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Qualitative Analysis of Indigo Precursors from Woad by HPLC and HPLC-MS

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A qualitative method has been devised to analyse the indigo precursors in leaf extracts of woad (*Isatis* spp.) using HPLC coupled to an evaporative light scattering detector and confirmed using HPLC coupled, on line, to a particle beam mass spectrometer. Using this technique, indican has been positively identified in shoot extracts of European (*I. tinctoria*) and Chinese (*I. indigotica*) woad. Greater amounts of indican were found in the Chinese compared with the European woad. Copyright © 2000 John Wiley & Sons, Ltd.

Keywords: woad; indican; isatan B; indole; indigo; high performance liquid chromatography; evaporative light scattering detector; mass spectrometry; *Isatis tinctoria*; *Isatis indigotica*.

INTRODUCTION

Historically, *Isatis tinctoria* (European woad) was used for the production of the blue dye indigo. The plant *I. indigotica* (Chinese woad or tein-cheing) is closely related to its European counterpart (Fortune, 1846), although it has not been used as widely. Indigo is derived from the plant in the form of precursors. In most cases, the indigo precursor, indoxyl, is found in plants as the glucoside, indican [indoxyl- β -D-glucoside; Fig. 1(a)]. Initially, it was thought that indican was the only precursor in plants (Schunk, 1855, 1858), but it was later suggested that, in woad, the precursor was not indican but an ester of indoxyl, isatan B [indoxyl-5-ketogluconate; Fig. 1(b)] as described in Epstein *et al.* (1967). Subsequently, indican was positively identified in roots and shoots of woad (Strobel and Gröger, 1989).

New methods for the analysis of the precursors are now available and several methods for the analysis of indigo have been published in recent years (Fischer *et al.*, 1990; Wouters and Verhecken, 1991). However, these methods have been devised for the qualitative analysis of dyes used in historical artefacts and to identify natural and synthetic dyes in textiles. Such methods do not provide quantitative means for the determination of the potential of a plant for the production of indigo. Recently, TLC has been used routinely to analyse the ratio of indican to isatan B in woad (Strobel and Gröger, 1989).

In the present work, a rapid, qualitative HPLC method is described for the determination of precursors of indigo in extracts of the indigo producing plant, woad.

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EXPERIMENTAL

Plant culture. Woad, *Isatis tinctoria* (European woad) and *I. indigotica* (Chinese woad), was grown from seed in pots containing John Innes No. 3 compost, under glass with supplementary lighting to simulate a minimum 16 h day at 18°C day and 15°C night. Plants were grown for approximately 6 weeks before the first samples were taken.

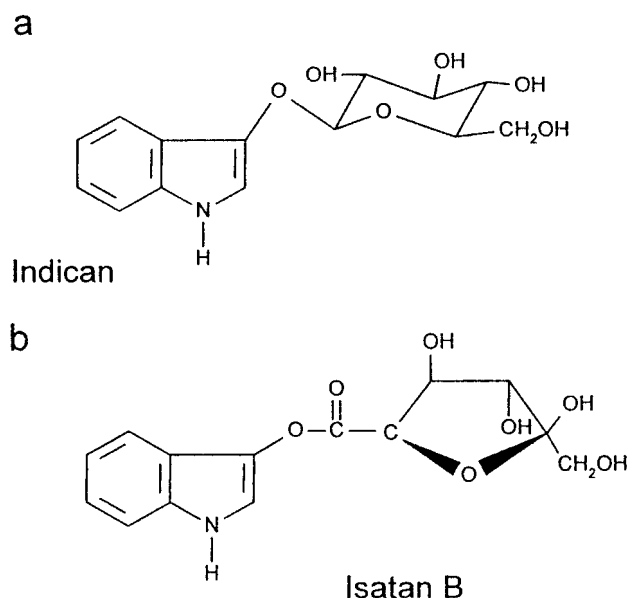


Figure 1. Indigo precursors (a) indican (indoxyl- β -D-glucoside; found in indigo-producing plants), and (b) isatan B (indoxyl-5-ketogluconate; found only in woad). Both of these molecules can be hydrolysed to form indoxyl, two molecules of which combine to form indigo.

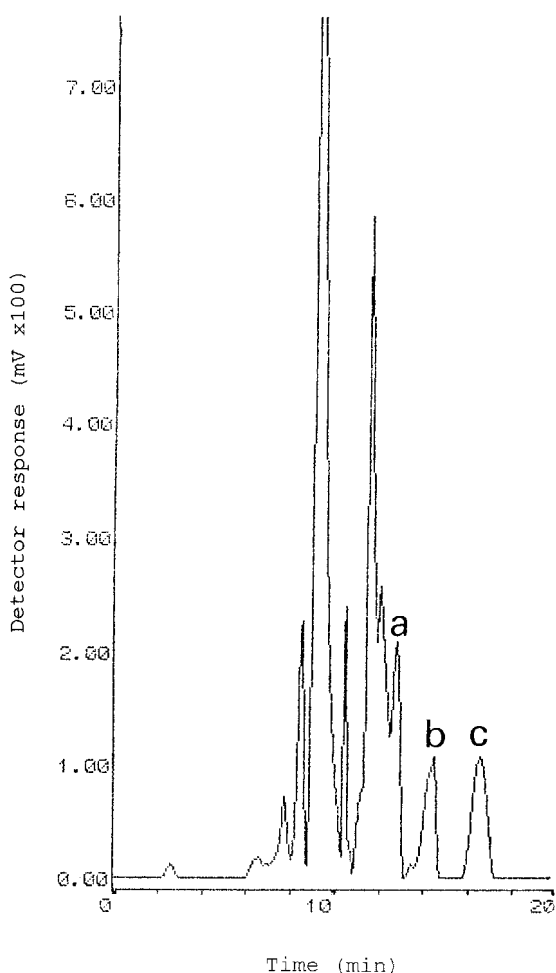


Figure 2. HPLC separation of an aqueous extract of woad showing peaks of indigo (a), indican (b) and isatan B (c), all of which were positively identified (see Experimental section for details).

Extraction technique. Duplicate leaf discs (1 cm diameter) were taken from middle-sized leaves and extracted in 5 mL of boiling water for 5 min. The resulting extract was either filtered through a 0.22 μ m Millipore membrane filter or centrifuged (5 min; 10000g), diluted as necessary and analysed directly by HPLC with an evaporative light scattering detector (ELSD) or by HPLC coupled to a mass spectrometer.

HPLC-ELSD. A Varian (Walnut Creek, CA, USA) Vista model 5500 HPLC system was coupled to an ELSD (Alltech, Carnforth, UK) from which data was collected using a Ramona 5 (Raytest, Sheffield, UK) data logger. The methanol extract (20 μ L) was injected via a Rheodyne (Cotati, CA, USA) valve onto an Econosphere (Alltech) C18 column (250 \times 4.6 mm i.d.; 5 μ) coupled to a second Econosphere C18 column (150 \times 4.6 mm i.d.; 3 μ) protected by a 5 μ guard column. The compounds were detected by ELSD with the nitrogen flow set at 2.00 standard litres/min and the drift tube temperature stabilised at 92°C. An isocratic solvent

system was used comprising methanol:water:acetonitrile (15:60:25) containing 0.2% formic acid (final concentration) with the flow rate being 0.4 mL/min throughout the run.

HPLC-MS. An LDC-Milton Roy (Riviera Beach, FL, USA) HPLC was used coupled to a Thermoquest (Finnigan Mass, San Jose, CA, USA) LCQ mass spectrometer. The atmospheric pressure chemical ionization source was used on the LCQ with a vapourizer temperature of 450°C and a capillary temperature of 150°C; the source voltage was 2 kV. Methanol extracts (20 μ L) were injected into the HPLC-MS using the same columns and gradient system as described above. Spectra were acquired in the negative ion mode over the range 50–800 amu.

Quantification of indican. Serial dilutions of an indican standard (1 mg/mL) were determined sequentially using HPLC-ELSD to obtain a standard curve. From this, the amount of indican in water extracts from woad leaf disc samples was determined.

Preparation of isatan B standard. Crude isatan B (for use as a standard) was prepared from fresh leaves according to the method of Kokubun *et al.*, (1998), which was modified by initially extracting woad leaves with boiling water (5 min), cooling rapidly to 25°C and acidifying with acetic acid (1% final volume). The extract was characterized by NMR and HPLC-MS.

RESULTS AND DISCUSSION

The conditions for the separation of indigo precursors by TLC (Strobel and Gröger, 1989) were used as the basis for the development of an HPLC-ELSD method for the routine detection of indigo precursors in woad extracts. However, as no standard was available for isatan B, it was necessary to produce a crude isatan B extract and characterize it using NMR and HPLC-MS. Analysis by negative ion MS of the crude isatan B revealed a mass peak of 308 which was compatible with the $[M-1]^-$ ion of isatan B (mass 309), which, together with NMR analysis, positively identified the compound as isatan B.

It was possible to separate, with baseline resolution, standards of the putative indigo precursors, indican and isatan B by HPLC-ELSD (data not shown). Aqueous extracts of woad leaf material were analysed using HPLC-ELSD. The traces obtained from this type of analysis showed that indican, isatan B and indigo were all present in plant extracts, identified by direct comparison of their retention times with the standard compounds (Fig. 2). HPLC-ELSD data indicated that both European and Chinese woad contain indican and isatan B. This work represents the first analytical procedure, using HPLC, which identifies indican and isatan B from woad leaf extracts.

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