The Production of Natural Indigo with a High Purity

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1. Introduction

When, at the end of the 19th century, synthetic indigo replaced natural indigo for large-scale dyeing, one of the reasons was that the synthetic product was not only cheaper but was consistently purer (Perkin, 1900). Now, with a renewed and increasing interest in the naturally sourced product, the purity of natural indigo becomes once again an important consideration in determining the extent to which the dyeing industry will take up the natural product.

We have been working on a European Commission-funded research and development project: Sustainable Production of Plant-derived Indigo (Spindigo) (www.spindigo.net), which aims to reintroduce indigo-yielding crops to European agriculture. The project has 9 partners in Spain, Finland, Germany, Italy and the UK. The principal crops the project has examined are woad (*Isatis tinctoria*) and *Polygonum tinctorium*. Woad grows and yields well in all countries involved. *P. tinctorium* is better suited to Northern/Central Italy and to Germany. From both crops, extraction of indigo involves steeping the leaf material at an optimum temperature to allow the indigo precursors to leach out of the leaves. Indigo is formed when indoxyl is released from the precursors and the solution well oxygenated. The main precursor in woad readily releases indoxyl on addition of alkali (generally potassium or calcium hydroxides), while the main precursor in *P. tinctorium* releases indoxyl on hydrolysis catalysed by endogenous or added ß-glucosidase (Angelini et al., 2003).

One of the objectives of the Spindigo project is to provide a means for European farmers to produce natural indigo with a purity of at least 90%. We have found over the last three years of indigo extraction that the purity of the indigo produced by the method outlined above varies widely, from just 5% to a ceiling value of about 60%. These values compare with the purity of the 19th century product obtained from the tropical *Indigofera tinctoria*, which varied from 20% to 90% purity (Perkin, 1900). The collapse of the market for the natural product came about with the commercialisation of a synthetic product that had a purity consistently greater than 90% (Perkin, 1900). Moving forward a century, Stoker et al., (1998) reported the purity of indigo extracted from woad to be in the range of 20% to 40%; and Bechtold (2002) reported that the purity of indigo extracted from *P. tinctorium* to vary from <2% to 9.2% (determined by photometry) or to 12.3 % (determined by dyeing).

The present paper considers the nature and origin of the impurities in natural indigo.

2. Materials and Methods

Indigo was extracted from freshly harvested woad leaves, which were immersed in water (0.5 kg/l) at 75 °C for 7.5 min, the extract was cooled to 25 °C with an ice bath, passed through a 1 μm filter, then sufficient KOH added to bring the solution to pH 9. After stirring for 30 min, saturated citric acid was added to bring the solution to pH 3, the indigo was collected by centrifugation, rinsed and dried. Indigo purity was determined by a microgravimetric method in which weighed samples were extracted with acetone to remove indirubin, and with 1-methyl-2-pyrrolidinone (NMP) to remove indigo. Indirubin and indigo were determined by loss of weight on extraction.

Indigo was formed from indoxyl acetate (10 mM) by adding KOH (20 mM); the mixture was stirred for 30 min, and 0.2 ml samples were taken for indigo (614 nm peak) and indirubin (570 nm shoulder) determinations in NMP (90%) containing 0.5 % butylated hydroxytoluene (BHT) and 13 mM citric acid. The soil used was a silt loam of known composition from a field in Pisa, Italy on which *P. tinctorium* was grown. When woad extract was added in these experiments it was present at a concentration equivalent to what would have been present if we had started from leaves rather than from indoxyl acetate. The indigo precursor content of the woad extracts is low (about 10%) compared with the concentration of indoxyl acetate used.

3. Results and Discussion

There are two possible sources of contamination of the indigo formed from plant leaves: the leaf material itself, and soil or other extraneous matter such as insect parts that could adhere to the leaf surface. The amount of impurity originating from soil will vary depending upon the extent to which the leaves are contaminated by soil material from rain splash and dust. To take just one example, we were able to wash from 950 g of typical woad leaves 6.1g of soil. The pure indigo extracted from these leaves amounted to 0.26 g. Thus if all the soil had adhered to the indigo and accompanied it, the raw indigo product would have been 96% soil.

Soil is a complicated and variable entity, but what is understood of the interaction of organic compounds with soil makes it likely that indigo would be tightly bound to soil particles. Both the crystal structure of indigo (Süsse et al., 1988) and its binding to cellulose in dyeing cotton demonstrate its propensity for forming intermolecular H bonds. As Schulten and Leinweber (2000) concluded in their review of the structure of organic soil complexes: “H bonding, dipole/dipole interactions and trapping of biological substances emerge as essential features in the chemistry of organic matter in soils. All models of organic matter in water and soils showed H bonds as a crucial feature”. This provides the basis for our belief that indigo is likely to be tightly bound to any soil particles which are present when the indigo is formed. The elimination of this contaminant is thus best accomplished by preventing as far as is practical the presence of soil when indigo is formed from the indoxyl precursors extracted from the leaves. Thus the harvested leaves are washed in cold water, and the water into which the leaves are extracted is filtered.

To determine the specific effect on indigo purity of the soluble and colloidal materials that originate in the leaf, we have modelled indigo formation in the

laboratory using an alkali-catalysed hydrolysis of indoxyl acetate. The predominant precursor in woad leaves, isatan B, indoxyl-3-(5- ketogluconate) (Epstein et al., 1967), is relatively unstable and not available commercially. However alkali catalyses the hydrolytic release of indoxyl from both indoxyl acetate and isatan B, and thus we have used the commercially available indoxyl acetate. With this system we have observed that there is a loss of indigo yield, and a lowering of indigo purity when either soil or woad extract are introduced (Table I). We have included kaempferol in these experiments because of earlier suggestions (Perkin 1906) that kaempferol might be responsible for reducing yield and generating impurities in natural indigo. In our experiments we have noted that kaempferol reduced yield in proportion to the kaempferol concentration added, but it did not reduce indigo purity, at least when the (variable) content of indirubin was accounted for (Table I). In the experiments in which indoxyl acetate is used as a model precursor, indirubin was formed in amounts that varied from one experiment to another, and which were greater than would normally be measured in the indigo formed from woad extracts.

Table I Effect of kaempferol, soil and woad extract on yield and purity of indigo made from indoxyl acetate hydrolysed with KOH. Kaempferol (3 mM), soil (5 mg/ml) and woad extract were added as indicated. Indigoids comprise indigo plus indirubin.

Additions	Indigo product (mg)	Indigo content (mg)	Indirubin content (mg)	Indigoids	
				(mg)	(%)
None	128	71.0 ± 0.9	49.9 ± 0.9	122 ± 1.8	95 ± 1.4
Kaempferol	71	43.8 ± 1.1	25.8 ± 2.2	69.6 ± 3.3	98 ± 4.6
Soil	110	46.6 ± 1.7	31.2 ± 2.8	77.6 ± 4.5	71 ± 4.0
Woad extract	103	68.7 ± 2.1	0	68.7 ± 2.1	67 ± 2.1

An obvious point perhaps, but one that needs to be made explicit, in considering the purity of the indigo produced from plants is that the greater the indigo precursor content of the leaves, the greater the purity. Contamination from the plant material and residual soil is relatively constant for particular extraction conditions, while the precursor content can vary greatly: older leaves, and leaves harvested early in the year contain relatively low precursor levels. Thus we observed that in extractions from 300 - 600 g batches of leaves, a harvest in February gave low yields of indigo and a purities of 3-4% with unwashed and 18-24% for washed leaves. Leaves harvested in April gave higher yields and purities of 32-36% for unwashed and 50-56% for washed leaves. The highest purities were obtained when leaves were harvested in the period July to September. Harvesting in this period and selecting smaller, cleaner leaves, the purities of 8 extractions varied from 57% to 91 % (mean of 74% +/- 10 SEM) with an average yield of 0.5 (+/- 0.08 SEM) g per kg fresh weight leaves.

To obtain the highest purity, we found it necessary to sediment the indigo in an acid medium containing citric acid, or to wash the sedimented indigo with acid subsequently. In a series of three experiments, sedimentation in acid compared to that in alkali gave rise to improvements in purity from 17% to 56%, 43% to 68% and 15% to 60%. Similarly, washing the raw indigo in dilute HCl after grinding the dried product provided dramatic increases in purity. For example, the purities of three large-

scale preparations of indigo were raised by a wash in 2M HCl from 21% to 71%; from 0.6% to 63 % and from 19% to 72%.

To understand the basis of the interaction of natural indigo with the impurities, it is necessary to consider not merely the chemical nature of the impurities but also their physical relationship with the indigo. In experiments using indoxyl acetate as a model substrate we have shown (P. Garcia Macias and P. John, in preparation) that both soil and woad extract provide nuclei for the formation of sedimentable indigo particles. The reason for this nucleation effect is likely to be the adsorption of indoxyl to colloidal particles. As a result of this adsorption, when indigo is formed from the indoxyl it does so as a coating around the particles. Thus we have a situation where the impurities are effectively "protected" by a layer of indigo from simple water washes that might otherwise remove them from the indigo product, and raise the product purity. In addition to this coating effect, microscopic examination of indigo preparations shows larger contaminating particles trapped within a matrix of indigo. Again, this would create difficulties for a simple wash procedure for indigo purification. The nucleation effect of the soil particles and colloidal plant materials immediately suggested to us that a purer product could be obtained by using indigo itself to "seed" the nucleation process. However we have found that neither synthetic nor natural indigo is effective in this way, presumably because the affinity of the negatively charged indoxyl groups for uncharged indigo is lower than their affinity for appropriately charged colloidal particles.

In conclusion, indigo can be extracted from woad with a purity of 90% if three conditions are met: the leaves contain a sufficiently high yield of indigo precursors; the leaves are rinsed free of soil; and the indigo is sedimented in an acid medium.

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5. References

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