap drive at 53.4. Details on method development and validation procedures for the PLE/LC-MS protocol have been reported [17].

Quantitative analysis

A solution of 10 mM ammonium formate containing sinigrin (2 mg/L) as internal standard (IS solution) was used for all dilution steps. Stock solutions (1 mg/mL) of all reference compounds were prepared in IS solution, serial dilutions covered a concentration range of 20 to 0.1 μg/mL. Each standard solution was measured in triplicate.

The dried extracts, obtained by extraction of 1.0 g frozen, powdered leaf material, were dissolved in 100.0 mL IS solution. Furthermore, a dilution of 1 : 100 was prepared with IS solution. All solutions were centrifuged (5 min, 1600 × *g*, 4 °C) prior to LC-MS analysis. Measurements were made in triplicate.

Supporting information

Figures representing seasonal changes for individual glucosinolates of *Isatis tinctoria* accessions, and *Isatis indigotica* are available as Supporting Information.

Results and Discussion

Analysis of glucosinolates **1 – 9** (**Fig. 1**) was carried out with a recently developed and validated PLE protocol and ion-pair HPLC coupled with ESI-MS detection in negative mode [17]. Calibration curves were found to be quadratic functions over a concentration range of 0.1 to 20 μg/mL. Regression coefficients ranged between 0.9995 and 1. **Fig. 2** shows the extracted ion chromatogram (EIC) of the reference compounds **1 – 9**, the base peak chromatogram (BPC) of the extracts, and the EIC used for peak integration of the identified glucosinolates.

Leaf extracts of *I. tinctoria* and *I. indigotica* showed significant qualitative and quantitative differences in their glucosinolate pattern (**Fig. 2**). Glucobrassicin (**5**) and sulfoglucobrassicin (**8**), the major glucosinolates of *I. tinctoria*, could not be identified in the taxiconomically closely related species *I. indigotica.* Progoitrin (**3**) and 4-hydroxyglucobrassicin (**6**) constituted the main glucosinolates in *I. indigotica*. The aliphatic glucosinolates epiprogoitrin (**1**) and epiprogoitrin (**3**) were present in higher concentrations than in leaves of *I. tinctoria* (Supporting Information **Fig. 8S**). The total glucosinolate content in *I. indigotica* (28.6 μmol/g dry weight) was only about 50% of the average total glucosinolate content in *I. tinctoria* (accession "Jenaer Waid") (50.5 μmol/g dry weight). Until recently, *I. indigotica* was considered to be a subspecies of *I. tinctoria* , but amplified fragment length polymorphism (AFLP) analysis showed that they have to be considered as two different species [18]. The differences in glucosinolate pattern corroborate these findings.

The analysis of leaves from different *I. tinctoria* accessions showed that the glucosinolate pattern was subject to seasonal changes. The aliphatic glucosinolates examined here were gluconapin (**4**), progoitrin (**3**), and its epimer epiprogoitrin (**1**). Although these compounds constitute minor glucosinolates in *Isatis* leaves (0.1 – 1.3 μmol/g for **1** and **3**, and 0.01 – 0.04 μmol/g for **4**) compared to the indole glucosinolates, changes within the growth period were observed. Samples collected on six different dates showed that four out of five accessions reached highest concentrations of aliphatic glucosinolates towards end of June, whereas peak concentrations were earlier in the case of "French woad" (**Fig. 3**). A steady decline was observed in the later dates of sampling. Sampling before the June 11 date was not possible, since the plantlets were too small. June represents the earlier part of the vegetation period for woad in its first year culture (rosette stage). Similar decrease of aliphatic glucosinolate concentration during the vegetative stage has been observed for other cruciferous species, such as rape (*Brassica napus*) [19], [20].

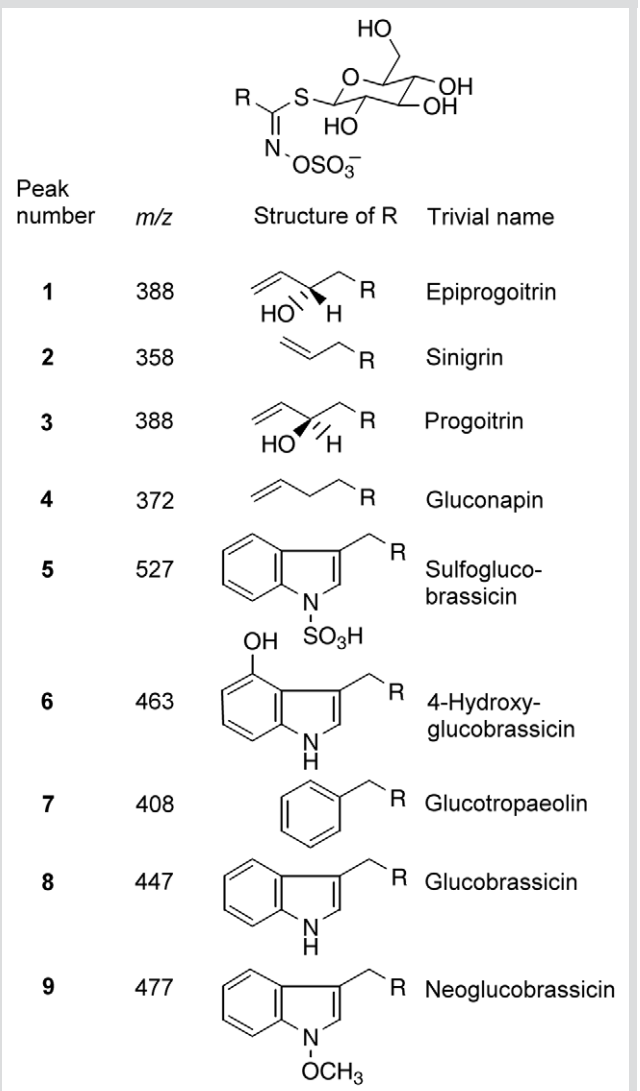

Fig. 1 Structures of glucosinolates **1 – 9**.

Glucobrassicin (**8**), and its derivatives sulfoglucobrassicin (**5**), 4-hydroxyglucobrassicin (**6**) and neoglucobrassicin (**9**) are indolic glucosinolates in *I. tinctoria*. The concentrations of the major compounds **5** and **8** were approx. 100-fold higher than those of aliphatic glucosinolates epiprogoitrin (**1**) and progoitrin (**3**). For the five accessions in our study, the concentrations of total indole glucosinolates were lowest in the young plantlets, and increased several fold to reach a maximum in July/August (**Fig. 4**). A closer analysis of data revealed that the increase was due to sulfoglucobrassicin (**5**), whereas the concentration of glucobrassicin (**8**) remained fairly constant throughout the vegetation season. This trend is illustrated here with the example of the accession "Thüringer Waid" (**Fig. 5**). The other indole glucosinolates in leaves, 4-hydroxyglucobrassicin (**6**) and neoglucobrassicin (**9**) occurred in approx. 100-fold lower concentrations (Supporting Information, **Figs. 4S** and **7S**), and did not show a consistent pattern of seasonal change. Neither glucotropaeolin (**7**), a simple aromatic glu-

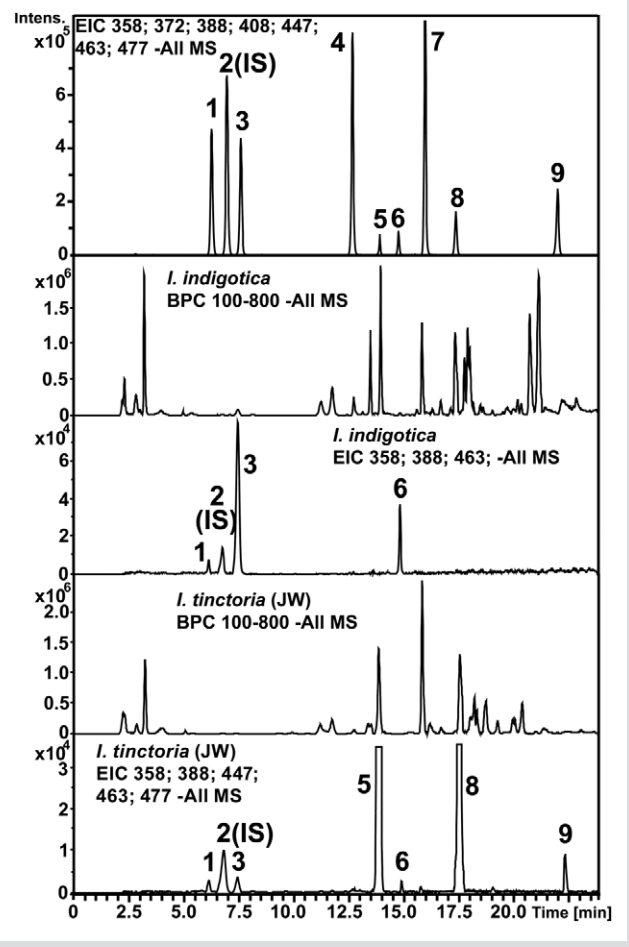

Fig. 2 HPLC separation of a mixture of reference compounds **1 – 9** (top). Sinigrin (**IS**) is used as internal standard. Base peak chromatograms (BPC) and Extracted ion chromatograms (EIC) of the major glucosinolates are shown below for leaf extracts of *Isatis tinctoria* "Jenaer Waid" and *Isatis indigotica*.

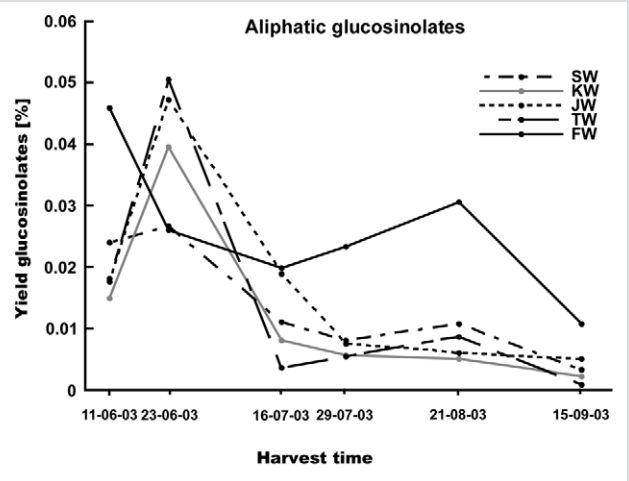

Fig. 3 Concentration of total aliphatic glucosinolates epiprogoitrin (**1**), progoitrin (**3**) and gluconapin (**4**) in shock frozen, freeze-dried leaves of *Isatis tinctoria* accessions "Swiss woad" (SW), "Kieler Waid" (KW), "Jenaer Waid" (JW), "Thüringer Waid" (TW) and "French woad" (FW). The datapoints represent the sum calculated for dried plant material at the respective dates of sampling. Data for individual glucosinolates are given as Supporting Information.

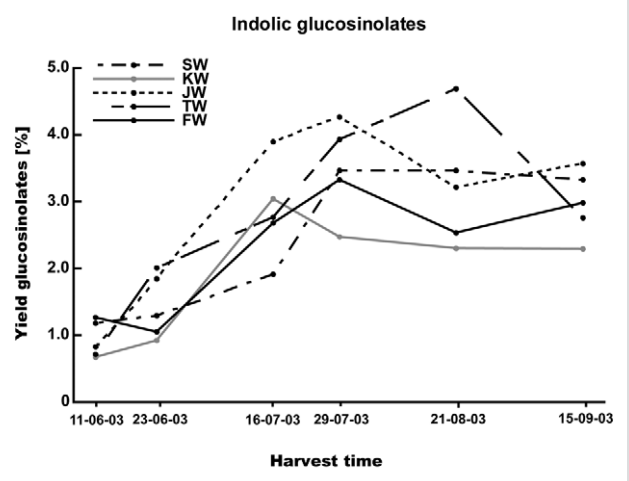

Fig. 4 Concentration of total indolic glucosinolates sulfoglucobrassicin (**5**), 4-hydroxyglucobrassicin (**6**), glucobrassicin (**8**), and neoglucobrassicin (**9**) in shock frozen, freeze-dried leaves of *Isatis tinctoria* accessions "Swiss woad" (SW), "Kieler Waid" (KW), "Jenaer Waid" (JW), "Thüringer Waid" (TW) and "French woad" (FW). The data points represent the sum calculated for dried plant material at the respective dates of sampling. Data for individual glucosinolates are given as Supporting Information.

cosinolate, nor further indolic glucosinolates reported from *I. tinctoria* seeds [16] were detected in any of the leaf samples.

The comparison of five different *I. tinctoria* accessions revealed some differences in their glucosinolate patterns. Leaf samples of the strain "Kieler Waid" had the lowest total glucosinolate concentration. The highest concentration of the major compound sulfoglucobrassicin (**5**) was found in "Thüringer Waid" which, on the other hand, exhibited the least diverse spectrum in other glucosinolates. Gluconapin (**4**) and neoglucobrassicin (**9**) were not detectable in "Jenaer Waid and "Thüringer Waid", and the latter also did not contain 4-hydroxyglucobrassicin (**6**) (Supporting Information, **Figs. 1S–7S**). The differences in glucosinolate pattern seem to reflect the relatively high degree of genetic diversity and varying phenotypic characteristics that were recently reported for European *I. tinctoria* landraces [18].

The effect of repeated harvesting on the qualitative and quantitative composition in glucosinolates was studied with the accessions "Jenaer Waid" and "Thüringer Waid". On one half of the experimental plots plants were left untouched, and only small leaf samples were taken for analysis. On the other part of the plot, the fully grown leaf rosettes were cut with a mowing machine on June 24 and July 31, in order to simulate subsequent harvests during the vegetation season. From the regrown leaf rosettes of these plants, small leaf samples were then taken for analysis at the indicated dates (**Fig. 6**). A comparison of glucosinolate concentrations in these leaf samples revealed no substantial differences between the untouched plants and those with a newly regrown leaf rosette. As shown for sulfoglucobrassicin (**5**) (**Fig. 6**), repeated harvest did not have a major effect on the glucosinolate concentration of newly grown leaves. Data for epiprogoitrin (**1**), progoitrin (**3**) and glucobrassicin (**8**) are given as Supporting Information, **Figs. 9S–11S**.

This study is the first to address seasonal changes of glucosinolates in *Isatis* sp., and in particular in *I. tinctoria*. Sulfoglucobrassicin (**5**), the main indole glucosinolate, accumulated in the leaves over most of the vegetation period. Biosynthetic studies on indolic glucosinolates suggest that glucobrassicin (**8**) is con-

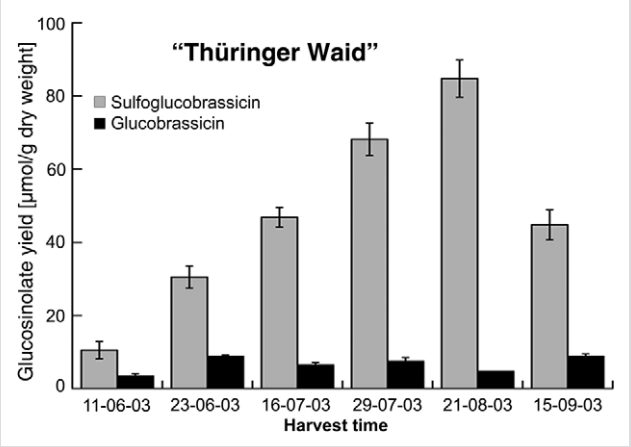

Fig. 5 Concentration of the major glucosinolates sulfoglucobrassicin (**5**) and glucobrassicin (**8**) in the *Isatis tinctoria* accession "Thüringer Waid" harvested at the indicated dates.

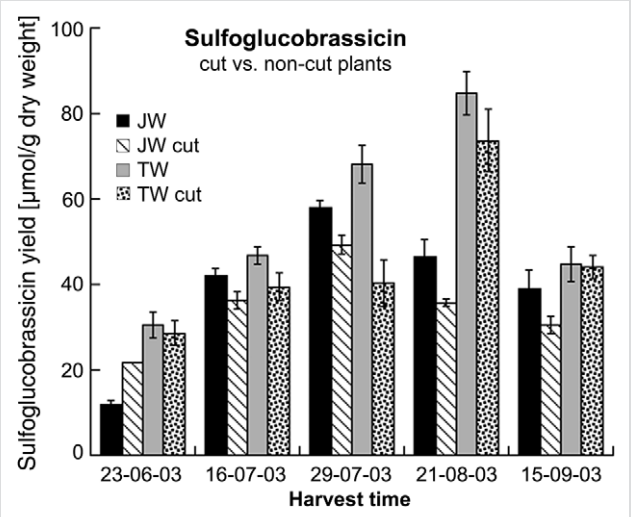

Fig. 6 Concentration (µmol/g) of sulfoglucobrassicin (**5**) in leaf samples of *Isatis tinctoria* accessions "Jenaer Waid" (JW) and "Thüringer Waid" (TW) at the indicated harvest dates, compared to sulfoglucobrassicin concentration in leaf material from plants which were subjected to periodical mowing (JW cut) and (TW cut).

verted to sulfoglucobrassicin (**5**), neoglucobrassicin (**9**) and 4-hydroxyglucobrassicin (**5**) before the initiation of a *de novo* biosynthesis of glucobrassicin (**8**) [21].

Glucosinolates are considered as typical defense compounds of Brassicaceae [22], and their synthesis can be induced to a certain extent by stress. We, therefore, initially thought that glucosinolate concentrations in the regrown leave rosette of the mowed plants would be possibly higher than in the control plants. Increase of indole glucosinolates after puncturing leaves with needles had been reported with rape and mustard cotyledons [23]. However, this upregulation of biosynthesis was only a transient phenomenon with a maximum one day after wounding, and two weeks later the concentration had dropped to the level of untreated control plants. Considering the mowing and sampling regime in our study, the observed lack of marked changes in glucosinolate pattern is in line with these findings. Multiple harvesting of leaf rosette would increase the overall yield of plant material without significantly modifying the phytochemical composition of the leaves, at least for glucosinolates.

As for indigo precursors [10] and tryptanthrin [11], we investigated the influence of the drying procedure on glucosinolate content. In contrast to the shock frozen freeze dried leaf samples, none of the glucosinolates including the major compounds sulfoglucobrassicin (**5**) and glucobrassicin (**8**), could be detected in plant material that had been dried at ambient temperature or at 40 °C (data not shown). However, we were not able to detect any of the known breakdown products of indolic glucosinolates such as indole-3-carbinols [(1*H*-indol-3-yl)methanol and derivatives], indole-3-acetonitriles [2-(1*H*-indol-3-yl)acetonitrile and derivatives] and its corresponding isothiocyanates as well as dimeric compounds as 3,3′-diindolylmethanes [bis(1*H*-indol-3-yl)methane and derivatives] or indolyl ascorbigens (indol-3-ylascorbigen and derivatives) described in earlier studies [24], [25], [26], [27]. The degradation products of indol glucosinolates in woad are presently unknown, and it remains to be clarified whether they contribute in any form to the post-harvest formation of indol-derived compounds such as tryptanthrin.

We previously analyzed the indigo precursors isatan A, isatan B and indican in the same *Isatis* samples [10]. The concentration of total indigo precursors was approximately in the same range as that of the indole glucosinolates (3 – 5 % of dry weight). Hence, tryptophan-derived secondary metabolites clearly dominate in woad. However, the concentration of indole glucosinolates was higher in *I. tinctoria* than in *I. indigotica*, whereas the indigo precursors were higher in *I. indigotica*. This suggests that the biosynthesis of these compounds may be limited by the supply in the precursor amino acid, and secondary metabolism of *I. tinctoria* may tend more towards indole glucosinolate synthesis, and *I. indigotica* towards production of indigo precursors.

Acknowledgements

We thank Dr. A. Vetter and A. Biertümpfel, Thüringische Landesanstalt für Landwirtschaft (TLL), Jena and Dornburg, for the provision of woad samples from the experimental field plots in Dornburg. The major part of the study was carried out as the Master Thesis of K. Suter.

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Supporting Information to:

Seasonal Changes and Effect of Harvest on Glucosinolates in *Isatis* leaves

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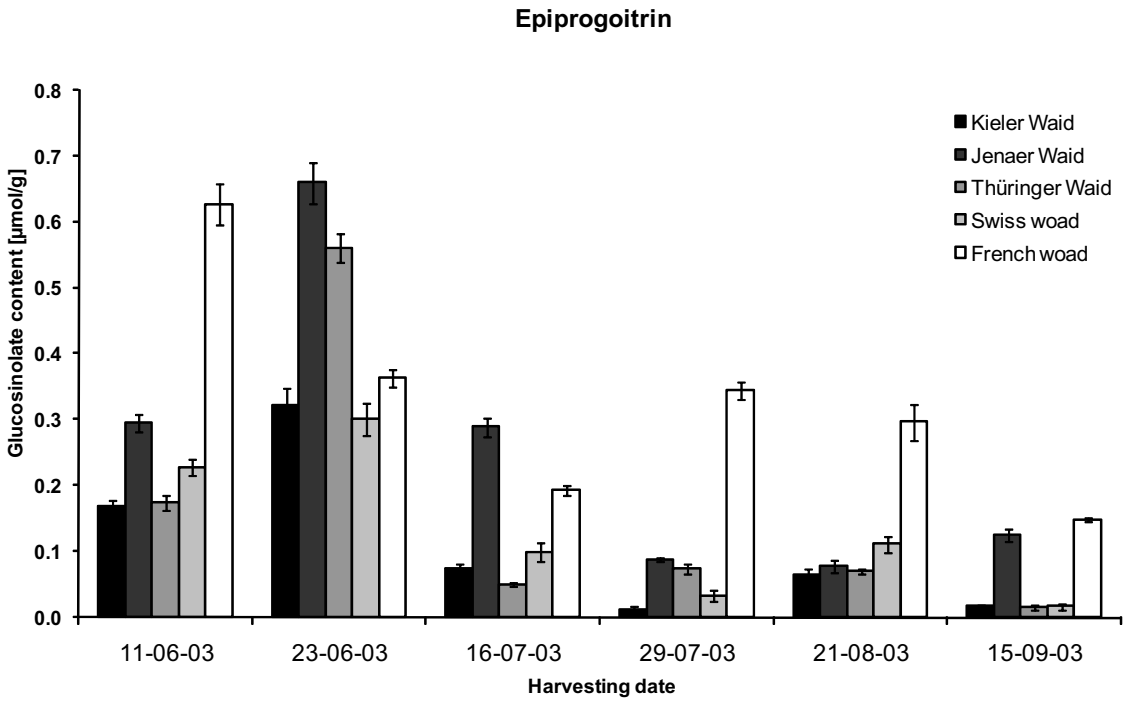

Fig. **1S** Concentration of epiprogoitrin (**1**), in shock frozen, freeze-dried leaves of *Isatis tinctoria* accessions. The dates of sampling are indicated in the graph.

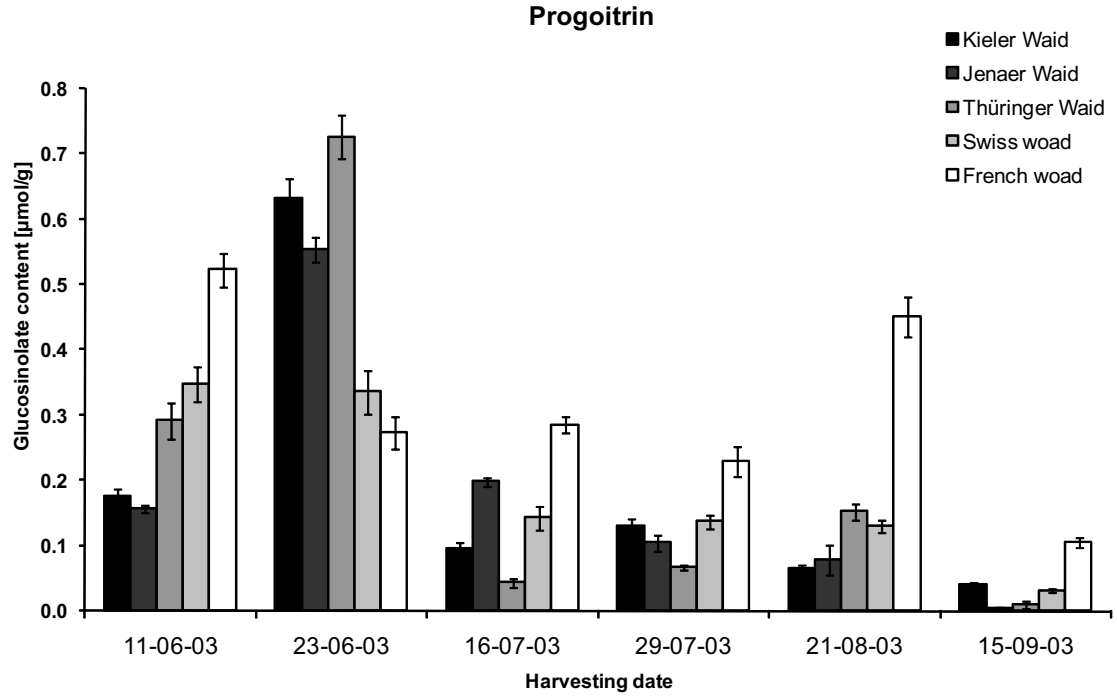

Fig. **2S** Concentration of progoitrin (**3**), in shock frozen, freeze-dried leaves of *Isatis tinctoria* accessions. The dates of sampling are indicated in the graph.

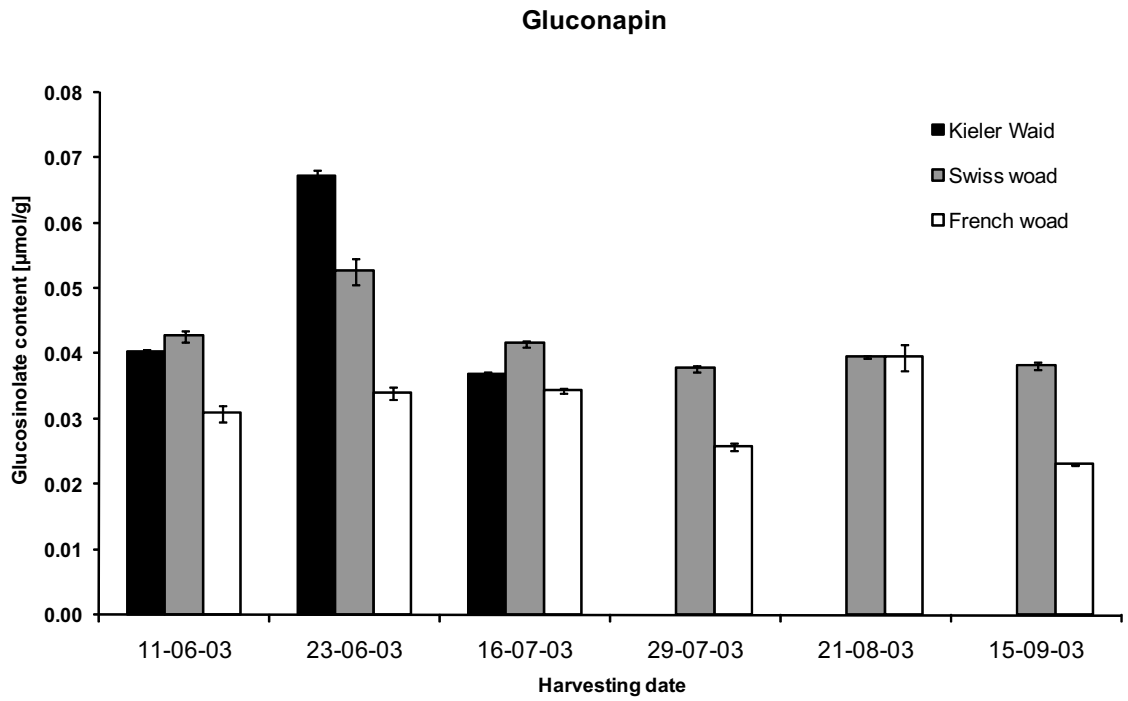

Fig. **3S** Concentration of gluconapin (**4**), in shock frozen, freeze-dried leaves of *Isatis tinctoria* accessions. The dates of sampling are indicated in the graph. The compound was below detection limit in the accessions “Jenaer Waid” and “Thüringer Waid”.

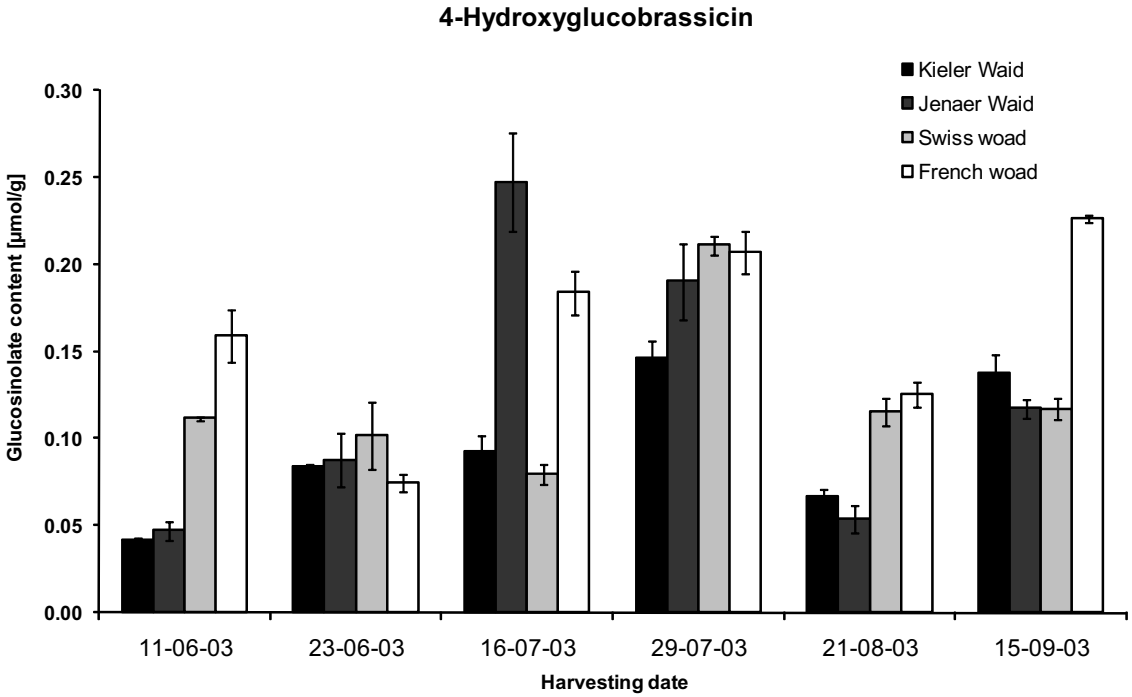

Fig. **4S** Concentration of 4-hydroxyglucobrassicin (**6**), in shock frozen, freeze-dried leaves of *Isatis tinctoria* accessions. The dates of sampling are indicated in the graph. The compound was below detection limit in the accession “Thüringer Waid”.

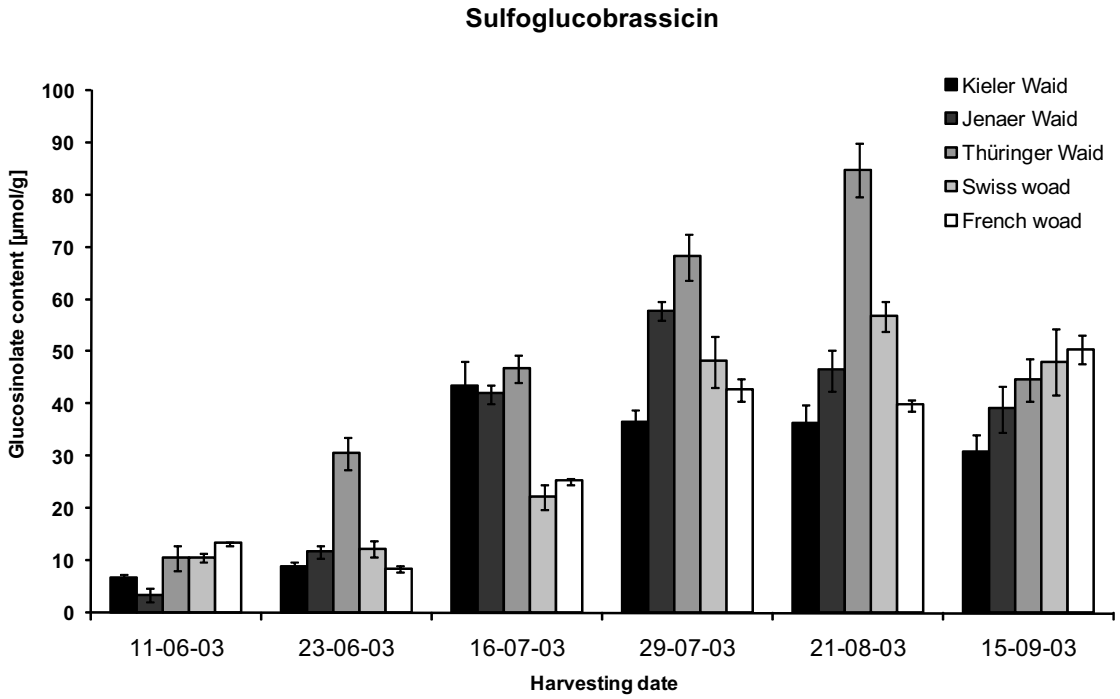

Fig. **5S** Concentration of sulfoglucobrassicin (**5**), in shock frozen, freeze-dried leaves of *Isatis tinctoria* accessions. The dates of sampling are indicated in the graph.

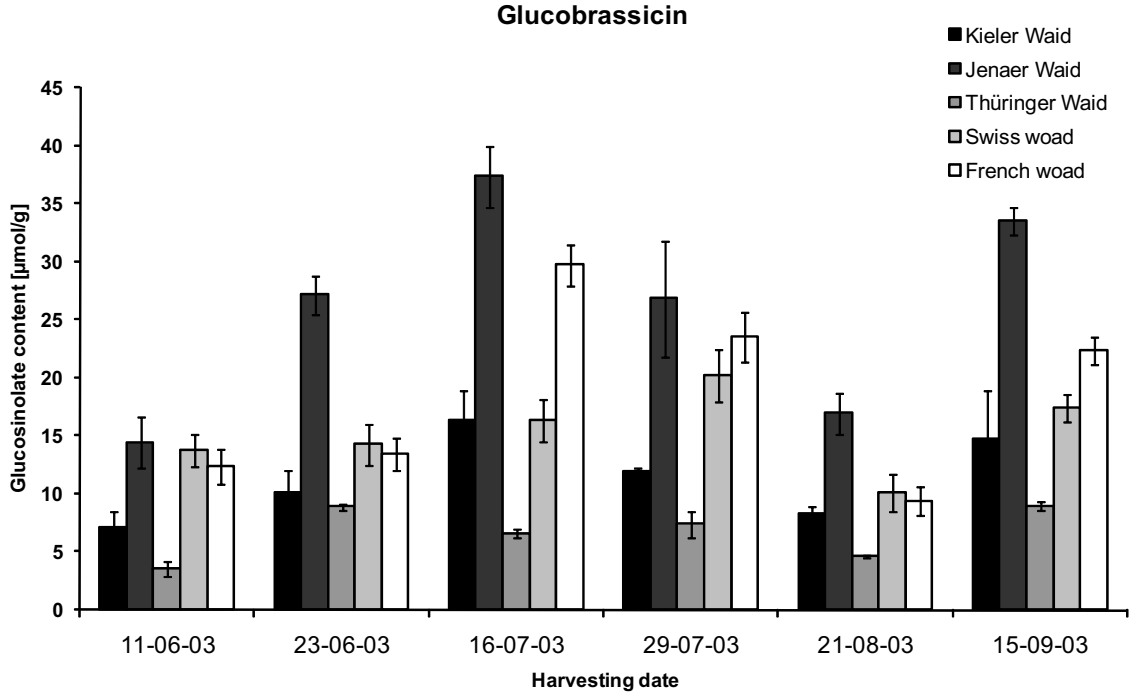

Fig. **6S** Concentration of glucobrassicin (**8**), in shock frozen, freeze-dried leaves of *Isatis tinctoria* accessions. The dates of sampling are indicated in the graph.

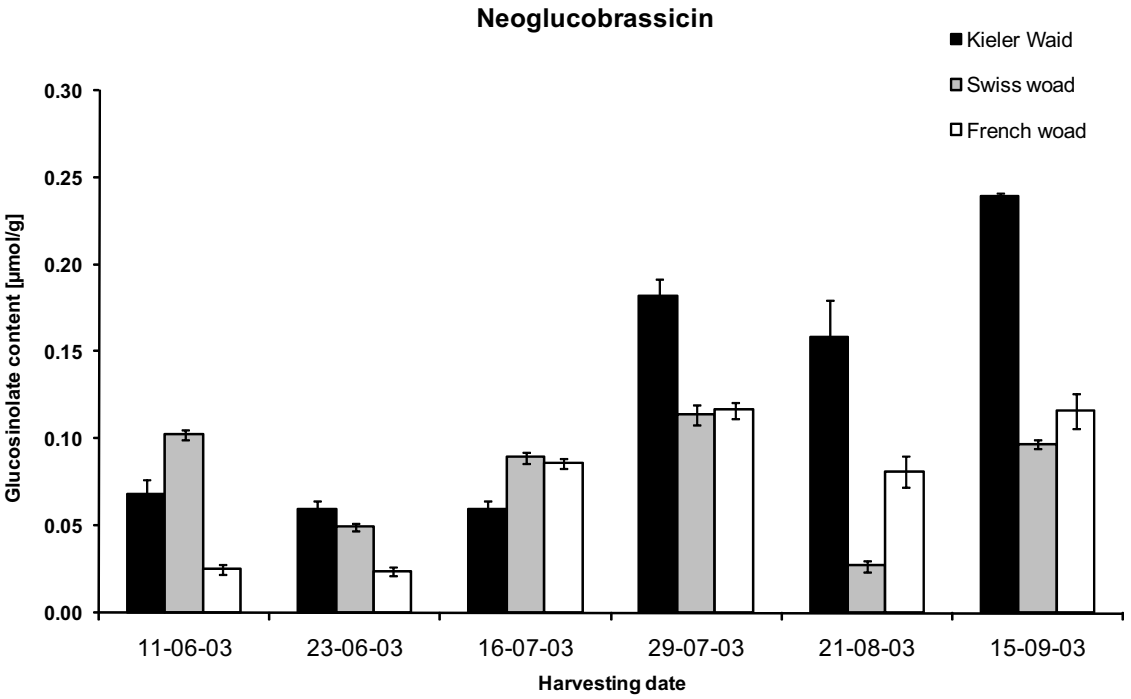

Fig. **7S** Concentration of neoglucobrassicin (**9**), in shock frozen, freeze-dried leaves of *Isatis tinctoria* accessions. The dates of sampling are indicated in the graph. The compound was below detection limit in the accessions “Jenaer Waid” and “Thüringer Waid”.

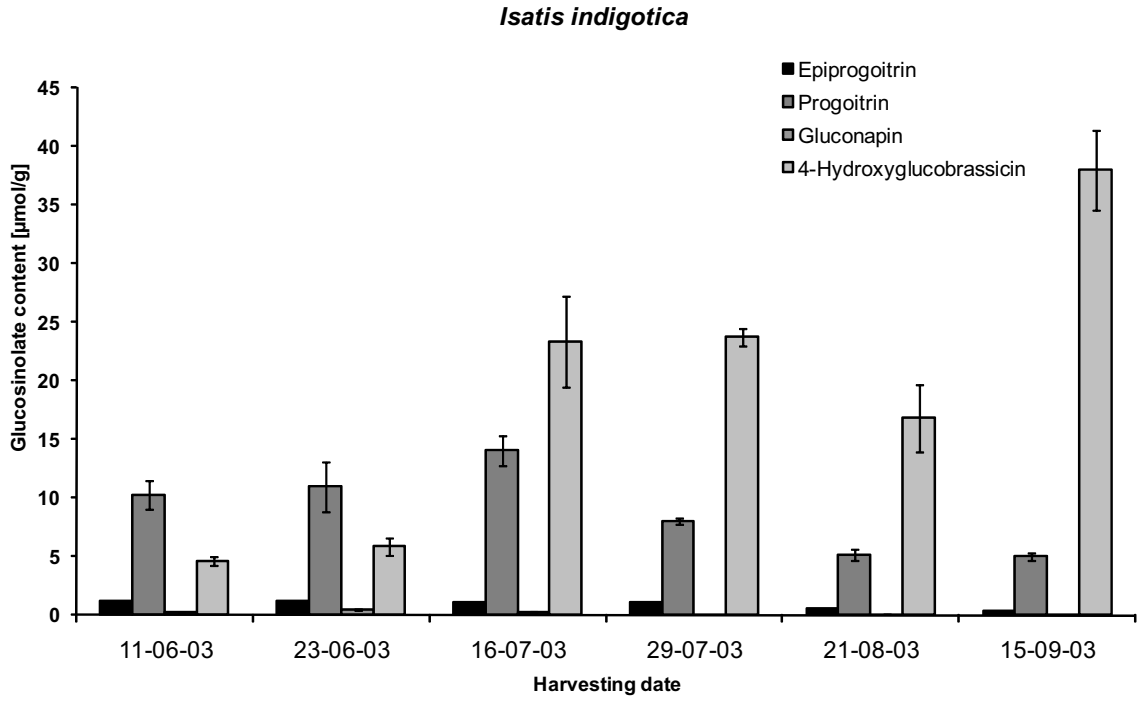

Fig. **8S** Concentration of epiprogoitrin (**1**), progoitrin (**3**), gluconapin (**4**) and 4-hydroxyglucobrassicin (**6**) in shock frozen, freeze-dried leaves of *Isatis indigotica.* The dates of sampling are indicated in the graph.

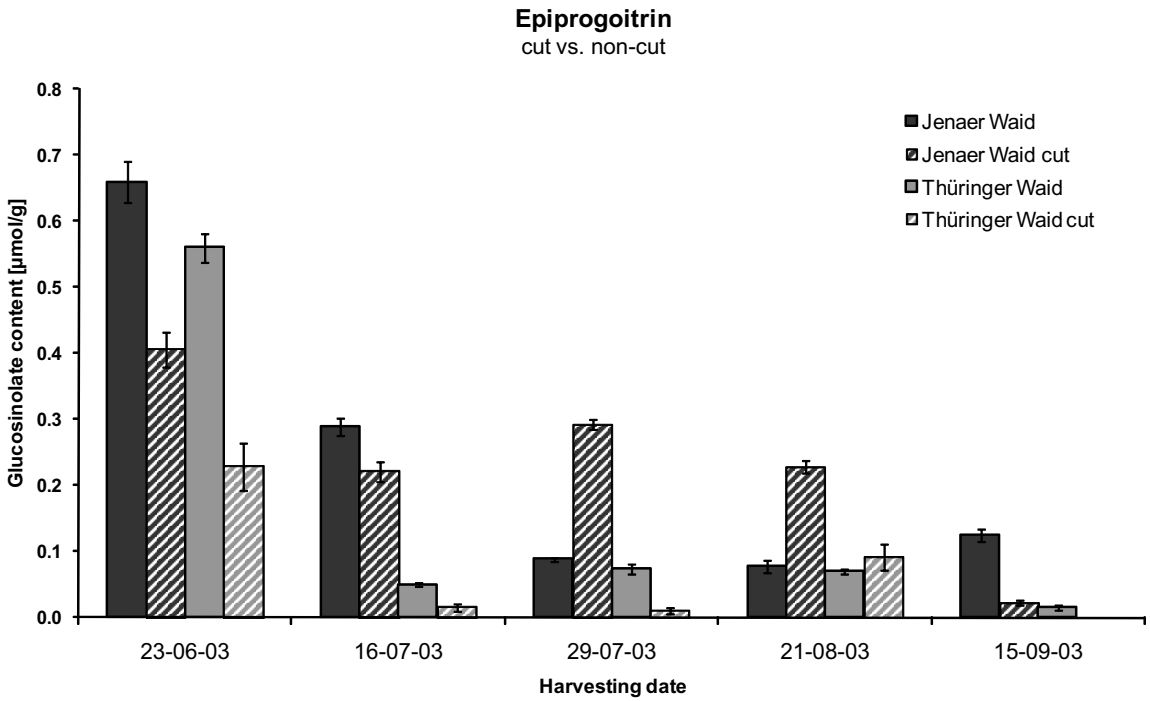

Fig. **9S** Concentration (µmol/g) of epiprogoitrin (**1**) in leaf samples of *Isatis tinctoria* accessions “Jenaer Waid” (JW) and “Thüringer Waid” (TW) at the indicated harvest dates, compared to epiprogoitrin concentration in leaf material from plants which were subjected to periodical mowing (JW cut) and (TW cut).

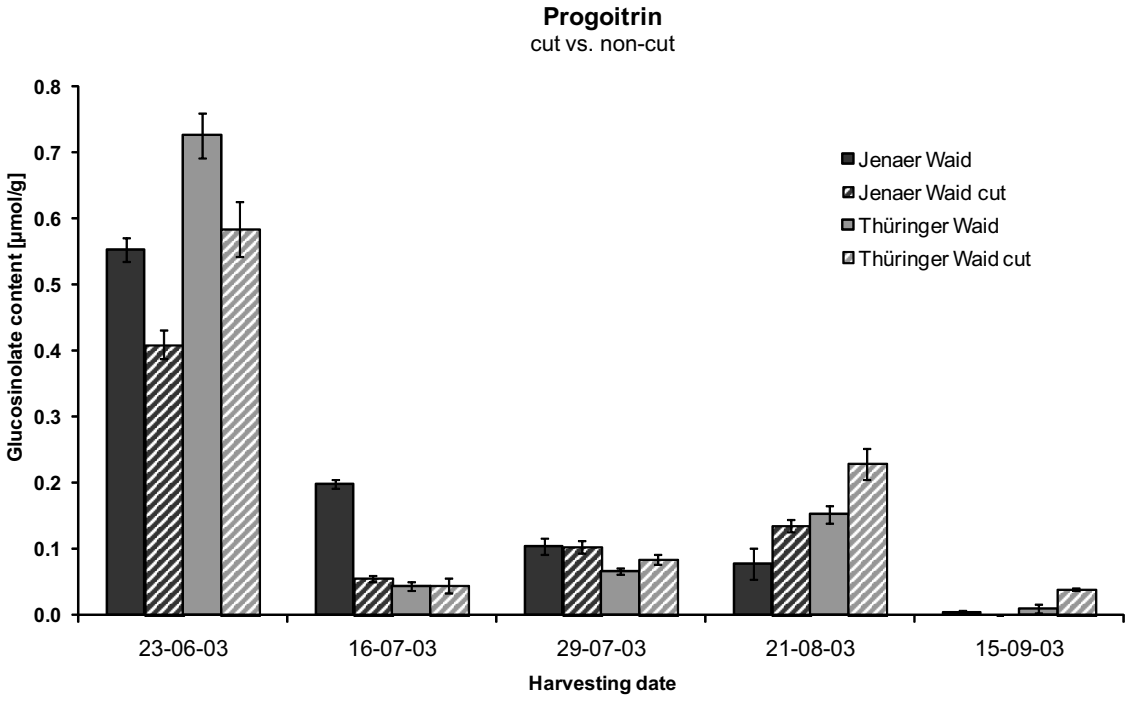

Fig. **10S** Concentration (µmol/g) of progoitrin (**3**) in leaf samples of *Isatis tinctoria* accessions “Jenaer Waid” (JW) and “Thüringer Waid” (TW) at the indicated harvest dates, compared to progoitrin concentration in leaf material from plants which were subjected to periodical mowing (JW cut) and (TW cut).

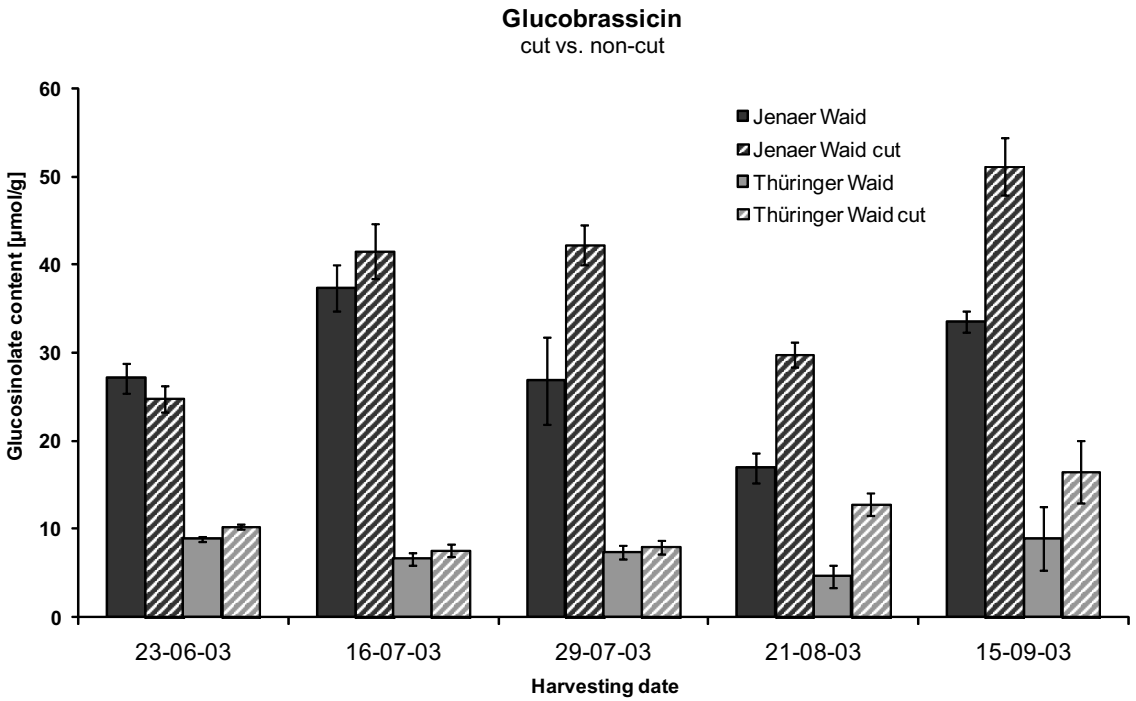

Fig. **11S** Concentration (µmol/g) of glucobrassicin (**8**) in leaf samples of *Isatis tinctoria* accessions “Jenaer Waid” (JW) and “Thüringer Waid” (TW) at the indicated harvest dates, compared to glucobrassicin concentration in leaf material from plants which were subjected to periodical mowing (JW cut) and (TW cut).

3.5 Glucosinolate pattern in *Isatis tinctoria* and *I. indigotica* seeds

(Mohn et al., Planta Med 2008; 74:885-888).

Glucosinolate Pattern in *Isatis tinctoria* and *I. indigotica* Seeds

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Abstract

The glucosinolate patterns in seeds of five *Isatis tinctoria* and two *Isatis indigotica* accessions were assessed with a recently developed and validated LC-MS assay for direct analysis of glucosinolates without prior desulfatation. Glucosinolate peaks were identified with in-source fragmentation and detection of the sulfate anion ($m/z = 97$), and by MS/MS experiments. The glucosinolate patterns of the seeds showed characteristic differences compared to leaves. Glucoisatisin and epiglucoisatisin were diagnostic of seed samples. Qualitative and quantitative differences in glucosinolate patterns between both *Isatis* species were found for seed samples, enabling a differentiation of the two medicinal plants at the level of seed material.

Key words

Isatis tinctoria · *Isatis indigotica* · Brassicaceae · pressurized liquid extraction · LC-MS · glucosinolates

Abbreviations

BPC: base peak chromatogram
COX-2: cyclooxygenase-2
EIC: extracted ion chromatogram
5-LOX: 5-lipoxygenase
PLE: pressurized liquid extraction

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

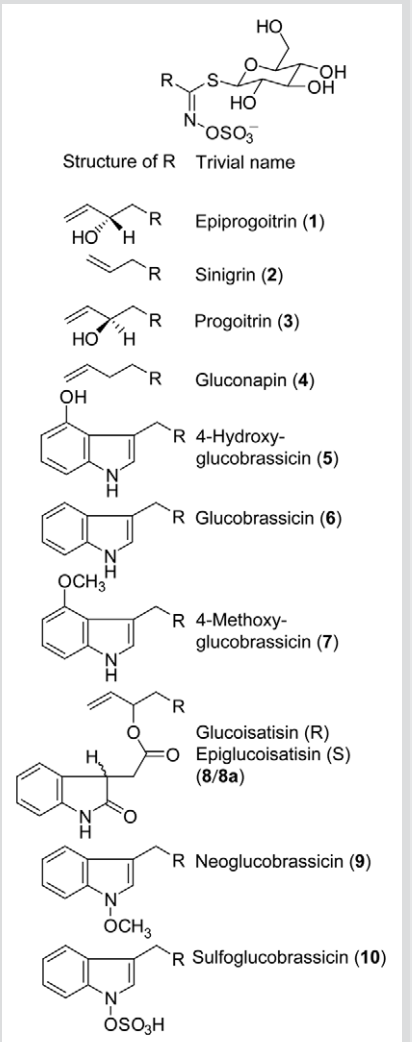

Fig. 1 Structures of compounds **1 – 10**.

Isatis tinctoria L. and *I. indigotica* Fort. (Brassicaceae) are ancient indigo dyes and medicinal plants in Europe and in Traditional Chinese Medicine (TCM), respectively [1]. In recent years, the two species were intensively investigated from a phytochemical and pharmacological viewpoint [2], [3], [4], [5], [6], [7], and in a clinical pilot study [8]. Seasonal variation of the two most abundant classes of secondary metabolites in *Isatis* leaves, indigo precursors and glucosinolates, and changes in metabolite profiles during post-harvest treatment have been studied [9], [10], [11]. Characteristic differences in glucosinolate pattern were observed in leaves of *I. tinctoria* and *I. indigotica*, corroborating the recent separation into two distinct species [12].

Comparatively little is known about the glucosinolates in seeds [13], [14], [15]. We here report on the identification of further indole glucosinolates in seeds of five different *I. tinctoria* and two *I. indigotica* accessions. First, analysis of glucosinolates (structures see **Fig. 1**) was undertaken with a recently developed and validated pressurized liquid extraction (PLE) protocol and ion-pair HPLC coupled with ESI-MS detection in negative ion mode under full scan conditions [16]. In a second HPLC run, peaks corresponding to glucosinolates were earmarked via in-source fragmentation and detection of the fragment ion $m/z = 97$ ($[SO_4H]^-$). In addition, MS/MS experiments were carried out to observe the elimination of the sulfate anion. **Fig. 2** shows the base peak and extracted ion chromatograms of compounds **1 – 6**, **8/8a** and **9** in *I. tinctoria* seeds, the ion trace of the $m/z = 97$ fragment, and ESI-MS recorded under non-fragmenting conditions. LC-TOF-MS in negative mode was used to determine the accurate molecular mass of compounds (conditions see Mohn et al. [16]). Methods for the identification of glucosinolates by MS/MS [17] and quantification by multiple reaction monitoring (MRM) have been recently described [18], [19], [20]. For a rapid search for glucosinolates, we found in-source fragmentation and detection of the characteristic $m/z = 97$ $[SO_4H]^-$ fragment ion to be more sensitive and easy to perform than MS/MS fragmentation. Absolute peak intensities were 10 to 33 times higher for individual glucosinolates caused by in-source fragmentation while MS/MS fragmentation gave equal or lower relative peak intensities for the $m/z = 97$ $[SO_4H]^-$ fragment ion (**Table 1**). These findings are in agreement with observations of Mellon et al. [18].

Characteristic differences in the glucosinolate patterns between leaves and seeds were found. Sulfoglucobrassicin (**10**), the major glucosinolate in *I. tinctoria* leaves, was absent in the seed extracts of either species. Glucoisatisin and epiglucoisatisin (**8/8a**; calcd. 562.0927; found 562.0930 and 562.0932) were found in

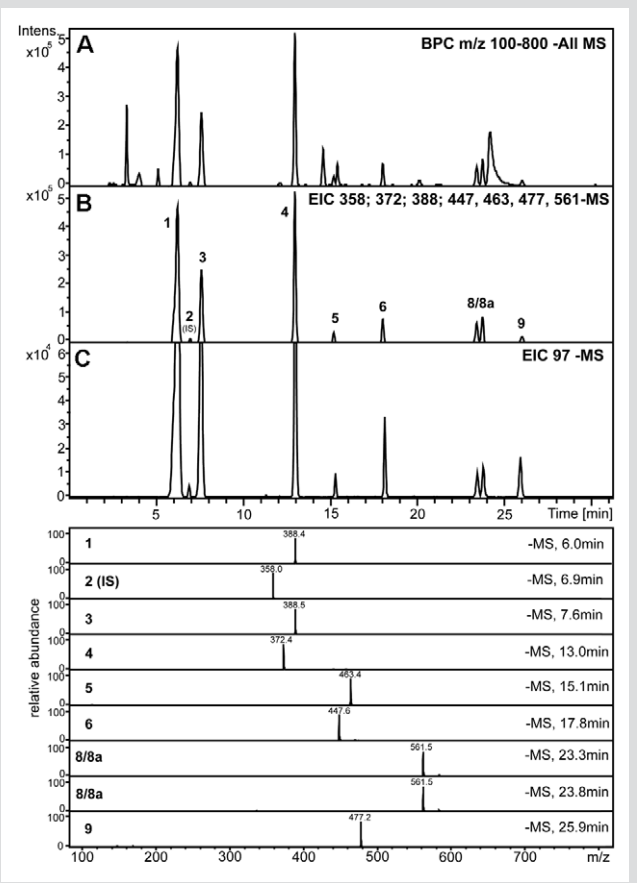

Fig. 2 Glucosinolates in *Isatis* seed extracts. Base peak chromatogram (BPC; **A)** and extracted ion chromatogram (EIC; **B**) of major glucosinolates are shown for *Isatis tinctoria* accession "Thüringer Waid". Sinigrin (**2**) was used as internal standard (IS). The trace of ion 97 *m/z* ($[SO_4H]^-$) resulting from in-source fragmentation was used to selectively detect all glucosinolate peaks (**C**). Mass spectra of compounds **1 – 9** recorded under non-fragmenting conditions are shown below (**C**).

the seeds of both *Isatis* species. These two compounds have been recently identified as an unseparable epimeric mixture from *I. tinctoria* seeds [14]. The two compounds were not found in leaves of the two species [11] and thus can be considered as markers of seed material. Two other glucosinolates reported by Frechard et al. [14], 3′-hydroxyglucoisatisin and 3′-hydroxyepiglucoisatisin, could not be detected in any of the *Isatis* seed samples. A further indolic glucosinolate, 4-methoxyglucobrassicin (**7;** calcd. 478.0716, found: 478.0713), was detected as a trace compound in both *I. indigotica* seed samples, but was absent from any of the *I. tinctoria* accessions.

A recent comparison of glucosinolate patterns in leaves revealed significant qualitative and quantitative differences between the two *Isatis* sp., but comparativey small differences between the *I. tinctoria* accessions [11]. A similar observation could be made in case of the seeds. Comparison of HPLC profiles (**Fig. 3**, and **Fig. 1S** in Supporting Information) showed that glucobrassicin (**6**) and neoglucobrassicin (**9**) were only present in *I. tinctoria* seeds, whereas 4-methoxyglucobrassicin (**7**) was only found in *I. indigotica.*

Only one study (Elliott and Stowe [13]) has been carried out to investigate the distribution of glucosinolates in seeds and leaves of *I. tinctoria*. They found glucobrassicin (**6**) to be the only glucosinolate in the seeds while glucobrassicin (**6**), neoglucobrassicin (**9**) and sulfoglucobrassicin (**10**) were identified in the leaves in considerable amounts. Our results support these findings, with the exception of neoglucobrassicin (**9**) which was present in all strains of *Isatis tinctoria* seeds. However, Elliott and Stowe did not analyze other indolic glucosinolates in their study and did not investigate differences between the *Isatis* species. The content of total glucosinolates in seeds of both species was lower than in the corresponding leaf samples (**Table 2**, and **Table 1S** in Supporting Information). *Isatis tincoria* seeds contained mainly aliphatic glucosinolates, whereas indolic glucosinolates were

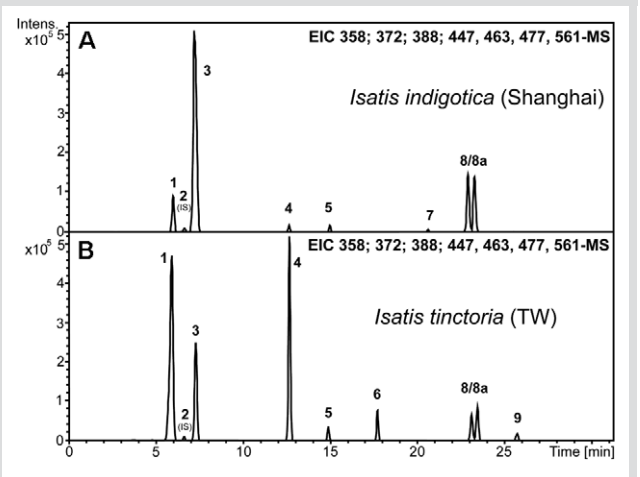

Fig. 3 Comparison of glucosinolates in *Isatis* seed extracts. Extracted ion chromatograms (EIC) of glucosinolates in *Isatis indigotica* (Shanghai) and of the *Isatis tinctoria* accession "Thüringer Waid" (TW). Sinigrin (**2**) was used as internal standard (IS).

Table 1 Comparison of fragmentation conditions. In-source fragmentation gave equal or higher relative signal intensities compared to fragmentation by MS/MS, and the absolute signal intensity was clearly higher for the sulfate fragment *m/z* = 97 via in-source fragmentation

Compound	Relative signal intensity of the m/z = 97 $[SO_4H]^-$fragment ion		Absolute signal intensity ratio in-source vs. MS/MS fragmentation
	In-source fragmentation	Fragmentation by MS/MS	
Epiprogoitrin (**1**)	100%	100%	16:1
Progoitrin (**3**)	100%	100%	12:1
Gluconapin (**4**)	100%	100%	33:1
4-Hydroxyglucobrassicin (**5**)	51%	24%	10:1
Glucobrassicin (**6**)	79%	62%	16:1
Glucoisatisin/Epiglucoisatisin (**8**/**8a**)	100%	16%	20:1
Neoglucobrassicin (**9**)	100%	15%	10:1

Table 2 Differences of the glucosinolate content between *Isatis tinctoria* and *Isatis indigotica* seeds

Glucosinolate	*Isatis tinctoria* ("Thüringer Waid") amount[b] in mg/100 g dw	*Isatis indigotica* (Shanghai) amount[b] in mg/100 g dw
Epiprogoitrin (**1**)	130	50
Progoitrin (**3**)	90	140
Gluconapin (**4**)	55	10
Sulfoglucobrassicin (**10**)	n. d.	n. d.
4-Hydroxyglucobrassicin (**5**)	15	10
Glucobrassicin (**6**)	45	n. d.
4-Methoxyglucobrassicin (**7**)	n. d.	traces
Glucoisatisin (**8/8a**)[a]	90	120
Neoglucobrassicin (**9**)	10	n. d.

n. d. = not detected.

[a] Both isomers together.

[b] Values estimated.

dominant in the leaves [11]. In summary, our findings show organ-specific differences in glucosinolates. In combination with our previously reported results [11] these findings support the molecular taxonomy [12] showing that *Isatis tinctoria* and *Isatis indigotica* should be considered as separate species. From a practical viewpoint, seed material of *Isatis* species can be easily differentiated on the basis of characteristic glucosinolate pattern.

Materials and Methods

Seeds of defined strains of *Isatis tinctoria* ("Thüringer Waid", "Jenaer Waid", "Kieler Waid", "French Woad" and "Swiss Woad") were obtained from the Agricultural Research Station of Thuringia (TLL), Dornburg, Germany. *Isatis indigotica* seeds were purchased from Sand Mountain Herbs ("*Isatis indigotica* USA") and from the Shanghai Institute of Materia Medica, Shanghai, China ("*Isatis indigotica* Shanghai"). Voucher specimens are kept at the Institute of Pharmaceutical Biology, University of Basel under accession numbers 153 PG 1 (Thüringer Waid), 153 PG 4 (Swiss woad), 153 PG 5 (French woad), 153 PG 9 (Kieler Waid), 153 PG 10 (Jenaer Waid), 153 PG 20 (*Isatis indigotica* USA) and 163 PG 21 (*Isatis indigotica* Shanghai).

Glucosinolate analysis was carried out according to a published protocol [16] with small modifications in the HPLC gradient. The mobile phase consisted of an aqueous solution of 10 mM ammonium formate (adjusted to pH 6.4 with formic acid; eluent A) and acetonitrile (eluent B). The gradient of B was as follows: from 1% to 3% in 7 min, 3% to 15% (5 min), 15% to 17% (3 min), held at 17% for 2 min, 17% to 19% (4 min), held at 19% for 3 min, 19% to 24% (6 min). Flow rate was 1.0 mL/min. A split ratio of 1 : 4 was used with the ESI interface. Separations were carried out on an Aqua C18 125 Å column (5 μm, 250 × 4.5 mm I.D.; Phenomenex) equipped with a guard column (4.0 × 3.0 mm I.D.) at 20.0 °C. The sample injection volume was 20 μL. LC-TOF-MS in negative mode was used to confirm the molecular formula of glucosinolates (see [16]).

For the detection of the fragment ion m/z = 97 ($[SO_4H]^-$) mass spectrometric conditions of the Esquire 3000 plus ion trap MS were optimized as follows: ion charge control: (ICC 30 000), scan speed: 13 000 *m/z*/s, gauss filter width: 0.2 *m/z*, nitrogen as drying gas (10 L/min) and nebulizing gas (30 psi), nebulizer temperature: 300 °C, capillary voltage: 4107 V, endplate offset: –500 V, skimmer voltage: –87.5 V and trap drive: 28.4. Spectra were recorded in the range of 70 to 650 *m/z*.

MS^n experiments were performed on the glucosinolate $[M - H]^-$ ions using helium as collision gas. The isolation width was set to 4.0 *m/z* and the fragmentation amplitude was set to 1.00 V in the `smart fragmentation' mode. ESI source voltages were as follows: capillary voltage at 4500 V, endplate offset at –500 V, capillary exit offset at –115 V, skimmer voltage at –40.0 V and trap drive at 53.4. The scan range for mass spectra was 80 *m/z* to 800 *m/z*.

Supporting information

EIC spectra of glucosinolates from *Isatis* seeds and concentration of glucosinolates in *Isatis* seeds in comparison to published data of *Isatis* leaf material and seeds are available as Supporting Information.

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Supporting Information

Glucosinolate pattern in *Isatis tinctoria* and *I. indigotica* seeds

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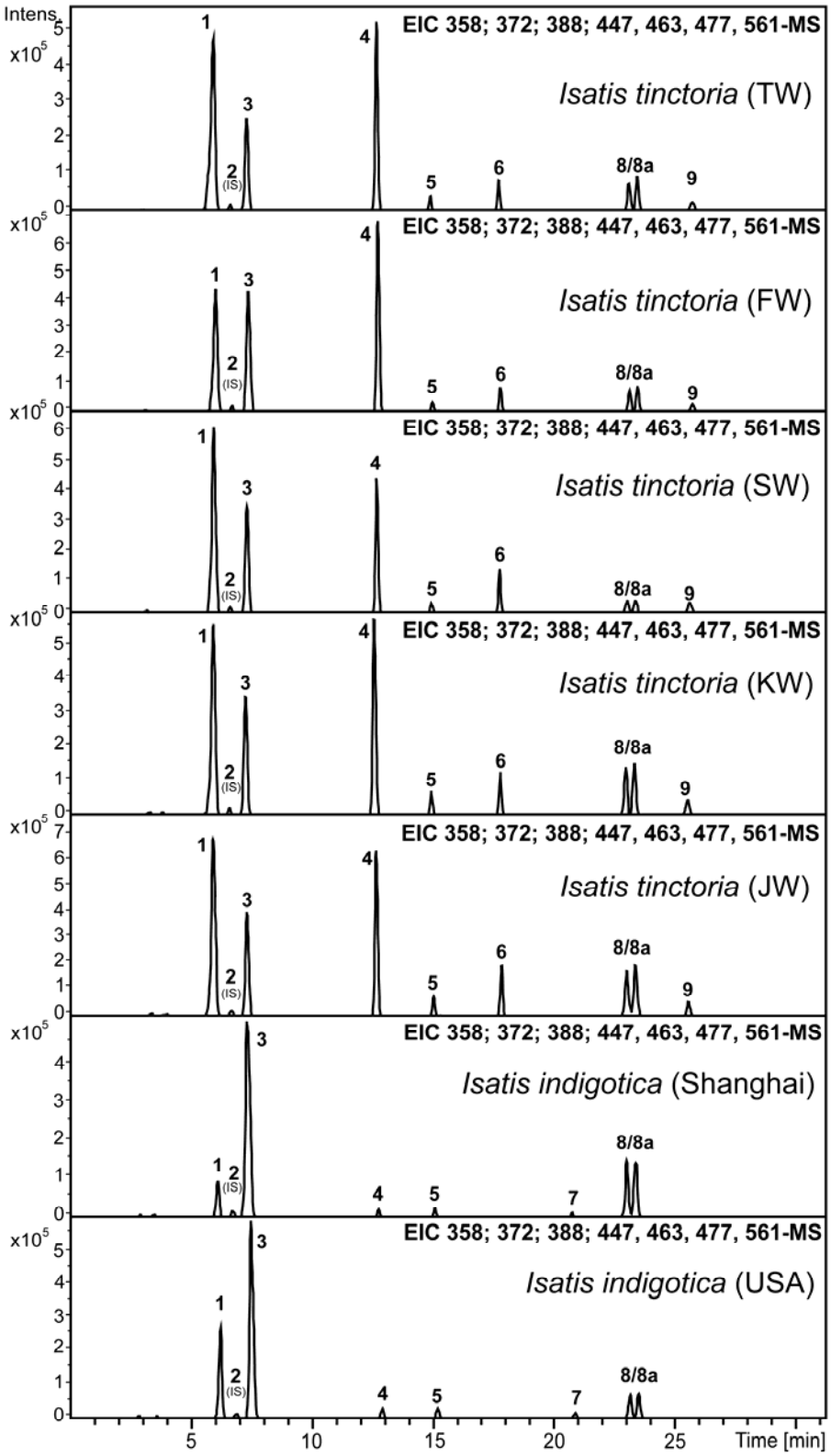

Figure **1**. Glucosinolates in *Isatis* seeds. Extracted ion chromatograms (EIC) of identified glucosinolates of the *Isatis tinctoria* accessions “Thüringer Waid” (TW), “French Woad” (FW), “Swiss Woad” (SW), “Kieler Waid” (KW), “Jenaer Waid” (JW) and of “*Isatis indigotica* “Shanghai” and “*Isatis indigotica* USA” are shown. Sinigrin (**2**) was used as internal standard (IS).

Table **1**. Concentration of glucosinolates in *Isatis* seeds in comparison to published data of *Isatis* leaf material and seeds

Leaves

Data from Mohn et al. [16] (amount of glucosinolates calculated in mg / 100 g dry weight)

***Isatis tinctoria* ("Thüringer Waid")**

glucosinolate	amount in mg / 100 g dw min[a]	max[a]
Epiprogoitrin (**1**)	0.574	21.8
Progoitrin (**3**)	0.374	28.3
Gluconapin (**4**)	n.d.	0.5
Sulfoglucobrassicin (**10**)	553.9	4482.6
4-Hydroxyglucobrassicin (**5**)	n.d.	n.d.
Glucobrassicin (**6**)	157.3	401.7
4-Methoxyglucobrassicin (**7**)	n.d.	n.d.
Glucoisatisin (**8/8a**)	n.d.	n.d.
Neoglucobrassicin (**9**)	n.d.	2.7

Isatis indigotica

glucosinolate	amount in mg / 100 g dw min[a]	max[a]
Epiprogoitrin (**1**)	11.9	45.3
Progoitrin (**3**)	194.5	546.6
Gluconapin (**4**)	0.3	16.7
Sulfoglucobrassicin (**10**)	n.d.	n.d.
4-Hydroxyglucobrassicin (**5**)	213.8	1762.7
Glucobrassicin (**6**)	n.d.	n.d.
4-Methoxyglucobrassicin (**7**)	n.d.	n.d.
Glucoisatisin (**8/8a**)	n.d.	n.d.
Neoglucobrassicin (**9**)	n.d.	n.d.

Data from Elliott and Stowe [13]

Isatis tinctoria

glucosinolate	amount in mg /100 g dw Light grown shoots	Etiolated shoots
Sulfoglucobrassicin (**10**)	50.2	11
4-Hydroxyglucobrassicin (**5**)	n.d.	n.d.
Glucobrassicin (**6**)	175.6	160
Neoglucobrassicin (**9**)	3.4	132

Seeds

***Isatis tinctoria* ("Thüringer Waid")**

glucosinolate	amount[b] in mg / 100 g dw
Epiprogoitrin (**1**)	130
Progoitrin (**3**)	90
Gluconapin (**4**)	55
Sulfoglucobrassicin (**10**)	n.d.
4-Hydroxyglucobrassicin (**5**)	15
Glucobrassicin (**6**)	45
4-Methoxyglucobrassicin (**7**)	n.d.
Glucoisatisin (**8/8a**) (both isomers together)	90
Neoglucobrassicin (**9**)	10

***Isatis indigotica* (Shanghai)**

glucosinolate	amount[b] in mg / 100 g dw
Epiprogoitrin (**1**)	50
Progoitrin (**3**)	140
Gluconapin (**4**)	10
Sulfoglucobrassicin (**10**)	n.d.
4-Hydroxyglucobrassicin (**5**)	10
Glucobrassicin (**6**)	n.d.
4-Methoxyglucobrassicin (**7**)	traces
Glucoisatisin (**8/8a**) (both isomers together)	120
Neoglucobrassicin (**9**)	n.d.

Data from Elliott and Stowe [13]

Isatis tinctoria

glucosinolate	amount in mg /100 g dw
Sulfoglucobrassicin (**10**)	n.d.
4-Hydroxyglucobrassicin (**5**)	n.d.
Glucobrassicin (**6**)	230
Neoglucobrassicin (**9**)	n.d.

n.d. = not detected

[a] amount glucosinolates depending on seasonal variation

[b] values estimated

4. CONCLUSION AND OUTLOOK

The future aim is to establish a new phytomedicinal product based on a lipophilic woad extract as a “New Herbal Entity” (NHE = herbal remedy without traditional use). As for the vast majority of phytomedicines, the known active compounds in woad amount only to a small proportion of the total extract, while the composition of the extract matrix remains unclear. A systematic investigation of the metabolite profile of *Isatis tinctoria* leaf extracts was therefore carried out using a combination of HPLC-based MS and NMR profiling techniques. Approximately 90% of the pharmacologically active dichloromethane extract could be assigned to particular compounds or compound classes (by taking the relative peak areas of the ELSD profile as a measure). This is comparable or better than the state of knowledge for well-characterised special extracts, such as EGb 761 from *Gingko biloba* L. and ZE 331 from *Petasites hybridus* L. which are active ingredients in well-known phytopharmaceuticals. The applied analytical methods enable a profiling of *Isatis* extracts in a holistic manner and will provide a means of standardisation and quality control of *Isatis* based on its entire composition. To our knowledge, this was the first time in natural product analysis that such a broad range of detectors/detection modes were used in parallel. It enabled the detection of a structurally highly diverse spectrum of metabolites. Our approach is of general interest for any profiling/metabolomics study.

The sourcing of homogenous raw material represents another challenge in the development of a new phytomedicine. Our investigations on five different *I. tinctoria* strains revealed some differences in the secondary metabolite patterns due to a relative high degree of genetic diversity. With the used profiling strategies, it will be possible to select the best suitable *Isatis* variety and to standardise the plant cultivation process in order to develop an active herbal preparation that can be produced in an industrial scale.

It is still unclear which precursor molecules are involved in the formation of tryptanthrin during harvest and post-harvest treatment. Clear evidences for a contribution of indole

glucosinolates are still missing so far. Isotope labelling of the indole unit of putative precursors, and the subsequent identification of the labelled indole units in tryptanthrin by NMR would provide direct evidence. However, this approach would be costly and time-consuming. Continuing experiments for the search of other possible candidates as precursor molecules should also be carried out. Whether previously identified precursors of indigo like isatan A and B are involved in the formation of tryptanthrin and other indole alkaloids in woad should be investigated. Knowledge on the formation of tryptanthrin would facilitate the optimisation of cultivation and post-harvest conditions.

Besides the cultivation aspects, more investigations are needed to develop a woad based phytomedicine. The mode of action of the active principles is known only in part. Previous studies showed that tryptanthrin inhibits cycloxygenase-2 (COX-2) and lipoxygenase-5 (5-LOX) but the binding sites are still unknown. Studies analysing the interaction between the involved enzymes and tryptanthrin (e.g. analysed by SPR) would be suitable to reveal the binding sites. Further experiments are also needed to understand the mode of action of (*E*)-3-(3',5'-dimethoxy-4'-hydroxy-benzylidene)-2-indolinone on a molecular level.

Moreover, pharmacological synergies, through compounds acting on different targets involved in inflammation, seem to occur in woad. However, experimental evidence is lacking and needs to be provided for *Isatis.* Such findings would be of interest for phytomedicines in general.

Finally, the bioavailability of active substances in a standardised woad extract has to be investigated, and toxicological studies in animals need to be carried out before legal authorities would allow first clinical efficacy studies.

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Publications, Short lectures and Awards

Publications:

1. Basalo C, **Mohn T**, Hamburger M. Are extraction methods on quantitative assays of pharmacopoiea monographs exhaustive? A comparison with pressurized liquid extraction. Planta Med 2006; 72:1157-62.
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Short lectures:

- **Mohn T**, Rüster U, Hamburger M. Extraction and analysis of non-derivatized glucosinolates in plant extracts – a validated PLE/LC-MS protocol. 55th International congress and annual meeting of the Society of Medicinal Plant Research, Graz, Austria, September 2-6, 2007.
- **Mohn T**, Plitzko I, Hamburger M. A comprehensive metabolite profiling of *Isatis tinctoria* leaf extracts. International Conference on LC-NMR and related techniques: Challenges in biological systems. Jena, Germany, August 27 – 29, 2008.

Awards:

09.2007: Winner of the '**young researcher award**' of the International Society of Medicinal Plant Research.