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Optimisation of ESI-MS detection for the HPLC of anthraquinone dyes

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Abstract

HPLC analysis of dyes from ancient objects is often difficult because of the small quantity of colorant extracted; their identification, usually based on DAD, is limited to commercially available compounds. It was shown that the structural characterisation of unknown compounds could be achieved using HPLC–MS and could be finely tuned to enable both detection and characterisation. Optimisation was carried out using source parameter step-adjustment, mobile phase composition and post-column additive testing; dye characterisation trial was carried out using MS^3 pattern study. The results clearly show advantages in comparison to DAD namely improved molecular identification and better selectivity. Unfortunately, as several compounds had the same molecular mass and MS^3 fragmentation, their retention parameters were very useful for adequate structure recognition. The optimised conditions were successfully applied to the identification of components from a small sample of wool thread dyed with madder (*Rubia tinctorum*) and Our Lady's bedstraw (*Galium verum*).
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Keywords: Anthraquinone; Natural dyestuff; HPLC–DAD; ESI-MS; *Rubia tinctorum*; *Galium verum*

1. Introduction

The analysis of dyes in ancient artefacts is an integral part of the scientific investigation of ancient works of art that is essential as it enables us to improve our knowledge of the raw materials and their use down the centuries. The dyes used up to the 19th century were either of vegetable (weld, madder, indigo, etc.) or animal origin (cochineal, shellfish, etc.) and belonged to various chemical types, such as flavonoids (yellow), anthraquinones (red) and indigoids (blue and violet) [1]. Of these chemical types, anthraquinoid dyes provide the most important red dyes and lakes used in artistic paintings. Whilst these molecules, because of their great stability, are very often encountered in even extremely ancient artefacts, the possibility of characterising many of them is very difficult as few of them are available as standards and the quantities obtained from, often tiny samples do not enable their isolation in sufficient quantity for spectrometric analyses (particularly NMR). The analysis of dyes is generally carried out via the combined use of separative and spectral methods. Separation is often carried out using reversed-phase liquid chromatography (RPLC) [1–11] and, for detection, UV–visible spectroscopy has historically been the most frequently used method [1–3,5–7,9,10,12]. Dyes are thus detectable, but their absolute identification very often remains difficult in the absence of standards analysed under similar conditions; indeed, the UV–visible spectra of compounds with related structures are relatively similar and can suggest that they belong to the same family of dye (flavones, flavanones, anthraquinone, etc.). Numerous compounds are still unreferenced and very few pure standards are commercially available for comparison. Detection threshold is also a problem, as samples originating from archaeological and historical objects are often tiny and the dyes have very often deteriorated [13]. Of contemporary

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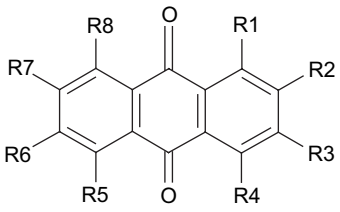
Table 1
The structures and origins of considered compounds



Compound (and it's abbreviation)	Origin	R1	R2	R3	R4	R5	R6	R7	R8
Tectoquinone (Tec)	*Tecoma ipé*	−H	$-CH_3$	−H	−H	−H	−H	−H	−H
Alizarin (Ali)	*Rubia* spp., *Galium* spp., *Oldenlandia umbellata*, *Asperula tinctoria*, *Asperula ciliata*, *Crucianella maritima*, *Hedyotis auricularia*, *Morinda umbellata*, *Morinda citrifolia*	−OH	−OH	−H	−H	−H	−H	−H	−H
Hystazarin (Hys)	Synthetic	−H	−OH	−OH	−H	−H	−H	−H	−H
Quinizarin (Qza)	*Rubia tinctorum*	−OH	−H	−H	−OH	−H	−H	−H	−H
Xanthopurpurin (purpuroxanthin) (Xpu)	*Galium* spp., *Rubia tinctorum*, *Rubia cordifolia*, *Rubia sikkimensis*, *Morinda umbellate*	−OH	−H	−OH	−H	−H	−H	−H	−H
3-MeO-hystazarin (Moh)	*Oldenlandia umbellate*	−H	−OH	$-OCH_3$	−H	−H	−H	−H	−H
Chrysophanol (Chr)	*Rheum* spp., *Rumex* spp., *Rhamnus* spp., *Cassia* spp., etc.	−OH	−H	$-CH_3$	−H	−H	−H	−H	−OH
Anthragallol (Agl)	*Rubia tinctorum*, *Coprosma lucida*, *Hymenodictyon excelsum*	−OH	−OH	−OH	−H	−H	−H	−H	−H
Purpurin (Pur)	*Galium* spp., *Rubia tinctorum*, *Rubia cordifolia*, *Rubia cordifolia* var. *munjista*, *Rubia sikkimensis*, *Rubia tetragona*, *Relbunium hypocarpium*	−OH	−OH	−H	−OH	−H	−H	−H	−H
Aloe-emodin (Ale)	*Aloe* spp., *Rheum* spp., *Rhamnus* spp., *Cassia* spp.	−OH	−H	$-CH_2OH$	−H	−H	−H	−H	−OH
Emodin (Emo)	*Polygonum* spp., *Rumex* spp., *Rheum* spp., *Rhamnus* spp., *Cassia* spp., *Dermocybe semisanguinea*	−OH	−H	−OH	−H	−H	$-CH_3$	−H	−OH
Quinalizarin (Qlz)	Synthetic	−OH	−OH	−H	−H	−OH	−H	−H	−OH
Flavokermesic acid (laccaic acid D) (Flk)	*Porphyrophora* spp., *Dactylopius coccus*, *Kermes vermilio*	$-CH_3$	−COOH	−OH	−H	−OH	−OH	−H	−OH
Kermesic acid (Ker)	*Porphyrophora* spp., *Dactylopius coccus*, *Kermes vermilio*	$-CH_3$	−COOH	−OH	−H	−OH	−OH	−H	−OH
Frangulin (Fra)	*Rhamnus catharticus*	−OH	−H	−*O*-Rhamnose	−H	−H	−OH	−H	−OH
Carminic acid (Car)	*Porphyrophora* spp., *Dactylopius coccus*	−OH	−Glucose	−OH	−OH	−H	−OH	−COOH	$-CH_3$
Ruberythric acid (Rba)	*Rubia tinctorum*, *Rubia akane*, *Oldenlandia umbellata*	−OH	−H	−*O*-Primeverose	−H	−H	−H	−H	−H
Laccaic acids (LaA, LaB, LaC, LaE)	*Kerria lacca*	−OH	See Table 2	−OH	−OH	−H	−COOH	−COOH	−COOH

detection methods that have been developed over the last few years, whilst fluorescence spectroscopy has been used for some coloured compounds [9], mass spectrometry (MS) is the most important [2,4,6–10,14,15] although very few systematic studies using MS as detection method have been carried out on anthraquinoid dyes.

This work comprises a detailed study of anthraquinoid dyes using high performance liquid chromatography/mass spectrometry (HPLC–MS and HPLC–MS–MS) using standards obtained directly/indirectly from various chemical suppliers as well as purified raw extracts and those containing acids (kermesic acid, flavokermesic acid, carminic acid, laccaic acid and ruberythric acid). The purpose of this study was to apply a developed analytical protocol to samples of dyed fibres and to samples from ancient objects so as to identify the origin of the natural dye used.

2. Experimental

2.1. Chemicals and samples

In total 18 compounds (Tables 1 [16] and 2) were used, four of which were commercial, namely alizarin, purpurin, emodin and quinalizarin (Acros Organics, Noisy-le-Grand, France). The remaining compounds, of varying purity, were obtained from the Helmut Schweppe collection (BASF Laboratory, Ludwigshafen, Germany). The laccaic acid standard was an extract from the *Kerria lacca* insect (Table 2). The 18 standard compounds were dissolved in a MeOH/H_2O 50/50 blend at a concentration of ~12 ppm.

Wool reference samples dyed with madder (*Rubia tinctorum*) and Our Lady's bedstraw (*Galium verum*) were obtained from D. Cardon at the University of Lyons 2 CNRS, France. The samples were prepared as follows: for each dyeing, about 10 g of wool in thread was preliminarily mordanted using 1.5 g of potassium-aluminium sulphate (alum) in hot deionised water. After cooling and rinsing in tap water, the samples were dyed with the hot water extract obtained with 10 g of dried and chopped roots of the respective plants. As the samples were prepared some 10 years ago, detailed information of the precise dyeing method used is unavailable.

The extraction of the dye was carried out using 1 mg of dyed thread in 200 μL of conc. HCl/MeOH/H_2O 2/1/1, which was vacuum dried and then solubilised in 50 μL MeOH/H_2O 1/1 mix and filtered [17].

Methanol (MeOH, HPLC gradient grade), acetonitrile (MeCN, HPLC gradient grade), isopropanol (^{i}PrOH) and formic acid (HCOOH for the analysis) were obtained from Carlo Erba (Milan, Italy) and supplied by SDS. Water for chromatography, acetone (CH_3COCH_3 for the HPLC), acetic acid (CH_3COOH for the analysis) and hydrochloric acid (37% fuming HCl) were supplied by Merck (Darmstadt, Germany).

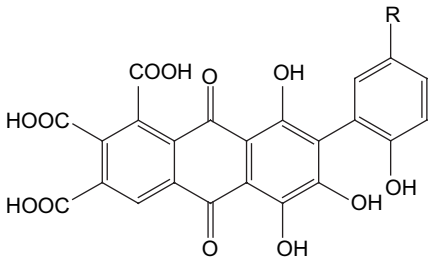
2.2. Instruments and methods

For the separations, a Hypersil BDS C_{18}, 100 mm × 2.1 mm, 3 μm column equipped with a 10 mm × 2.1 mm pre-column with an identical filling up purchased from Thermo (Runcorn, UK) was used at room temperature. The mobile phase used comprised (A) H_2O (for chromatography) and (B) MeCN, with acetic or formic acid at 0.05–0.15% being present in A. A linear gradient was used: 0–1 min 5% B, a 1–41 min linear gradient up to 60% B, 41–50 min 60% B; a flow rate of 0.3 mL/min was used.

The HPLC–DAD analyses were carried out with a quaternary pump and a Surveyor Diode Array Detector from Thermo (200–600 nm). The LC–MS system was a TSP Spectra System that included a SN4000 controller, P1000XR quaternary pump, SCM1000 degasser and an ion trap mass spectrometer (LCQ) equipped with an ESI (electrospray ionisation) source (Thermo, Runcorn, UK). The data were acquired using a Finnigan Xcalibur acquisition system.

The ESI source was used in negative mode with the following parameters: sheath gas flow rate: 80 arbitrary units (approximately 1.2 L/min), auxiliary gas flow rate: 45 arbitrary units (approximately 13.5 L/min), spray voltage: 4.5 kV. The parameters shown in Table 3 were applied to the interface between the source and the analyser. For the post-column additions, a P1000XR (Thermo) quaternary pump was used and connected between the end of the column and the ESI source

Table 2
The structures of laccaic acids (from *Kerria lacca* insect)



Type (and abbreviation)	R
A (LaA)	$-CH_2CH_2NHCOCH_3$
B (LaB)	$-CH_2CH_2OH$
C (LaC)	$-CH_2CH(NH_2)COOH$
D (Flk)	See flavokermesic acid (Table 1)
E (LaE)	$-CH_2CH_2NH_2$

Table 3
Experimental conditions used for optimisation of MS detector signal

Conditions	Step 1	Step 2	Step 3
Fixed parameters	Capillary voltage and tube lens offset	Tube lens offset and optimal spray voltage (from step 1)	Optimal spray voltage and capillary parameters (from steps 1 and 2)
Optimised parameters	Spray voltage	Capillary voltage	Tube lens offset
Range	2–5 kV	0–30 V	−10–15 V
Increments	0.5 kV	5 V	5 V

Table 4
Molar mass, pseudo-molecular ions and fragmentation of studied compounds

Compound	Abbreviation	M (g/mol)	$[M-H]^-$ (amu)	MS^n (amu)
Tectoquinone	Tec	222	Not detected	–
Alizarin	Ali	240	239	239 → 211 (−28) → 183 (−28)
Hystazarin	Hys	240	239	239 → 211 (−28) → 167 (−44)
Quinizarin	Qza	240	239	239 → 211 (−28) → 167 (−44)
Xanthopurpurin (purpuroxanthin)	Xpu	240	239	239 → 211 (−28) → 167 (−44)
3-MeO-hystazarin	Moh	254	253	253 → 238 (−15) → 210 (−28)
Chrysophanol	Chr	254	253	253 → 225 (−28) and 238 (−15) → 210 (−28)
Anthragallol	Agl	256	255	255 → 227 (−28) → 183 (−44)
Purpurin	Pur	256	255	255 → 227 (−28) → 171 (−56) and 199 (−28)
Aloe-emodin	Ale	270	269	269 → 240 (−29) → 239 (−1)
Emodin	Emo	270	269	269 → 225 (−44) → 181 (−44) and 210 (−15) → 182 (−28)
Quinalizarin	Qlz	272	271	271 → 243 (−28) → 199 (−44) and 215 (−28)
Flavokermesic acid (laccaic acid D)	Flk	314	313	–
Kermesic acid	Ker	330	329	329 → 285 (−44) → 257 (−28) → 213 (−44) → 185 (−28)
Frangulin	Fra	416	415	415 → 269 (−146) → 241 (−28) and 225 (−44)
Carminic acid	Car	492	491	491 → 447 (−44) → 357 (−90) → 339 (−18) → 321 (−18)
Laccaic acid E	LaE	495	494	494 → 450 (−44)
Laccaic acid B	LaB	496	495	495 → 451 (−44)
Ruberythric acid	Rba	534	533	533 → 239 (−294)
Laccaic acid A	LaA	537	536	536 → 492 (−44)
Laccaic acid C	LaC	539	538	–

entry with a PEEK T. Variable post-column flow ranging from 0.1 to 0.3 mL/min. The optimisation of the parameters specific to the mass spectrometer was carried out using two steps:

- a study using direct introduction (flow injection analysis: FIA) in which the sample is introduced in the presence of the MeCN/H_2O 30/70 (%V/%V) mobile phase + 0.1% acetic acid, without a chromatographic column, directly into the mass spectrometer.
- HPLC–MS using the previously described chromatographic conditions.

3. Results and discussion

The first step of the study consisted in acquiring the mass spectra of the various available standards using FIA mode. For all the standards except tectoquinone, the obtained ion is mainly the pseudo-molecular $[M-H]^-$ ion (Table 4) in accordance with previous research [6]. The ESI source was not adapted to the detection of tectoquinone, the less polar compound of the standards, which produced no signal.

Fragmentation experiments (MS^n) were carried out on the pseudo-molecular ions (Table 4).

Table 5
Choice criteria for a practical anthraquinones classification. The compounds chosen for the optimisation are in bold caracters.

Groups and choice criteria		Name
Group A: 2 hydroxyl groups in 1 and 8 positions (possible 2 hydrogen bonds with the same carbonyl function)		Aloe-emodin **Frangulin** Chrysophanol Emodin
Group B: carboxyl group in 2 position and hydroxyl groups in 3, 5, 6 and 8 positions		Carminic acid **Kermesic acid** Laccaic acids Flavokermesic acid
Group C: only hydroxyl groups in 1, 2, 5 and 8 positions		**Quinalizarin**
Group D: no ionisable group		**Tectoquinone**
Group E: functional groups only on one aromatic ring	*E1*: no hydrogen intramolecular bonds with carbonyl function	Hystazarin **3-MeO-hystazarin**
	E2: at least 2 hydroxyl groups in 1 and 4 positions (hydrogen bonds with each carbonyl function)	**Purpurin** Quinizarin
	E3: 2 or 3 hydroxyl groups but only one in position 1 (1 hydrogen bond with carbonyl function)	**Alizarin** Anthragallol Ruberythric acid Xanthopurpurin

For most of the anthraquinones, a loss of 28 amu was observed, corresponding to a loss of CO. The daughter ions at (M−H−28) amu fragment and display in most cases a peak at *m/e* (M−1−28−44) corresponding to a loss of 44 amu ($-CO_2$) [2,14].

In the case of anthraquinones including an −*O*-glycoside (frangulin, ruberythric acid), the first observed rupture (−146 amu, loss of rhamnose from frangulin, −294 amu loss of primeverose from ruberythric acid) corresponds to the loss of one or more glycoside units. The fragmentations of −*C*-glycosides do not lead to the loss of glycoside (see carminic acid, for example). In the case of anthraquinones with an acid group (COOH), the loss of 44 amu ($-CO_2$) is observed at first (kermesic acid, carminic acid, laccaic acid). These fragmentation experiments first of all lead to suggest a membership group for an unknown anthraquinone. However, if isomeric compounds, (such as $M = 240$ g/mol: xanthopurpurin, quinizarin and hystazarin) are taken into account, the various observed fragmentations (up to MS^3) do not permit a differentiation. The data provided by mass spectrometry alone are therefore not sufficient to identify the structure of an unknown anthraquinone. Further chromatographic information will therefore be necessary to establish structure propositions.

Bearing in mind that the final objective is to identify anthraquinones that are present in minute quantities in the objects, it is important to work in mass spectrometry with optimised parameters to provide maximum responses (maximum *S*/*N* ratio).

The gas flows were set to values close to those provided by the literature for the same equipment [6] and which happened to be those recommended by the manufacturer. The other parameters were varied around the mean values recommended by the manufacturer.

Table 3 describes the step by step development of the optimisation method that was used.

Steps 1−3 were carried out for three capillary temperatures (170, 200 and 250 °C) and provided three sets of optimal conditions (one for each capillary temperature). The results were then compared and an ''absolute'' optimum was obtained for each compound: a capillary temperature, spray voltage, capillary voltage and tube lens offset.

This optimisation study was carried out using direct introduction via FIA: the sample was introduced in the presence of a mobile phase of MeCN/H_2O 30/70 (v/v) + 0.1% of acetic acid, without a chromatographic column, directly into the mass spectrometer. The monitoring was carried out in SIM mode on the pseudo-molecular ion $[M-H]^-$.

Table 6
Optimal conditions

	Capillary temperature (°C)	Spray voltage (kV)	Capillary voltage (V)	Tube lens offset (V)
Derksen [6]	200	4.5	−12	
Our optimisation	200	4.5	−15	−5

Considering that these compounds belong to a similar chemical family and that they are likely to behave in a similar manner, it was sought to reduce the number of solutions on which the optimisation was carried out. It was carried out on six probes representative of a classification of the 18 standards. Because the physico-chemical parameters (pK_a, hydrogen bond donor or acceptor property, hydrocarbonated volume…) of the compounds which would have enabled a classification were unavailable, the regrouping was carried out by studying the various anthraquinone substitutes: their number, their polarity, their position, their capacity or their incapacity to instigate intramolecular hydrogen bonds with quinonic groups.

Table 5 indicates groups, choice criteria and the compounds chosen for the optimisation in bold characters.

This study enabled us to obtain five different sets of optimal conditions for the six studied compounds. In order to verify on one hand the pertinence of the groups and on the other hand to

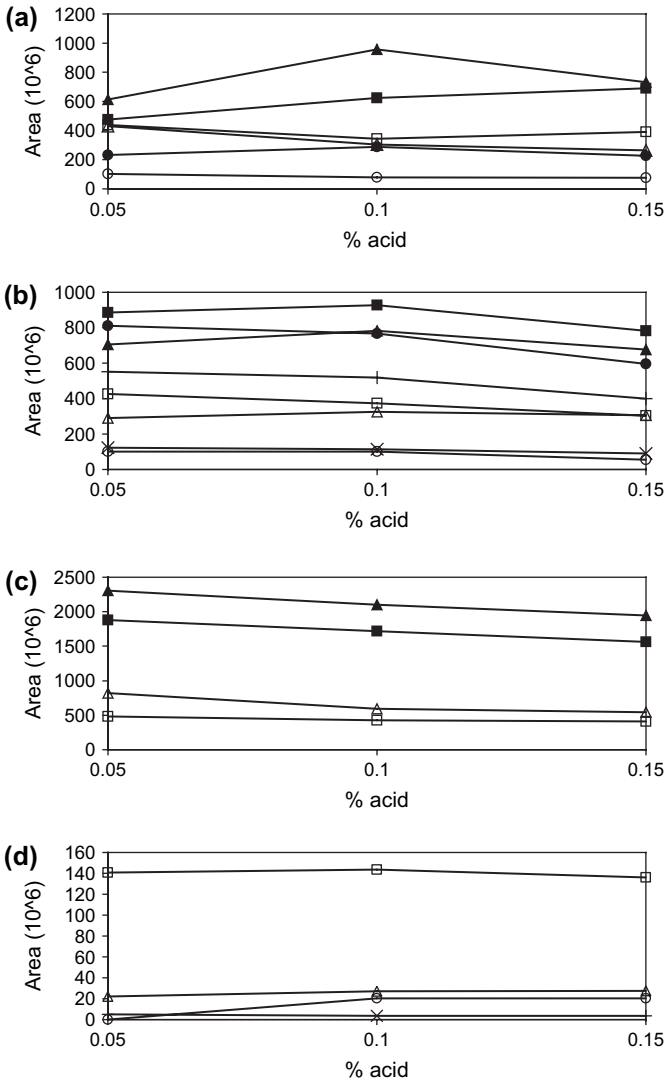


Fig. 1. Compounds' peak areas as a function of acid content in the mobile phase. ''Filled'' symbols and '+': acetic acid; ''empty'' symbols and '×': formic acid. (a) ■ and □: Car; ▲ and △: Ker; ● and ○: Flk; (b) ■ and □: Hys; ▲ and △: Agl; ● and ○: Moh; + and ×: Pur; (c) ■ and □: Xpu; ▲ and △: Emo; (d) □: LaA; △: LaB; ○: LaC; ×: LaE.

select the best of the five optimal conditions, the following step involved the injection of the 18 available standards in the HPLC–MS coupling with each of the previously mentioned optimal conditions.

The resulting mean optimal conditions are provided in Table 6 in which the parameters used by Derksen with the same equipment [6] also appear. The adequacy of the different values shows that the proposed classification and the reduction of the number of compounds to optimise is judicious, except in the case of ruberythric acid which had been placed in group E3 and whose behaviour is akin to that of group B. Spray voltage and tube lens offset are the most influential parameters on the response of the various compounds. The reliability of the developed method was tested. A repeatability study showed a 5% RSD. A time reproducibility study was carried out and provided RSDs ranging from 3 to 8%. The optimum for the various parameters is very similar for all of the compounds including acids (only the tube lens offset changes: +5 V for acids). This validates the use of " mean " conditions (Table 6) for the rest of the study.

In HPLC–MS, other than the optimisation of the parameters specific to the mass spectrometer, it is important to look into the composition of the eluent coming into the source of the mass spectrometer.

Actually, with the nature and the concentration of the studied compound, the composition of the mobile phase, i.e. the percentage of organic solvent, it's nature, the pH and the concentration of eventual additives influence mass spectrometry responses [18]. All this must be in agreement with the requirements brought about by chromatographic separation. In the case of the separation of anthraquinones carried out in columns with C_{18} bonded silica, it is necessary to work with a mobile phase rich in water to obtain appropriate retentions, given the polarity of the compounds. During the use of an ESI source, it is mentioned that the intensity of the signal increases with the proportion of organic solvent coming into the source [4,18].

In order to reconcile the requirements of the separative system (necessity for a mobile phase rich in water) with those of the detection (necessity for a mobile phase poor in water), it was decided to supplement the chromatographic mobile phase with various organic solvents with a post-column addition in order to increase the mass spectrometry response of each compound.

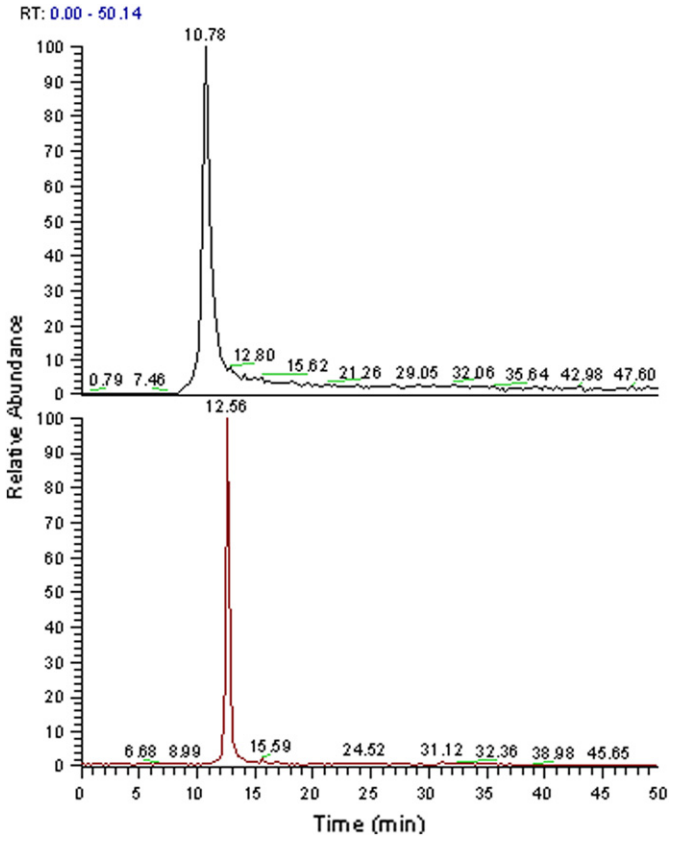


Fig. 2. Chromatogram of carminic acid in presence of 0.1% acetic acid (up) and of 0.1% formic acid (down).

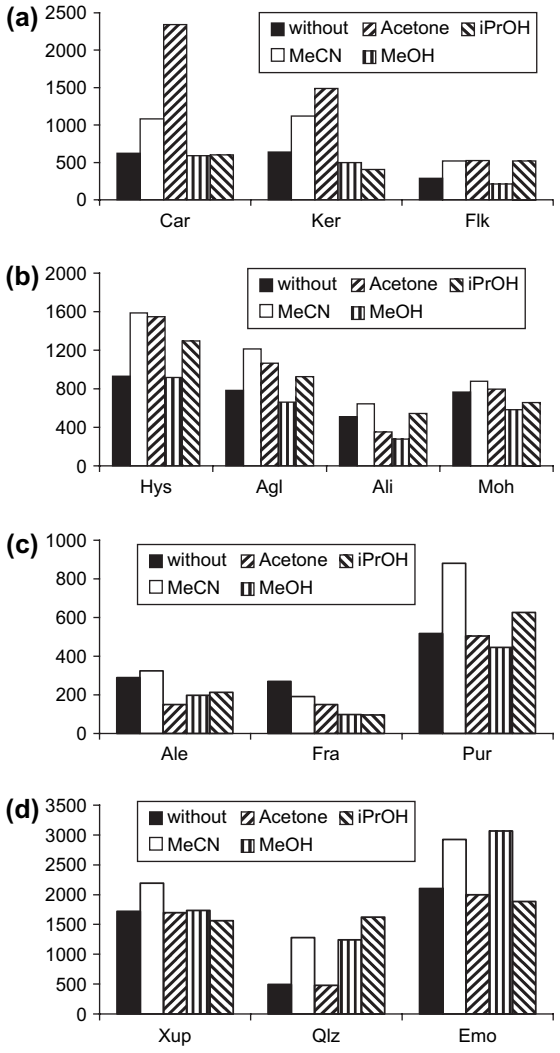


Fig. 3. Peak areas' dependence on post-column solvent addition. Full rectangle: without post-column addition; open rectangle: with MeCN; rectangle with upward lines: with acetone; rectangle with vertical lines: with MeOH; rectangle with downward lines: with ^{i}PrOH.

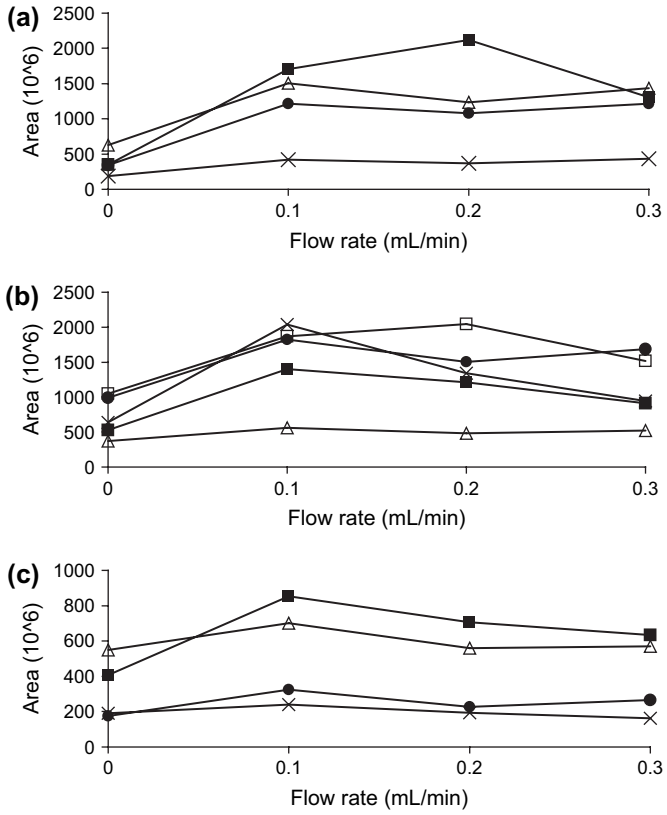


Fig. 4. Compounds' peak areas as a function of post-column flow. (a) ■: Car; △: Hys; ●: Ker; ×: Flk; (b) ■: Agl; △: Ali; ●: Xpu; ×: Qlz; □: Emo; (c) ■: Moh; △: Pur; ●: Ale; ×: Fra.

Furthermore, chromatography requires the presence of a small percentage of acid in the mobile phase to retrograde the acid–base equilibrium of the solvated compounds. Working with a regulated pH enables to have all of the compounds in a neutral form, particularly those which possess acid groups. As the presence of this acid could influence the MS response, we tested various acids with various concentrations.

3.1. Effect of the presence of acid in the mobile phase

Five main acids modulating the separation of anthraquinones are mentioned in literature: acetic acid, formic acid, methanesulfonic acid, trifluoroacetic acid and orthophosphoric acid [3,4]. As the latter three raise compatibility problems with the mass detector, the study was limited to the use of acetic and formic acids.

The effect of the percentage of acid in the mobile phase on the area of the peaks is provided in Fig. 1 for a number of compounds, representing all of the variations for the 18 standards.

Without acid in the mobile phase, the compounds hardly respond. The presence of acid is therefore necessary, for the separation as well as for the detection.

For most of the compounds, except for laccaic acids, the response is better with acetic acid than with formic acid, the optimum being with a value ranging from 0.05 to 0.1% for both acids (Figs. 1a–c).

However, no response was obtained for laccaic acids in the presence of acetic acid whereas they are detected in the presence of formic acid (Fig. 1d).

Moreover, for the anthraquinones with an acid group (carminic acid, kermesic acid, flavokermesic acid), an increase in the retention time was observed when switching from acetic acid to formic acid (carminic acid $t_r = 10.7$–12.8 min, kermesic acid $t_r = 19.8$–22.2 min, flavokermesic acid $t_r = 20.1$–22.5 min), while the other anthraquinones show a constant retention time. This increase is also systematically accompanied with a sharpening of the peak (Fig. 2). The pH of the aqueous phase in the presence of formic acid is lower than that of the aqueous phase containing acetic acid. There is a more important retrogradation of the acid–base equilibrium towards the acid form, and it is neutral, for the species with a weak pK_a, i.e. anthraquinones with an acid group. In the case of a pH regulated with acetic acid, the percentage of the base form of these acids is significant in comparison with their neutral form. The fact that the solute is present in the solution in two forms in equilibrium leads on one hand to a large peak [19] and on the other hand to a form globally more polar than the neutral one, which leads to a weaker global retention.

This can be taken advantage of for the identification of anthraquinones with an unknown structure. An analysis in the presence of acetic acid and another in the presence of formic acid can make it possible to detect anthraquinones with a weak pK_a, because of the retention time shift and the modification of the shapes of the peaks (narrower peaks).

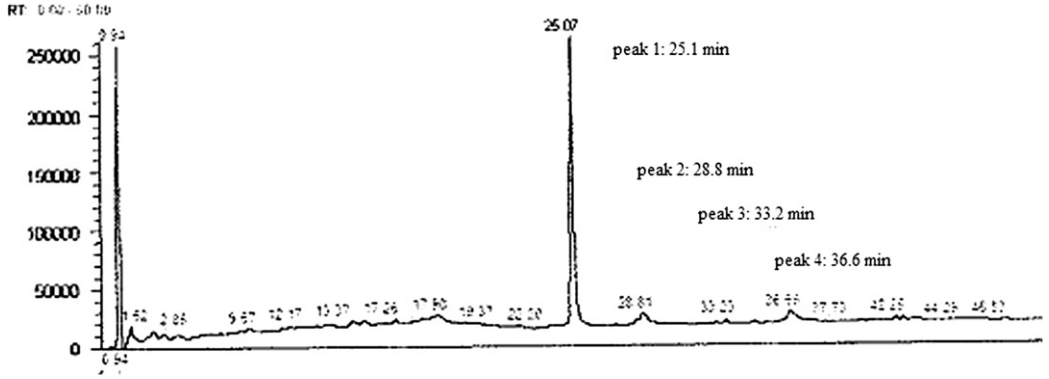


Fig. 5. Chromatogram of *Rubia tinctorum* sample obtained by HPLC–DAD. Peak 1: 25.1 min; peak 2: 28.8 min; peak 3: 33.2 min; peak 4: 36.6 min.

3.2. Effects of the post-column addition of organic solvents

Four solvents were studied: protic solvents (MeOH and ^{i}PrOH) and aprotic solvents (MeCN and acetone). In order not to modify chromatographic retention, they were added in post-column mode thanks to a PEEK T situated at the end of the column.

The obtained results are reported in Fig. 3.

For most of the anthraquinones except for frangulin, the acetonitrile that was added post-column provokes an increase of the peak areas. Methanol only increases the signal for two anthraquinones. In the case of some anthraquinones, response decreases. Isopropanol, just like acetone, has contrasted effects in as much as it increases the response for some compounds and decreases it for others.

Globally, the two aprotic solvents provide a beneficial effect for detection. Because acetonitrile was the solvent which increased the response for most of the anthraquinones, it was chosen for the rest of the study.

Because an important flow (>0.6 mL/min) is unfavourable with the ESI source, a study of the flow of solvent added post-column was carried out in order to find the best compromise between the addition of organic solvent, the flow of the chromatographic column and the flow compatible with the ESI source. The chromatographic flow has been kept constant at 0.3 mL/min and that of the post-column solvent was modified within a range from 0 to 0.3 mL/min (Fig. 4). An optimum can be found for most of the compounds for a post-column flow from 0.1 to 0.2 mL/min. A total flow of 0.6 mL/min is unfavourable for the response of some of the anthraquinones.

The optimisation of the parameters specific to the mass spectrometer and the choice of the eluent coming into the source are essential steps to define the best detection conditions, this being all the more crucial as the samples in which are the anthraquinones to be analysed can be in very small quantity. It is therefore desirable to highlight all the compounds of interest with a minimum of analyses.

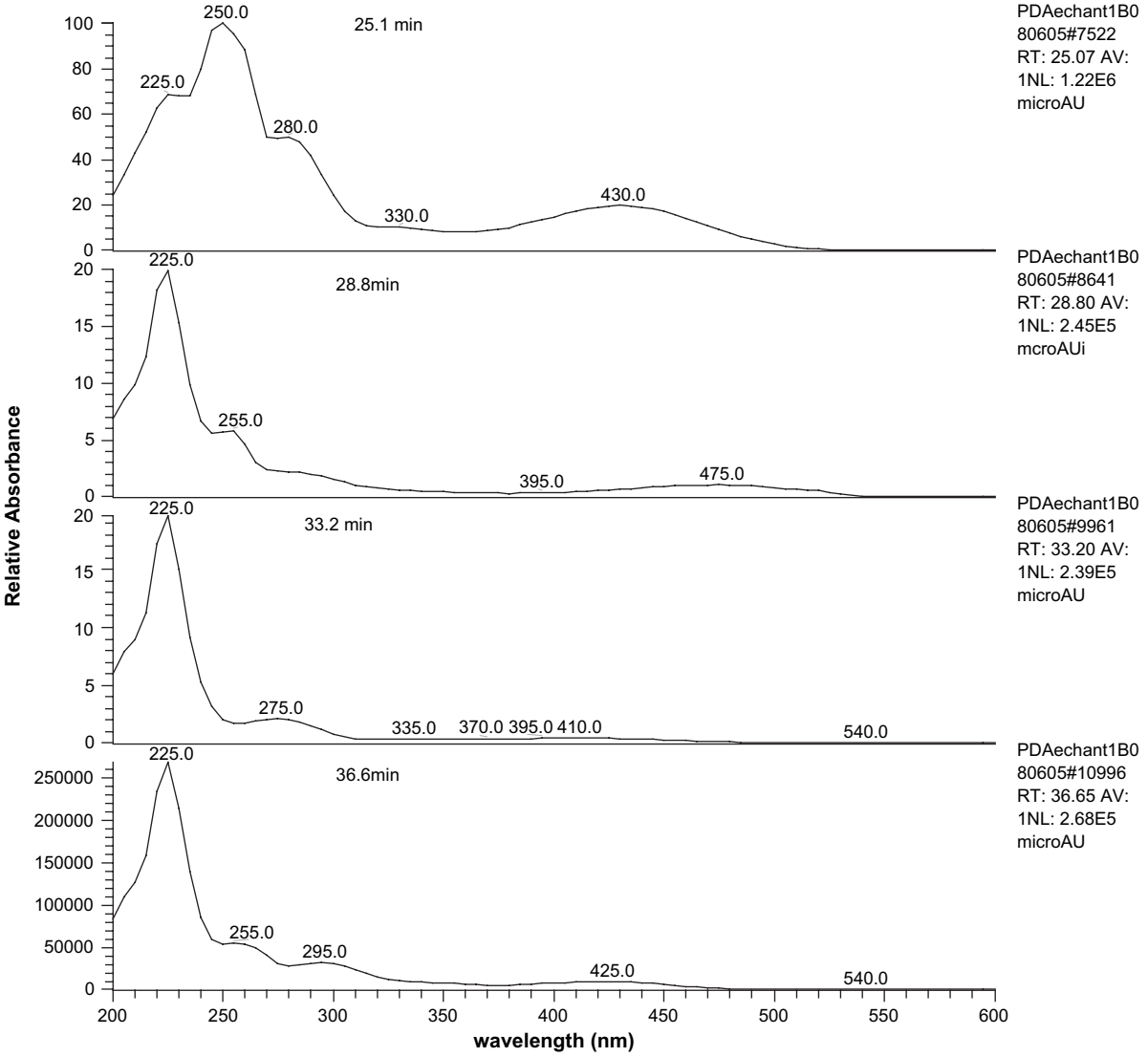


Fig. 6. UV–visible spectra of separated anthraquinones from *Rubia tinctorum* sample. Peaks at 25.1, 28.8, 33.2 and 36.6 min from chromatogram Fig. 5.

3.3. Application to a sample of wool dyed with madder (*R. tinctorum*)

The procedure that was previously used was applied to a sample of wool dyed with madder. The conditions of analysis result from the previous optimisation: column and gradient as defined in the Section 2, MeCN/H_2O + 0.1% acetic acid or 0.1% formic acid gradient, flow of 0.3 mL/min, MeCN in post-column at 0.15 mL/min.

This dye has already been the object of studies found in literature. Cardon [20] indicates that 28 anthraquinonic derivatives have been identified in this plant. Alizarin, which is the main colorant, pseudo-purpurin, rubiadin, munjistin (as well as heterosides of the last three anthraquinones), christophin and a lucidin heteroside have all been detected in fresh roots. In dried roots purpurin, xanthopurpurin, nordamnacanthal, quinizarin and two of it's derivatives, anthragallol and one of it's derivatives, alizarin 1-methylether and rubiadin 1-methylether can also be found.

Derksen et al. [21] analysed an extract of *R. tinctorum* with an HPLC–UV at 250 nm. They highlighted the presence of the primeveroside of lucidin, ruberythric acid, alizarin and purpurin. With an HPLC–MS and an HPLC–DAD, alizarin, purpurin and ruberythric acid were detected by other authors [7]. The primeveroside of lucidin, ruberythric acid, pseudo-purpurin and munjistin may also be present [6,7,10,22].

It therefore seems that, depending on the technique used, the anthraquinones that are detected are not the same and that we are far from a complete detection. The importance of a systematic study of their separation and of an optimisation of their detection to increase sensitivity becomes manifested here.

The extraction method that was applied is presently the most widespread among researchers working on the analysis of dyes in patrimonial objects, but it presents drawbacks in relationship to the original composition of the dyes extracted from sources [1,23]. Because of the acid hydrolysis carried out during the preparation of the sample, glycosides are not visible anymore. Thus, neither ruberythric acid nor lucidin primeveroside mentioned in the literature are detected after extraction.

A first study was carried out with the diode array detector. The chromatogram in Fig. 5 shows four peaks the UV–visible spectra of which are given in Fig. 6. The comparison of the chromatographic information (retention time) and spectroscopic information (UV–visible spectra) with the anthraquinone standards led without ambiguity to the attribution of two of the four peaks to alizarin and purpurin.

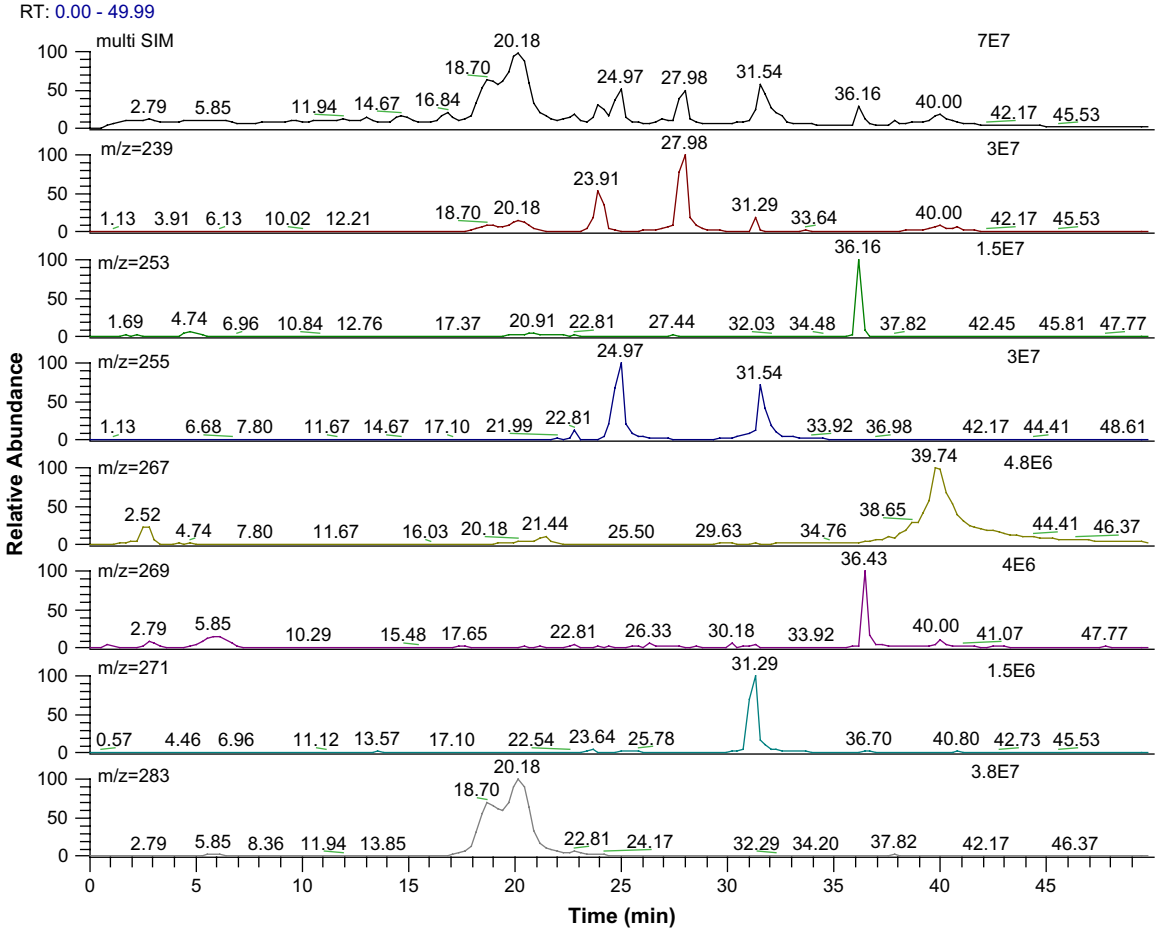


Fig. 7. Chromatogram (multi-SIM) (upper trace) and extracted ion current at *m/z*: 239, 253, 255, 267, 269, 271 and 283 (up to down) of *Rubia tinctorum* sample obtained by LC–MS.

The chromatogram obtained with the HPLC–MS Fig. 7 displays more peaks. This brings to light the interest of coupling mass spectrometry and diode array detection for the analysis of complex mixtures where a great number of anthraquinones are in small quantities.

The comparison of the retention times and of the values of the pseudo-molecular ion $[M-H]^-$ of the compounds of the chromatogram with those of the standards enabled an identification of the first nine anthraquinones listed in Table 7. Given the small quantity of sample available, fragmentation could only be carried out to MS^2. The use of the tandem MS providing the first filiation enabled to confirm the attributions carried out on the two criteria of retention time and value of the mass of the $[M-H]^-$ adduct. Actually, the observed loss is −28 amu or −44 amu. In the case of the peak attributed to kermesic acid, the loss of 44 amu is coherent with the fact that this anthraquinone shows an acid group (as previously discussed (Table 4)). It is important to note that the presence of this acid is rather unexpected in madder dye. This compound is usually present in reds of animal origin (insects; as major in: *Kermes vermilio* and as minor in *Dactylopius coccus* and those belonging to the *Porphyrophora* family). This molecule is probably present in our reference in a ''parasite'' manner, due to the hazardous ''pollution'' during dyeing (for example: reuse of the vessel used for another dyeing).

An ambiguity remains concerning the presence of the peak attributed to quinalizarin because the obtained fragmentation does not correspond to the available standard.

The UV–visible spectrum as well as the presence of a 253 amu *m/z* peak seems to be in favour of the presence of rubiadin (Table 8) potentially expected in this sample, but for which a standard is unavailable. This is not sufficient to ascertain the presence of this anthraquinone. However, the fragmentation carried out on this ion led to $m/z = 225$ amu (loss of 28 amu) as well as to an ion at $m/z = 209$ amu (loss of 44 amu) coherent with the fragmentations observed in our available anthraquinone standards. Finally, this compound shows a retention higher than that of xanthopurpurin with the same polarity, but which has one less CH_3 group, i.e. possessing a smaller hydrocarbonated volume. This is coherent with the expected elution order in RPLC (reversed-phase liquid chromatography).

A double peak at $m/z = 283$ amu is detected at the beginning of the chromatogram with a fragmentation of (283 → 239 (−44) → 211 (−28)) in agreement with that of anthraquinones with an acid group (initial loss of 44 amu). The study carried out by replacing the acetic acid with formic acid in the mobile phase led to the chromatograms in Fig. 8. The increase of the retention time as well as a finer peak in the presence of formic acid confirms the presence of an acid group in the structure. All of these data suggest the presence of munjistin (Table 8).

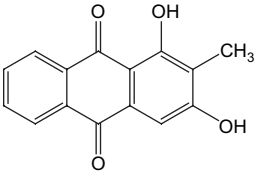
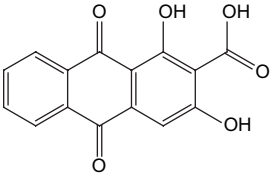
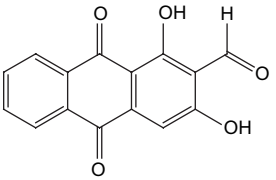
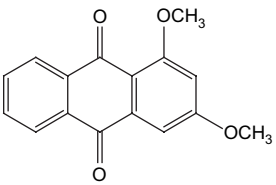
A peak is observed towards 40 min with $m/z = 267$ amu, therefore corresponding to $M = 268$ g/mol. This leads us to suggest nordamnacanthal or purpuroxanthin dimethyl ether, the formulae of which are in Table 8. In both cases, retention time is coherent with the expected RPLC elution order. Actually, the retention of nordamnacanthal can be compared to that of xanthopurpurin. The presence of the aldehyde group in position 2, on one hand increases the hydrocarbonated volume of the molecule and on the other hand decreases it's polarity through the formation of intramolecular hydrogen bonds with the hydroxyl groups in positions 1 and 3. This leads to an increase in retention with regard to xanthopurpurin as observed. In the same way, the retention of purpuroxanthin dimethyl ether can also be compared to that of xanthopurpurin.

Table 7
The anthraquinones identified in *Rubia tinctorum* reference dyeing

Compound	$[M-H]^-$ (uma)	t_r[a] (min)	t_r[b] (min)	MS^2
Kermesic acid	329	20.2	22.2	329 → 285 (−44)
Hystazarin	239	23.9	24.0	239 → 211 (−28)
Anthragallol	255	25.0	24.8	255 → 227 (−28)
Alizarin	239	28.0	28.0	239 → 211 (−28)
Quinalizarin	271	31.3	31.2	271 → 225 (−46)
Xanthopurpurin	239	31.3	31.3	239 → 211 (−28)
Purpurin	255	31.5	31.4	255 → 227 (−28)
Emodin	269	36.4	36.3	269 → 225 (−44)
Quinizarin	239	40.0	40.0	239 → 211 (−28)
Rubiadin	253	36.2	36.2	253 → 225 (−28)
Munjistin	283	18.7–20.2 (two peaks)	22.7	283 → 239 (−44)
Nordamnacanthal	267	40.0	39.8	267 → 237 (−30)

[a] Mobile phase with acetic acid.
[b] Mobile phase with formic acid.

Table 8
The structures of anthraquinones identified by MS^n without standards in *Rubia tinctorum* reference dyeing

Compound	Molar mass (g/mol)	Structure
Rubiadin	254	
Munjistin	284	
Nordamnacanthal	268	
Purpuroxanthin dimethyl ether	268	

The replacement of the two hydroxyl groups in positions 1 and 3 by methoxy groups increases the hydrocarbonated volume, decreases the polarity of the molecule also leading to an increase of the retention with regard to xanthopurpurin. The chromatographic information alone does not allow to decide between the two compounds. The fragmentation obtained for this peak (267 → 237) leads to a loss of 30 amu, which is in favour of the presence of the compound with an aldehyde group (loss of $H_2C{=}O$) and not of the compound with methoxy groups the expected fragmentation of which would be −15 amu (loss of CH_3) as already observed in the case of 3-MeO-hystazarin. These observations lead to suggest the presence of nordamnacanthal.

The different compounds identified in this sample are listed in Table 7.

3.4. Application to an extract of Our Lady's bedstraw (G. verum)

The same method as the one described for the previous sample was followed. The study with HPLC–DAD lead to highlighting three peaks, two of which were intense, whereas the HPLC–MS coupling showed many more peaks (Fig. 9). The comparison of the retention times, of the $[M-H]^-$ values and of the fragmentations with the available standards leads to suggest the presence of alizarin, purpurin, hystazarin, anthragallol, quinizarin and xanthopurpurin. The presence of rubiadin was highlighted in a similar way as with the other sample, by combining the information relative to the retention time, to the HPLC elution order, to the $[M-H]^-$ value and to the observed ruptures. The study in both acids (acetic and formic) as well as the above mentioned data lead to suggest the presence of munjistin. These identifications are in agreement with data from the literature [20], particularly as purpurin and rubiadin are the main components of *G. verum*.

The only information from the UV–visible spectra is not sufficient for identification of the anthraquinones present in a given sample. Similarly, the only information of the mass of the $[M-H]^-$ ion enables to refine the hypotheses but there remains an ambiguity as to the compound, in as much as numerous anthraquinones are isobars. However, the use of MS^n technique enables to attribute the structures more precisely following the interpretation of the observed ruptures and the comparison with those of standard substances analysed in the same conditions. It even enables the attribution of structures to peaks which did not match any available standard. The combination of spectral information (and more particularly the (MS^2) fragmentations) and of chromatographic data

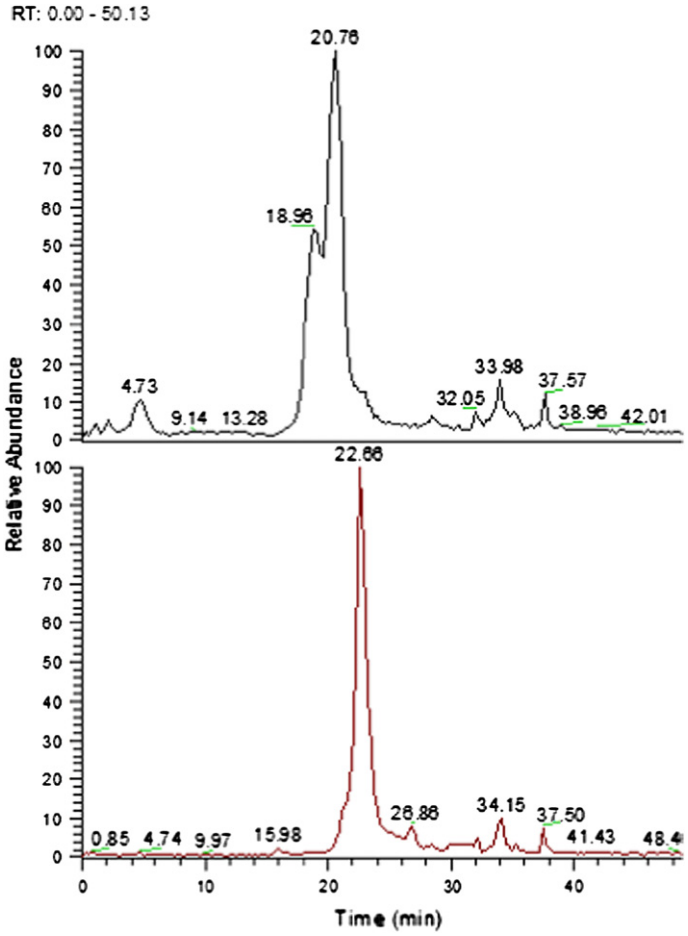


Fig. 8. Peak shape extracted from chromatogram of *Rubia tinctorum* sample in presence of 0.1% acetic acid (up) and of 0.1% formic acid (down).

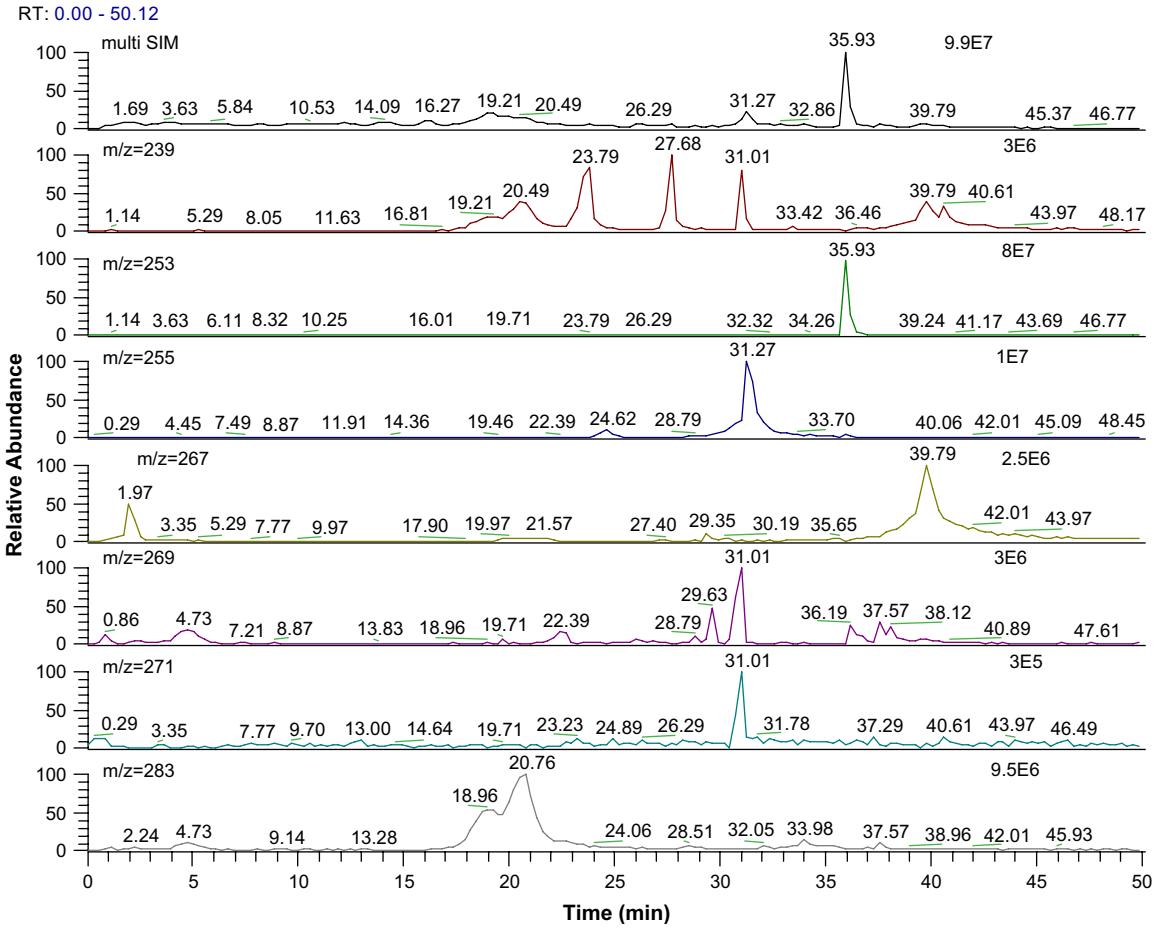


Fig. 9. Chromatogram (multi-SIM) (upper trace) and extracted ion current at *m/z*: 239, 253, 255, 267, 269, 271 and 283 (up to down) of *Galium verum* sample obtained by LC–MS.

(retention, modification of the retentions in the presence of formic acid) led to the identification of the anthraquinones in two samples, *R. tinctorum* and *G. verum*.

4. Conclusions

The analysis of archaeological or historical materials poses specific problems: their size (very small quantity available) and their complexity. MS providing structural information enables the "in line" detection and identification of unknown compounds. The optimisation of the MS detection parameters enables to work with a very small quantity of matter and thus to reach a "compromise" in analysis conditions which enable to detect all of the compounds of interest in a single analysis, with an adequate sensitivity. Furthermore, the excellent resolution of the method enables to highlight co-eluted compounds, even if one of them is a minority, which is not an easy task in the case of a detection using UV–visible (DAD) spectroscopy.

The complementarity of separative techniques (retention time, elution order, shape of the peaks) and spectroscopic techniques (UV–visible, MS–MS) is essential for the identification of anthraquinones and in particular enables the identification of anthraquinones that are not available as standards.

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