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Liquid Chromatography

W. Nowik, Laboratoire de Recherche des Monuments Historiques, Champs-sur-Marne, France

Introduction

Natural dyes are organic matter made up of coloured compounds originating from natural living sources such as plants and animals. These compounds can be found either directly in the extracts or become coloured by hydrolysis, oxidation, condensation, etc., from extracted colourless precursors. Some of them change colour following such reactions or by complexation with certain metallic cations.

These dyestuffs are generally employed for dyeing fibres and fabrics (usually protein and cellulosic), staining several organic and mineral materials (histological preparations, wood, hair, feathers, Easter egg shells), producing organic pigments (painting, printing) and colouring of food, beverages, cosmetics and pharmaceutical products. They are used in analytical chemistry as well as acid–base indicators or complexation agents. A large number of naturally occurring coloured substances are chemically labile. This excludes their direct use principally because of poor light-fastness. Some dyestuffs, in addition to their colouring qualities, have therapeutic properties like regulation of metabolism, anti-inflammatory treatment and anti-cancer prevention among others.

Used frequently around the world in traditional societies, starting probably from the Stone Age, natural dyes were rapidly replaced by synthetic dyes from the second half of the nineteenth century onwards. Nowadays, natural dyes have a growing importance in our lives since almost all of them are hypoallergenic and nontoxic for humans, which is a significant advantage over many synthetic dyes.

Chemical classification of dyestuffs follows the general arrangement of organic compounds and depends on the generic, basic structure. There are over 20 family structures which many hundreds of compounds belong to. Examples of the most frequently encountered structures are shown in **Table 1**.

The most important application of dyes, from a historical point of view, is textile dyeing. For this reason, the most common classification of dyestuffs is determined by their dyeing properties and dyeing technology. According to their dyeing properties, natural dyes belong to four principal groups: direct dyes (e.g. curcumin, a diaryloylmethane from *Curcuma longa*), mordant dyes (e.g. alizarin and other anthraquinones from various madders), reactive dyes (e.g. depsides and depsidones from lichens) and vat dyes (e.g. indigotine from indigo, woad, dyer's knotweed and others).

Membership to one of the dye families is determined by the type of 'connection' between colorant and support. However, many dyestuffs may show more than one dyeing mechanism, simultaneously or according to applied dyeing conditions. The type of connection determines the method of liberation of dyestuff prior to analysis. Identification of type and origin of dyestuffs requires an understanding of composition.

Extraction

Direct Raw Material Extraction

Many natural dyestuffs or their precursors (e.g. indican and isatan B for indigotine) are water-soluble. Their extraction from raw plant or animal material can be achieved by water, sometimes heated to facilitate penetration into the cells. This process, along with possible cell destruction, can also be accelerated by the use of an ultrasound bath. In several cases, the addition of water-miscible solvents (e.g. methanol, ethanol or acetonitrile) improves the recovery of relatively hydrophobic compounds, such as curcumin, certain antraquinons, etc. Moderately polar solvents

Table 1 Some important chemical families of dyestuffs

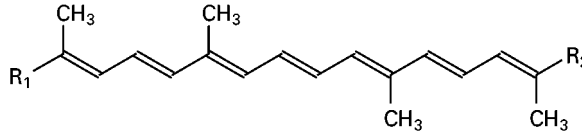
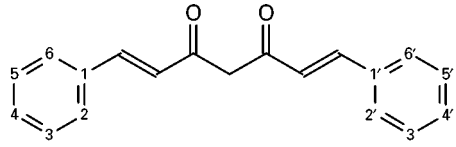
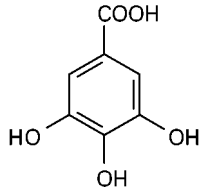
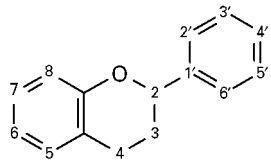
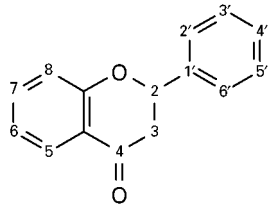
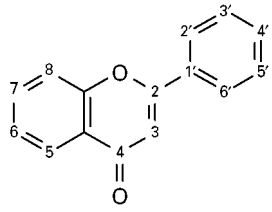
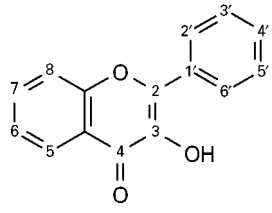
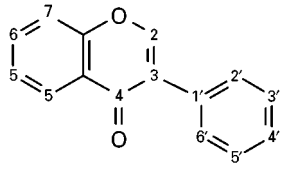
Generic name	*Generic structure*	*Example*	
		Name	*Usual name*
Carotenoid		Carotenoid-dicarboxylic acid (R_1, R_2 = COOH)	Crocetin
Diaryloylmethane		Diferuoylmethane (4,4′ = –OH, 5,5′ = –OMe)	Curcumine
Gallotannin		Digaloylhamamelose	Hamamelitannin
Flavane		3,3′,4′,5,7-Pentahydroxyflavane	Catechin
Flavanone		3,3′,4′,7-Tetrahydroxyflavanone	Fustin
Flavone		4′,5,7-Trihydroxyflavone	Apigenin
Flavonol		3,4′,5,7-Tetrahydroxyflavone	Kaempferol
Isoflavone		3′,4′,5-Trihydroxy-7-methoxyisoflavone	Santal

Table 1 *Continued*

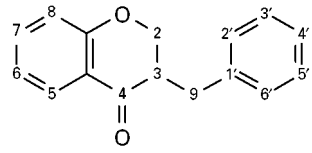
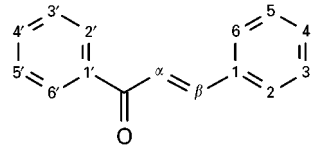
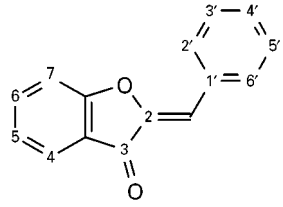
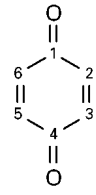
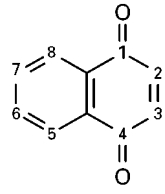
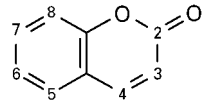
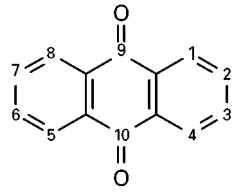
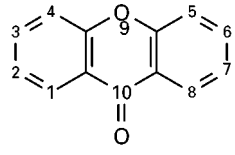
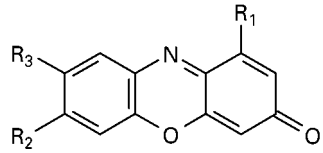
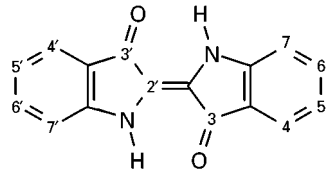
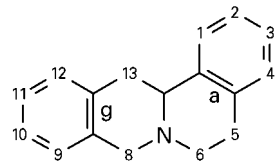
Generic name	*Generic structure*	*Example*	
		Name	*Usual name*
Homoisoflavone		3,4′,5′,7-Tetrahydroxyhomoisoflavone	Sappanon A
Chalcone		3,4,4′,5,6′-Pentahydroxychalcone	Robtein
Aurone		3′,4′,6-Trihydroxyaurone-6-glucoside	Sulfurein
Benzoquinone		3,6-Dihydroxy-2,5-di(*p*-hydroxyphenyl) benzoquinone	Atromentine
Naphthoquinone		2-Hydroxynaphtoquinone	Lawsone
Coumarin		7,8-Dihydroxycoumarin-7-β-D-glucoside	Daphnin
Anthraquinone		1,3-Hydroxy-2-carboxyanthraquinone	Munjistin
Xanthone		1,7-Dihydroxy-9H-xanthen-9-on-7-D-glucuronide	Euxanthic acid

Table 1 *Continued*

Generic name	*Generic structure*	*Example*	
		Name	*Usual name*
Phenoxazone		α-Aminoorcein (R_1 = Me, R_2 = NH_2, R_3 = orcinol)	Orcein[a]
Indole		6,6′-Dibromoindigotine	Purple[a]
Berbine		5,6-Dihydro-3-hydroxy-2,9,10-trimethoxy dibenzo[*a*,*g*] quinolizinium hydroxide	Berberine

[a]The name of a natural mixture of related compounds. It's applied here when the particular usual name of the compound does not exist.

such as ethyl acetate have been proposed for general extraction purposes. The choice of extraction solvent and conditions affects the quantitative aspects of dyestuff analysis.

Even the use of water, and especially warm water – traditionally used for the recovery of colouring matter – may also introduce composition changes. Many natural dyestuffs are heterosides (gluco-, rhamno-, primeverosides and other) and can become aglycone forms through the action of enzymes (e.g. glucosidases) present in living organisms. Their activity can be inhibited either by excessive heating (boiling) or by the addition of denaturation factors (alcohols, strong electrolytes).

For quantification purposes, especially in pharmaceutical and cosmetic laboratories, extracts are analysed after acid hydrolysis. The hydrolysis of heterosides transforms them into related aglycones (e.g. ruberythric acid to alizarin). This treatment simplifies the chromatogram, because one aglycone can be obtained from several monosaccharide or disaccharide derivatives and the number of chromatographic peaks decreases (**Figure 1**).

Coloured Matter Samples Extraction

Acid hydrolysis has remained a quasi-universal method of natural dyestuff extraction from dyed and stained supports. The extraction medium is usually composed of an aqueous mineral acid solution (e.g. HCl or H_2SO_4), sometimes with the addition of methanol to prevent possible precipitation of some compounds. This hydrolysis destroys chemical ionic and coordination links between dye molecules and support or mordant complexes. Fixed extraction conditions are the goal of repetitive results, because of possible partial structural change or destruction of several compounds. In applied acid solutions a depolymerization of oligomeric and polymeric molecules (e.g. gallotanins to gallic acid, lacmus to orceines) can be observed. The amount of acid usually employed in the extraction medium is also enough to catalyse the oxidation of homoisoflavonoids (e.g. haematein to haematoxylin). The major transformation remains, nevertheless, the aglyconeization of the heterosides. Decomplexation with ligands stronger than the dyestuffs has been proposed to improve the recovery of complex dyes by avoiding hydrolysis of heterosides.

In the special case of a non-soluble matrix, as for organic pigments in drying oil layers from paintings, the direct derivatization with *m*-(trifluoromethyl) phenyltrimethylammonium hydroxide (TMTFTH), followed by methylation of paint medium with boron trifluoride/methanol (BF_3/MeOH) is employed. This method provides good recovery of dyestuffs, principally anthraquinoids. Its main inconvenience is the formation of multiple methylated derivatives for some compounds.

Vat dye constituents are very sparingly soluble in aqueous solutions. Some indigoids are soluble in

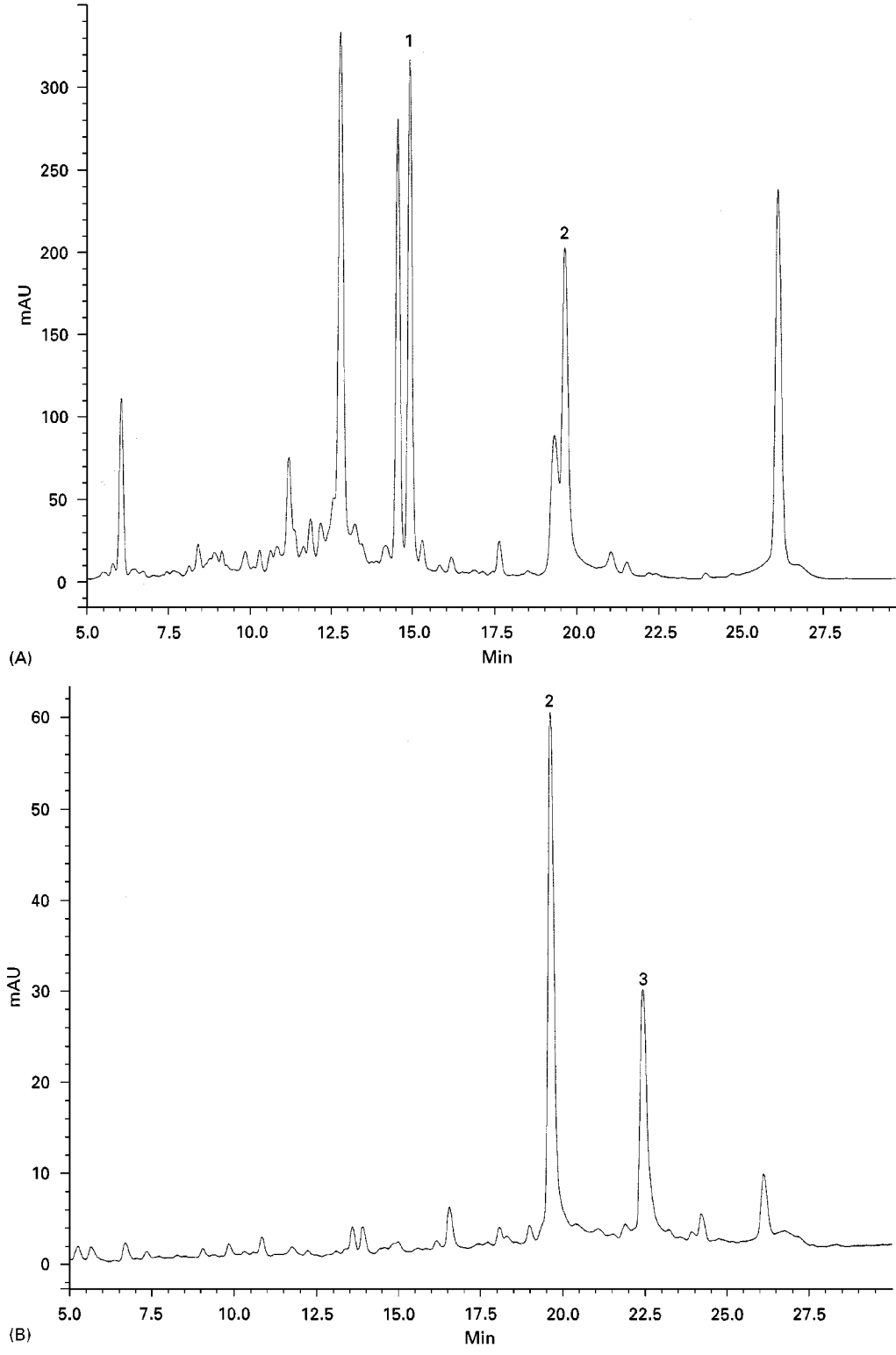


Figure 1 Influence of hydrolysis on composition of dyestuff extracted from root of Madder (*Rubia tinctorium* L.). (A) Chromatogram of fresh aqueous extract; (B) the same extract after acid hydrolysis. Compounds: 1 ruberythric acid (β-2-alizarin primeveroside), 2 alizarin, 3 purpurin.

chloroform, but one of them – 6,6′-dibromoindigotine from so called 'true' or 'Tyrian' purple – is well known as a 'soluble-in-nothing' compound. In fact, for a satisfactory extraction of all compounds from vat dyes, heated pyridine, dimethylformamide (DMF) or dimethyl sulfoxide (DMSO) is necessary. Pyridine

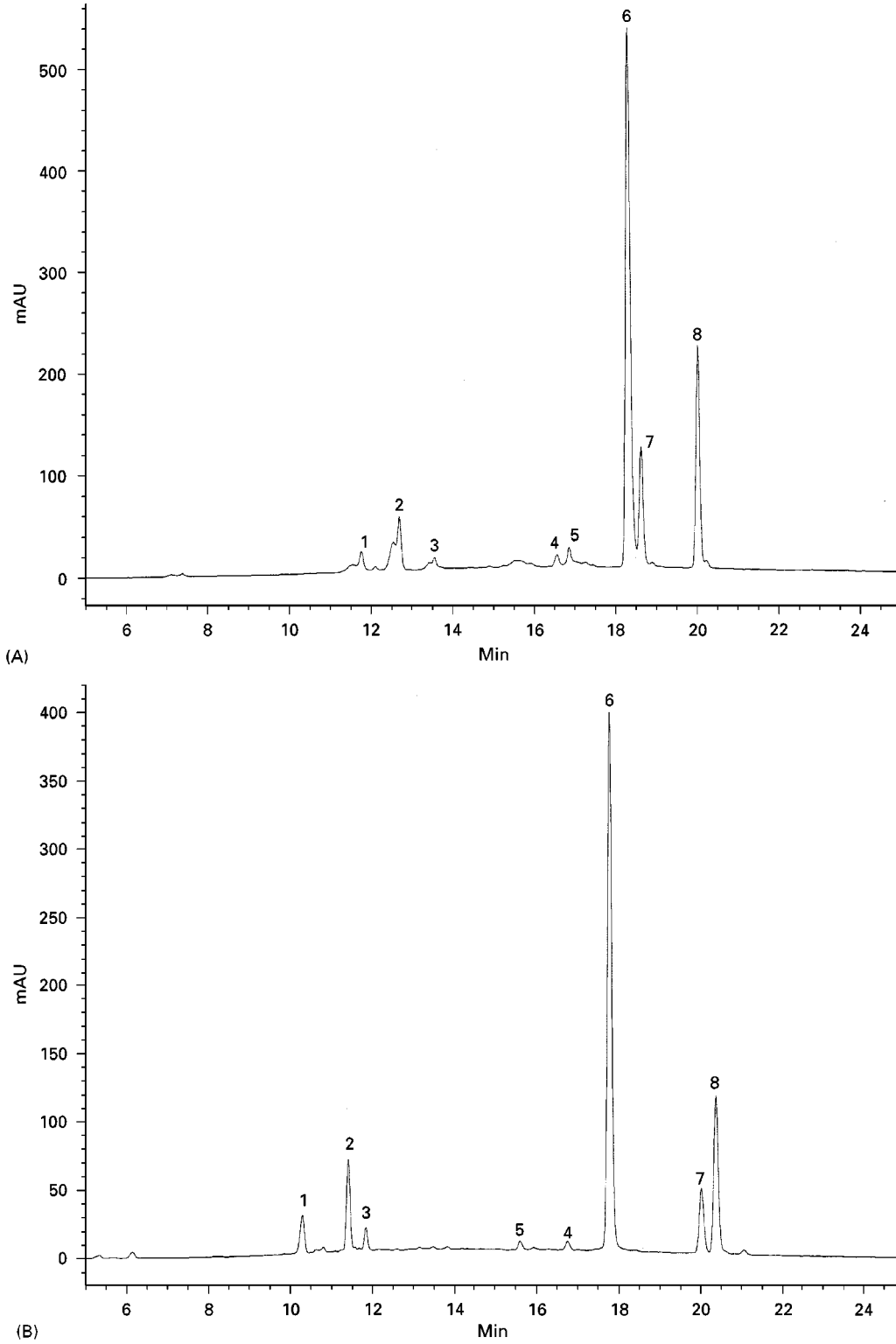


Figure 2 Influence of aqueous mobile phase composition and RP-18 stationary phase brand on separation of dyestuff from Dyers' Broom (*Genista tinctoria* L.). (A) Hypersil BDS, 3 μm, 100 × 4.6 mm; 80% H_2O–10% MeOH–10% of 0.5% H_3PO_4, from 1 to 40 mn gradient MeOH 90%. (B) Hypersil BDS, 3 μm, 100 × 4.6 mm, 85% H_2O–5% MeCN–10% of 1% MSA, from 1 to 40 mn gradient MeCN 50%. (C) Adsorbosphere HS, 3 μm, 100 × 4.6 mm; 85% H_2O–5% MeCN–10% of 1% MSA, from 1 to 40 mn gradient MeCN 50%. Compounds: 1–5 nonidentified, 6 luteolin, 7 genistein, 8 apigenin.

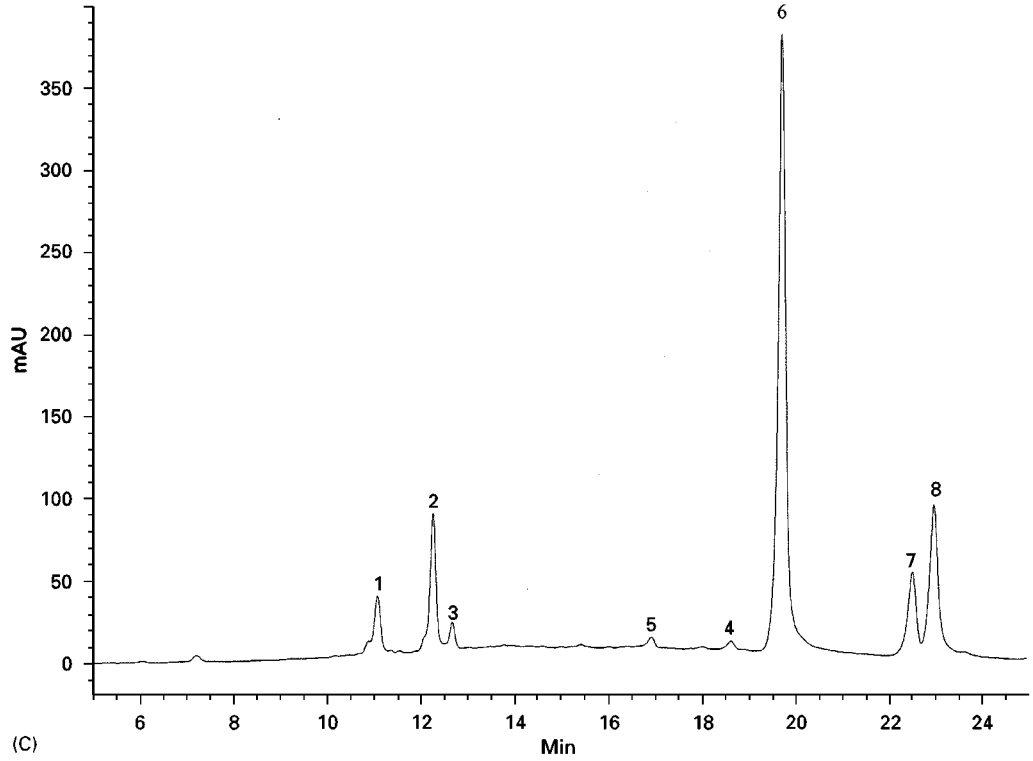


Figure 2 *Continued*

is quite toxic and apparently destroys alkyl-bonded silica stationary phase when extracts are separated. The indigotins in DMF solutions isomerize to related indirubins with the help of ultraviolet light. DMSO is very viscous and displays mixing problems (known as 'viscous fingering') when the aqueous mobile phase is employed and affects stationary phase efficiency (ranging from poor peak shape to multiple peaks). It appears that DMF is the best medium, under conditions of time- and temperature-controlled extraction, even if its viscosity makes large volume injections difficult.

Chromatography

Mobile Phases

The solubility of natural dyestuffs covers practically the entire range of solvent polarities, from water to *n*-hexane. Still, many of them are soluble in both moderately polar organic solvents (e.g. chloroform) and water. These properties are linked to the compounds' structures. In addition to their generic, low polar, aromatic or isoprene structures, they usually contain polar groups, especially proton-releasing ones such as hydroxyl and carboxyl. Practice shows that the use of water with some organic water-miscible solvents solubilizes almost all classes of dyestuffs.

Such behaviour of natural dyes determines the type of chromatographic system used. Apart from the tentative applications of a normal phase (silica) and a nonpolar eluent, reversed-phase conditions are most often applied.

The solvent system is commonly composed of water, an organic modifier and an ion suppressor or counterion.

Many ion suppression reagents are used to control pH. At pH around 2.5 most dyestuffs are unionized but some basic compounds become ionized (e.g. berberine, which is fortunately rather rarely encountered). The most popular acids are *o*-phosphoric (H_3PO_4) and acetic (CH_3COOH, AcH), but in the case of mass spectrometry (MS) on-line detection they should be replaced by volatile trifluoroacetic acid (TFA). To avoid the formation of poorly soluble salts of basic compounds and/or to improve peak shapes, methanesulfonic acid (MSA) has been proposed (**Figure 2**, compare A and B).

The use of counterions is less habitual owing to the considerable equilibrium stabilization time needed for the stationary phase in gradient analysis.

Analysis time in both isocratic and gradient elution is usually between 15 to 45 min, depending on the type and the number of compounds requiring separation.

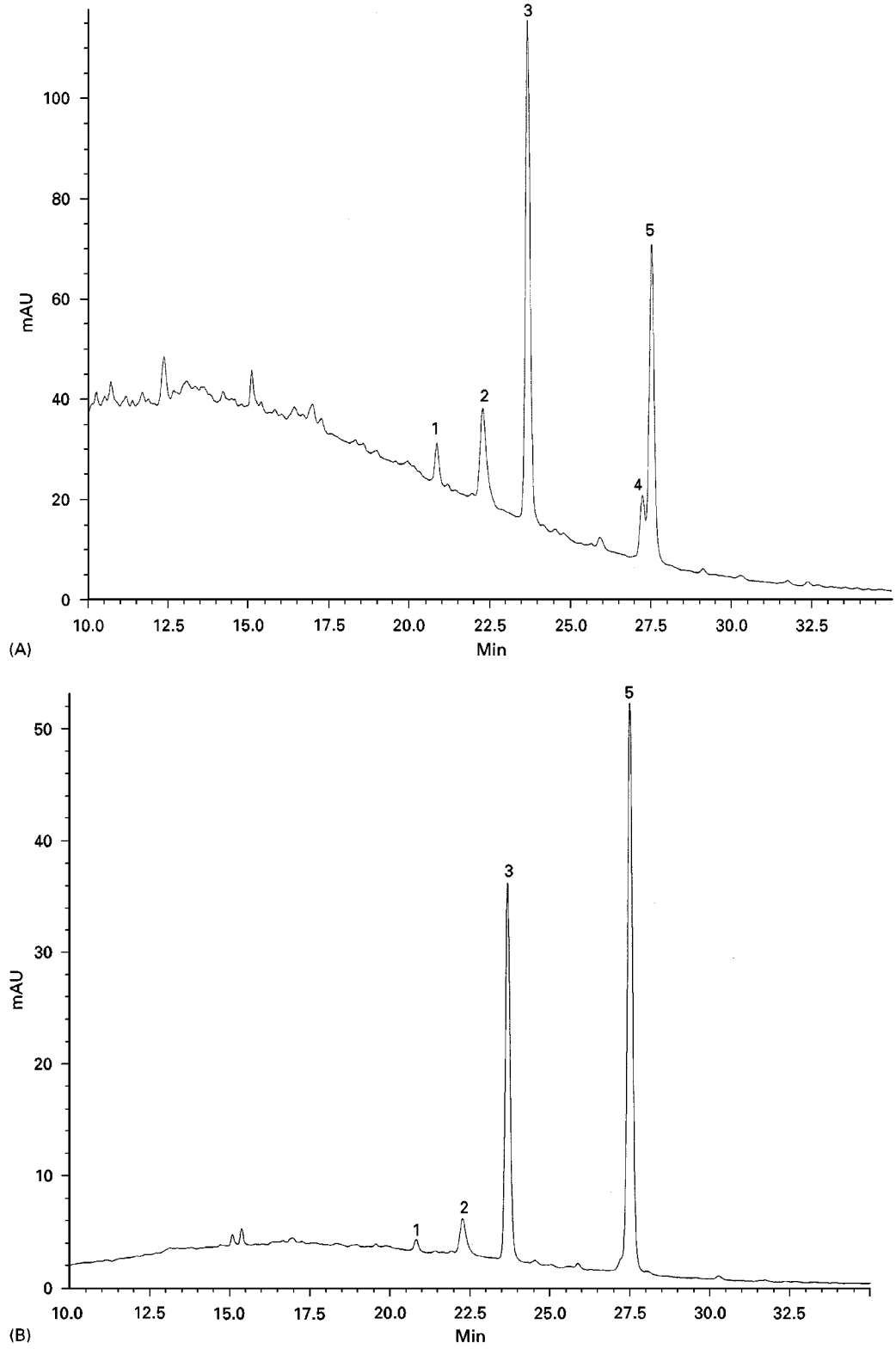


Figure 3 General and selective UV-vis channel detection of violet dyed wool sample (Coptic textile, 6th century). (A) General detection at 285 nm; (B) specific detection of red components corresponding to Madder at 450 nm; (C) specific detection of indigo blue at 615 nm. Compounds: 1 anthragallol, 2 pseudopurpurin, 3 alizarin, 4 indigotin, 5 purpurin.

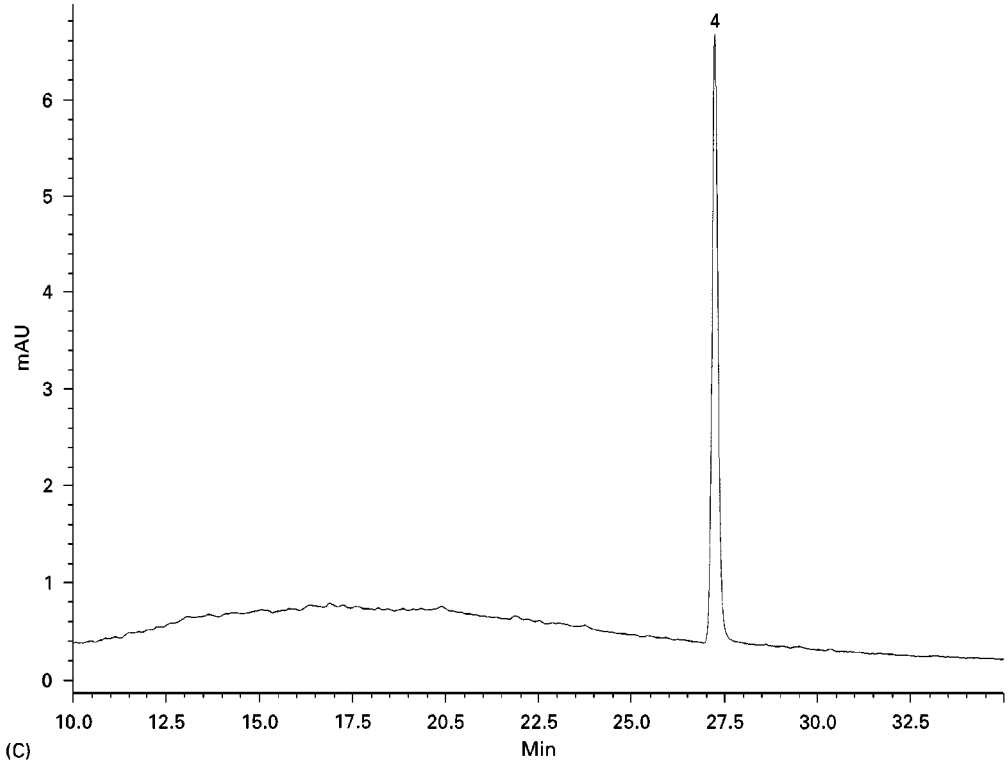


Figure 3 *Continued*

Stationary Phases

Among the stationary phases used in natural dyestuffs analysis, the major part of applications is achieved on octadecyl (called RP 18, or C 18) bonded silica. This type of phase is the most versatile; however, different brands bring about important differences in separation characteristics (Figure 2, compare B and C). The octyl (RP 8) and phenyl (Ph) phases have some specific applications and can offer other, sometimes more satisfactory separations than RP 18 in specific cases.

Advances in natural dyes chromatography follow general trends: a growing efficiency of separations and reduced separation time as well as increased sensitivity of detection. This is the reason for the constant testing of the types of stationary phases, and of the rising use of smaller and more uniform particles and also of shorter and narrower columns.

Detection and Identification

Detectors

Dyestuff components show strong selective absorption in the UV-vis spectrum. This effect is responsible for their coloration and makes absorption detection simple. In the case of single or multichannel detectors, the recognition of compounds is based on retention time and, if possible, on absorption at characteristic wavelengths, usually in the visible (e.g. 360 nm for yellows, 485 nm for reds, 650 nm for blues, etc., with a suitably large bandwidth) (**Figure 3**). The photodiode array (PDA) gives the possibility of confirmation of compound identity by the corresponding peak spectra (**Figure 4**).

The principal inconvenience of UV-vis detection is the recognition of only those substances entered as references in the database. Even the PDA gives only partial structure recognition because of limited UV-vis information contained in the spectra and their possible alteration in different mobile phase compositions. New compounds have to be collected at the detector exit and analysed by other, usually spectrometric, methods. This problem can be avoided by application of on-line mass spectrometry, giving more precise information about the molecular structure. An interesting approach to detection can be in-line tandem of PDA and a mass-selective detector (MSD) permitting pre-selection of coloured compounds and thus their detailed analysis.

Sources Recognition Criteria

High-performance liquid chromatography analysis of natural dyes gives an identification of compounds. From this information, as a first step, a chemical class or dyeing group can be deduced. Moreover, the

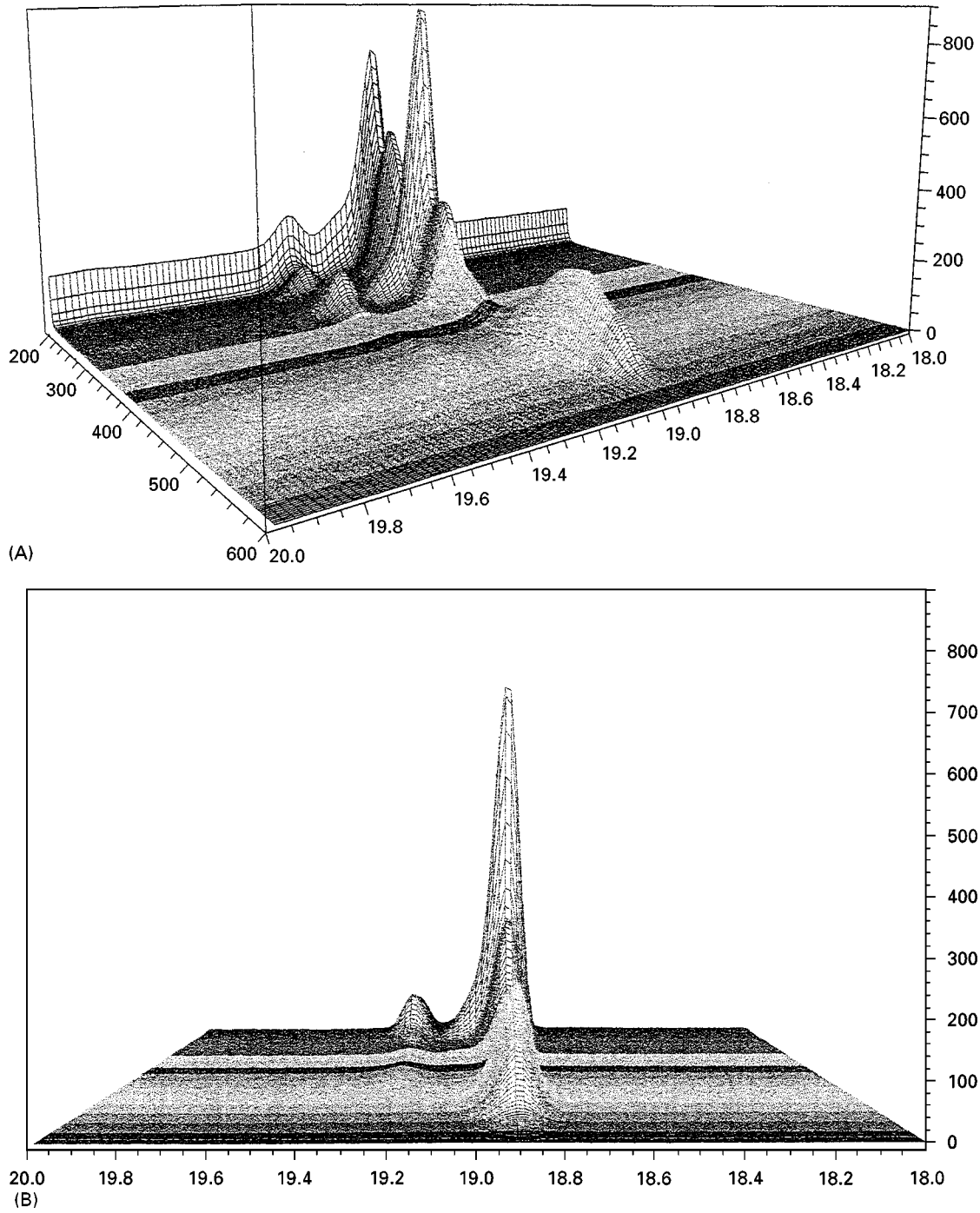


Figure 4 Representation of a fragment of data collected from PAD during the analysis of extract from females of Kermes (*Kermes vermilio* PLANCH). Kermesic acid (higher absorption and flavokermesic acid (lower absorption). (A) General view (axes: x = time, y = wavelength, z = absorption); (B) Chromatograms cut display (x, z). (C) Spectra cut display (y, z).

qualitative and quantitative information about the compounds detected in a chromatogram can help in dyestuff source recognition. This is the aim of chemotaxonomy, employed for many purposes: in biology, in the history of civilizations, in forensic sciences, etc. In general, analysis of colorants from plant and animal material give a few major and a lot of minor peaks on chromatograms recorded at the colour-characteristic wavelengths. The corresponding characteristic compounds are adequate for the identification of botanical or zoological phylem, family, subfamily, group or genus, but can also be common for several, quite different species (**Figure 5**). Species identification is based on the relative

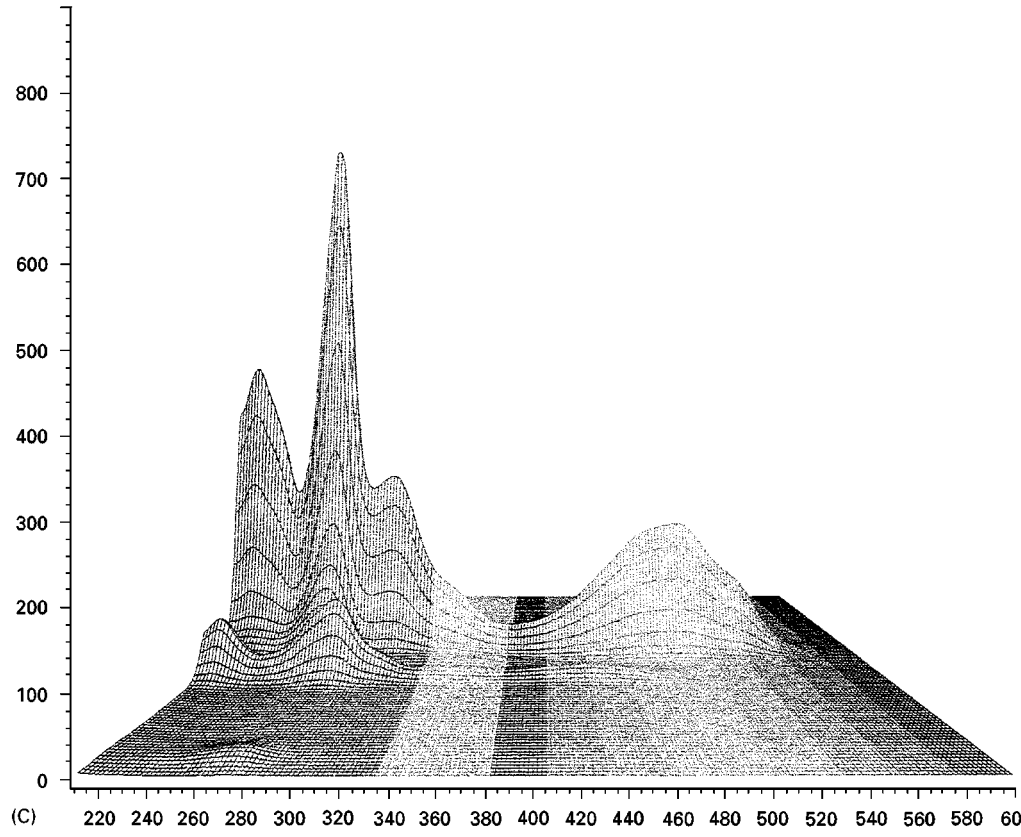


Figure 4 *Continued*

quantity and identity of marker compounds. Their presence or absence is characteristic for only one species. Given the complexity of colorants originating from natural sources, as is the case for other natural substances, sometimes it should be satisfactory to obtain and to compare just their fingerprints.

The chromatographic fingerprints appearance depends on sample preparation, separation conditions and detection principles. That is why the constitution of one's own database containing the results of analysis of reference samples according to established, invariable and repeatable procedure is required.

Extra-Analysis Factors Affecting Dyes Composition

The analytical results obtained from real, coloured objects sometimes show important differences in dye composition in comparison with reference samples from one's own database or published data. These differences limit a clear interpretation and source identification. Such a situation is a result of one or more factors, of which the most important are quantity of sample, method of dyestuff preparation and ageing of the dye.

The quantity of coloured sample necessary for analysis depends on the total contents of the dyestuff in the sample. In the first approach this can be evaluated as a function of colour intensity. Of course, precise evaluation is more complex and should consider the substrate (or matrix) material properties such as surface-to-mass ratio, parameters of penetration of dyestuff in mass of matrix and others. Substrate physicochemical behaviour can influence the sample preparation method prior to analysis and extraction efficiency. Preparatory treatment may modify dyestuff component proportions.

By diminishing sample size, the analytical information about dyestuff composition is progressively lost, starting with 'disappearance' from chromatograms of trace substances and continuing through the vague appearance of minor components until the identification of principal compounds is finally uncertain. As a result source recognition precision declines and becomes more difficult to perform. Still, this effect does not affect the relative quantity of dyestuff components. The lower limit of detection depends on performances of the chromatographic system and detector used.

Another modifying factor, dyestuff preparation, affects many stages, from raw material extraction to deposition on substrate. For each step, a multitude of recipes were invented using diverse plant parts and

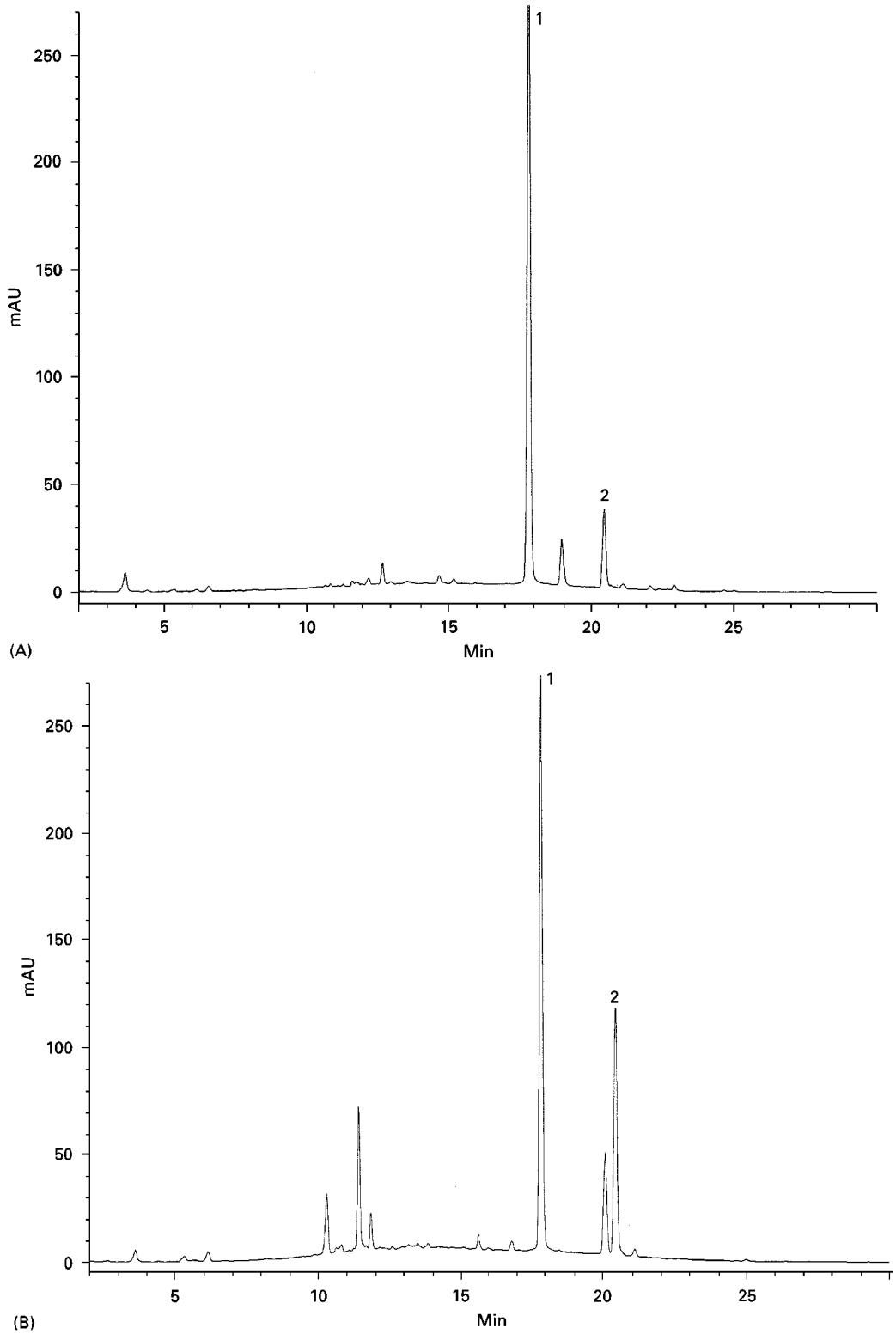


Figure 5 Some reference samples dyed with Eurpoean plant extracts containing both luteolin (1) and apigenin (2) (detection at 345 nm). (A) Saw-wort (*Serratula tinctoria* L.); (B) Dyer's Broom (*Genista tinctoria* L.); (C) Weld (*Reseda luteola* L.); (D) *Daphne gnidium* L.

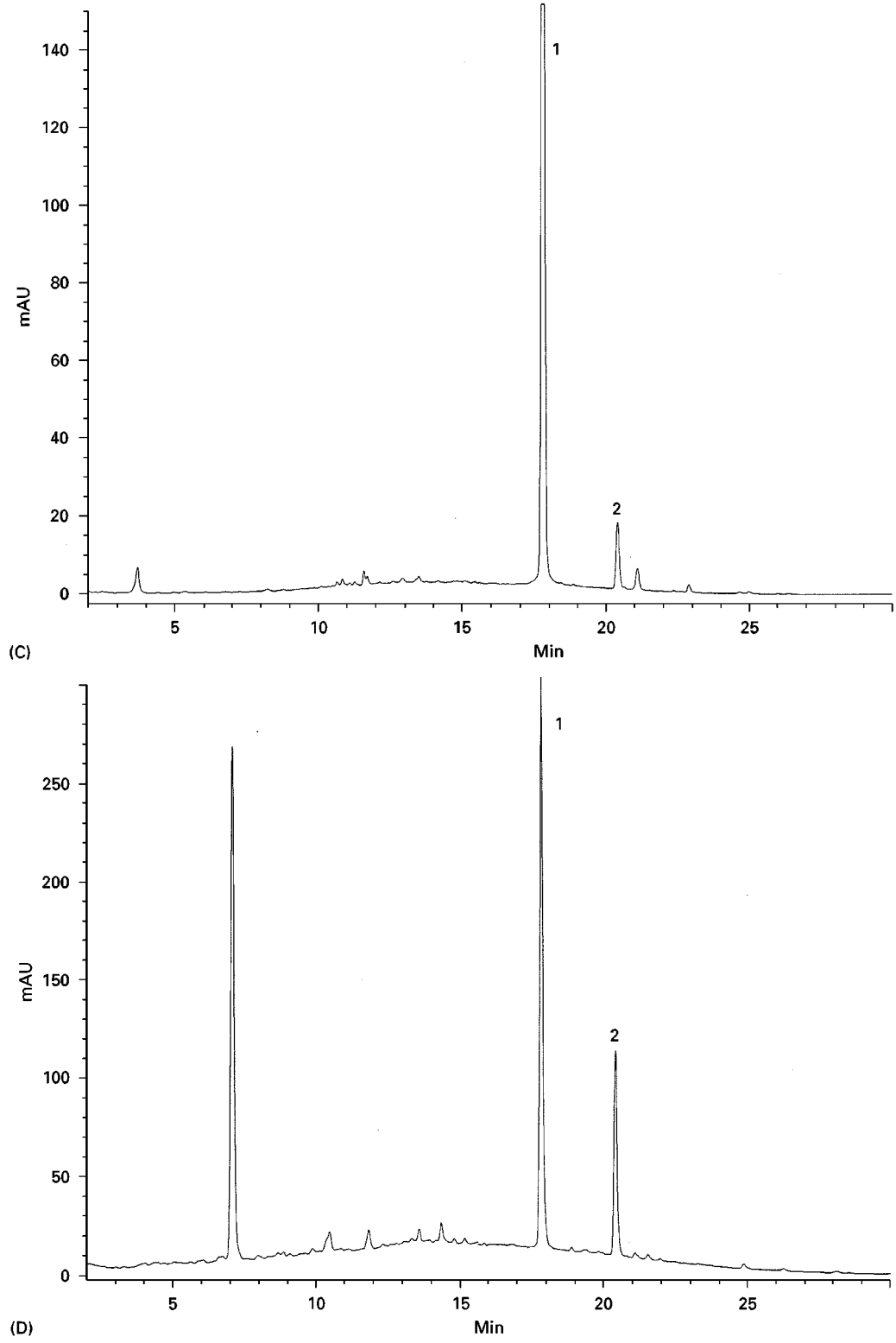


Figure 5 *Continued*

varying operation procedures, as well as the addition of different organic and mineral substances to the dyestuffs extracts and dyebaths (**Figure 6**). Lack of recent systematic study of the influence of different operations and additives on dye composition restricts interpretation.

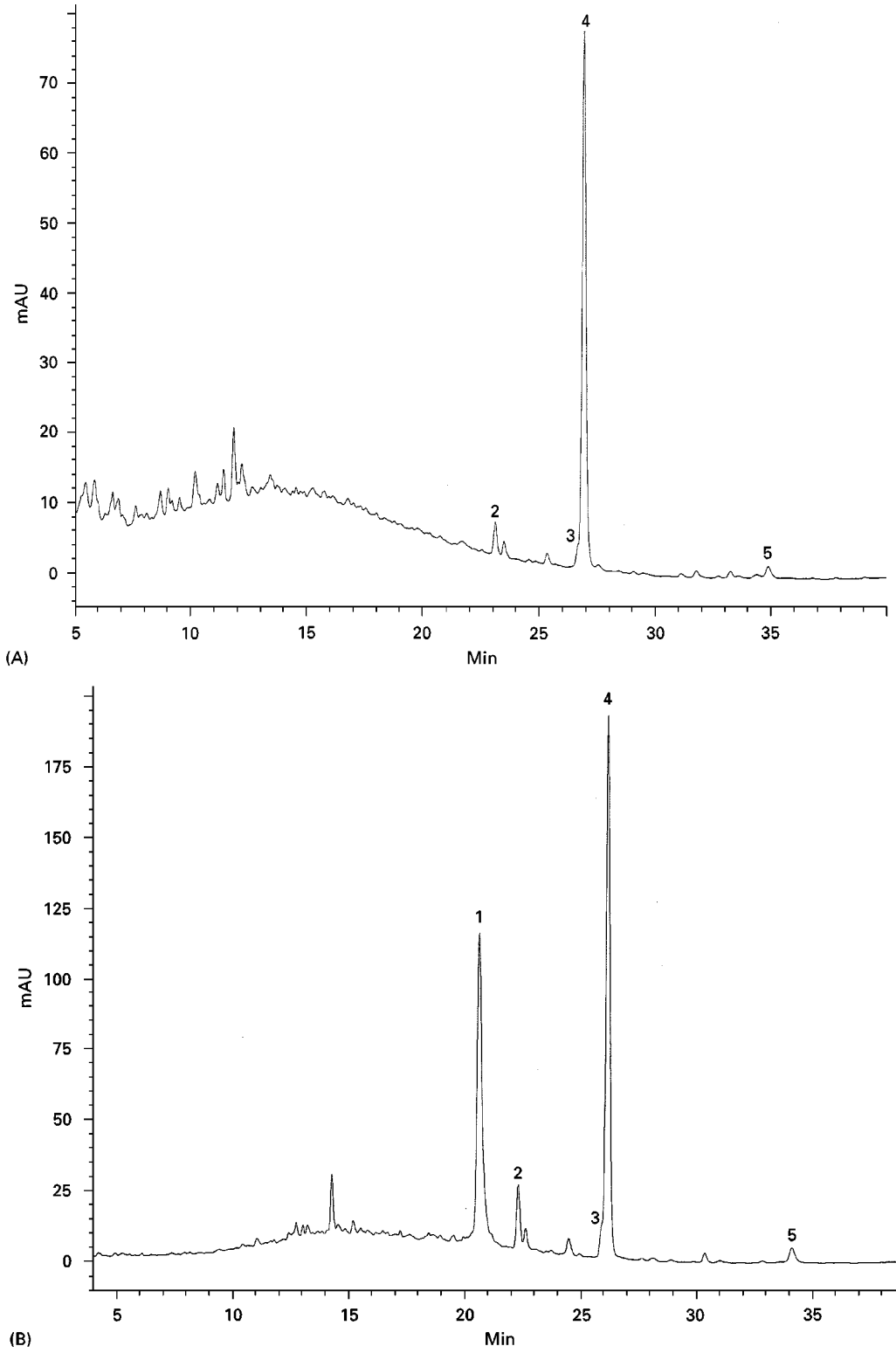


Figure 6 Four contemporary reference samples of wool dyed with Madder (*Rubia tinctorium* L.) obtained from different dyers (detection at 254 nm). Compounds: 1 pseudopurpurin, 2 alizarin, 3 xanthopurpurin, 4 purpurin, 5 unidentified anthraquinone.

A third possible dyestuff composition change determinant, which is very important in historical samples, is ageing (**Figure 7**). The generic name 'ageing' enfolds the conjugated impacts of many physical and chemical factors. Light is considered as the most important of them in dyestuffs degradation. In

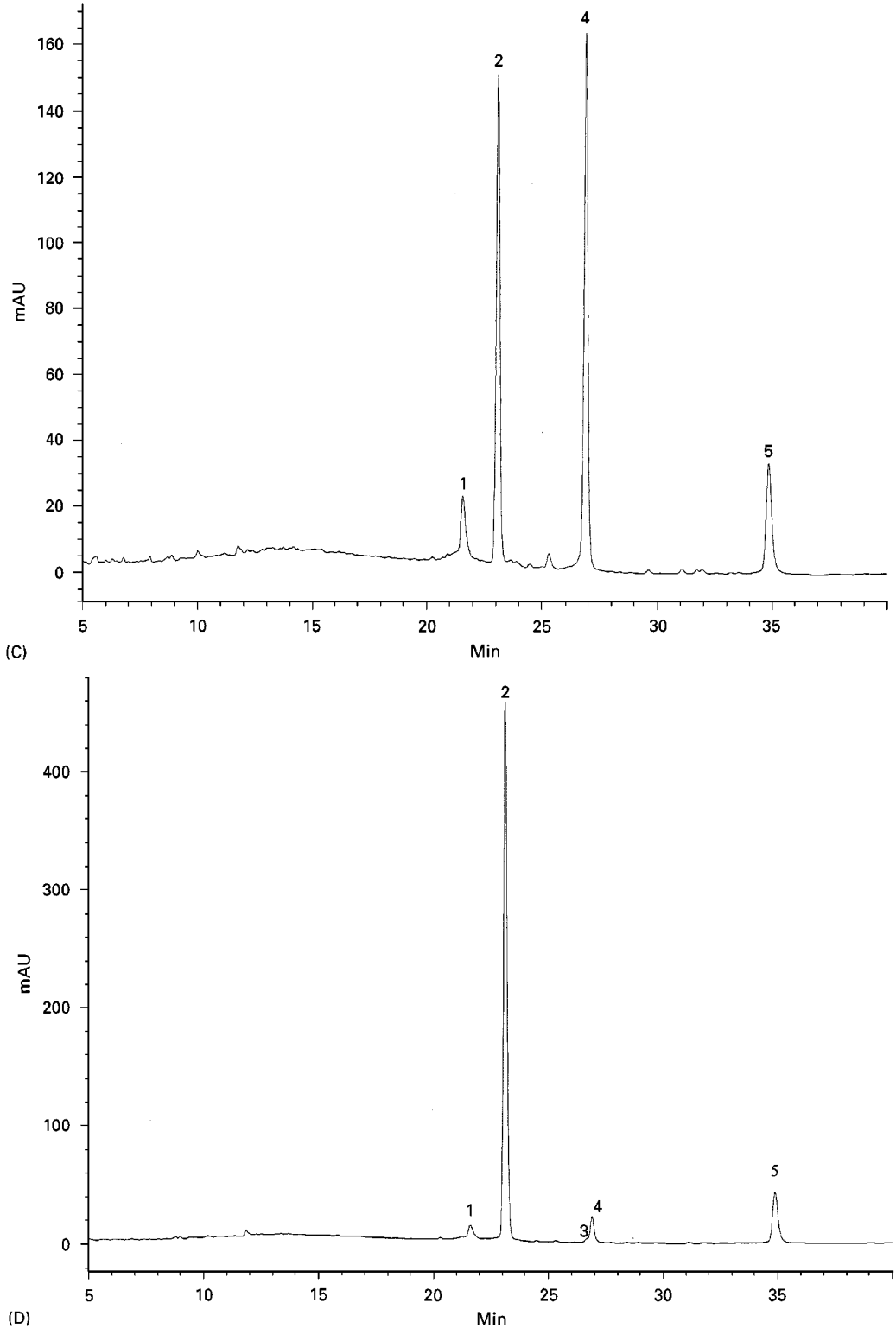


Figure 6 *Continued*

comparison with other colouring matter (e.g. mineral pigments), the stability of dyes' molecular structures under visible light, and especially ultraviolet excitation is poor. Other important factors are water (even as air humidity), air constituents (e.g. ozone), air pollutants (above all sulfur and nitrogen oxides) and,

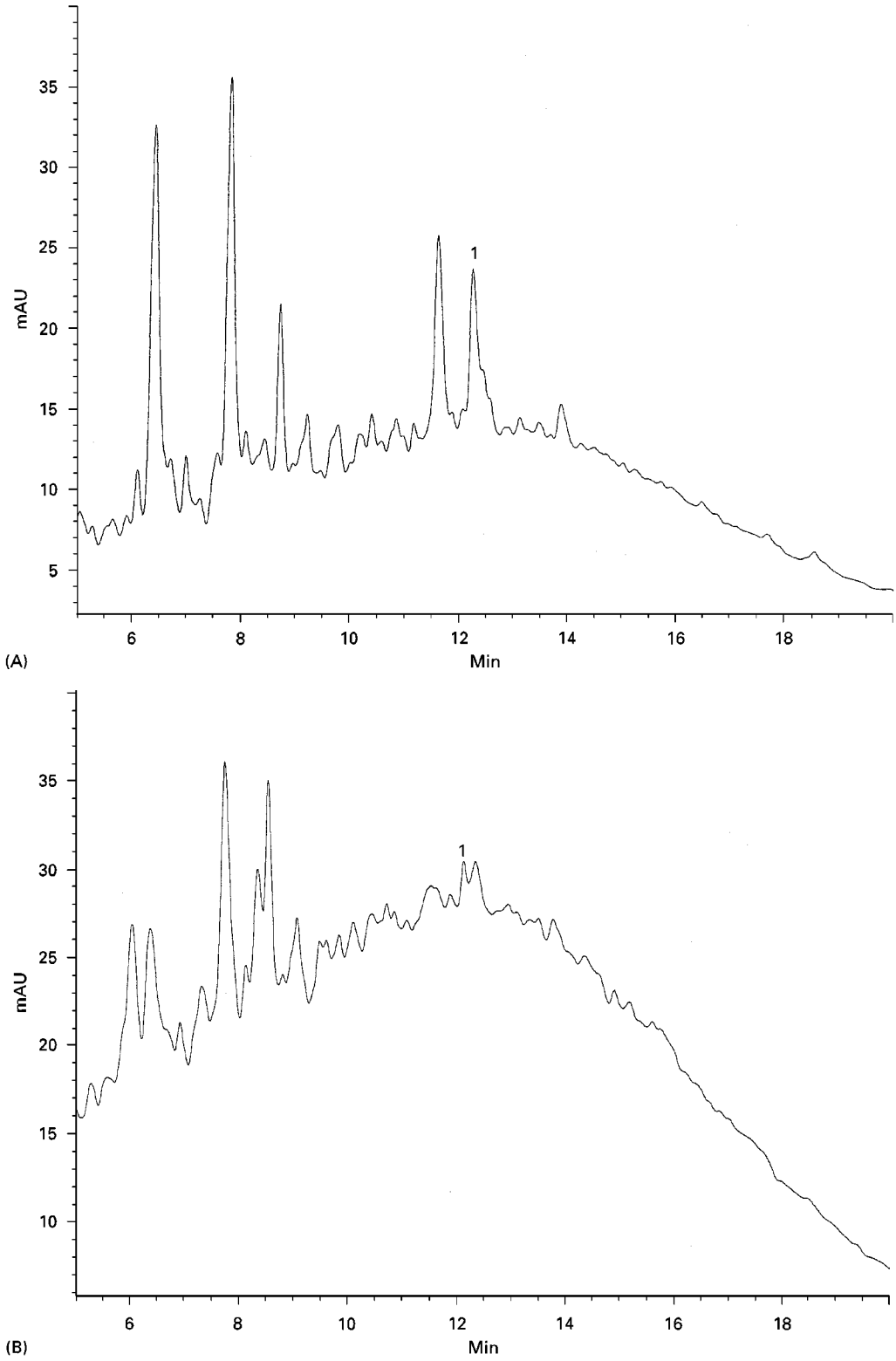


Figure 7 Accelerated ageing of dyestuff extracted from Brazil (*Caesalpinia sappan* L.) (detection at 285 nm) (A) Freshly dyed wool; (B) Sample after standardized accelerated ageing. Compound 1: brazilein derivative formed during acid hydrolysis.

for archaeological excavated objects, mineral salts solutions giving dangerous pH values. Each of the ageing elements presented represents just a part of the global process. Determination of their particular roles is possible but does not allow exact modelling or reproduction because of probable synergy between

them. In any case, the methods of 'artificial' ageing (also called 'accelerated' ageing) give a good approximation, sufficient for comparative colour fastness testing.

For textile fabrics it is important to mention the influence of washing and solvent cleaning on the presence of dyestuffs. Their resistance to wet cleaning methods (wash-fastness) is principally related to the dyeing group and washing conditions. Some dyestuffs or their components are sensitive to washing. Such textile handling also belongs to 'ageing', but the adjective 'natural' is in this case probably less adequate.

During ageing two effects are generally observed: hue change and fading. Change of hue is a result of partial dyestuff fading, because of different degradation rates of dye components. Chromatograms obtained from such samples show qualitative and quantitative differences from the original dyestuff. Real sample composition can be ambiguous and similar to several sources, especially if the markers are absent: *in extremis* it can be so far from any known reference that the question of a 'new', not yet identified source can appear. This kind of hypothesis always merits scrupulous verification.

Fading usually signifies transformation of coloured compounds into colourless products. Recent developments in HPLC-MS provides a way to identify original dyestuffs in discoloured samples from their degradation products, even in small amounts. This approach corresponds to expectations of specialists in various domains and will certainly be developed in the future.

Conclusion

In spite of many historic and recent scientific works concerning the chemistry and analysis of natural dyestuffs, our knowledge of this subject still seems to be far from exhaustive. The contribution of HPLC in advances of chemotaxonomy of dyestuffs of plant and animal origin has attracted growing interest in recent years. This technique is relatively easy to perform and does not need any complex sample preparation. Its performances are sufficient for good separation of many dyestuff components in a reasonable time. The HPLC technique provides the possibility of both qualitative and quantitative analysis of separated compounds by employing detectors considered as standard in liquid chromatography (UV, UV-Vis, PDA) or recently adapted, powerful techniques of detection (MSD).

Problems still remaining are directly linked to the biosynthesis of natural dyes (possible pathway variation) and to their properties (stability, transformation and degradation). They are the principal phenomena affecting chromatogram interpretation. Many of the compounds separated have not yet been identified. Their identification will thus certainly be the objective of future works.

See also: **II/Chromatography: Liquid:** Detectors: Ultraviolet and Visible Detection. **Extraction:** Solvent Based Separation.

Further Reading

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