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Determination of natural colorants in plant extracts by high-performance liquid chromatography

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Abstract: The determination of the colouring compounds apigenin (**1**), lawsone (**2**), juglone (**3**) and indigotin (**4**) in plant extracts using HPLC–UV/Vis methods is reported. The methods were applied to the analysis of **1**–**4** in ethanolic and propylene glycolic extracts originating, respectively, from chamomile (*Chamomilla recutita* [L] Rauschert, Asteraceae), henna (*Lawsonia inermis* L., Lythraceae), walnut (*Juglans regia* L., Juglandaceae) and natural indigo (*Indigofera* sp., Fabaceae). In the case of the indigo extracts, an optimized acid hydrolysis was applied. HPLC separations were performed on a Hypersil ODS RP18 column using linear gradient elution programs. The detection limits for **1**–**4** were 0.11, 0.6, 0.10, 0.089 μg mL^{-1}, respectively. The procedure did not involve any sample "clean-up" methods. The amounts of the colouring compounds ranged from 0.006 (**3**) to 0.13 mg mL^{-1} (**4**) in the ethanolic extracts and from 0.22 (**2**) to 1.44 mg mL^{-1} (**4**) in propylene glycolic extracts. The proposed HPLC methods are advantageous in terms of sample preparation and the selective separation of the compounds. The plant dye extracts are commonly used in hair colouring formulations. The results indicate that the methods developed may serve for the quantitative control of dying plants and cosmetic products.

Keywords: apigenin; indigotin; juglone; lawsone; high-performance liquid chromatography.

INTRODUCTION

Until the mid-19th century, natural dyes were the only source of available colours. They were used for many purposes: to colour natural fibres, to produce inks, watercolours and artist's paints. They also served to colour cosmetic products.[1] Synthetic dyes, being less expensive than natural ones, experienced great progress in a short time. Nevertheless, their utilization presents some risks for the health of consumers. Natural dyes are less toxic, less polluting, less health hazardous and non-carcinogenic. Recently, remarkable interest in natural dyes has

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been witnessed in the cosmetic industry. The manufactures offer plant dye extracts, powder pigments and pigment pastes for use in hair colouring and hair highlights. The plant dyes, which meet the growing needs of hair care formulations, such as dyes, shampoos and conditioners, were designed as part of a new range of "environmentally responsible" cosmetic ingredients. Vegetal yellow dyes are flavonoids compounds and notably both luteolin and apigenin (**1**) are largely found in *Reseda luteola*, *Reseda lutea*, *Genista tinctoria*, *Chamomilla recutita*, and *Solidago* spp.[1,2] Natural henna (*Lawsonia inermis*) produced the most important source of a red dye for hair colouring, *i.e*., lawsone (2-hydroxy-1,4--naphthoquinone, **2**).[3–5] Brown to dark brown colour was obtained mainly from *Juglans regia*; the effective substance is juglone (5-hydroxy-1,4-naphthoquinone, **3**).[3] Natural indigo provided the most common source of blue pigments. Indigo refers to several species of *Indigofera*. In tropical and sub-tropical areas, the plants most widely used for indigo production were *Indigofera* spp., of which there are over 350 species.[1,6,7] In temperate climates, the most commonly used species was *Isatis tinctoria* or dyers woad.[1,8] No natural dye is a pure product and often the exact natural source of a given dye can only be derived from the presence of minor dye components. Toxicological properties of indigo are reported in the literature.[9] Lawsone has genotoxicity/mutagenicity potential *in vitro* and *in vivo* and no safe threshold for lawsone can be established.[10] The analytical control of dyes is of considerable importance in the cosmetic industry and scientific methods are required to control the quality of hair care formulations. The presence of chemical markers guarantees identity and efficacy of the plant colouring extracts.

In previous analytical studies of plant dyes various methods were used in commercial textile and archaeological dyeing of fibres,[1,2,11–17] pharmaceuticals,[18] food samples,[19] and colouring compounds in molluscs.[20–22] Several HPLC methods for analyzing cosmetic colorants[23–26] and dye precursors of oxidative hair dyes[4,26] are available in the literature but they were applied to the analysis of synthetic colorants in commercial hair dyeing formulations. Moreover, although HPLC methods for analyzing indigotin (**4**) in different matrices have been published,[2,11–15,17,20–22] only a few studied on the HPLC analysis of *Isatis tinctoria* leaves and *I. indigotica* roots and leaves have been reported.[8,27,28]

Reversed-phase liquid chromatography with UV/Vis diode-array detection (DAD) has been used for the identification of **4** in natural organic pigments used in historical art objects.[12,14,15] Karapanagiotis *et al*. (2006) and Koren (2008) studied molluscan blue and red-purple indigoid vat dyes by HPLC/DAD.[21,22] Mass spectrometry was applied for the identification of indigoid compounds extracted from objects of historical interest using direct inlet into different ion sources: atmospheric pressure chemical ionization (APCI)[14] and electrospray ionization (ESI–MS)[13,17] after their separation by HPLC. Puchalska *et al*. identified

indigoid compounds (indigo, indirubin, isoindigo, isoindirubin) of natural dyestuffs and their natural or synthetic precursors (indican, isatin, indoxyl, 2-indolinone) by LC/ESI-MS in textile fibres of art samples.[13] A liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) method was developed to determine indican, isatin, indirubin and indigotin in the roots and leaves of *I. indigotica*.[28] A qualitative method was devised to analyse indigotin precursors (indican) in leaf extracts of European (*I. tinctoria*) and Chinese (*I. indigotica*) woad using HPLC coupled to an evaporative light scattering detector (ELSD).[8]

The chamomile phenolic fraction is most commonly analyzed by HPLC/ /DAD and HPLC/MS.[29,30] In spite of their promising perspectives, HPLC/NMR, capillary electrophoresis (CE) and capillary electrochromatography (CEC) still face some limitations regarding resolution, expensive instruments and time-consuming procedures.[30–32] High performance liquid chromatography with UV detection is the most commonly used method for the determination of the naphthoquinones juglone in walnut leaves[33–35] and fruits,[36,37] and lawsone in natural henna.[3–5,38] An HPLC/DAD method was used for the simultaneous determination of the naphthoquinones: 1,4-naphthoquinone, lawsone, juglone and plumbagin in plants (*Dionaea muscipula*, *Drosera rotundifolia*, *Drosera spathulata*, *Drosera capensis* and *Paulownia tomentosa*).[34,35] The quantitative analysis of **2** and henna glycosides can be performed by HPLC[34,35] and HPTLC.[39,40] Recently, two chromatographic methods were developed to determine the chemical fingerprinting of *L. inermis*: HPTLC identification followed by densitometric measurements and RP-HPLC.[5] HPTLC was proposed to control the quality of raw plant materials and formulations based on the title plant. Differential pulse voltammetry (DPV) coupled with a hanging mercury drop electrode (HMDE) and micro flow device were utilized to determine the content of juglone in leaves *of J. regia*.[41]

Only a few HPLC methods have hitherto been appropriately validated.[19,28,31,33,34] Several methods for analyzing plant extract colorants by HPLC are available in the literature, but none of them has been applied to the analysis of **1**–**4** in propylene glycolic plant extracts. However, **1** has been previously analyzed in so-called "glycolic" extracts (consisting of 92.7 % propylene glycol, 2 % glycerine and 5 % ethanol, w/w) of *Chamomilla recutita* by CE and CEC[31,32] but the methods used were different from the one described herein. The present work is the first attempt made to use HPLC/UV for the identification of the chemical markers **1**–**4** in plant colouring extracts for hair care formulations.

The aim of this study was the identification and quantitative determination of the most important colouring components in ethanolic and propylene glycolic plant extracts: chamomile (*Chamomilla recutita*, Asteraceae) extract and apigenin; henna (*Lawsonia inermis*, Lythraceae) extract and lawsone; walnut (*Juglans regia*, Juglandaceae) extract and juglone; natural indigo (*Indigofera* sp., Faba-

ceae) extract and indigotin. For these purposes, it was necessary to optimize the analysis of the commonly used cosmetic colorants by a simple and reliable HPLC method coupled with UV/Vis detection. The ethanolic and propylene glycolic extracts were supplied by a Bulgarian company that manufactures herbal extracts for cosmetic uses. The HPLC methods described herein are of practical interest, allowing the determination of colouring compounds present in hair care formulations defined as "natural", which should only contain plant extract colorants.

EXPERIMENTAL

Chemicals and reagents

The standard of apigenin was purchased from Extrasynthese (Genay, France) and those of lawsone (97 %), juglone (97 %) and indigotin (95 %) from Sigma–Aldrich (Deisenhofen, Germany). HPLC-grade solvents and analytical-grade chemicals were provided by Merck (Darmstadt, Germany). The water was double distilled. Solvents were filtered through a 0.45 μm filter (Millipore, Bedford, MA, USA) and degassed in an ultrasonic bath before use. Stock solutions (0.4 mg mL^{-1}) of **1**–**3** were prepared by dissolving 20 mg of each powder in 50 ml methanol. The solutions were stored in a refrigerator. The working standard solutions of appropriate concentration were prepared daily by diluting the stock standard solutions with methanol. The solutions of **4** were freshly prepared in the range 0.2–0.001 mg mL^{-1} in methanol–dioxane (1:1, v/v) and kept in vials preventing light penetration to avoid decomposition of indigotin.

Extracts

The ethanolic and propylene glycolic extracts from chamomile (*Chamomilla recutita*), henna (*Lawsonia inermis*), walnut (*Juglans regia*) and natural indigo (*Indigofera* sp.) were received directly from a Bulgarian producer. According to the provider, the alcoholic extracts were obtained with 70 % ethanolic–aqueous solution. The ethanolic extracts (10 mL) were evaporated under vacuum at 40 °C and reconstituted with 10 mL methanol. The propylene glycolic extracts were diluted appropriately in MeOH (1 mL extract to 5 mL solvent). All extracts were then submitted to sonication at room temperature for 5 min using a RK 52 H Sonicator (Bandelin electronic, Berlin, Germany) at 120 W, 35 kHz and centrifuged at 10000 rpm for 10 min. The supernatant was collected and filtered through a 0.45 μm filter (Millipore, Bedford, MA, USA) prior to injection.

Acid hydrolysis of the indigo extracts

The samples of natural indigo (1 ml of methanolic or methanolic–propylene glycolic extract, see Section *Extracts*) were hydrolyzed for 15 min at 100 °C in 3 mL of a mixture of 37 % HCl–water (2:1). The extracts were rapidly cooled and centrifuged at 10000 rpm for 10 min. The blue residue after acid hydrolysis indicated the presence of indigotin, which was redissolved in 1 mL methanol/dioxane (1:1, v/v) and filtered through a 0.45 μm filter (Millipore) prior to injection. Indigotin was detected in freshly obtained samples.

HPLC analysis

The chromatographic analyses were performed on a Varian (Walnut Creek, California USA) chromatographic system equipped with a tertiary pump Model 9012, a rheodyne injector with a 20 μl sample loop, a UV/Vis detector Model 9050 set at 335, 340, 249 and 288 nm according to the UV absorption maxima of the compounds **1**–**4**, respectively. A Varian Star Chromatography workstation and computer software (version 4.5) for controlling the

system and collecting the data were used. A reversed phase Hypersil ODS RP18, 5 μm, 250×4.6 mm I.D., Shandon (Runcom, England) column equipped with a precolumn 30 mm×4.6 mm (Varian, USA) filled with the same stationary phase was used.

Gradient program I. The chromatographic separation of **1** was realised using a mobile phase consisting of A) acetonitrile, B) methanol and C) 20 mM potassium dihydrogen phosphate buffer adjusted to pH 3.20 with orthophosphoric acid. The elution program commenced at 15 % A:5 % B:80 % C followed by a linear gradient for 20 min to 30 % A:10 % B:60 % C. The flow rate was 1 mL min^{-1}.

Gradient program II. The chromatographic separation of **2** was performed using a binary solvent system consisting of: A) 3 % methanol in a 20 mM potassium dihydrogen phosphate buffer (adjusted to pH 3.20 with orthophosphoric acid) and B) 45 % methanol in 20 mM potassium dihydrogen phosphate buffer (adjusted to pH 3.20 with orthophosphoric acid). The elution program was from 100 % A to 100 % B in 55 min. The flow rate was 1.3 mL min^{-1}.

Gradient program III. The chromatographic separation of **3** was performed using a binary solvent system consisting of: A) 3 % methanol in a 20 mM potassium dihydrogen phosphate buffer (adjusted to pH 3.20 with orthophosphoric acid) and B) 45 % methanol in 20 mM potassium dihydrogen phosphate buffer (adjusted to pH 3.20 with orthophosphoric acid). The elution program was from 90 % A:10 % B to 10 % A:90 % B in 45 min. The flow rate was 1.2 mL min^{-1}.

Gradient program IV. Chromatographic separation of **4** was carried out using a mobile phase consisted of A) acetonitrile, B) methanol and C) 100 mM ammonium acetate buffer with 30 mM dibutylamine adjusted to pH 6.8 with acetic acid. The elution program commenced at 10 % A: 35 % B: 55 % C followed by linear gradient for 25 min to 10 % A:65 % B:25 % C. The flow rate was 1 mL min^{-1}.

For all programs, the mobile phase was returned to the initial conditions in 5 min and the column was equilibrated for 10 min. The oven temperature was set at 27 °C.

Quantitative analysis

The analysis of the assayed compounds (**1**–**4**) was performed using the external standard method. Working solutions containing 0.2, 0.1, 0.05, 0.02, 0.01 and 0.001 mg mL^{-1} of **1**–**3** were prepared from stock solution, 0.4 mg mL^{-1} in methanol, respectively. The employed concentrations of **4** were 0.2, 0.1, 0.05, 0.02, 0.01 and 0.001 mg mL^{-1} and the solutions were prepared in dioxane–methanol (1:1, v/v).

Triplicate analyses were performed for each concentration and the peak area was detected at 335, 340, 249 and 288 nm for **1**–**4**, respectively. Calibration curves were constructed from the peak areas *vs*. analyte concentrations. Slope, intercept and other statistics of the calibration lines were calculated by linear regression using the Analytik-Software (Leer, Germany) STL statistics program. The regression equations were, respectively, for compounds **1**–**4**: $y = 3.38\times10^7x + 7.43\times10^4$ ($r^2 = 0.9969$); $y = 6.40\times10^6x + 4.19\times10^4$ ($r^2 = 0.9990$); $y = 3.70\times10^7x + 1.05\times10^6$ ($r^2 = 0.9936$) and $y = 4.09\times10^6x - 1719$ ($r^2 = 0.9999$).

For every sample, the complete assay procedure was performed in triplicate and the standard deviation calculated.

RESULTS AND DISCUSSION

The principal flavonoid colouring matter is apigenin. It produces the most vibrant and lightfast yellow colour. For standardization purposes of chamomile extracts, apigenin, to which hair colouring property and several therapeutic ac-

tivity have been associated, is the flavonoid of choice.[1,32,42] The content of this flavonoid in extracts of chamomile flowers was found in a heterogeneous range of values: 106 (methanolic extract), 77 (ethanolic extract) and 11 μg g^{-1} (glycolic extract).[31] Lawsone and juglone are the most commonly occurring naphthoquinones.[3] Natural henna is known to contain 1–2 % lawsone, which is responsible for colouring orange–red. The amount of juglone detected in walnut leaves was about 0.2–0.4 % (fresh weight).[41] Naphthoquinones are not a precursor of oxidative hair dyes. The vegetal blue indigotin is not synthesized directly by the plant; it is a product derived from indole glucoside precursors, which are secondary metabolites.[1,6,28] *Indigofera* spp. contain a yellowish glycoside indican (indoxyl-*β*-D-glucoside), which is readily hydrolyzed in aqueous solution by dilute mineral acid or by enzymes to the respective aglycon indoxyl and a sugar. To form indigotin from the precursors, the carbohydrate moiety is cleaved from the indoxyl group and two of the resulting indoxyl molecules combine oxidatively to produce an indigotin molecule. In practice, once the molecule has been hydrolyzed, this combination occurs spontaneously under aerobic conditions and indigotin precipitates from solution and deposits as a blue sediment.

Due to the presence of the glycoside indican in the extracts from indigo (*Indigofera* spp.), its transformation to indigotin was achieved by acid hydrolysis. The final dissolution of indigotin was realised with a mixture of dioxan and methanol (1:1, v/v), which resulted in an increase in the dissolution and stability, in comparison with those previously reported.[13,19] The limited solubility of indigotin in organic solvents[15] and its instability in these media[13,19,43] should be mentioned. A study of the solubility of indigotin was performed by Blanc *et al.*[15] It was found that indigotin is soluble in acetic acid and pyridine. To the commonly used methanolic–acidic extraction method for natural indigo in textile fibres, an additional methanol/DMF,[14] DMSO,[13] acetic acid/SDS,[15] or warm pyridine[44] extraction step was applied. Moreover, indigotin is unstable in acidic, basic medium and under daylight.[13,45] The stability of indigotin solutions in DMSO was examined by Puchalska *et al*. It was found that in solutions exposed to daylight at room temperature, a degradation of 50 % of indigotin to isatin was completed after 7 days; after 30 days, only the degradation product could be found.[13] In a previous study, Altinos *et al*. reported that working solutions (10 mg in 100 mL) of indigotin were prepared in methanol–water (40:60, v/v) for a HPLC method and that the solutions were stable for at least 12 h.[19] In the present study, indigotin was detected in freshly prepared dioxane solutions and extracts and they were stable for at least 3 days (over this period, the *RSD* (area) was 3.16 %). In addition, dioxane is less toxic in comparison with pyridine and DMF, currently evaluated by the International Agency for Research on Cancer (IARC) as carcinogens.

The HPLC analyses were directly performed on the total extracts of chamomile, henna and walnut without any manipulation of the samples. Some authors have reported a sample "clean-up" procedure by solid-phase extraction[32] of both ethanolic and propylene glycolic extracts of chamomile as the presence of propylene glycol in the matrix inhibited the absorption of the compounds to the stationary phase. The present method does not involve any sample purification step, the propylene glycolic extracts were diluted appropriately in MeOH and no inhibition of the partitioning caused by the presence of propylene glycol was found.

Preliminary RP-HPLC experiments for the separation of the chamomile extract showed that certain pairs of major aglycones, such as luteolin and quercetin, apigenin and isorhamnetin, were not well resolved by currently used systems consisting of an organic phase (either methanol or acetonitrile) and water/TFA or water/formic acid.[29,30] Therefore, the effects of various proportions of methanol (A) and acetonitrile (B), ranging from 5 % A:10 % B to 20 % A:30 % B were tested for the separation of the analytes. The proposed HPLC method (gradient program I) enabled the determination of **1** with advantages in terms of retention time (24 min in comparison with 40 min[29]) and the selective separation of the above-mentioned aglycones and methylated aglycones axillarin and chrysosplenol, well known for their presence in chamomile extracts.[29]

The structural difference between **2** and **3** is small, originating only from the position of the hydroxyl group. Initial chromatographic runs were gradients that ranged from 3 % methanol to either 30 or 45 % over 45 or 55 min. Eight binary solvent systems were investigated consisting of methanol and either phosphate buffer (20 mM potassium dihydrogen phosphate buffer (adjusted to pH 3.20 with orthophosphoric acid) or an aqueous phase, containing 0.1 % phosphoric acid. The HPLC methods (gradient program II and III) for the separation and quantification of **2** in henna extracts and **3** in walnut extracts were based on a binary gradient system consisting of phosphate buffer containing 3 % methanol and 45 % methanol over 55 and 45 min, respectively. It should be mentioned that the previously reported chemical fingerprinting of *L. inermis* using HPLC enabled the evaluation and comparison of raw plant material only by peak groups at specific and significant t_R values.[5] The method developed in this study successfully separated **3** from phenolic compounds (phenolic acids, tannins, flavonoids), while in the HPLC method for the simultaneous determination of naphthoquinones in plants,[34] the retention times of **2** and **3** were 3 and 5 min, respectively; they were not completely distinguished from the interfering above-mentioned phenolic compounds.

In this report, an HPLC system for the determination of **3** where the chosen mobile phase is compatible with determination of **2** and **1** is reported. The binary mobile phase in gradient programs II and III could be rapidly applied to the determination of **2** and **3**, either as single compounds or in a mixture.

The development of HPLC methods for **1**–**3** was performed using a mobile phase consisting of organic modifier(s) and pH 3.2 phosphate buffer (gradient programs I–III). The pH was chosen in order to prevent ionization of the phenolic hydroxyl groups of apigenin and naphthoquinones; it was presumed that the studied compounds are neutral molecules at pH 3.2. Indigotin is unstable in acidic and basic medium, hence a buffer of pH 6.8 was chosen for the analysis. Additionally, in the HPLC of indigotin dissolved in dioxane–methanol (1:1, v/v) as eluent with phosphate buffer without dibutylamine, double peaks were observed for each analyte. The addition of dibutylamine to the ammonium acetate buffer at pH 6.8 improved the partition of the analytes between the stationary and mobile phase (gradient program IV). The proposed HPLC method enables the determination of indigotin with advantages in terms of retention time (15 min in comparison with 35 min[15] and 52 min[27]).

For the HPLC–UV analysis, four wavelengths were specified: indigotin has maximum absorbance at 288 nm, lawsone at 340 nm, juglone at 249 nm and apigenin at 335 nm. These wavelengths allow a sufficient sensitivity of detection for the determined compounds to be obtained. In the case of the natural indigo extracts obtained because of acid hydrolysis, a broad elevation of the baseline was observed; this was assigned to some product of acid hydrolysis of the extract. Peaks of the chemical markers **1**–**4** were assigned in the HPLC chromatograms by comparing individual peak retention times with those of authentic reference standards and by the spiking technique.

The repeatability was established by injecting standard solutions of the assayed compounds (0.01 mg mL^{-1}) six times. The reproducibility was determined over 10 days by three injections per day of the same solutions. The relative standard deviations (*RSD*s) of the repeatability and the reproducibility were $\leq$ 2.42 % and $\leq$ 5.71 %, respectively. The precision of the retention times was evaluated taking into account a triplicate analysis of both standards and plant samples, and the obtained mean values were derived with standard deviation (Table I). In the calibration experiments, the interval of linear response covered the concentration range from 0.001 to 0.4 mg mL^{-1} (except for indigotin, for which the upper limit was 0.2 mg mL^{-1}). All compounds showed acceptable linearity with correlation coefficients (r^2) higher than 0.993 within the range of concentrations investigated. The average precision of the entire analytical procedure expressed by the relative standard deviations (*RSD*s) of parallel measurements (n = 3) was estimated by measuring the within-day repeatability, being in all cases in the range 1.00–8.48 %. The *RSD* of **3** determined in the walnut extract exceeded 5 %. Taking account of the low amount present in the ethanolic–aqueous extract ($0.0059\pm5.03\times10^{-4}$), an *RSD* value of 8 % is acceptable.[46] The limit of detection (*LOD*) and the limit of quantification (*LOQ*) were calculated according to ICH guidelines based on the standard deviation of the regres-

sion lines of specific calibration curves and their slope using analyte concentrations in the range of the *LOD* and *LOQ*.[47] The detection (*LOD*) and quantification (*LOQ*) limits determined would permit the quantification of the compounds assayed at ppm levels, since the *LOD*s and *LOQ*s ranged from 0.089 to 0.6 μg mL^{-1} and from 0.32 to 1.8 μg mL^{-1} for indigotin and lawsone, respectively.

TABLE I. Precision, limit of detection (*LOD*) and limit of quantification (*LOQ*) for the assayed compounds

Compound	(t_R±*SD*) / min	*RSD*[a] / %	*RSD*[b] / %	*LOD* / μg mL^{-1}	*LOQ* / μg mL^{-1}
1	24.24±0.39	0.06	2.47	0.11	0.36
2	45.04±0.11	2.42	4.49	0.60	1.80
3	38.09±0.27	2.16	5.71	0.10	0.32
4	15.00±0.19	1.54	4.06	0.089	0.32

[a]Repeatability (n = 10); [b]reproducibility tested over 10 days (n = 3)

Mulinacci *et al*. described a semi-quantitative study of the flavonoid content of chamomile extracts.[29] The *LOD* and *LOQ* of a capillary electrophoretic and a capillary electrochromatographic method for the quantification of **1** in chamomile extracts were 3.80 and 11.5 μg mL^{-1}, and 35 and 150 μg mL^{-1}, respectively.[31,32] The proposed HPLC method using UV detection enabled the sensitivity to be increased thirty-fold, whereby values of 0.11 and 0.36 μg mL^{-1} were achieved, respectively. The simultaneous analyses of naphthoquinones by HPLC/ /DAD[34] and DPV[41] were performed by Babula *et al*. and the *LOD*s and *LOQ*s obtained for **2** were 65 (217) and 5 (16) ng mL^{-1}, respectively, and for **3**, 75 (252) and 18 (60) ng mL^{-1}, respectively. The obtained detection limits for **2** and **3** in the present study were in the same range as those realised in the literature,[34] but DPV characteristics were ten-folds better. The sensitivity obtained for **4** with the developed method was higher than that Altinos and Toptan achieved by HPLC with UV detection at 480 nm (0.2 μg mL^{-1}).[19]

The optimized methods were consequently applied to identify and quantify the colouring compounds **1**–**4** present in plant extracts originating from extraction with 70 % ethanol and propylene glycol as solvents. The obtained chromatographic profiles are shown in Fig. 1. Although very similar peak profiles were obtained, the amount of the colouring compounds differed considerably among these extracts. The concentrations of the compounds assayed were found to be lower in the ethanolic–aqueous extracts than in propylene glycolic extracts. The amounts of the colouring compounds assayed ranged from 0.006 (**3**) to 0.13 mg mL^{-1} (**4**) in the ethanolic extracts and from 0.22 (**2**) to 1.44 mg mL^{-1} (**4**) in the propylene glycolic extracts (Table II).

The chamomile extract used in hair care formulations is standardized on more than 1.2 % total apigenins; an anti-inflammatory test supports that cha-

momile extract has good anti-inflammatory properties and that it protects cells against UV stress at dosages of 0.1–1.0 %.

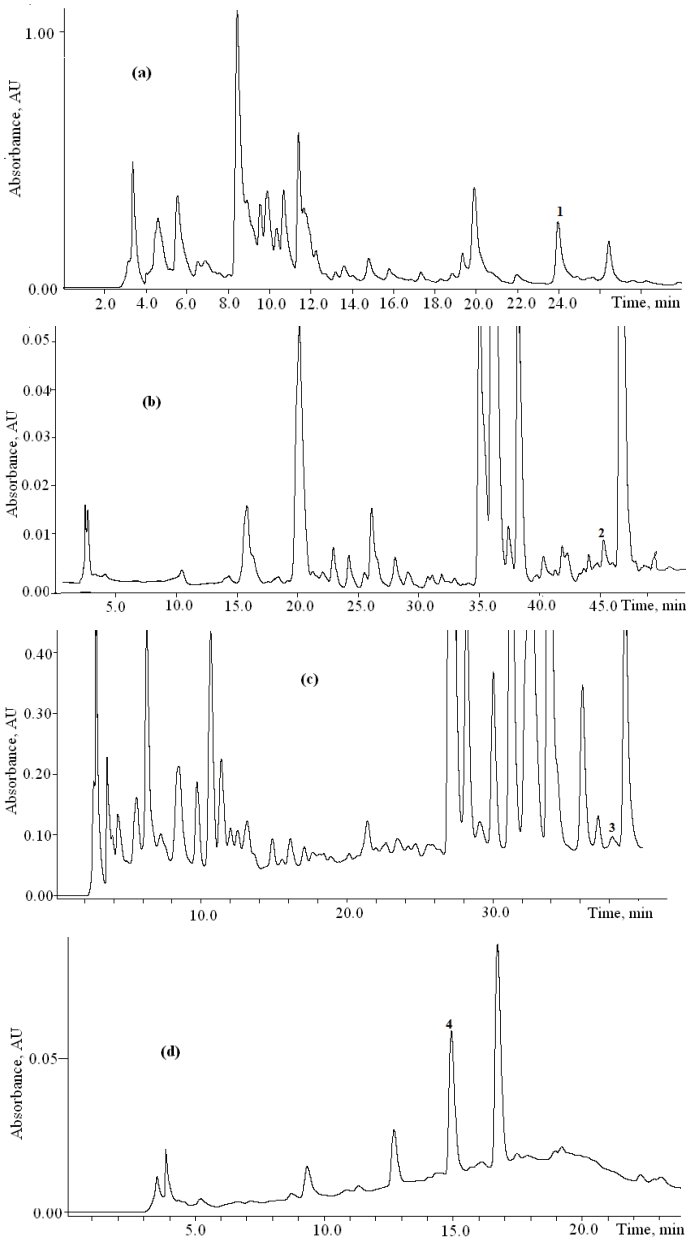

Fig. 1. HPLC chromatograms of a) chamomile propylene glycolic, b) henna ethanolic, c) walnut propylene glycolic and d) natural indigo ethanolic extracts. **1** – apigenin; **2** – lawsone; **3** – juglone and **4** – indigotin. See text for chromatographic conditions.

TABLE II. Content (mg mL^{-1}) of the compounds assayed in ethanolic and propylene glycolic extracts

Compound	Ethanolic extract±*SD* mg mL^{-1}	*RSD* / %	Propylene glycolic extract±*SD* mg mL^{-1}	*RSD* / %
1	0.0387±0.0023	5.82	0.5238±0.0358	6.83
2	0.0297±8.02x10^{-4}	2.70	0.2192±0.0066	3.03
3	0.0059±5.03x10^{-4}	8.48	0.3797±0.0035	1.00
4	0.1250±0.006	5.08	1.4400±0.006	4.00

Martinez *et al*. reported in the literature that the total apigenin content in a shampoo was equivalent to 105 ppm (0.0105 %) of a chamomile extract standardized on 1.5 % total apigenins.[42] Despite its low content in complex formulations (shampoos), the developed specific analytical method was able to successfully quantify the traces of apigenin. It has been proposed that lawsone be used as a non-oxidizing hair colouring agent at a maximum concentration of 1.5 % (typical concentration 1.26%) in the finished cosmetic product. Henna products were found to contain 0.24 % lawsone.[4] In this study, the concentrations of juglone were found to be very low in all samples. Due to polymerization phenomena, juglone is reported to occur in dry leaves only in vestigial amounts.[48] A literature survey revealed no data for the presence of juglone and indigotin in hair colouring formulations.

In conclusion, the proposed HPLC methods enable the routine determination of the most employed natural colorants **1**–**4**, originated from chamomile, henna, walnut and natural indigo extracts, with advantages in terms of sample preparation and the selective separation of the compounds. They could be used for quantitative analysis and quality control of extracts and hair care formulations.

ИЗВОД

ОДРЕЂИВАЊЕ ПРИРОДНИХ БОЈА У ЕКСТРАКТИМА БИЉАКА МЕТОДОМ HPLC

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Одређивана су бојена једињења апигенин (**1**), лосон (**2**), југлон (**3**) и индиготин (**4**) у биљним екстрактима методом HPLC–UV/Vis. Метода је примењена на анализу једињења **1**–**4** у етанолном и пропиленгликолном екстракту биљака камилица (*Chamomilla recutita* [L] Rauschert, Asteraceae), кана (*Lawsonia inermis* L., Lythraceae), орах (*Juglans regia* L., Juglandaceae) и природном индигу (*Indigofera* sp., Fabaceae). У случају екстракта индига, примењена је оптимизована кисела хидролиза. HPLC раздвајање је изведено на колони Hypersil ODS RP18, користећи линеарни елуциони градијент. Детекционе границе за **1**–**4** су 0,11; 0,6; 0,10 и 0,09 μg mL^{-1}. Процедура није захтевала методе претходног пречишћавања. Концентрација бојених једињења се кретала од 0,006 (**3**) до 0,13 mg mL^{-1} (**4**) у етанолном екстракту и од 0,22 (**2**) до 1,44 mg mL^{-1} (**4**) у пропиленгликолном екстракту. Предложени HPLC метод има предности у односу на друге методе у припреми узорака и селективном раздвајању једињења. Екстракти биљних боја се користе за бојење косе. Резултати овог рада показују да разви-

јена метода може имати примену у квантитативној контроли биљака за бојење и козметичких производа.

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