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Identification of degradation products of indigoids by tandem mass spectrometry

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The study concerns identification of photodegradation products of indigotin, indirubin and isoindigo. Experimental methodology consists of degradation of standard solutions of indigoids in a solar box and analysis of samples taken at different aging time by using capillary high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometric and spectrophotometric detectors. Identification of the formed compounds was based on careful interpretation of the electrospray ionization MS/MS spectra. Apart from the well-known degradation products of indigoids: isatin, isatoic anhydride and anthranilic acid, another seven species were also identified, and their proposed structures were confirmed by high-resolution molecular masses measurements; according to the best knowledge of authors, they have not been reported so far. The obtained results formed the basis for postulating mechanism of the process. Moreover, the MRM (Multiple Reaction Monitoring) method was developed for the identification of natural dyes and their degradation products in textiles of historical value. Apart from such colorants as indigotin and flavonoids, also presence of degradation products of indigoids was confirmed. Copyright © 2015 John Wiley & Sons, Ltd.

Keywords: degradation of indigoid dyestuffs; photostability of indigoids; high-performance liquid chromatography; tandem electrospray mass spectrometry

Introduction

Natural dyes were used for thousands of years for painting and dyeing of textiles. They had been used until the end of the 19th century, when in short time they were replaced with cheaper synthetic dyes, produced in many colors and shades. Colorants were obtained from various parts of plants or animals.[1] One of the most widely used natural dyes is indigo.[2] Its most popular sources were *Isatis Tinctoria*, *Indigofera Tinctoria*, *Indigofera Suffruticosa*, *Indigoferra Arrecta* and *Polygonum Tinctorium*.[3] Plants contain precursors of color compounds: indican and isatan B, which can be converted to indigotin by mild oxidation.[4] Apart from indigotin, isoindigotin, indirubin and isatin are also formed in this process.[5]

Along a time, works of art containing colorants undergo degradation under an influence of many external factors, e.g. oxygen, light, humidity and temperature.[6] Exposure to light is a main factor of fading that resulted from structural changes of color compounds.[7,8] Understanding of the mechanism of the process is important for conservators, as it allows to choose the best restoration procedure.[9,10]

Indigo is one of the most photostable natural dyes. This is due to absence of a photoinduced trans ➔ cis isomerization (twisting of the central C = C bond).[11] Nevertheless, after enough time, degradation of indigo is also observed.

Till now, only few studies concerning identification of indigo degradation products have been published. In model procedure examined, preparations were aged and formed degradation products were separated and identified.[12] Several methods of accelerated aging were proposed; usually exposure to the light in a solar box,[13] but also enzymatic[14] or ozone treatment.[15] There are a few well-known degradation products of indigoids: isatin,[13,15,16] isatoic anhydride[13,15,16] and anthranilic acid.[13,16] Less common, 2-benzyl-2-indole,[15] as well as small organic compounds[17] were also reported. Similar process is observed in the case of indigo carmine (sulfonated derivative of indigotin) where sulfonic isatin and sulfonic isatoic anhydride are identified as the main degradation products.[18,19]

Because of lack of standards of formed compounds, for their identification, mainly mass spectrometry with electrospray ionization[19–21] or with electron impact ionization[13,16] were used, also in tandem configuration.[13,18,19] For the separation of complicated mixture of degradation products, prior to MS detection, usually, liquid chromatography was recommended.[17,19,21] Gas chromatography was also used, but with additional derivatization step.[16,17,20,22] More information concerning molecular structures of the formed species offered tandem mass spectrometry.[23] The observed main neutral losses were H_2O, CO, CO_2 and HCN, and they allowed to identify functional groups present in degradation products.[24,25]

The aims of the study were to identify photodegradation products of selected indigoids, color constituents of natural indigo (Fig. 1), as well as to elaborate method for indirect identification of natural dyestuffs based on identification of their degradation products.

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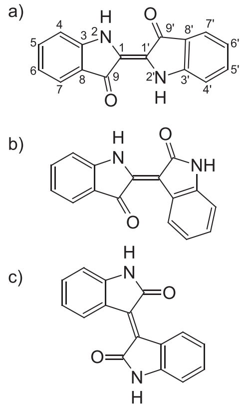

Figure 1. Molecular structures of: (a) indigotin, (b) indirubin and (c) isoindigotin.

Materials and methods

Instrumentation

Capillary high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (HPLC ESI MS/MS) analysis were performed with the use of Agilent 1200 series chromatograph (Agilent Technology, Waldbronn, Germany) equipped with a binary capillary pump, degasser, autosampler, thermostatically controlled column compartment, diode array detector with 80 μl flow cell and electrospray ionization triple quadrupole mass spectrometer (Agilent 6460 Triple Quad LC/MS, Agilent Technologies, Santa Clara, CA, USA). All the operations, acquiring and analysis of data were processed by MassHunter Software (Agilent Technology, USA). Conditions for separation and detection are shown in the Additional Supporting Information 1 (ASI 1). Parameters of the developed MRM method for each reaction are shown in Table 1.

Ultra-performance liquid chromatograph (UPLC) ACQUITY I-Class (Waters Inc) coupled with Synapt G2-S HDMS (High Definition Mass Spectrometry) (Waters Inc) mass spectrometer equipped with an electrospray ion source and Q ToF (Quadrupole/Time of Flight) type mass analyzer was used. The instrument was controlled, and

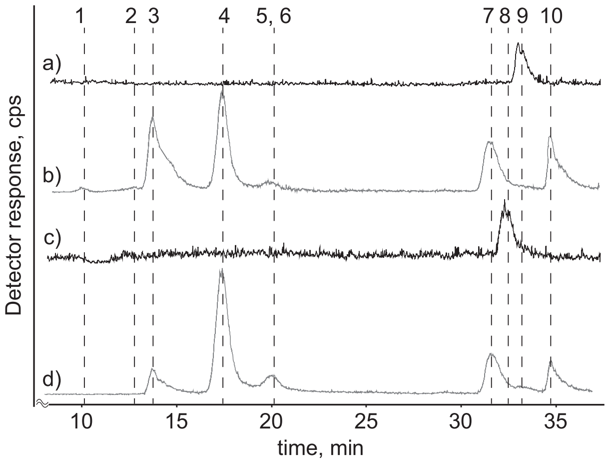

Figure 2. μ-High-performance liquid chromatography electrospray ionization MS chromatograms of indigotin degradation products. Samples taken after (a) 5 h; (b) 15 h; (c) 30 h; (d) 30 h. Black lines – registration in positive ion mode, gray ones – negative ion mode.

Table 1. Characteristics of Multiple Reaction Monitoring method for the identification of colorants and degradation products by μ-high-performance liquid chromatography electrospray ionization MS/MS

Tr, min	Compound	Ion mode	Precursor ion, *m/z*	Fragmentator energy, V	Fragment ions, *m/z* (CE,V)
10,8	Degradation product 1	–	166	80	122 (6), 92 (13)
10,8	Isatin	–	146	80	118 (7)
12,5	Degradation product 3	–	162	80	118 (8)
16,1	dcII	–	475	100	431 (14), 341 (23), 311 (24), 282 (41)
16,6	Degradation product 4	–	164	80	136 (5), 120 (10), 92 (20)
16,8	Carminic acid	–	491	100	447 (15), 357 (24), 327 (25)
16,8	Degradation product 5	–	194	90	150 (8), 136 (10,) 120 (15), 92 (20)
17,8	Luteolin-7-O-glucoside	–	447	140	285 (24)
18,1	Degradation product 6	–	178	80	136 (5), 134 (5), 92 (15)
19,0	Apigenin-7-O-glucoside	–	431	140	268 (31)
19,3	dcIV	–	491	100	447 (15), 357 (24), 327 (26), 284 (32)*
20,9	dcVII	–	491	100	447 (15), 357 (24), 327 (26), 284 (32)*
21,8	Degradation product 7	–	194	60	162 (2), 118 (12)
22,2	Degradation product 8	+	235	100	217 (25), 206 (40), 190 (40)
22,2	Luteolin	–	285	120	241 (11), 135 (12), 133 (20)
22,9	Degradation product 9	+	293	100	265 (10), 146 (25), 90 (30)
23,0	Flavokermesic acid	–	313	80	269 (5), 257 (17)*
23,6	Apigenin	–	269	100	151 (17), 149 (17), 117 (32)
23,6	Kermesic acid	–	329	80	285 (4), 257 (18)
24,8	Alizarin	–	239	140	211 (19), 210 (26), 167 (22)
25,5	Indigotin	+	263	60	235 (22), 219 (23), 206 (38), 132 (45), 77 (52)
26,0	Degradation product 10	–	327	100	162 (5), 136 (10), 118 (30)
26,6	Purpurin	–	255	140	227 (18), 171 (26), 129 (32)

recorded data were processed using MassLynx V4.1 software package (Waters Inc).

Accelerated aging of the dyestuffs was performed with the use of a solar box SunTest CPS+ (Atlas, Chicago, USA) equipped in a xenon lamp (200–900 nm; 500 W/m^2) with an outdoor filter. Solutions for analysis were sampled every 5 h, up to 60 hs of aging.

Chemicals

Indigotin was purchased from Fluka. Indirubin and isoindigotin have been synthesized according to Puchalska.[26] Formic acid of LC/MS purity from Fisher Scientific (Fair Lawn, NJ, USA) was obtained. Methanol (LC-MS grade) and dimethyl sulfoxide (HPLC grade) were purchased from POCH (Gliwice, Poland). Demineralized water from Milli-Q system Model Milipore Elix 3 (France) was used throughout.

Standard stock solutions (100 µg/ml) of indigotin and isoindigotin were prepared by dissolving 1 mg of each preparation in 10 ml of dimethyl sulfoxide (DMSO), and indirubin – in methanol. The obtained solutions were filtered over a 0.45 µm syringe filter (Supelco, Sigma-Aldrich, Bellefonte, PA, USA).

Historical samples

The examined fibers were taken from tapestry 'Scenes from the Opera' – collection of the Museum of King John III's Palace at Wilanów (Poland). They were extracted with 50 µl of DMSO and formic acid (97:3, v/v). The obtained solutions were kept in an ultrasonic bath for 5 min and in a water bath at 80 °C for 5 min. Then, they were filtered over a 0.22 µm PET (polyester) syringe filter and diluted with 50 µl of methanol and water (2:3, v/v) followed by analysis.

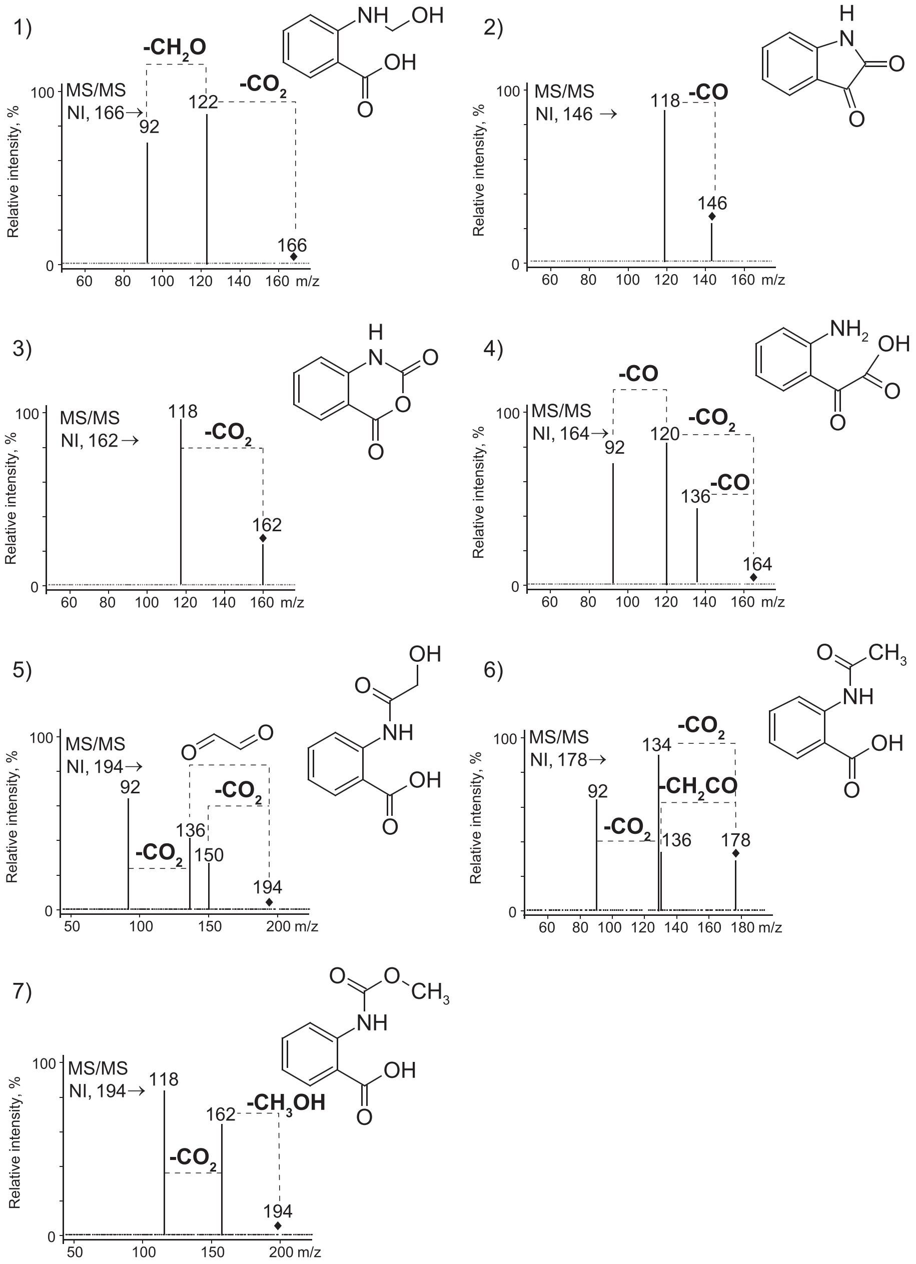

Figure 3. Electrospray ionization tandem mass spectrometry (MS/MS) spectra of small degradation products of indigotin. Numbers describing spectra reflect elution order of identified compounds (cf. Fig. 2).

Results and discussion

Products of indigotin degradation – MS/MS study

Samples taken after different aging time were analyzed by using capillary μ-HPLC UV Vis ESI QqQ MS. On the obtained chromatograms, ten peaks were observed (Fig. 2); signals of compounds **1–7** and **10** could be registered when ESI MS detection was performed in negative ion mode and **8** and **9** – exclusively in positive ions mode. Only protonated ions were observed in positive ion mode. It has to be mentioned, that compounds **6** and **8** were not observed, when UV detection was used.

The most abundant MS signals were attributed to the potential quasi-molecular ions and analyzed in product ion mode. The obtained MS/MS spectra of separated degradation products allowed identification of lost fragments and, in consequence, propose their structures.

Molecules of compounds **1–7** are relatively small and, according to nitrogen rule, contain one nitrogen atom (Fig. 3); they are formed by cleavage of the double bond between indol moieties occurring in indigotin structure. Careful evaluation of their fragmentation patterns creates the basis for drawing hypotheses concerning molecular structures. Typical observed losses were CO_2 and CO from carboxyl and carbonyl groups. Lost more characteristic fragments: formaldehyde (compound **1**), oxalic aldehyde (**5**), ethylenone (**6**) as well as methanol (**7**), allowed to propose more complicated structures. Identification of compounds **2** and **3** was confirmed by analysis of their standards, as they are well-known degradation products of indigotin: isatin (**2**) and isatoic anhydride (**3**). Moreover, in order to confirm identity of compound **7**, synthesis of N-methoxycarbonyl-anthranilic acid according to Erdmann [27] was performed. Its retention time (tr = 32.1 min) registered on chromatogram with UV detection at 254 nm, as well as MS/MS spectrum, were identical with those of the compound **7**. Addition

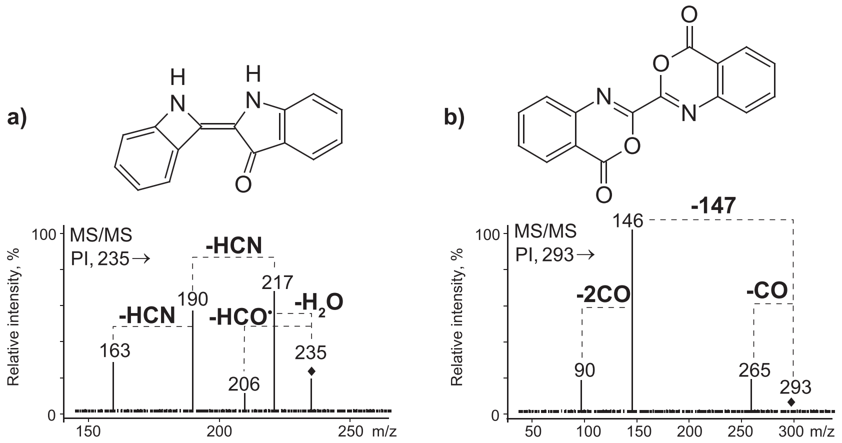

Figure 4. Electrospray ionization tandem mass spectrometry (MS/MS) spectra of compounds eluted in peaks **8** (a) and **9** (b) in chromatogram of indigotin degradation products (cf. Fig. 2).

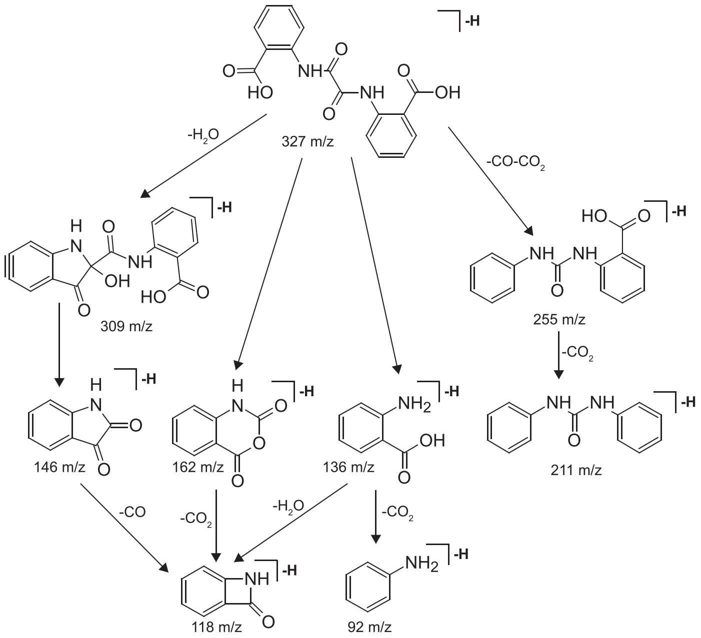

Figure 5. Fragmentation pathway of the ion at m/z 327 (compound eluted in peak **10** in the chromatogram of indigotin degradation products).

of the synthesized acid to the sample of photodegraded indigo resulted in the increase of the peak 7, as well.

In contrast to what was previously discussed, molecules of compounds **8–10** are bigger and, according to nitrogen rule, contain two nitrogen atoms. MS/MS spectrum of compound **8** is shown in Fig. 4(a). The registered fragment ions are the same as those observed in spectrum of indigotin.[28] Moreover, ion at *m/z* 235 (parent for compound **8**) was identified in spectrum of indigotin as fragment obtained by loss of carbon oxide. Hence, we would like to propose that compound **8** most probably is decarbonylated indigotin.

Molecular masses of compounds **9** and **10** are higher than mass of indigotin and it creates basis for the hypothesis, that they are products of photooxidation of the latter. In MS/MS spectrum of compound **9,** the precursor ion at 293 *m/z* is observed (Fig. 4(b)). The ion registered at *m/z* 146 is formed by loss of the fragment of *m/z* 147, what suggests cleavage of the bond between rings within molecule of symmetrical structure. Formation of the ion at *m/z* 90 by further losses of two carbon oxide molecules confirm presence of two oxygen atoms in each 'half molecule'.

MS/MS spectrum of compound **10** is much more complicated. Its careful interpretation allowed to propose fragmentation pathway of the precursor ion at 327 *m/z* shown in Fig. 5. In the scheme, two directions are distinguished: loss small molecules, e.g. carbon mono or dioxide or water (ions at *m/z* 309, 255, 211, 193), and cleavage of the bond between rings within molecules (ions at *m/z* 162, 146, 136). Further fragmentation of obtained ions by loss of small molecules was also observed.

The above hypothesis concerning structures of quasi-molecular and fragment ions of degradation products of indigotin was confirmed by their high-resolution spectra registered by using UHPLC-ESI Q TOF MS. Accurate molecular masses were measured and elemental compositions of the separated species were settled as well (Additional Supporting Information 2, ASI 2).

Mechanism of indigotin degradation

There are many theories concerning photodegradation of organic compounds as well as the role of oxygen in this process. They generally agree that the first step of degradation involves photoexcitation of species present in examined solution (e.g. analyte or solvent molecules),[8] As a result of further energy transfers, different reactive species may be formed,[8,29–31] among others OH$^\bullet$ free radicals. Under experimental conditions, their active role seems to be the most probable. However, also, another degradation pathway cannot be excluded, as in the present study, only species detectable with the use of UV-Vis and ESI MS were investigated.

Assuming, that OH$^\bullet$ free radicals (or another oxygen species activated by light) attack an aromatic ring or unsaturated bond or cause abstraction of a hydrogen atom,[32] allows to propose probable mechanism of indigotin degradation (Fig. 6).

Compounds identified in solutions containing indigotin degradation products (cf. Figs 3–5) were probably formed as the result of cleavage of the following bonds (numbering of atoms in indigotin molecule is shown on Fig. 1):

- C1–C1′ and C1-C9: compounds **1** and **7**;
- C1–C1′: compounds **2** and **3**;
- C1–C9, C1′–C9′ and C1′–C2′: compound **6**.

Primary degradation products of indigotin may undergo further transformations. Hence:

- Cleavage of bond C1–N2 in compound **2** causes formation of compound **4**;
- Oxidation of compound **6** (attack of hydroxyl radical on C atom in amide group) leads to compound **5**.

More complicated seem to be the mechanisms of the formation of compounds **8**, **9** and **10**, as here no cleavage of the bond C1–C1′

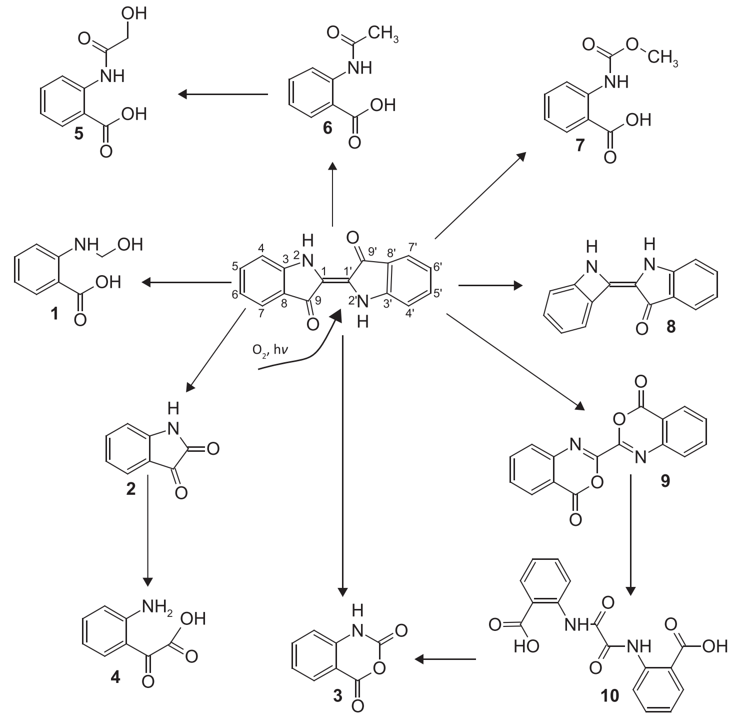

Figure 6. Proposed mechanism of degradation of indigotin.

Table 2. Colorants and degradation products identified in historical samples

	Color of fiber	Colorants	Degradation products
3–1	Dark brown	**Carminic acid**	3
		dcII	5
		dcVII	10
		Kermesic acid	
		Flavokermesic acid	
		Indigotin	
		Isatin	
		Luteolin-7-O-glucoside	
3–4	Dark brown	**Carminic acid**	3
		Alizarin	4
		Purpurin	5
		Indigotin	7
		Apigenin	10
		Luteolin	
		Apigenin-7-O-glucoside	
		Luteolin-7-O-glucoside	
		Kermesic acid	
		Flavokermesic acid	
		dcII	
		dcIV	
		dcVII	
		Isatin	
3–10	Green	**Luteolin-7-O-glucoside**	3
		Luteolin	5
		Indigotin	7
		Apigenin-7-O-glucoside	10
		Apigenin	

Major colorants.
Minor colorants.
Trace colorants.

is observed. Compound **9** is probably formed as the result of the attack of hydroxyl radical on C1–C1′ bond, but without its cleavage and formation of six-member rings after incorporation of oxygen atoms (similar mechanism of degradation of Indigo Carmine – indigotindisulfonate – has already been proposed).[18] Further oxidation of **9** leads to structure **10**.

Formation of compound **8** is rather unexpected and cannot be explained by simple red-ox mechanism involving participation of hydroxyl radical. Nevertheless, its spectrum obtained with use of Q ToF MS confirms such structure.

Other indigoids – indirubin and isoindigotin

Indigotin and indirubin after 5 h of aging are completely degraded; in the case of isoindigotin, even after 15 h of exposition to light, it is still present in examined sample among its degradation products. On chromatograms of solutions containing aging products of idigotin, indirubin or isoindigo, all **1–7** compounds are registered. However, they can be easily differentiated, as among degradation products of indirubin, compounds **8** and **9** are not observed and among these of isoindigo – compound **10** (characteristics of degradation products of indigoids is presented in Additional Supporting Information 2).

Historical samples

The examined fibers taken from the tapestry ‘Scenes from the Opera’ were extracted and analyzed by MRM method by using μ-HPLC ESI MS/MS. The obtained results are shown in Table 2.

On the chromatogram of the extract from green fiber (Fig. 7), there are nine peaks observed. Major peaks correspond to luteolin, apigenin, their glucosides and indigotin. In this case, green color of the fiber was obtained by mixing blue and yellow dyestuffs. Indigotin could originate from either woad (*Isatis tinctoria* L.) or indigo (*Indigofera tinctoria* L.). The identified flavonoids indicate that the used yellow dyestuff was weld (*Reseda luteola* L.). In the chromatogram, there are also peaks of degradation products of indigoids **3**, **5** and **10**.

Extract of analyzed brown fiber contained indigotin and red colorants – carminic acid and kermesic acid. It indicated that this textile was dyed with cochineal (*Dactylopius coccus* Costa) and indigo (or woad). Another fiber contained also anthraquinones: alizarin and purpurin. In this case, probably, madder (*Rubia tinctorum*) was also a component of the dyeing mixture.

All extracts from the examined fibers contained degradation products of indigoids, mainly compounds **3** and **10**, but also **4**, 5 and 7 were identified. It proved that the developed methodology (MRM method) can be a useful tool for identification of natural dyes based on their degradation products, also in the case when examined work of art is totally discolored.

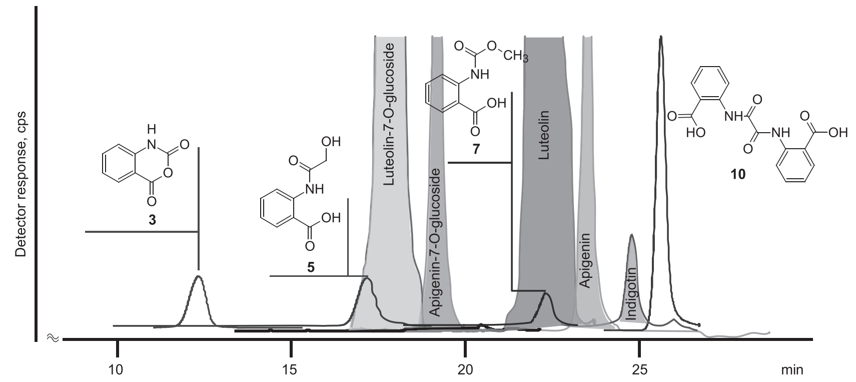

Figure 7. μ-High-performance liquid chromatography electrospray ionization tandem mass spectrometry (MS/MS) chromatogram of the extract from the green fiber (MRM detection).

Conclusions

Capillary HPLC coupled with ESI MS/MS, first time (according to the best knowledge of the authors) used for identification of natural colorants and products of their degradation obtained during artificial aging, was proven to be an efficient tool in this field, allowing to analyze samples as small as of volume 0.1 μl.

The developed methodology allows to differentiate indigotin, indirubin and isoindigo; as a result of photodegradation of each compound, different number of products is formed. Moreover, the MRM method can be used for the examination of even discolored textiles, as it allows to identify indigoid colorants, thanks to the identification of products of their degradation.

Acknowledgement

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Supporting Information

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