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High performance liquid chromatography of slightly soluble brominated indigoids from Tyrian purple

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ABSTRACT

Attempts at identification of mollusc species producing Tyrian purple from archaeological material are usually done with high performance liquid chromatography in the reversed-phase system, but the peaks obtained are often wide and asymmetric. This is due to the low solubility of the indigoids and their brominated derivatives in the mobile phase, especially 6,6′-dibromoindigotin, which is soluble in only few, particular solvents. Our study focused on improving both symmetry and peak height for more precise quantification. The influence of various factors was evaluated: stationary phase characteristics, mobile phase composition, elution gradient parameters and temperature on the peak shape of the main components of Tyrian purple. The best results were obtained using highly retentive, but moderately bonded ODS stationary phases (about 2.8 μmol m^{-2}), percolated with gradient of acetonitrile with acidified aqueous mobile phases (0.1% strong acid) at elevated temperatures (70 °C). The upper quantification limit for 6,6′-dibromoindigotin was improved by over 350%, between standard and optimised systems. Using them, the detection and quantification of trace Tyrian purple components (less than 0.15%) aside from major indigoids becomes possible. Consequently, for the first time, the new analogues of brominated and unbrominated indirubins were found in the shellfish purple from *Hexaplex trunculus*.

1. Introduction

Tyrian purple (or Royal purple), the mythic indigoid dyestuff, can be obtained from the hypobranchial glands of some marine molluscs, such as Mediterranean *Hexaplex trunculus* L. and *Bolinus brandaris* L., Central American *Plicopurpura pansa* Gould, North Atlantic *Nucella lapillus* L., and others [1,2].

The precursors of the indigoids of Tyrian purple – brominated indoxyl sulfates – are present in molluscs in very small amounts [3]. For this reason thousands of snails must be collected to obtain even a few grams of dye, making it extremely expensive. The dyestuff was used in ancient times for both textile dyeing and pigment preparation [4,5]. The purple of a characteristic dark blue to violet shades is made up of some compounds from the series of indigotin related compounds – some isatins, indigotins and indirubins were identified in this dye.

The detection of Royal purple in the objects of cultural heritage and the further identification of dyeing shellfish specie could give important information on dyeing or staining technology and on the object history. It could give also information on the social status of a person, when excavated objects – coloured with purple such as textiles and ceramics – are associated with human remains in graves. When the objects are isolated or found independently, as wall paintings, manuscripts, textiles and ceramics, their exceptional quality is often reinforced by the presence of Tyrian purple on them.

The analysis of Tyrian purple by high performance liquid chromatography does not require any particular sample preparation (e.g. derivatisation), and can be very specific and accurate [6].

The elution of indigotins and indirubins present in Purple may be achieved in reasonable time in isocratic conditions [7]. It's necessary to use the quasi-isocratic elution with harsh gradient analysis beginning when isatins detection is desired [8]. Also, the ending conditions should be modified (gradient of organic modifier and solvent flow rate) when the elution of 6,6′-dibromoindirubin, the last eluted purple compound, is expected in less than 30 min. Both quoted systems used methanol as organic modifier and phosphoric acid as acidifier.

Other separations are proposed in linear gradient elution with methanol and phosphoric acid, with PDA detection [9–11], or acetonitrile and trifluoroacetic (or formic) acid for MS identification [12–14]. The impact of trifluoroacetic acid concentrations of 0.1 and 0.001% in the mobile phase on detection of purple components in

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PDA and APCI-MS was studied recently [15]. According to the published results, higher trifluoroacetic acid concentration improves the detection just a little in the visible region and makes the MS signal drop dramatically.

The use of methanol [9] or acetonitrile [15] seems to modify the system selectivity towards purple components.

In most published chromatograms the detected absorption signals of separated Purple components are usually minute. It is not only because of the small size of samples available from real archaeological artefacts. At the higher, but still quite low purple concentration a problem of peak shape occurs for the indigotins, especially for the 6,6′-dibromoindigotin. Many real samples from historic objects contain important amounts of Purple leading to peaks distortion, sometimes sizeable [16–19]. Tyrian purple is well known to be insoluble in most solvents except hot pyridine, dimethylformamide or dimethylsulfoxide [3]. These solvents should be used for dye extraction from archaeological material, prior to RPLC analysis using octadecyl bonded silica with water/methanol or acetonitrile mobile phase [9–13]. However, the indigotins from purple are very slightly soluble in partially aqueous mobile phase used in the RPLC. Due to the difference of their solubility in the extraction and the elution solvents, the peak tailing could occur during chromatographic analysis.

In those conditions the determination of Purple components peaks end position on the baseline as well as peak resolution are poor.

The improvement of solubility of indigoids thus seems crucial for accurate peak area calculations and evaluation of the relative quantity of all purple components. The injection of relatively high dye concentrations in the chromatographic system should also enable the presence of minor compounds without losing the semi-quantitative approach to all components. This is rationalized by the fact that the peak area ratio of indigoids present in Royal purple dye was actually used by different authors for mollusc species identification [3,7,12,16,20].

In order to obtain symmetrical peaks and increasing the possible injected amount we have studied in this work the influence both of the nature of stationary phase, the mobile phase composition as well as the influence of the increase of temperature.

The examination of the real contribution of each factor in the solubility improvement and retention of analysed compounds needs to be evaluated experimentally which was the aim of this work. Thus, all parameters were evaluated to some extent.

2. Materials and methods

2.1. Standards and samples

The standards of indigotin (Ind), 6-bromoindigotin (6-BrInd), 6,6′-dibromoindigotin (6,6′-2BrInd), 6,6′-dibromo-*iso*-indigotin (6,6′-2Br-*iso*-Ind), indirubin (Inr) and 6′-bromoindirubin (6′-Inr) were obtained from Dr. Christopher J. Cooksey (Watford, United Kingdom). Additional 6-bromoindirubin (6-BrInr) and 6,6′-dibromoindirubin (6,6′-2BrInr) were kindly shared by Dr. Ioannis Karapanagiotis (Ormylia Art Diagnosis Center, Greece). As the quantities of these standards were extremely small, they were only used qualitatively for initial records of their retention times and spectra. Furthermore, two samples of Tyrian purple were used for experiments: wool yearns dyed with 6,6′-2BrInd from Dr. Helmut Schweppe (Frankenthal, Germany) and pigment obtained with shellfish *H. trunculus* on talc substrate from Dr. Jana Sanyova (IRPA/KIK, Bruxelles, Belgium). Isatin (Isa) and bromoisatin (BrIsa) were identified according to their published UV–vis spectra [6] in the chromatograms of extract from the pigment and used for further identifications. The structures of studied compounds are given in Fig. 1a–d.

2.2. Chemicals

The purple components standards and the dye were solubilised with dimethylsulfoxide (*DMSO*, for gas chromatography) from Merck (Darmstadt, Germany).

Chromatographic separations were done using methanol (*MeOH*, G Chromasolv) and acetonitrile (*MeCN*, Chromasolv) purchased from Sigma–Aldrich (Stainheim, Germany). The mobile phases were acidified with formic acid (*FA*, 99% +, for analysis), methanesulfonic acid (*MSAH*, 99%) both obtained from Acros (Geel, Belgium) or trifluoroacetic acid (*TFA*, 99.8+%, HiPerSolv ChromaNorm BDH Prolabo) from VWR (Fontenay-sous-Bois, France).

The ultra pure water used for all purposes was obtained with a Milli-Q Plus system from Millipore (Guyancourt, France).

2.3. Chromatography hard- and software

The analyses were performed on a HP 1100 HPLC-PDA system from Agilent Technologies (Wallbronn, Germany) composed of a vacuum solvent degasser, quaternary pump, autosampler, column oven and PDA 190–900 nm, all controlled by ChemStation software (ver. B.03.01).

2.4. Columns

A series of chromatographic columns, packed with various C18 bonded silica stationary phases were used for method development. The detailed data obtained from the manufacturers and calculated bonding density (*BD*) is reported in Table 1. The phases were selected according to Tanaka approach [21] and cover a large range of chromatographic properties.

The bonding density (*BD* [μmol m^{-2}]) was calculated using manufacturer phase characteristics: carbon load (P_c [%]) and specific surface (S [m^2 g^{-1}]), according to Berendsen-de Galan equation (Eq. (1)) [22]:

$$BD = \frac{10^6 P_c}{[1200 n_c - P_c(M-1)]S} \quad (1)$$

where M is molar weight and n_c is number of carbons in bonded species.

This calculation does not take into account the TMS end-capping.

For easier performance comparison, all columns were 100 mm × 2.1 mm i.d. size, filled with 3 μm spherical particles. The narrow-bore columns with small particle size guarantee better sensitivity and efficiency [23,24] compared to columns used in former studies [6,7,11–16]. The only except ion was made for Onyx Monolithic 100 mm × 3.0 mm rod of 2 μm macropore size, not available in narrow-bore format.

2.5. Operating conditions

Most separations were performed with a mobile phase composed of water (A), acetonitrile (B) and 1% of methanesulfonic acid in water (C). The linear gradient programme was used from 10% B, 10% C at 0 min to 90% B, 10% C at 35 min, A (H2O) *ad* 100%. Flow rate was set at 0.3 ml/min and column temperature at 30 °C in standard conditions.

The influence of column length on solubility through higher retention was tested with Onyx Monolithic columns. The column lengths were set at 100 mm, 200 mm and 400 mm, the last two by the coupling of 2 or 4 columns 100 mm each. Thanks to the excellent permeability of monolithic columns the maximal backpressure generated by the longest configuration at FL = 1 ml min^{-1} for 20%

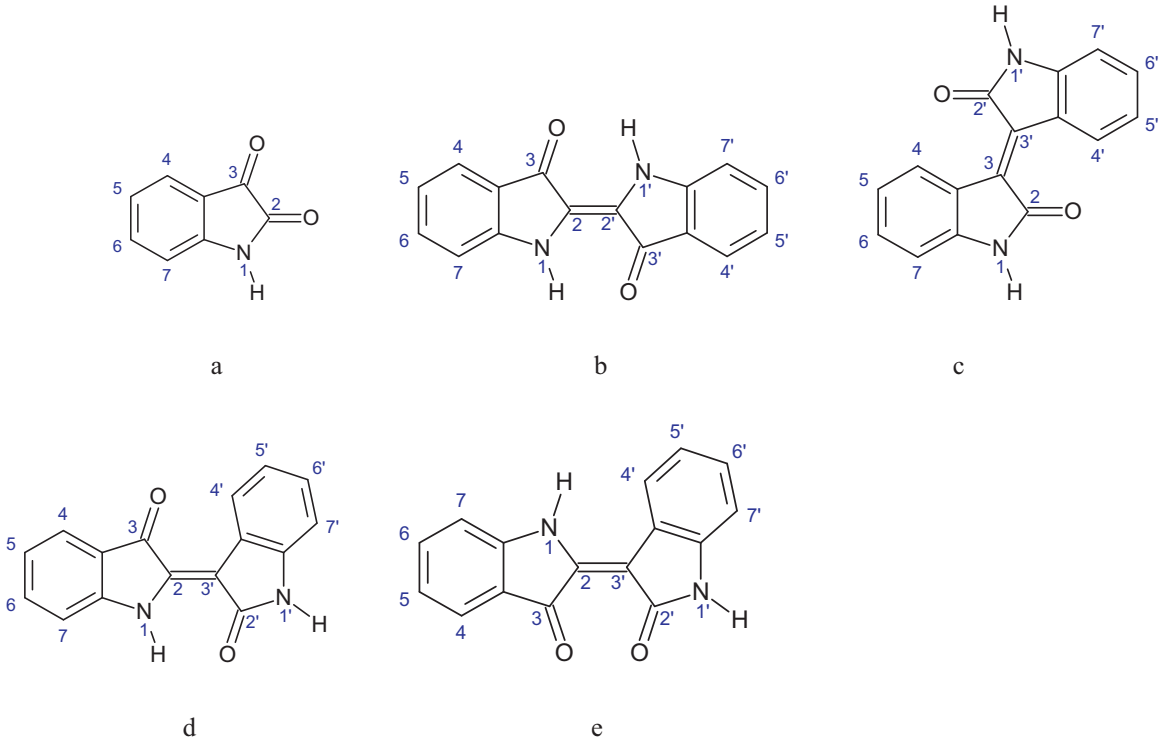

Fig. 1. Core structures of isatins, indigotins and indirubins: (a) isatin, (b) indigotin, (c) *iso*-indigotin, (d) indirubin, (e) *cis*-indirubin. Their brominated derivatives are substituted in positions 6 and 6′.

B was only 165 bar for $T = 60\,^{\circ}C$. The gradient parameters were: B (MeCN) from 5% at 0 min to 90% at 35 min, C (MSAH 1% in H2O) constant 10%, A (H2O) *ad* 100%.

The influence of temperature was tested between 30 °C and 70 °C at intervals of 10 °C. The temperature limit of the oven used was 80 °C. We have chosen for the experiences the Alltech Alltima C18 stationary phase standing with 80 °C heating, according to supplier data. We kept the upper limit temperature of the series below the limit allowed to prevent grafting hydrolysis by strong acid present in the mobile phase. However, no special stability tests were performed. The pre-heating of the mobile phase was done by the 6 μl coil integrated in the heating block of the column oven (G1316A). The post-column cooling was ensured by 400 mm × 0.12 mm i.d. flexible stainless steel capillary tubing (Agilent cat. #5021-1823) at ambient temperature. The gradient elution was done with acetonitril (B) and a 1% water solution of methanesulfonic acid (C) according to the programme: 0–1 min 5% B and 10% C, then linear gradient, finally 41–42 min 90% C and 10% C.

The chromatograms were in monitored UV–vis at 285, 308, 548 and 608 nm.

2.6. Sample preparation

Generally, the standards of indigoids of ca. 0.1 mg were solubilised in DMSO (50 μl) during 10 min with an ultrasonic bath at room temperature. They were filtered through disposable 0.2 μm pore diameter PTFE filters and aliquots of 5 μl injected on the chromatographic system. The stock solutions of Ind, 6-BrInd and 6,6′-2BrInd were saturated – a lot of precipitate remained on filters after filtration.

The stock solutions were diluted further in DMSO if necessary.

For the columns overload check, the stock solution of 6,6′-2BrInd, defined as c_{sat}, was diluted from 2× to 32× to obtain the concentration fractions equal $0.5c_{sat}$, $0.25c_{sat}$, $0.125c_{sat}$, $0.0625c_{sat}$ and $0.03125c_{sat}$.

The concentrations of dye in two samples: pigment and dyed wool yarn were unknown and the sample quantities giving concentrated dye extracts were fixed experimentally at ca. 1 mg each. Purple dye was thus extracted with DMSO (100 μl) during 10 min in the ultrasonic bath, filtered as previously and 5 μl of obtained solutions were taken to analysis.

Table 1
Characteristics of C18 (ODS) stationary phases (suppliers' data). Bonding density (*BD*) calculated according to equation (Eq. (1)).

Phase	Supplier	Carbon load (P_c) [%]	Pore size [Å]	Specific surface area (S) [m^2/g]	Bonding density (BD) [$\mu mol/m^2$]	Bonding type	End-capping
Platinum EPS	Grace	5	100	200	1.11	Monomeric	No
Platinum	Grace	6	100	200	1.34	Monomeric	Yes
Hypersil BDS	Thermo	11	130	170	3.09	Monomeric	Yes
Aquasil	Thermo	12	100	310	1.88	Monomeric	Hydrophilic
Alltima	Grace	16	100	340	2.41	Polymeric	Yes
Onyx Monolithic	Phenomenex	18	130	300	3.16	No data	Yes
Alltima HP HL	Grace	24	100	450	3.09	Monomeric	Yes

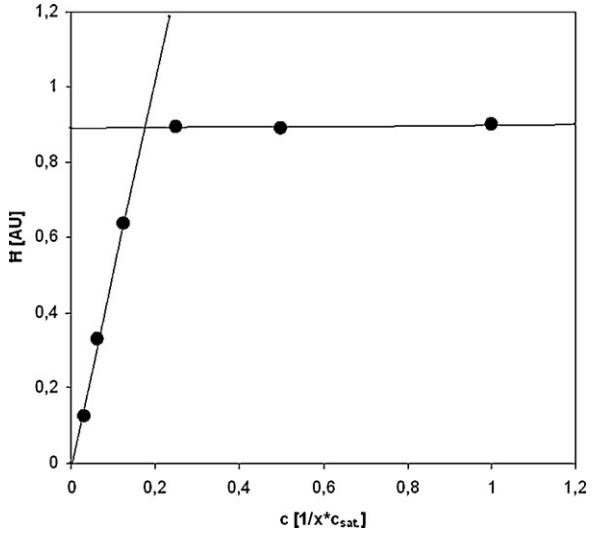

Fig. 2. Peak height (H) depending on concentration (c) of 6,6′-2Br-Ind and "plateau" corresponding to the solubility limit ($H_{lim} \equiv \bar{H}$). Saturated stock solution ($c_{sat.}$) is considered as 1. Successive dilutions give the fractions of that concentration. Column Hypersil BDS C18, T = 30 °C, detection wavelength 608 nm.

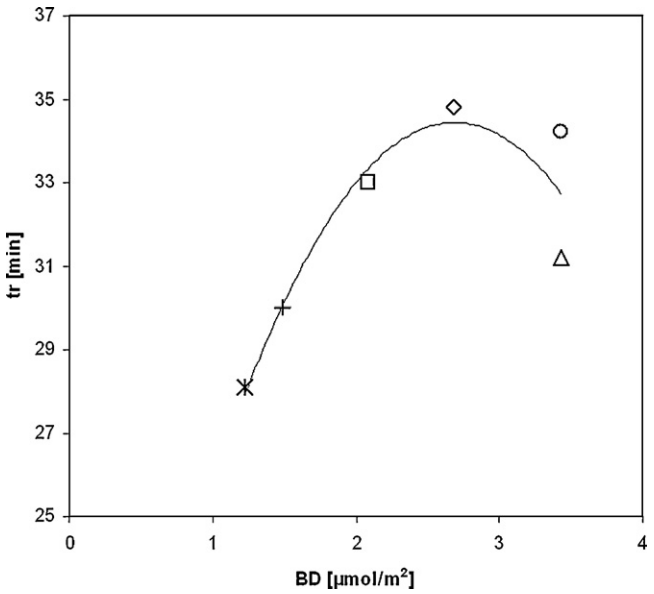

Fig. 3. Dependance of retention (tr) on bonding density (BD). (*) Platinum, (+) Platinum EPS, (Δ) Hypersil BDS, (□) Aquasil, (◊) Alltima, and (○) Alltima HP HL.

3. Results and discussion

It could be observed that the increasing the quantity of injected 6,6′-2BrInd leads to dramatic tailing. At growing concentrations of purple dye the same appears also for 6′-BrInd and finally for Ind. The observed peak distortion seems to appear with the respective solubility of indigotins. The solubility of indigoids decreases with the number of bromine atoms attached to their structure in positions 6 and 6′ [25]. Also, indigotins are less soluble than indirubins because of the presence of intramolecular hydrogen bonds between the hydrogen from amino groups and oxygen from ketone groups [26,27]. In indigotins, these bonds are formed on both sides of the molecule of the most thermodynamically stable *trans* isomer while in indirubins only one hydrogen bond is sterically possible, leaving other polar groups accessible for solvents.

Two reasons are possible for this behaviour: the dye precipitation on the stationary phase or the formation of dispersion in the mobile phase by the association of dye molecules.

If the observed tailing is due to the very limited solubility of indigotins in the aqueous mobile phase used, much lower than in the injection solvents used, the precipitation of compounds at the column entrance may occur after mixing of the injection solvent with the mobile phase. This precipitate can then be slowly solubilised by the incoming mobile phase, forming the tailing part of the peak.

If molecular association takes place, the peak tailing may be the elution of a series of aggregated molecules of indigotins. It was similarly observed that the molecules of indigotin exist in associated forms in solvents, even as powerful as DMSO, DMF, pyridine, dichloromethane or di- and tetrachloroethane [25,28–32]. In this case, the tailing part may be in fact the overlay of peaks corresponding to the growing aggregate size.

The preliminary measurements done with a concentrated DMSO extract from the pigment and its successive dilutions show the existence of this limit of solubility expressed in peak height. The height of the peak of 6,6′-dibromoindigotin grows with concentration until a "plateau" is reached (Fig. 2). It could be supposed, that this limit corresponds to the saturation of the mobile phase by solvated solute molecules in applied conditions. The further increase of concentration results only in proportional tailing. This effect is also observed for 6-monobromoindigotin and indigotin, but to a lesser extent, as their mobile phase saturation concentrations are respectively higher.

The constant peak height of 6,6′-2BrInd, the less soluble Tyrian purple compound, may thus be practically used as a criterion for the evaluation of the optimised system. This maximum "plateau" height will be assigned for convenience as "$\bar{H}$ " in the rest of this paper. It corresponds to the maximal compound load giving a peak that does not tail.

The spectra of the 4 min large tailing peak of 6,6′-2BrInd were taken on both slopes at about 10% of peak height. Their comparison shows that the visible range maximum exhibits a very slight hypsochromic (or "blue"!) shift on the descending slope compared to the front of the peak. The maximum absorption wavelength changed by 2 nm and no supplementary maxima were detected. This remark is more consistent with the spectra modification due to the mobile phase enrichment with MeCN than to the aggregation of indigoid molecules described in the literature [29]. Thus, the peak tailing is most probably an effect of the precipitation of dye components on the column entrance and not of their molecular aggregation in the solvent.

3.1. Column characteristics

Many octadecyl bonded silica of different characteristics have been already used for Purple components separation [7,9,10,12–14,16,33], but systematic studies can provide better understanding of retention behaviour and facilitate suitable phase selection.

The goal of the following work is to find the most retentive stationary phase. The rationale is that in gradient elution conditions the compounds could be then eluted from the more retentive column at a higher content of organic modifier in the mobile phase. Thus, the solubilisation limit of separated compounds should be improved and, consecutively, their peaks should be higher and more symmetrical.

3.1.1. Stationary phase properties

The retention of 6,6′-2BrInd seems to depend on the density of bonding (BD) (Fig. 3). The empirical parabolic shape presents a maximum located around the BD value of 2.7 $\mu mol\,m^{-2}$ (or 2.9 $\mu mol\,m^{-2}$, if Hypersil BDS is excluded). The higher densities

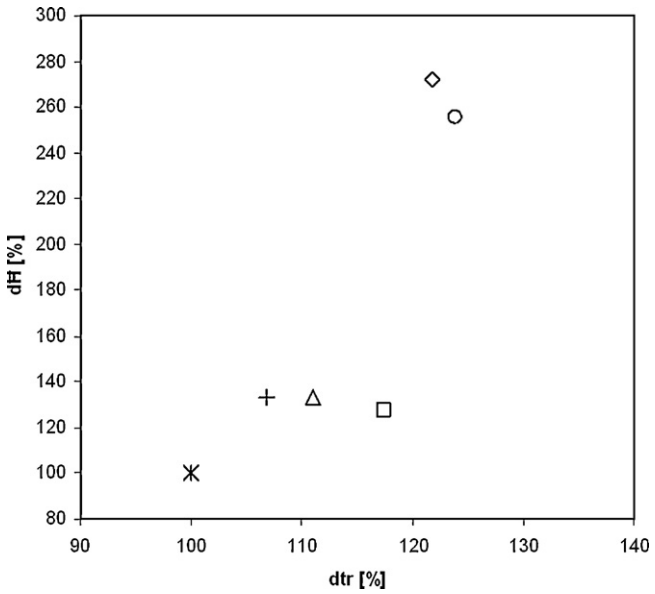

Fig. 4. Relationship of between relative "plateau" height (d^{H}) and relative retention (d*tr*). (*) Platinum, (+) Platinum EPS, (Δ) Hypersil BDS, (□) Aquasil, (◊) Alltima, (○) Alltima HP HL.

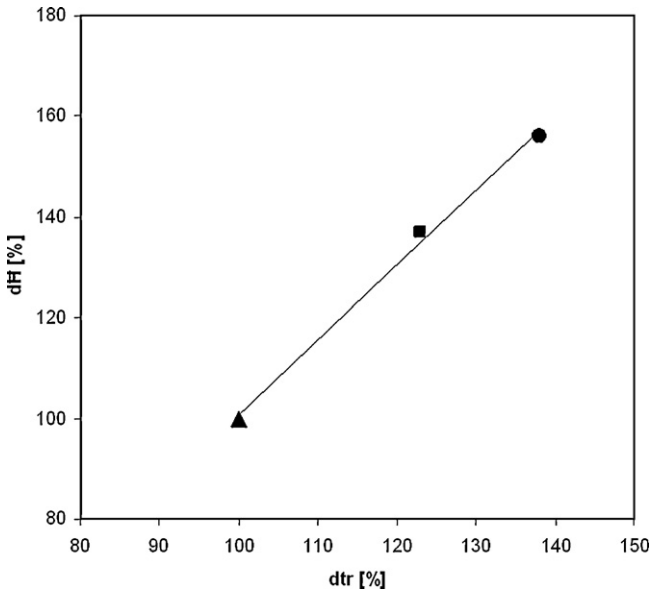

Fig. 5. Correlation between relative "plateau" height (d^{H}) and relative retention (d*tr*) for different column length [mm]: (▲) 100, (■) 200, (●) 400. Columns Onyx Monolithic, $T = 30\,^{\circ}C$.

do not contribute to retention improvement. Considering the configuration of 6,6′-2BrInd the existence of the maximum retention should correspond to the maximum hydrocarbon surface available for interaction with this solute. In more densely bonded phases the sterical hindrance plays the important part of solute–stationary phase interactions because of grafts proximity. In those conditions slipping of solute molecules between alkyl chains should be prevented increasingly and retention reduced.

The correlation of relative plateau height on relative retention is not obvious and should depend on additional, particular properties of each column (Fig. 4). The proportionality between relative plateau height (d^{H}) and relative retention time (d*tr*) seems to be valuable for the majority of columns. Again, the Hypersil BDS C18 seems fall out also from this correlation. The particularity of this phase compared to others is its low specific surface (Table 1). Alltima and Alltima HP HL give similar retention and plateau height and are clearly superior to other evaluated phases.

So, the appropriate C18 stationary phase should have a moderate bonding density, but its real suitability need to be checked experimentally by comparison with, for example, one of the Alltima phases used in this study. This experience shows that adjusting stationary phase silica bonding parameters allows the gain of H by 2.5 times (250%).

3.1.2. Column length

The retention time (*tr*) increases logarithmically with column length for the same gradient slope and starting composition [34,35]. H follows the *tr* increase, and the proportionality between *tr* and H is observed. The results, expressed as relative change of starting values (given as 100%) for relative "plateau" height (d^{H}) and relative retention (d*tr*), in function of column length are showed in Fig. 5.

Doubling column length gave an experimental gain of d^{H} of 37%; quadrupling it gave a gain of 56%. The d*tr* rose at the same time by about 23% and 37%.

3.2. Mobile phase composition

The solubility of compounds can depend on the mobile phase solvatation properties. In this part of the study some typical chromatographic solvents and buffers were evaluated. Also, the addition of DMSO to the mobile phase was tried.

Three gradients were set: the first from 10 to 90% of MeOH, the second from 5 to 90% of MeCN and the last of 5% DMSO–95% MeCN mixture from 5 to 90%. For all of them duration was 40 min and led with or without 0.1% (v/v) of acid in the mobile phase. The tested acids were formic and trifluoroacetic for their compatibility with MS detection, and methanesulfonic for its lower absorption in the UV region and weak ion-pairing properties. The use of strong acids like trifluoroacetic and methanesulfonic along with acetonitrile improves peak shape, and thus, resolution of all indigoids. Yet, the acid addition or its nature (mostly p*K*a) has proportionally little influence on separation and peak shape compared to other factors. That is in agreement with statement of Karapanagiotis [15]. He observed the slight improvement of peak height for Ind, Inr and 6,6′-2BrInd in UV–vis detection with trifluoroacetic acid when concentration changed from 0.001% to 0.1%. A small increase of selectivity of indirubins compared to indigotins simultaneous to a broadening of all peaks was observed when methanol was used as organic modifier. The addition of 5% DMSO to MeCN does not result in noticeable peak shape improvement. As the cut-off wavelength for DMSO 268 nm and viscosity is about 2 cP at 20 °C, no tests with higher DMSO concentrations were done.

So, the mobile phase, containing acetonitrile and methanesulfonic acid, was used for the further studies.

3.3. Temperature

The published separations of purple components were usually performed at room temperature or temperature was not given which presumably means the same. In one case it was mentioned that the system was thermostated at 40 °C [15]. However, any temperature effect on purple analysis was described.

The influence of temperature on retention and separation in HPLC is known through various effects [36–38]. The improvement of mass transfer kinetics between stationary and mobile phases

Table 2
Compounds detected in *Hexaplex trunculus* pigment extract. Column Alltima C18, $T = 70\,^{\circ}C$.

Compound	Retention time [min]	Spectrum maxima [nm]	% of total peaks area[a]	% of total peaks area[b]
Isa	4.8	242, 300, 420	1.17	2.22
BrIsa	9.1	256, 312, 412	3.06	5.71
cis-Inr	10.9	286, 380, 554	0.00	0.04
6′-Br-*cis*-Inr	13.1	288, 382, 564	0.00	0.06
6-Br-*cis*-Inr	13.7	298, 386, 544	0.00	0.11
6,6′-2Br-*cis*-Inr	15.5	288, 300, 382, 554	0.00	0.12
trans-Inr	19.8	290, 362, 544	2.17	1.78
6-Br-*trans*-Inr	23.5	298, 366, 536	2.57	1.28
6′-Br-*trans*-Inr	23.9	296, 362, 554	4.24	2.48
6,6′-2Br-*trans*-Inr	27.2	302, 366, 546	8.48	3.38
trans-Ind	18.6	286, 334, 614	5.97	7.51
Br-*trans*-Ind	22.5	288, 344, 606	34.81	37.07
6,6′-2Br-*iso*-Ind	22.9	278, 392, 486	0.00	0.00
6,6′-2Br-*trans*-Ind	25.9	292, 304, 348, 602	37.53	38.24

[a] Integrated @ 298 nm (all compounds).
[b] Integrated @ 308 nm (isatins), @ 392 nm (*iso*-indigotins), @ 548 nm (indirubins) and @ 608 nm (indigotins).

appears through sharper and more symmetrical peaks. The higher temperature also influences the partition coefficient and shortens retention time in fixed conditions or allows keeping the same retention using less organic modifier. In a gradient elution, the retention time depends on temperature according to a semi-empirical equation (Eq. (2)) [34]:

$$tr = a' + b'T \tag{2}$$

where a' and b' are characteristic constants for a solute and T is temperature (K). These constants can be found experimentally, after plotting tr versus T, by the resolution of linear equation of type: $y = -ax + b$.

In our case, the parallel improvement of solubility with temperature is an interesting issue.

The limiting parameters of high temperature separations are oven temperature limits, plus the thermal stability of the analysed compounds and the stationary phase. For more efficient temperature transfer from heating elements to the mobile phase, the mobile phase preheating and the columns of narrow internal diameter, 2.1 mm or less, are preferred. To take account of the lowering of absorption with temperature showed for indigotin [29] and the thermal resistance of PDA cells, it is necessary to let the mobile phase cool down before it enters the detector.

The plot of the relative "plateau height" $d^{\mathcal{H}}$ [%] versus relative retention time dtr [%] of 6,6′-2BrInd (Fig. 6) shows the temperature impact on solubility gain and retention. The correlation of absolute values between T and tr gives for 6,6′-2BrInd values of $a' = 38.23$, and of $b' = 0.117$ for equation (Eq. (2)) with correlation $r^2 = 0.9986$.

A 10 °C step gives the average solubility gain of 30% whereas dtr reduces of about 3%. Temperature change of 40 °C allows to solubilise 2.2 times more of 6,6′-2BrInd. Also, at the moment of the 6,6′-2BrInd peak maximum detection, the mobile phase is 7.9% less rich in MeCN compared to standard conditions (30 °C). This assumption is based on the retention time decrease of 3.7 min (from 34.8 to 30.1 min) and gradient slope 2.125% per min (0.708% per ml).

The solubilisation effect is also observed for the peak of 6-BrInd where concentration was clearly below $^{\mathcal{H}}$ value. This compound displays peak height rising with temperature, but the peak tails at all temperatures tested and no baseline resolution is observed from the next peak, the 6,6′-2BrInd. The rise of temperature accelerates the exchange of solutes between stationary and mobile phases tending to lead to more and more symmetrical peaks.

The indigoids are considered as chemically and thermally stable. They are sensitive for oxidizing agents and strong acids. Indigotin and its derivatives are also sensitive to reduction in strongly alkaline solution, producing *leuco* form. The decomposition temperatures of indigoids are above 190 °C.

From other hand, the purple dye recovery methods use solvent heating from 70 °C to 150 °C during 1–15 min [6,8–10,12,13,15,16,18]. None of these points were exceeded in our experiences. However, the comparison of obtained peak area for dye extract analysis at 30 °C and at 70 °C indicated the loss of about 30% for brominated indigotins and 50% for indigotin, and the gain of about 20% for isatins formed by decomposition of these indigotins. The relatively well soluble indirubins do not give tailing peaks and do not change peak height or area with temperature in a significant way. This observation is important for the purpose of comparison of results obtained with purple dye by different analytical approaches.

The relative standard deviation (RSD) of peak area integration has not exceeded 4.7% for 30 °C and 5.2% for 70 °C for peaks with signal per noise (S/N) ratio about 20. That value was averaged from 5 injections, which confirms excellent repeatability of analyses in both temperatures.

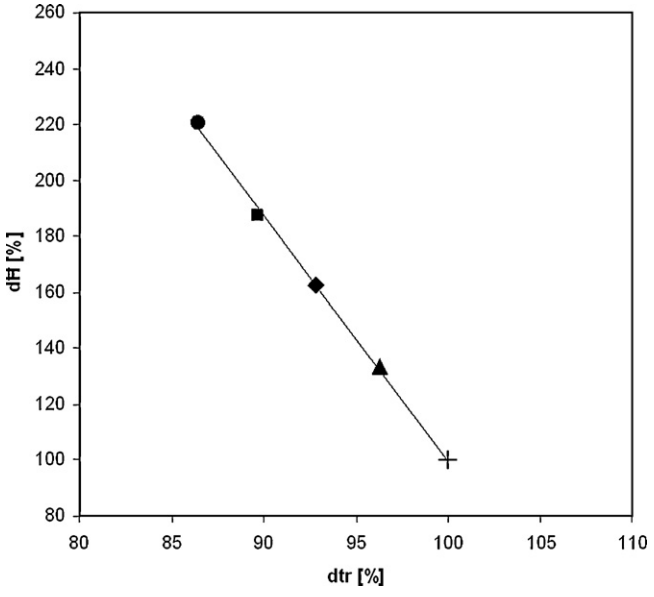

Fig. 6. Relative change of $d^{\mathcal{H}}$ versus relative dtr in function of temperature [°C]: (+) 30, (▲) 40, (♦) 50, (■) 60, (●) 70. Alltima C18 column.

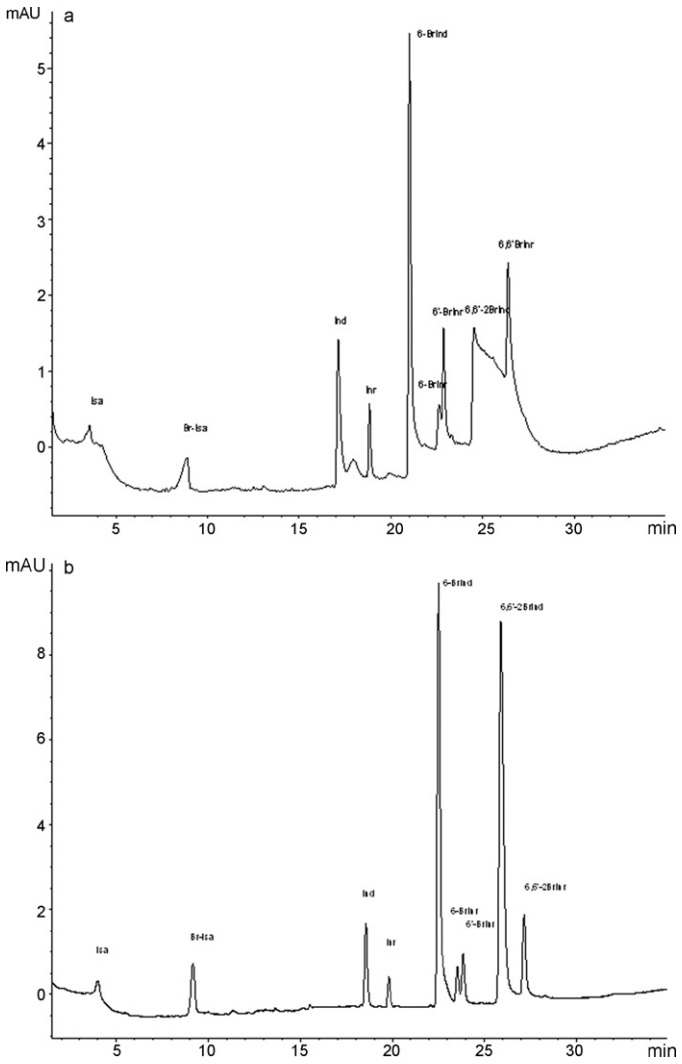

Fig. 7. Chromatogram of *Hexaplex trunculus* pigment at 298 nm detection wavelength. Column Hypersil BDS C18, $T = 30\,^{\circ}C$; column Alltima C18, $T = 70\,^{\circ}C$. Isa, isatin; BrIsa, 6-bromoisatin; Ind, indigotin; Inr, indirubin; 6-BrInd, 6-bromoindigotin; 6-BrInr, 6-bromoindirubin; 6′-BrInr, 6′-bromoindirubin; 6,6′-2BrInd, 6,6′-dibromoindigotin; 6,6′-2BrInr, 6,6′-dibromoindirubin.

3.4. The analysis of H. trunculus purple pigment

To evaluate the impact of the above described analytical conditions in term of semi-quantitative approach, the analysis of the same extract of standard sample of Purple pigment was carried out using two systems: Hypersil BDS C18 at 30 °C and Alltima C18 at 70 °C. The elution gradient was identical with that described in Section 2.3 (chromatography hard- and soft-ware). The injected volume was 5 μl in both cases. The analysis on Hypersil column at 30 °C corresponded to standard conditions used in our laboratory for dyestuffs analysis [39–41].

The obtained chromatograms display the presence of a series of indigoids previously detected in the purple from this specie, namely isatin, indigotin and indirubin as well as their 6(6′)-mono- and 6,6′-dibromo-derivatives (Fig. 7) with the noticeable peak shape differences obtained with standard conditions (Hypersil BDS, 30 °C) and optimised ones (Alltima, 70 °C). The peaks of Ind, BrInd and 6,6′-2BrInd obtained with standard system (Fig. 7a) tail a lot and their precise area estimation is difficult because of poor resolution essentially of mono- and dibrominated indigotin. Also, the peak end determination for both compounds is ambiguous. The chromatogram obtained in the second experience (Fig. 7b) allows more precise peak areas calculation for all compounds. Their relative quantities are shown in Table 2. They are similar to published data, in the limits of specie and dyeing conditions variability [3,16].

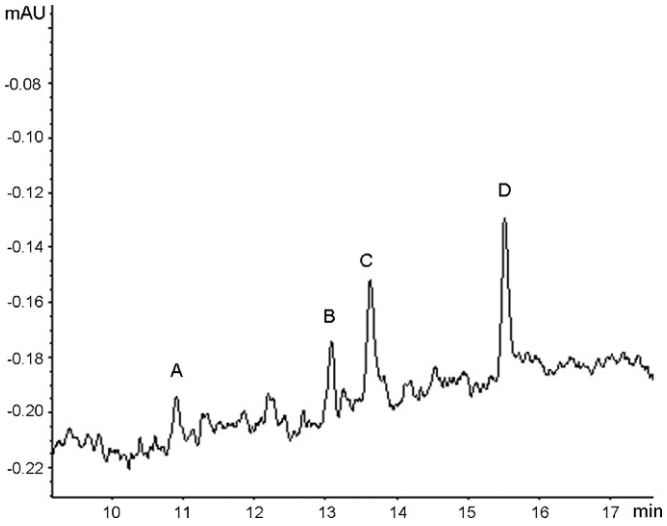

Fig. 8. Fragment of chromatogram (9–18 min) of *Hexaplex trunculus* pigment at 546 nm detection wavelength. Column Alltima C18, $T = 70\,^{\circ}C$.

However, compared to previously reported Purple composition, some additional peaks were detected for a first time at selective wavelength of 548 nm in a time range of 10–17 min (Fig. 8). Thanks to this indication, the closer look back on the chromatogram obtained at 30 °C allowed also to find the same compounds, but the improved analysis conditions allows their precise relative quantification along with the major compounds (Table 2). These compounds posses indirubin type absorption spectra (Fig. 9), well defined with injection of higher concentrations of pigment extract, and are less retained than regular indirubins. Their number (four) and relative retention follow the make-up of indirubins series and can correspond to one di-, two mono- and one unsubstituted indirubin derivative or isomer. Looking at their absolute retention times, they should be significantly less polar than regular indirubins. The change of bromine position or substitution by another halogene should not influence much the retention time in applied conditions, also the bromine in position 6 and 6′ seems the most logic – the formation of the Purple dyestuff involves 6-Br precursors.

So, the most probable reason of the diminution of retention should be the presence of polar groups in the structures of brominated and unbrominates indirubines.

The presence of supplementary polar groups in indirubin structure could be explained by the *cis*- form of indirubin (Fig. 1e). In those isomers, thermodynamically less stable, both ketone oxygens as well as both amine groups are free to interact with polar mobile phases. Their relative retention observed also suggests the identity as *cis*- forms on the basis of experiments done with unsubstituted indigotins and indirubins [42]. Additionally, their absorption maxima and batochromic (or "red"!) shift of about 10 nm is similar to that published for *cis*-indirubin in comparison to its corresponding *trans*- form [13].

Taking in account the above considerations we can propose the most probable identity of detected compounds: *cis*-Inr for compound (A), *cis*-6′-BrInr (B), *cis*-6-BrInr (C) and *cis*-6,6′-2BrInr (D). Also, according to the shape of corresponding spectra, it may be observed the inversion of elution order of monobromoindirubins: 6 then 6′ for *trans* isomers and 6′ then 6 for *cis* forms.

However, the shorter retention for these compounds in comparison with corresponding *trans*-indirubins seems too large

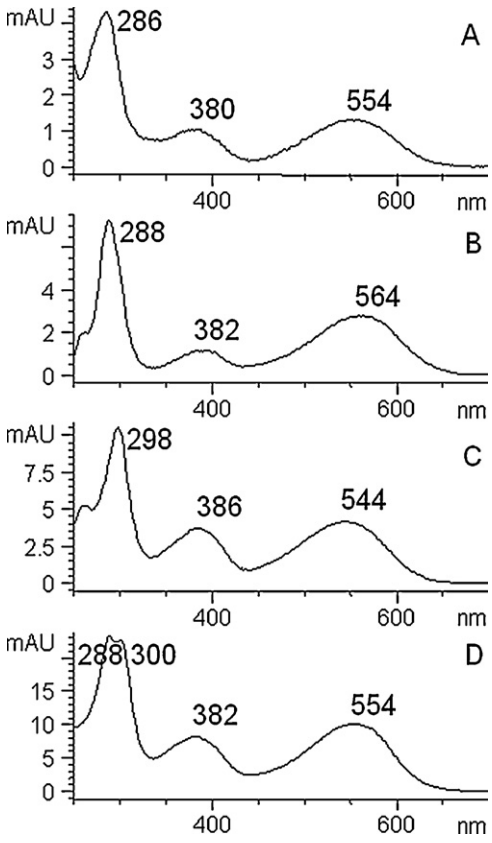

Fig. 9. UV–vis spectra of indirubin related compounds A–D: (A) unbrominated, (B) 6′-bromo, (C) 6-bromo, and (D) 6,6′-dibromo.

(about 10 min for corresponding *cis*-/*trans*- pairs) when, for example, 6,6′-dibromo-*iso*-indigotin and 6,6′-dibromoindigotin isomers retention difference, which correspond to the "liberation" of two amine groups, is relatively small (about 3 min).

The lesser hydrophobicity of detected compounds may be also explained by the presence of additional polar groups (e.g. –OH, $-SO_3H$) in positions other than 6(6′) occupied by bromine. According to obtained spectra, the positions 5(5′) and 7(7′) are privileged. The substitution in 4(4′) should modify the UV–vis spectra in a more significant way, comparably to what was already observed for indigotins [25,43].

These compounds were not detected previously in purple probably because too low quantity of dye extract injected, inappropriate detection wavelength or bandwidth, eventually different composition of the analysed individual Purple samples. The compounds could be easily missed as the ratio between the majors and the newly detected ones is very high, more than 100:1.

The proposed identity of the series of newly detected compounds, based on their UV–vis spectroscopic characteristics and chromatographic behaviour, need to be confirmed by MS or NMR.

4. Conclusion

This study gives some general directions for chromatographic system parameter selection concerning the stationary phase, mobile phase composition and analytical conditions for reverse-phase analysis of Tyrian purple. The correlation between maximum loading "plateau" height (H) and retention time of 6,6′-2BrInd in gradient elution shows clearly that this parameter is dependent mainly on solubility of dibromo indigotin in the mobile phase. The H parameter could be used for evaluation of further improvements of analytical conditions. The suitability of the system to obtain the highest peak with the best symmetry of brominated indigoids, determined as H, is greatly influenced by stationary phase parameters and temperature of separation. Other parameters, such as mobile phase composition and column length have a minor impact on the solubility of brominated indigoids.

Optimised analysis conditions may allow even 400% improvement of solubility compared to less retentive phases at near-ambient temperature, rendering quantitative analysis more accurate. The better solubility of critical compounds, and especially that of 6,6′-2BrInd, enables the analysis of concentrated solutions and detection of minor compounds as well as the parallel quantification of major and trace compounds which was not the case until now.

The described optimal analysis conditions were possible to perform using standard equipment, with obvious limitations, but can be easily extrapolated to more efficient systems. Further improvement, using higher temperatures, above 100 °C, is a potential possibility. However, it requires special high temperature resistant columns, secured ovens and the use of diode array detectors which might be complicated by the precipitation of compounds during pre-detection eluent cooling. Considering a certain sensitivity of indigoids in the mobile phase at elevated temperatures the further improvements may concern the analyses with shorter columns and rapid gradients to limit the journey of the compounds in chromatographic column.

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