Textile dyeing with a flavonoid dye: photo-stability and analytical chemistry methods

Alexandre Villela

Propositions

1. There are limitations to the yellow colour of wool dyed with weld.
(this thesis)
2. Using short UHPLC columns on conventional HPLC systems is an economical way of modernising HPLC-based analyses.
(this thesis)
3. People and planet are better taken care of through organic agriculture than through conventional agriculture.
(based on Reganold JP, Wachter JM. Nat. Plants 2016)
4. The release of genetically engineered organisms in natural, rural and urban areas is an ecocide.
5. The pursuit of excellence as human being must precede that of professional excellence.
6. Pride is fruit of short-sightedness.

Propositions belonging to the thesis entitled

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Thesis

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In memory of my grandmother Pérola (Voca)

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Chapter 1

General introduction

The contents of this chapter are, to a large extent, part of the following papers:

Villela A, van der Klift EJC, Mattheussens ESGM, Derksen GCH, Zuilhof H, van Beek TA. Fast chromatographic separation for the quantitation of the main flavone dyes in *Reseda luteola* (weld). J Chromatogr A 2011; 1218(47): 8544–50

Villela A, Derksen GCH, Zuilhof H, van Beek TA. Spectrophotometric comparison of the content of chlorophylls in weld (*Reseda luteola*). Anal Methods 2011; 3(6): 1424–7

Villela A, van Vuuren MSA, Willemsen HM, Derksen GCH, van Beek TA. Photo-stability of a flavonoid dye in presence of aluminium ions. Dyes Pigments 2019; 162: 222–31

Villela A, Derksen GCH, van Beek TA. Analysis of a natural yellow dye: an experiment for analytical organic chemistry. J Chem Educ 2014; 91(4): 566–9

1.1. Introduction

Coloured textiles have been used by mankind throughout times [1]. Dyes have been obtained from different natural sources, including plants, molluscs and insects [1] and were valuable in the past [2]. Although the use of natural dyes experienced a fast decline from the 1850s with the first synthetic dyes entering the market [3, 4], there has been a renewed interest. Currently, this is due to innovation-related, economical, personal, and ethical reasons [5]. Figure 1.1 depicts yarns dyed with natural dyes in various colours.

Figure 1.1. Yarns of wool dyed with natural dyes.¹

In addition, identification of dyestuff of historical textiles is of historical and conservational interest [2]. Figure 1.2 depicts a live mannequin wearing a costume dyed with a natural dye.

¹ Dyed by Judy Hardman (United Kingdom); picture taken in La Rochelle/France (2011).

Figure 1.2. Valérie Fischbach acting as a live mannequin wearing a costume in *Incroyables et Merveilleuses* style dyed with weld (*Reseda luteola* L.).²

It should be noted, however, that the use of natural dyes for textiles is not synonymous with environmentally friendly dyeing [5]. Different factors must be addressed for it to be an ecologically sound industrial activity [6]. Naturally, techno-economic aspects of production also require attention [7].

Weld (*Reseda luteola* L.) used to be an important vegetable source of dye for textiles in Europe and the Mediterranean area [1]. The main colouring compounds of the plant belong to the class of flavonoids [1]. The flavone luteolin (**lut**) and two of its glycosyl conjugates, **lut**-7-*O*-glucoside (**lut** monoglucoside, **lmg**) and **lut**-7,3'-*O*-diglucoside (**lut** diglucoside, **ldg**), are the main flavonoids of the plant [8] (Fig. 1.3). Seven other minor ones include the aglycones apigenin and chrysoeriol, together with mono and diglycosides of all three aglycones [8, 9].

² *Les Incroyables et Merveilleuses* was a French fashion trend of the end of the 18th century; made by students preparing for a degree in arts and crafts at *lycée polyvalent Jules Verne de Sartrouville*—a high school in France—together with their teachers; picture taken in La Rochelle/France (2011).

Figure 1.3. Structure of the flavone luteolin (**lut**), the aglycone of the main flavonoids of weld (*Reseda luteola* L.).

RP-HPLC–UV (reversed phase high-performance liquid chromatography with spectrophotometric detection in the ultraviolet range of the electromagnetic spectrum) is a very suitable technique for the separation and detection of these non-volatile moderately polar UV-active analytes, and is available in most laboratories. Various assays have been published for the quantitation of flavonoids in weld, by different analytical techniques. RP-HPLC–DAD (RP-HPLC–diode-array detector) has been used to quantify the aglycones quercetin, luteolin, and apigenin in weld after simultaneous extraction-hydrolysis of the glycosides [10]. Other groups used RP-HPLC–UV to quantify only luteolin [11, 12]. Cristea *et al.* [13] extracted weld with methanol–water 8:2 at room temperature and under reflux and, afterwards, analysed the three main flavones by the same technique with external standardisation. Moiteiro *et al.* [14] also used methanol–water 8:2, but extracted the flavonoids from weld by 10 min sonication at room temperature. In addition to **ldg**, **lmg**, and **lut**, they also quantified luteolin-4'-*O*-glucoside. Since there was no simple, validated RP-HPLC–UV method for the quantitation of **ldg**, **lmg**, and **lut** in samples of weld, the development of such a method—and its validation for the purpose—was aimed at. The method that was developed is described in **chapter 2**.

According to the industrial partner of the project, one of the problems to overcome was a greenish hue that accompanies the yellow colour after dyeing alum (dodecahydrate aluminium potassium sulphate) pre-treated textiles with weld. Such a greenish hue would be undesirable, and it was hypothesised that chlorophylls *a* and *b* are potential sources of it. Thus, for the purposes of raw material characterisation, the development of a simple analytical method for the quantitation of chlorophylls in samples of weld was aimed at. The method that was developed envisages to compare the content of porphyrin ring-containing pigments in different weld plant materials, and is described in **chapter 3**.

Weld has been used to dye both animal fibres—such as wool—and vegetable fibres, after they have been treated (mordanted³) with alum in facultative combination with the dye-assistent tartar [1]. Alum-premordanted wool dyed with weld leads to yellow colours and, generally, the yellow colours of textiles dyed with natural dyes are poorly resistant to light [1]. Considering today's requirement of photo-resistance (light-fastness) of the colours of dyed textiles, this is

³ The word mordant comes from the Latin word *mordere*, which means “to bite”. Metal salts have been referred to as mordants in relation to a classical model of binding of dyes to fibres involving the metals [1].

also the case for the dye of weld. Therefore, the work reported in **chapter 4** aimed at finding out whether:⁴

- a progressive decrease of $[Al^{3+}]$ would progressively increase the photo-stability of the dye of weld;
- the endogenous glycosidase of weld should be inactivated before extracting the flavonoids of the plant in order to obtain the most photo-stable dye.

Because of its wide applicability, high-performance liquid chromatography (HPLC) is one of the analytical techniques with which the undergraduate student of life sciences should be acquainted. This is also true for the internal standard method [15], which is important in quantitative instrumental analysis [16, 17]. The experiment described in **chapter 5** aims at familiarising students with both. This is accomplished using the real-world application of natural dyes for textiles. Weld, the plant used in the experiment, and wool dyed by students with its extract are depicted in Figure 1.4.

Figure 1.4. Patch of weld (*Reseda luteola* L.) and alum-pretreated wool dyed with extract of weld.

⁴ The rationale for these aims is presented in section 4.1.

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Chapter 2

LC–UV method for the quantitation of the main flavonoids of weld

The content of this chapter is largely that of the following paper:

Villela A, van der Kluft EJC, Mattheussens ESGM, Derksen GCH, Zuilhof H, van Beek TA. Fast chromatographic separation for the quantitation of the main flavone dyes in *Reseda luteola* (weld). J Chromatogr A 2011; 1218(47): 8544–50.

A simple validated industrially-usable quantitative method to assess the flavone content of *R. luteola* samples is described in this chapter. The flavones were overnight-extracted from the dried and ground aerial parts of the plant at room temperature via maceration with methanol–water 8:2. Afterwards, they were quantified through internal standardisation against chrysin by RP-HPLC–UV at 345 nm. The efficiency of the one-step extraction was 95%. The limits of detection (LOD) and quantitation (LOQ) were ≤ 1 ng and ≤ 3 ng, respectively, providing ample sensitivity for the purpose. The precision expressed as relative standard deviation of the entire method was $< 6.5\%$ for the combined content of luteolin-7,3'-*O*-diglucoside, luteolin-7-*O*-glucoside, and luteolin. The average absolute recovery (accuracy) at three spiking levels was 102% (range: 98–107%) and the relative recovery ranged from 99 to 102%. The separation was initially carried out on a traditional 250 mm $\times$ 4.6 mm 5 μ m-particle size HPLC column (80 min run time, 35.9 mL MeOH). It was then speeded up by the use of a 50 mm $\times$ 3.0 mm 1.8 μ m-particle size UHPLC column (5 min run time, 1.4 mL MeCN), while still using a conventional HPLC system. Whereas the retention times on the UHPLC column were relatively less reproducible, cross-validation showed that the quantitation of luteolin-7,3'-*O*-diglucoside, luteolin-7-*O*-glucoside, and luteolin was not statistically significantly different, with comparable precision. The method is more sensitive when using the UHPLC column than when using the HPLC column. The analytical method described meets the demand for a very small manpower input per sample and uses standard laboratory equipment. Using a UHPLC column on a conventional HPLC system is a way of modernising HPLC-based phytochemical analyses inexpensively.

2.1. Introduction

In view of the expectation of having to analyse hundreds of samples in the course of the academic and industrial dye research of which this project is part, developing a method suitable for the simultaneous analysis of the three main flavones of the plant with as little manpower input as possible and using standard equipment was aimed at. According to the industrial partner of the project, **ldg**, **img**, and **lut** constitute about 80% of the total flavonoid content of *R. luteola* and the ratio between them and the minor flavonoids is fairly constant. Thus, quantifying only these three flavones, while being informative, keeps the LC-based method simple and cheap.

A validated RP-HPLC–UV method for the quantitation of **ldg**, **img**, and **lut** that satisfies the demand of the project in terms of low manpower input is reported in this chapter. Moreover, the chromatographic step on a 250 mm × 4.6 mm 5 µm-particle size HPLC column was speeded up and made low in solvent consumption by using a 50 mm × 3.0 mm 1.8 µm-particle size UHPLC column, while still using a conventional HPLC system.

2.2. Material and methods

2.2.1. Plant material, chemicals and reagents

Five plants were withdrawn from a bunch of dried *Reseda luteola* L. (cultivar code: H) plants. The plants used were grown in the North of the Netherlands and were harvested on August 2007. A voucher specimen was deposited at the Wageningen branch of the National Herbarium of the Netherlands (Gen. Foulkesweg 37, 6703 BL Wageningen, The Netherlands) and identified as W. Olsder s. n. (WAG, barcodes WAG0248296–WAG0248298). Drying took place by leaving the plants outdoors during daytime and indoors during the nights, for ~10 days. The aerial parts of the plants were ground with a cutting mill (Retsch GmbH, type SM1, Haan/Germany; 0.25 mm sieve). This combined sample is henceforth referred to as *R. luteola* H, and was stored in the dark.

Methanol (“HPLC grade” or similar), DMSO (99.9% for spectroscopy, Acros Organics), and acetonitrile (HPLC grade or p.a.) were used without further purification. Ultrapure water was prepared with an EasyPure UV system (Barnstead/Thermolyne, Dubuque/USA) or purchased from Merck (Lichrosolv water for chromatography). Demineralised water (Ph. Eur.) was purchased from VWR or prepared with a Seradest SD 2000 ion-exchanger (Seral Erich Alhäuser GmbH, Ransbach-Baumbach/Germany). Formic acid was from Acros Organics (98+%) and VWR (AnalaR Normapur 99–100%), ammonium formate was from Aldrich, Fluka or VWR, EDTA (dihydrate, either di- or tetrasodium) was from either Aldrich or VWR, and luteolin (**lut**) used for spiking was from Sigma (≥98%, TLC grade). Standard compounds luteolin-7,3'-di-*O*-glucoside (luteolin diglucoside, **ldg**), luteolin-7-*O*-glucoside (luteolin monoglucoside, **img**), and luteolin (**lut**) were all of analytical control grade (Extrasynthese). Their purity was determined by ¹H-NMR in DMSO-*d*₆ (99.5+ atom % D, Aldrich) with a 400 MHz NMR spectrometer (Bruker, Avance III, Fällanden/Switzerland) as 88, 94, and 98 and 94% for **ldg**, **img**, and **lut** (Extrasynthese and Sigma, respectively), respectively. Maleic acid (puriss., ≥99.0 % by HPLC, Fluka) was used as internal standard for the analyses by NMR [1]. Chrysin (97% grade) was from Aldrich. G24 Environmental and Innova 4080 Incubator Shakers (New Brunswick Scientific Co. Inc., Edison/USA) were used. A 15-stirring points

magnetic stirrer (Variomag Poly 15, H+P Labortechnik, Munich/Germany) was used. The sonication bath used was from Elma, type T 700 (Germany).

2.2.2. Sample preparation

(200.0 ± 0.9) mg of dried *R. luteola* H were weighed (Mettler AE 260 balance) in 50 mL Erlenmeyer flasks. A magnetic stir bar and 20 mL of methanol–water 8:2 (v/v) were added to each flask, after which they were closed with a rubber stopper. The flasks were placed either on a 15-stirring point magnetic stirrer or on individual magnetic stirrers. Samples were extracted for 16 h at room temperature, at 300 rpm. Then, 5.00 mL of chrysin stock solution were added to the Erlenmeyer flask and stirring continued for another 10–14 min. Subsequently, an aliquot of the solution was filtered via a 0.45 µm syringe filter (Minisart RC4, Sartorius Stedim Biotech, Goettingen/Germany)/polypropylene syringe (with rubber stopper) to a glass HPLC autosampler vial, discarding the first drops.

2.2.3. LC I (HPLC column)

HPLC analyses in Wageningen were performed on a Waters system consisting of a binary pump (1525 µ), a photodiode array detector (DAD) (996) and an autosampler (717 plus), and equipped with a column oven model 5CH. An Alltima 250 mm × 4.6 mm C18 5 µm-particle size column was used, in combination with a C18 guard column. The volumes of the syringe and loop installed were 250 and 200 µL, respectively. The system was controlled by Waters Empower 2 software. The draw rate of the autosampler was 1 µL sec⁻¹ and the injected volume, 20 µL. Mixtures of methanol–water and methanol only were used as needle wash solvent (the latter is preferred for keeping sample carryover below 1%). One of the relative recovery experiments was carried out in Steenbergen, on an analogous system (see section SI A.1.1, Appendix A). At both locations, a pH 3 buffer of 160 mM formic acid, 40 mM ammonium formate and 0.04 mM of EDTA in water (A), and methanol (B) were used as solvents. The flow was 1.00 mL min⁻¹ and the following linear gradient was used: 85–60% A (0–35 min), 60–36% A (35–47 min), 36–23% A (47–60 min), 23–0% A (60–65 min), 0% A (65–70 min). Subsequent injections could be made after 10 min of re-equilibration. The column was kept at 40 °C. The DAD was set to scan from 245 to 500 nm (Wageningen) and 200 to 595 nm (Steenbergen). The peak areas were based on the absorbance at 345 nm. LC–DAD–MS for peak purity checking was performed in Wageningen with the same column on a Thermo Scientific system consisting of an Eppendorf CH-30 column oven, a Finnigan Surveyor autosampler plus, MS pump plus, DAD plus, and a Finnigan LXQ linear ion trap MS with ESI probe working in (+)-ionisation mode in combination with Xcalibur software (version 2.0.5). Conditions were basically the same as above, except that solvent A contained no EDTA. About 10% of the flow was directed to the MS.

2.2.4. LC II (UHPLC column)

Analyses were performed on an Agilent system consisting of a binary pump (Agilent 1200 Series), a diode array detector (DAD), an autosampler, and a column oven (all Hewlett-Packard, Series 1100). An Agilent XDB-C18 50 mm × 3.0 mm 1.8 µm-particle size column without guard column was used. The system was controlled by HP ChemStation for LC 3D (Rev. A.06.03) software. The following modifications were done to reduce the internal volume of the system: (i) the mixer was replaced by a 7.5 mm × 3.0 mm Alltima C18 (5 µm-particle size) pre-

column, which now acted as the mixer and connected damper and injector; and (ii) shortening of the tubing from the injection system to the column and from the column to the detector. The injected volume was 2 μL . The column temperature was 35 $^{\circ}\text{C}$ (measured with a digital thermometer, model 3150, PeakTech). The DAD scan range was 245–500 nm and detection was carried out at 345 nm (bandwidth 4 nm), having 470 nm (bandwidth 40 nm) as reference λ . The following solvents were used as eluent: solvent A was the same as for the HPLC column, whereas solvent B was acetonitrile. The flow was 0.90 mL min^{-1} and the following linear gradient was used: 85–45% A (0–4.00 min), 45–85% A (4.00–4.01 min) and 85% A, isocratic (4.01–5.00 min). Injections were made every 5 min.

2.2.5. Calibration curves, linearity and determination of relative response factors (RRFs)

For the HPLC column, the stock solutions were prepared as follows: (i) 125 mg of chrysin (internal standard, **i.s.**; MW: 254.24 g mol^{-1}) were dissolved in methanol (0.5 mg mL^{-1} ; 2.0 mM); (ii) 2.0 mg of **ldg** (MW: 610.52 g mol^{-1}) were accurately weighed and dissolved in methanol–dimethyl sulfoxide 7:3 (v/v); (iii) 3.6 mg of **lmg** (MW: 448.38 g mol^{-1}) were accurately weighed and dissolved in methanol–dimethyl sulfoxide 7:3 (v/v); and (iv) 2.4 mg of **lut** (MW: 286.24 g mol^{-1}) were accurately weighed and dissolved in methanol. In the cases of **i.s.** and **lut**, the dissolution was assisted by the use of a shaker at room temperature. Generally, the exposure of the solutions of the flavones to light was minimised. As 10 mL volumetric flasks were used for **ldg**, **lmg**, and **lut**, the concentration of the stock solutions were 176 $\mu\text{g mL}^{-1}$ (0.29 mM), 341 $\mu\text{g mL}^{-1}$ (0.76 mM), and 233 $\mu\text{g mL}^{-1}$ (0.81 mM), respectively (taking into account the purity determined by NMR). The **i.s.** stock solution was stored at 4 $^{\circ}\text{C}$. The dilutions were made by adding 1.00 mL of the **i.s.** solution and different volumes of each of the stock solutions to 5 mL volumetric flasks. Five concentrations for **ldg** and **lmg**, and seven for **lut** (for which a 10x diluted stock solution was additionally required) were prepared by the use of volumetric pipettes, ranging as follows: (i) **ldg**: 7–141 $\mu\text{g mL}^{-1}$; (ii) **lmg**: 14–205 $\mu\text{g mL}^{-1}$; and (iii) **lut**: 2–116 $\mu\text{g mL}^{-1}$. This was done separately for each of the flavones. In all cases: (i) only methanol was added to the mark and (ii) each solution was injected once. For the UHPLC column, the stock and diluted solutions were prepared as above, except that 2.4 mg of **ldg**, 3.7 mg of **lmg**, and 2.3 mg of **lut** were weighed (thus, taking into account the purity determined by NMR, the concentrations of the stock solutions were: 215 $\mu\text{g mL}^{-1}$ or 0.35 mM, 340 $\mu\text{g mL}^{-1}$ or 0.76 mM, and 230 $\mu\text{g mL}^{-1}$ or 0.80 mM, respectively) and the concentrations of the diluted solutions ranged as follows: (i) **ldg**: 9–150 $\mu\text{g mL}^{-1}$; (ii) **lmg**: 14–238 $\mu\text{g mL}^{-1}$; and (iii) **lut**: 2–115 $\mu\text{g mL}^{-1}$. In all cases, each solution was injected once.

2.2.6. Evaluation of the extraction efficiency

R. luteola H was extracted according to the normal procedure (above). However, the work-up was done differently. The extract was filtered under reduced pressure by the use of an ordinary 60° filtration funnel and a suction flask. The filtrate was collected in a test tube placed inside the flask. It was then transferred to a 25 mL volumetric flask and methanol–water 8:2 (v/v) was added to the mark. The solution was syringe-filtered and analysed by HPLC. No **i.s.** solution was added as the experiment was run on a relative basis and the results were calculated based on the sum of the areas of the **ldg**, **lmg**, and **lut** peaks. The filter paper was transferred back to the Erlenmeyer flask. The powder of the plant material was removed from the filter paper with the aid of 20 mL of freshly added methanol–water 8:2 (v/v). The same extraction and work-up

procedures were carried out twice more. This experiment was carried out thrice. The extraction times of each cycle were: 1st 16 h; 2nd 24–26 h; and 3rd 53–56 h.

2.2.7. Precision of the entire method

For the HPLC column, three series of seven replicates of the usual extraction were carried out (sample = *R. luteola* H). They occurred on days 1, 3 and 5. Those of days 1 and 5 were carried out with a 15-stirring point magnetic stirrer, whereas those of day 3, by the use of the individual magnetic stirrers. For the UHPLC column, eight replicates of the usual extraction were carried out (sample = *R. luteola* H). After storage of samples at 4 °C or –20 °C, they were analysed on days 5, 5, and 6.

2.2.8. Recovery of the method

2.2.8.1. Relative recovery experiments, including evaluation of the stability of the compounds under the extraction conditions

The relative recovery experiment was conducted similarly to that described earlier [2]. In short: pre-purified samples were subjected anew to the entire analytical procedure. In this case, a 10-stirring points magnetic stirrer (RO 10 P, IKA Werke, Staufen/Germany) was used. The areas of the peaks before and after the analytical procedure, including extraction, were compared. Also, extracts were filtered through filter paper Whatman 42 and brought to a final volume of 25.0 mL with methanol–water 8:2 (v/v). In each case, the area of the peaks of **ldg**, **lmg**, and **lut** of syringe-filtered and non-syringe-filtered (above) samples were compared ($n = 3$). The experiments were carried out with three different amounts of plant material: ~100, 200, and 300 mg.

2.2.8.2. Absolute recovery by spiking experiment (accuracy)

Known amounts of each flavonoid of interest were added to the plant material (spiking). Then, the extraction and work-up proceeded as usual. Due to the poor solubility of the glycosides (**lmg** and **ldg**) in methanol, water, and their mixtures, this required a specific approach. Each flavonoid standard was accurately weighed and transferred to a round-bottom flask with methanol, which was then evaporated *in vacuo*. Methanol–water 8:2 (v/v) was added to the residue. This was followed by simultaneous agitation and sonication during 10 min. This solution was analysed ($n = 3$) with the internal standard method to determine the absolute amount of each flavone present. Next, 20.00 mL of the solution was added to 200 mg of plant material, followed by standard extraction, work-up, and analysis (above). The amounts of **ldg**, **lmg**, and **lut** present in 200 mg of *R. luteola* H were 707 µg (average) (3.3% = relative standard deviation), 1,298 µg (3.9%), and 195 µg (5.4%), respectively ($n = 12$). The spiking was carried out at three levels, as follows: low (two triplicates), medium (triplicate) and high (triplicate). The added absolute amounts of **ldg**, **lmg**, and **lut** in the spiking experiments were:

- (i) 392 µg (0.7%), 618 µg (0.2%), and 78.1 µg (0.6%), respectively, in the first triplicate of level low;
- (ii) 341 µg (0.2%), 623 µg (0.6%), and 118.2 µg (0.5%), respectively, in the second triplicate of level low;
- (iii) 726 µg (0.5%), 1,369 µg (0.4%), and 232.7 µg (0.4%), respectively, in the level medium;
- (iv) 929 µg (0.4%), 1,923 µg (0.2%) and 290.8 µg (0.3%), respectively, in the level high.

Thus, the spiking levels were: 40–61% (low), 103–119% (medium), and 131–149% (high), roughly corresponding to 50, 110, and 140% of the flavonoid content that is present in the plant material. The recovery (in %) was calculated as [3]:

$$\{[(\text{amount found}) - (\text{amount in plant})] / \text{amount spiked}\} \times 100\%$$

2.2.9. Stability of samples ready for analysis

All extracts of series 1 of the HPLC column precision experiment (above) were collected in four HPLC vials. One set of vials was loaded for analysis in the HPLC shortly after the work-up procedure. The second set of vials was also placed in the auto-sampler (room temperature and dark), but analysed 24 hrs after the previous series. The third set was placed at 4 °C, and the fourth, at –20 °C. They were analysed 72 h and 15 days after the first set of vials, respectively.

2.2.10. Data/statistical analysis

Values of the results presented were generally rounded up according to their uncertainties (standard deviation values), expressed with one significant digit. Unless stated otherwise, statistical analyses were carried out using the Mann–Whitney *U*-test ($\alpha = 5\%$, two-sided test) [4].

2.3. Results and discussion

2.3.1. Extraction

As the assay was expected to be used in an industrial setting for hundreds of samples during many years, the amount of labour per sample was of eminent importance. Earlier, a slow maceration-type extraction with introduction of **i.s.** before sampling followed by RP-HPLC proved itself very labour-efficient in the analysis of 750 *Taxus* samples [5, 6]. A similar approach (overnight maceration with stirring) was evaluated for *R. luteola* flavones.

Next, the solvent for extraction had to be selected. Cristea *et al.* [7] compared different solvents for the extraction of **ldg**, **img**, and **lut** from the aerial parts of *R. luteola*, with methanol–water 8:2 (v/v) giving the highest yield. The same authors observed 10–30% lower extraction yields of the flavonoids after extraction with methanol–water 8:2 (v/v) for 4 h at room temperature than after 15 min of refluxing [7]. This is consistent with our preliminary observations using a solvent–sample ratio of 20, with which—even after ~18 h of extraction at room temperature—lower yield of the flavones were obtained than after 15 min of reflux. However, when using a solvent–sample ratio of 100, the yields were observed not to be statistically significantly different (Mann–Whitney *U*-test, $\alpha = 10\%$). This fits with the observation by Cerrato *et al.* [8] that the yield of **lut**, upon extraction with methanol at room temperature, reaches an equilibrium after ~16 h. Considering that the aim was to develop a method requiring as little as possible manpower input per sample, the overnight extraction at room temperature is clearly advantageous over the 15 min extraction by reflux. The extraction, when carried out overnight and using a solvent–sample ratio of 100, gave 95% efficiency. A second and a third extraction yielded 4 and 1%, respectively. A fourth extraction step with additional sonication yielded no additional flavones. Performing a single extraction in this manner saves time, chemicals, and manpower input. As over 99% of the flavonoids are extracted in the first two cycles and s_r (relative standard deviation) values are low, it is an option

to correct the initial outcome by multiplying it by a factor of 100/95 to arrive at the true flavone content. In the tables, uncorrected values are presented. The manual labour input of the whole analytical procedure is minimal, consisting of weighing the previously ground plant material, adding solvent and stir bar, placing the flask on a magnetic stirrer, adding the **i.s.** solution, and syringe-filtering the sample into the HPLC vial.

2.3.2. Chromatography

The validation was performed on a traditional HPLC column. The solvents used with this 5 μm -particle size RP-HPLC 250 mm $\times$ 4.6 mm column were: (i) an aqueous buffer of pH 3 (solvent A) [9], and (ii) methanol (solvent B). The detection wavelength was selected to be 345 nm in view of the absorbance maxima of **ldg** (340 nm), **lmg** (350 nm), and **lut** (350 nm). No sample preparation other than filtration proved necessary (Fig. 1, top). The total run time, including equilibration, was 80 min, and 35.9 mL of methanol were needed. From the results of the precision experiment, retention times were observed to be precise, varying at most 0.4% over a period of 5 days. Within one day, the s_r values of the retention times varied from 0.004 to 0.05%.

The use of a 1.8 μm -particle size UHPLC 50 mm $\times$ 3.0 mm column saves time and solvents. The UHPLC column could be run with a flow of 0.9 mL min⁻¹ on a conventional HPLC system after minor adaption of the hardware and tubing, and changing the B solvent to acetonitrile (Fig. 1, bottom). This shortened the run time to 5 min, and only 1.4 mL of acetonitrile was needed. The resolution between **ldg** and **lmg** ($R_s = 5.4$) was lower than on the HPLC column ($R_s = 9.2$), but still more than sufficient for reliable integrations.

Although in absolute terms the retention time variation was lower on the UHPLC column, relatively (*i.e.*, precision), they were observed to be 25 to 180 $\times$ (**i.s.** and **ldg** cases, respectively) higher than with the 5 μm -particle size HPLC column. However, never did this influence the quantitation (Table 2.4). The higher relative retention time variation is likely caused by working near the maximum pressure of the HPLC pump (400 bar) and, additionally, by the much lower absolute retention times (**ldg**: varying from 0.85 to 0.93 min, and varying from 33.67 to 33.81 min for the 1.8 and 5 μm -particle size columns, respectively). This was supported by performing some trial analyses on a true UHPLC system. After increasing the flow to 1.4 mL min⁻¹ and adapting the gradient (max. pressure 525 bar), the run time was shortened to 2 min and the retention time variability was reduced to 0.05–0.73% (data not shown).

Figure 2.1. Top: HPLC profile of *R. luteola* H extract on 5 μm -particle size 250 mm $\times$ 4.6 mm HPLC column. Column temperature was 40 $^{\circ}\text{C}$ and injected volume was 20 μL . Identity of the main peaks is indicated: luteolin-7,3'-di-*O*-glucoside (luteolin diglucoside, **ldg**), luteolin-7-*O*-glucoside (luteolin monoglucoside, **img**), luteolin (**lut**) and chrysin (internal standard, **i.s.**). Bottom: Analysis of *R. luteola* H on 1.8 μm -particle size 50 mm $\times$ 3.0 mm UHPLC column. Column temperature was 35 $^{\circ}\text{C}$ and injected volume was 2 μL . Top and bottom: Detection at 345 nm.

2.3.3. Accuracy

The accuracy of the method was determined by an absolute recovery (spiking) experiment at three levels. Namely, by adding roughly 50%, 100%, and 140% of what is expected in the plant (Table 2.1). The s_r values of the results are equivalent to those of the usual procedure (Table 2.4), except for the low spiking level. The average recovery was not statistically different from 100% (assuming normal distribution of the results and considering $2s$), indicating good accuracy. To check for any constant zero-order losses due to adsorption on glass and filters which cannot be detected with spiking experiments, relative recovery experiments at three different concentrations were carried out. The stability of the compounds under the extraction conditions was simultaneously evaluated (Table 2.1). At all three concentrations, the relative recovery of the three flavonoids was not statistically different from 100% (assuming normal distribution of the results and considering $2s$), showing that—as expected—the extraction procedure is mild and that any losses due to sample manipulation, filtration, or adsorption to glass are negligible.

Table 2.1. Relative and absolute recoveries ^a of **ldg**, **lmg**, and **lut** ($n = 3$; sample = *R. luteola* H).

Level	Relative (%) ^{b,c}			Absolute (%) ^{b,d}		
	ldg	lmg	lut	ldg	lmg	lut
Low	(102 ± 2), 2	(99 ± 2), 2	(100 ± 3), 3	(99 ± 8), 8	(103 ± 7), 7	(106 ± 15), 14
Medium	(101.0 ± 0.7), 0.7	(99.7 ± 0.2), 0.2	(101 ± 1), 1	(98 ± 6), 6	(101 ± 6), 6	(107 ± 8), 7
High	(101 ± 2), 2	(99 ± 1), 1	(101 ± 1), 1	(98.7 ± 0.9), 1.0	(99.9 ± 0.2), 0.2	(102 ± 4), 4

^a Relative recovery is based on subjecting an extract corresponding to 100, 200, or 300 mg of plant material anew to the entire analytical procedure; absolute recovery is based on three spiking experiments at 50, 110, and 140%.

^b Expressed as (average ± standard deviation), s_r (relative standard deviation).

^c The (relative) recovery of **ldg**, **lmg**, and **lut** due to the syringe-filtration also ranged between 99 and 102%, with s_r values ranging between 0.1 and 2.6%.

^d Low level: $n = 6$.

2.3.4. Identification and peak purity

The identity of the three main flavonoids of *R. luteola* was confirmed to be **ldg**, **img**, and **lut** by having identical retention times and on-line UV–vis absorption spectra to those of authentic reference substances, and on-line mass spectrometric experiments (see section **SI A.1.2**).

Peak purity analysis (see section **SI A.1.2**) by LC–MS and LC–UV showed there is no significant co-elution of minor compounds absorbing at 345 nm of *R. luteola* and the **ldg**, **img**, **lut**, and **i.s.** peaks (see section **SI A.2.1**) on either the HPLC or the UHPLC column. Thus, the peak areas on both columns at 345 nm are representative for **ldg**, **img**, **lut**, and **i.s.**

2.3.5. Calibration curves and limits of detection and quantitation

Internal standardisation was the quantitation method selected. Out of four potential internal standards [3',4'-dihydroxyflavone, **lut** tetramethylether, chrysin (5,7-dihydroxyflavone) and acacetin (apigenin-4'-methylether)], chrysin was selected based on stability issues, price considerations (€ 0.01 assay⁻¹), and retention time, in spite of its relatively low absorbance at 345 nm.

The relative response factors (RRFs, slope of $A_x/A_{i.s.}$ plotted vs. $w_x/w_{i.s.}$) of **ldg**, **img**, and **lut** were determined and the linearity of the concentration of the solutions vs. area of the peaks was checked. The RRFs were 1.10 (**ldg**), 1.51 (**img**), and 2.37 (**lut**) with the HPLC column and 1.13 (**ldg**), 1.58 (**img**), and 2.29 (**lut**) with the UHPLC column. The areas of the peaks were observed to linearly correlate with the concentrations of the solutions over the range of concentrations studied (Table 2.2). These ranges are satisfactory for the quantitation of the compounds of interest at the concentrations in which they are present in *R. luteola* H and other samples.

Table 2.2. HPLC calibration curves and linearity of **ldg**, **img**, and **lut**. **Ldg** and **img**: Five point-curves; **Lut**: Seven point-curves.

Compound [x]	HPLC column ^a [x] (μg mL ⁻¹) vs. A _x (R ²)	UHPLC column ^a [x] (μg mL ⁻¹) vs. A _x (R ²)
ldg	y = 46,517x + 12,410 (0.9995)	y = 4.9919x + 0.1505 (0.9998)
img	y = 65,346x – 19,906 (0.9999)	y = 7.044x + 7.8141 (0.9998)
lut	y = 101,383x + 89,417 (0.9998)	y = 10.249x – 2.0982 (0.9992)

^a **ldg**, **img**, and **lut**: Each case, n = 1.

The limits of detection (LOD) and quantitation (LOQ) of the method were determined based on the slopes of the calibration curves and the *s* of the baseline noise of analyses of analytical blanks, and on the signal-to-noise ratio of injections of highly diluted solutions (Table 2.3). For more details on the determination of the LOD and LOQ values, see Appendix A. Assuming normal distribution of the results and considering both the *s* values of the results and, in this case, the uncertainty of the LOD values estimated based on the signal-to-noise ratio, the LOD values using the HPLC column are the same, regardless the procedure used for their estimation. Similarly, using the UHPLC column, except for **ldg**, the LOD values are also the same. Moreover, the method is more sensitive when using the UHPLC column than when using the HPLC column (Table 2.3).

Table 2.3. LOD and LOQ values of **ldg**, **img**, and **lut** using both HPLC and UHPLC columns, based on: (i) the slopes of the calibration curves and the *s* of the baseline noise of analyses of analytical blanks; and (ii) based on the signal-to-noise ratio.

Compound	LOD $\pm s$ (ng)	LOQ $\pm s$ (ng)	LOD (ng)
HPLC column (injection volume of 20 μ L)			
	i (n = 5)		ii (n = 1)
ldg	1.1 $\pm$ 0.3	3 $\pm$ 1	0.9
img	0.7 $\pm$ 0.2	2.2 $\pm$ 0.7	0.8
lut	0.25 $\pm$ 0.08	0.8 $\pm$ 0.2	0.4
UHPLC column (injection volume of 2 μ L)			
	i (n = 6)		ii (n = 1)
ldg	0.29 $\pm$ 0.05	0.9 $\pm$ 0.1	0.1
img	0.21 $\pm$ 0.04	0.6 $\pm$ 0.1	0.2
lut	0.14 $\pm$ 0.02	0.41 $\pm$ 0.07	0.1

The LOD and LOQ values of this method are low compared with those of earlier methods. Plazonić *et al.* and Kočevár *et al.* [10, 11] determined LOD and LOQ values for **img** and **lut** that are 6–20 $\times$ higher than the values reported here, when using the HPLC column, and 20–50 $\times$ higher than the values reported here, when using the UHPLC column. Gaspar *et al.* [12] determined LOD values <7 ng and LOQ values $\leq$ 11 ng for **ldg**, **img**, and **lut**, which are 6–12 $\times$ (LOD) and 4–10 $\times$ (LOQ) higher than the values reported here, using the HPLC column, and are 20–60 $\times$ (LOD) and 10–20 $\times$ (LOQ) higher than the values reported here, using the UHPLC column. The baselines of the UV detectors used in the work reported here were highly stable. On average, the *s* values of the baseline noise over large time intervals were only 0.04 ± 0.01 mAU (HPLC column) and 0.09 ± 0.01 mAU (UHPLC column) (see Appendix A for details). Baselines in earlier studies were possibly less stable. In addition, different methods were used to estimate those limits, as Plazonić *et al.* [10] used the residual *s* of the regression line and slope, and Kočevár *et al.* and Gaspar *et al.* [11, 12] based it on the signal-to-noise ratio.

2.3.6. Stability of samples

The stability of the samples ready for HPLC analysis was evaluated to verify whether the residence time in the autosampler would influence the outcome. This could, for example, be the case when analysing a large sample set. Moreover, it was evaluated whether the samples are stable upon storage at 4 $^{\circ}$ C for 3 days and at -20 $^{\circ}$ C for 15 days. Upon storage of the samples ready for HPLC analysis, the area of the chrysin peak was not statistically significantly different than that of the samples analysed shortly after the work-up procedure, indicating that neither precipitation of the **i.s.** nor sample concentration took place. Assuming normal distribution of the results and considering the *s*, the amounts of the flavones remained the same in all analysed sets of vials. Thus, the compounds in samples are stable upon storage under the aforementioned conditions.

2.3.7. Quantitative results and precision

The concentrations of **ldg**, **lmg**, and **lut** in *R. luteola* H, as determined on the 5 μm -particle size column, were 3.5 mg g^{-1} , 6.5 mg g^{-1} , and 1.0 mg g^{-1} , respectively. For the 1.8 μm -particle size column, these figures were 3.6 mg g^{-1} , 6.5 mg g^{-1} , and 1.0 mg g^{-1} (Table 2.4). These results are not statistically significantly different (**ldg**). This indicates that the HPLC system copes well with the small UHPLC injection volumes (2 μL). Furthermore, major advantages of the use of the UHPLC column are, of course, the 16 $\times$ faster analysis and the 25 $\times$ lower solvent consumption.

The precision of the entire method on the HPLC column was evaluated in terms of repeatability and intermediate precision. The s_r values for the whole analytical procedure were $\sim 5\%$ with the HPLC column, except for series 3 (Table 2.4). Still, the larger s_r values of series 3 did not influence the average outcome. The type of stirrer used (15-point, which heats itself up by 3 $^{\circ}\text{C}$ during operation, for series 1 and 3 vs. individual ones for series 2) does not seem to have any influence on the outcome, which suggests robustness of the method regarding small temperature variations. The s_r values of the quantitations carried out with the UHPLC column were 7% (**ldg**), 7% (**lmg**), and 10% (**lut**) (Table 2.4). These values are comparable to those of the analyses of the same samples carried out with the HPLC column, 7% (**ldg**), 7% (**lmg**), and 11% (**lut**) (footnote *d*, Table 2.4). Kočever *et al.* and Plazonić *et al.* [11] [10] reported s_r values for the repeatability and intermediate precision of the areas of **lmg** and **lut** peaks ranging from 0.9 to 4.3%, respectively, when analysing standard solutions. When analysing extracts, Kočever *et al.* [11] observed slightly higher s_r values, up to 5.4% for **lmg**. Gaspar *et al.* [12] evaluated the repeatability of the whole analytical procedure using two samples of *R. luteola* of extreme (high and low) contents of flavonoids. The observed average s_r values were 5.1% (**ldg**), 3.9% (**lmg**), and 4.9% (**lut**). The s_r values reported here are slightly higher than those of the aforementioned works. Possible reasons include the different experimental designs and data processing. Still, the proposed method determines the combined content of **ldg**, **lmg**, and **lut** with $s_r < 6.5\%$, which is fine for the intended purpose.

Table 2.4. Precision of the entire method, and that of the analyses using the UHPLC column, for the quantitation of **ldg**, **lmg**, and **lut** in *Reseda luteola* (sample = *R. luteola* H).

Series	Compound	HPLC column	UHPLC column
		Concentration (mg g ⁻¹) ^{a,b} (<i>s_r</i> in %)	Concentration (mg g ⁻¹) ^{c,d} (<i>s_r</i> in %)
1	ldg	3.6 (5.3)	3.6 (7.1)
	lmg	6.7 (5.2)	6.5 (7.2)
	lut	1.01 (5.8)	1.0 (10.4)
2	ldg	3.5 (3.8)	3.6 (6.2)
	lmg	6.5 (3.6)	6.5 (6.5)
	lut	1.00 (4.9)	1.01 (9.0)
3	ldg	3.5 (6.1)	3.6 (8.8)
	lmg	6.4 (6.5)	6.5 (8.5)
	lut	1.01 (8.5)	1.0 (11.5)

^a Extractions were carried out on three non-consecutive days (each day, *n* = 7).

^b Series 3: *s* of the quantitation of **ldg**, **lmg**, and **lut** were 0.2, 0.4 and 0.09, respectively; thus, the *s_r* of their combined amounts was 6.3%.

^c Extractions were carried out once (*n* = 8); then, stored samples were analysed in three series (see section 2.2.7).

^d Analysis of same samples (*n* = 8) using the HPLC column: Amount = 3.6 mg g⁻¹, *s_r* = 6.8% (**ldg**); 6.5 mg g⁻¹, 7.2% (**lmg**); and 1.0 mg g⁻¹, 10.7% (**lut**); for that, the following RRFs (determined anew for HPLC column, details not presented) were used: 1.09 (**ldg**); 1.51 (**lmg**); and 2.28 (**lut**).

2.4. Conclusion

The analytical method reported here is suitable for the quantitation of **ldg**, **lmg**, and **lut** in the aerial parts of *R. luteola*. According to the extensive validation (extraction efficiency, recovery, accuracy, precision, sensitivity, peak purity, sample stability), this is accomplished accurately. The precision reported (<6.5%) is adequate for the purpose. The industrial partner of the project reported, in daily practice, a standard deviation <4% when analysing over 120 *R. luteola* samples using this method. The method meets the need for very little manpower input per sample and uses standard laboratory equipment. Although the analytical method was developed for the quantitation of flavones in *R. luteola*, it is expected that, after additional validation, it could be applied on other flavone-rich plant materials too.

Two types of columns were used: For the validation, a traditional 4.6 mm column with 5 µm particles; then, the chromatographic step was speeded-up by the use of a short UHPLC column, while still using a conventional HPLC system. The use of the UHPLC column reduced the run time by a factor of 16 and the organic solvent consumption by a factor of 26. Retention times were relatively less reproducible with the UHPLC column than with the HPLC column. This could be remedied by either lowering the flow to 0.6 mL min⁻¹ or using a true UHPLC pump. Cross-validation showed that the quantitation of **ldg**, **lmg**, and **lut** was not statistically significantly different, with comparable precision. Moreover, the method is more sensitive with the UHPLC column than with the HPLC column. Using a UHPLC column on a conventional HPLC system is, thus, a way of modernising HPLC-based (phytochemical) analyses inexpensively.

2.5. Supplementary material

Appendix A contains information supplementary to that in this chapter. Sections **material and methods**, **results and discussion**, and **reference** are available.

2.6. References

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Chapter 3

Spectrophotometric comparison of the content of chlorophylls in weld

The content of this chapter is largely that of the following paper:

Villela A, Derksen GCH, Zuilhof H, van Beek TA. Spectrophotometric comparison of the content of chlorophylls in weld (*Reseda luteola*). Anal Methods 2011; 3(6): 1424–7.

An analytical method for the comparison of the content of chlorophylls and their structurally similar breakdown products in weld is described.

3.1. Introduction, material and methods, results and discussion, and conclusion

The greenish hue to which reference is made in the introductory chapter of this thesis could be caused by one of the two factors elaborated upon in this paragraph. First, it could be an intrinsic property of the use of the flavone-based dye; the colours of textiles dyed with mordant dyes are influenced by the metal salt used as mordant [1]. Second, chlorophylls *a* and *b* (the main and intensely green pigments of terrestrial plants) are present in the aerial parts of weld and it was hypothesised that they could bind to metal-treated fibres due to Lewis acid–Lewis base interactions. Based on this hypothesis, quantitation of chlorophylls in weld samples was of interest as part of the characterization of the raw material for production of the dye-extract. With this as background, the development of a simple analytical method to compare the relative amounts of chlorophylls and their breakdown products in different weld samples was aimed at. Because of the expected large number of samples, low manpower input was of paramount importance.

A first consideration in such a development is the observation that chlorophylls are unstable and can be broken down, *e.g.*, by enzymatic activity, light exposure or under acidic conditions [2, 3]. As harvested weld is dried under fairly rough conditions, it not only contains chlorophylls *a* and *b* (**chls** *a* and *b*), but also their breakdown products, including pheophytins (magnesium atom replaced by two hydrogen atoms), chlorophyllides (no phytol chain) and pheophorbides (magnesium atom replaced by two hydrogen atoms and no phytol chain) [4, 5]. To minimally modify the composition of **chls** and breakdown products of dried weld plant material, precautions should be taken during the analysis. The extraction needs to be fast, carried out in dim light, and use cold solvents of neutral pH [2, 3, 5]. The quantitation step either immediately follows the extract preparation [2, 3] or the compounds should be stable in the extract [5].

Chls can be detected by spectrophotometry, spectrofluorimetry or mass spectrometry [4, 6]. Whereas spectrofluorimetry is more sensitive than spectrophotometry, quenching of fluorescence takes place in highly concentrated solutions [7] and spectrophotometry (without a preceding HPLC step) is simpler and faster to use. In addition, it is expected that only **chls** and some of their breakdown products in weld absorb light at wavelengths >630 nm.

Chls *a* and *b*, chlorophyllides *a* and *b*, pheophytins *a* and *b*, and pheophorbides *a* and *b* display two main absorption bands: The blue one (Soret), at wavelengths <500 nm and the red one, between 640 and 670 nm [5]. Despite the main blue bands being more intense than red ones, the latter must be used due to increased selectivity. The red bands of **chls** and chlorophyllides are centered at nearly the same wavelength [5, 8]. Those of pheophytins and pheophorbides are 4 to 11 nm red-shifted and the molar absorption coefficients are about 40% smaller relative to **chls** [5, 8]. Thus, the red absorption band of extracts of weld (Figure SI B.1, appendix B) comprises the red bands of multiple compounds. The absorbance at its maximum was used to compare different samples.¹

¹ Although not done during this work, it could be tested whether weld samples containing different **chls**/chlorophyllides-to-“pheopigments” ratios could be compared after conversion of **chls** and chlorophyllides to corresponding “pheopigments” using HCl (see section “Pheophytinization under Defined Conditions”—and two preceding sections—of ref. 3 for elaboration on the conversion of **chls** to pheophytins using the acid).

The method for analysing of **chls** and their breakdown products in weld was evaluated. Simultaneous extraction of 20 samples followed by sequential absorbance measurements was observed to be unsuitable, as the absorbance of identical samples gradually increased going from sample 1 to sample 20 (appendix B). Absorbances of the extracts of samples that were put to steep (soak) for 2 h (4 °C) after dynamic extraction with acetone were higher than of those in which the measurements immediately followed the extraction (entries 1 through 4 of Table 3.1), indicating an on-going extraction. The 5 min centrifugation step after the extraction also increased the yield (appendix B). Thus, for a suitable comparison of the content of **chls** and breakdown products in weld samples, the contact time between sample and solvent has to remain constant.² Centrifugation followed by transfer of the supernatant to the cuvette without filtration was unsuitable. Results suggested scattering of light due to turbidity of the extracts (data not shown),³ indicating the need for sample filtration.

The yield of the 3 min vortexing extraction with acetone at room temperature is about 50% of what can be extracted after 24 h with this—for chlorophyll—not very efficient solvent (entries 5 and 7). Regarding the choice of solvent: Absolute ethanol extracts about 75% of what the superior DMF⁴ extracts but is more efficient than acetone (entries 7, 9, and 12). In addition, among the tested extraction procedures, vortexing is the fastest and probably mildest, and ethanol has the advantage of low toxicity and being environmentally friendly. Finally, the presence of water in the solvent leads to formation of hydroxylated allomers [11]. Also, water in the solvent leads to a red-shift of the main absorption bands of **chls** and reduction of their molar absorption coefficients [3].⁵ Thus, the final analytical procedure consists of a 3 min extraction with absolute ethanol by vortexing at room temperature, followed by syringe-filtration of the extract and absorbance measurement.⁶

² No difference in extraction efficiency among weld samples from different plant parts is expected. This is indicated by the linearity of the relation seen ahead (Figure 3.1 and preceding discussion).

³ Optical clarity of solutions was checked at 700 nm [9].

⁴ Toxicity of DMF has hampered its recommendation for routine work [5]. DMF is also viscous and has a “faint amine odour” [10].

⁵ Ethanol absolute is: (i) 3× more expensive than ethanol 96%, but still ~10% cheaper than methanol and (ii) highly hygroscopic [10].

⁶ Final analytical procedure: (125.0 ± 0.9) mg of sample are weighed in centrifuge tube (10 mL, Oak Ridge, PPCO, Thermo Scientific, Rochester/USA); 5 mL of absolute ethanol added via a 10 mL graduated cylinder; 3 min vortexing (MS2 Minishaker, IKA Works—Wilmington/USA; 2,500 min⁻¹) of each sample individually; syringe-filtration (5 mL PP syringe; syringe-filter, PTFE, 13 mm, 0.45 µm, Grace Davison Discovery Science) of extracts into either a 2 mm-pathlength disposable plastic cuvette (Eppendorf) or a 10 mm-pathlength disposable plastic cuvette (Greiner Bio-One); use of disposable plastic cuvette caps; absorbance of subsequent extracts was measured at 3 min intervals (Cary 100 Scan UV–Visible spectrophotometer—Varian/Australia; double beam mode; λs: 665 nm and 750 nm; spectral bandwidth: 1.5 nm; signal averaging time: 1 s; light beam: 10 mm high, starting at 15 mm from bottom of cuvette holder). Note: (i) Centrifuge tube: held in place during extraction with supports and clamps. (ii) First drops of syringe-filtered solutions: discarded to prevent possible changes in concentration. (iii) The 2 mm-pathlength plastic cuvette needs to be lifted up with the lever of the cuvette holder of the spectrophotometer and to be filled over the 500 µL-mark, as its optical surface is only 3 mm high and starts 7 mm from the base. (iv) Procedures were conducted under reduced light. (v) Possibility of further simplification by using an automatic pipette for the addition of the solvent—most likely with a concomitant gain in precision—and a vortex mixer equipped with a tube holder.

Table 3.1. Absorbance of extracts of weld samples due to chlorophylls and their structurally similar breakdown products obtained with different solvents and methods of extraction, using three types of cuvettes.^{a,b}

Entry	Plant material	n	Extraction procedure; ^c sample treatment and series of measurements	Solvent ^d	Absorbance (AU) and <i>S_r</i> (%) ^c
10 mm-pathlength quartz cuvette					
1	B	4	3 min vortexing; 5 min centrifugation	Acetone	0.146 (2.6)
2	B	4	3 min vortexing; 5 min centrifugation; 2 h (4 °C)	Acetone	0.161 (2.3)
3	B	4	10 min sonication	Acetone	0.162 (1.2)
4	B	4	10 min sonication; 2 h (4 °C)	Acetone	0.177 (3.0)
5	B	4	16 h shaking	Acetone	0.241 (0.7)
6	B	4	16 h shaking; 2 h (4 °C)	Acetone	0.227 (1.5)
7	B	3	3 min vortexing	Acetone	0.125 (4.9)
8	B	3	3 min vortexing	MeOH–H ₂ O (9:1)	0.150 (6.0)
9	B	2	3 min vortexing	Abs EtOH	0.163 (0.5)
10	B	3	3 min vortexing	EtOH 96%	0.168 (0.7)
11	B	3	3 min vortexing	MeOH	0.183 (4.8)
12	B	3	3 min vortexing	DMF	0.215 (0.6)
2 mm-pathlength plastic cuvette					
13	A	1	3 min vortexing; day 1	Abs EtOH	0.022
14	A	3	3 min vortexing; day 7 (morning)	Abs EtOH	0.023 (3.5)
15	A	4	3 min vortexing; day 7 (afternoon)	Abs EtOH	0.021 (2.5)
16	E	1	3 min vortexing; day 1	Abs EtOH	0.128
17	E	4	3 min vortexing; day 7 (morning)	Abs EtOH	0.129 (3.3)
18	E	4	3 min vortexing; day 7 (afternoon)	Abs EtOH	0.120 (1.4)
19	F	1	3 min vortexing; day 1	Abs EtOH	0.424
20	F	4	3 min vortexing; day 7 (morning)	Abs EtOH	0.420 (5.0)
21	F	3	3 min vortexing; day 7 (afternoon)	Abs EtOH	0.427 (2.8)
22	G	1	3 min vortexing; day 1	Abs EtOH	0.982
23	G	4	3 min vortexing; day 7 (morning)	Abs EtOH	0.936 (4.5)
24	G	4	3 min vortexing; day 7 (afternoon)	Abs EtOH	0.931 (2.4)
25	H	1	3 min vortexing; day 1	Abs EtOH	1.048
26	H	4	3 min vortexing; day 7 (morning)	Abs EtOH	0.976 (7.4)
27	H	4	3 min vortexing; day 7 (afternoon)	Abs EtOH	0.957 (5.8)
1 mm-pathlength quartz cuvette					
28	A	3	3 min vortexing; day 2 (n = 1) and day 9 (n = 2)	DMF	0.015 (2.6)
29	E	3	3 min vortexing; day 2 (n = 1) and day 9 (n = 2)	DMF	0.108 (2.1)
30	F	4	3 min vortexing; day 2 (n = 1), day 9 (n = 2), and day 9 (n = 1)	DMF	0.363 (7.3)
31	G	3	3 min vortexing; day 2 (n = 1) and day 9 (n = 2)	DMF	0.695 (1.1)
32	H	3	3 min vortexing; day 2 (n = 1) and day 9 (n = 2)	DMF	0.765 (0.2)

^a λ of detection: Entries 1 through 12 and 28 through 32, absorbance at 664 nm minus absorbance at 750 nm (664 – 750 nm); entries 13 through 27, 665 – 750 nm. Note: 1) Subtraction of absorbance at 750 nm needed due to

large cuvette-to-cuvette variation of 2 mm-pathlength plastic cuvettes (range of readings at 750 nm: -53–89 mAU); 2) Red-absorption maxima: Abs EtOH, 665 nm; other solvents, 664 nm.

^b **A, B, E, F, G, and H** are the codes of (dried and ground-sieved) weld samples in increasing concentration of **chls** and breakdown products. Note: 1) Differences among samples include cultivar and plant parts used;⁷ 2) Results of analyses of samples **C** and **D** are displayed in appendix B.

^c Always at room temperature and under reduced light.

^d MeOH = methanol, Abs EtOH = absolute ethanol, and DMF = *N,N*-dimethylformamide.

^e Average absorbance (s_r = relative standard deviation).

The repeatability {“precision under the same operating conditions over a short interval of time” [12]} of the method was assessed based on the relative standard deviation (s_r) of analyses of weld samples **A, E, F, G, and H**. Literature data from a study on the extraction of pigments from phytoplankton showed sonication in DMF to be a suitable reference method, as that method displayed excellent s_r values (0.4–6.0%, depending on the pigment) and fulfils nearly all criteria for a suitable extraction technique, including precision [5]. The s_r values of the assay presented here are typically 2–5%, and in all cases <7.5% (entries 13 through 27). Thus, the method displays good repeatability, more than meeting the demands for the intended purpose.

The results of the two series of measurements of each weld sample were compared (entries 14 vs. 15, 17 vs. 18, 20 vs. 21, 23 vs. 24, and 26 vs. 27). In the cases of samples **F, G, and H**, the difference was $\leq 2\%$. This is smaller than that observed for samples **A** (13%) and **E** (7%). However, the absolute differences in absorbance of these samples are very low (**A**: 2 mAU and **E**: 9 mAU). The s_r values are higher than those of samples **F, G, and H** due to the low absolute absorbance of the extracts of samples **A** and **E**. If increased precision is required for samples with absorbance ≤ 190 mAU, 10 mm-pathlength plastic cuvettes should be used (appendix B).

Based on the analysis of 81 weld samples, with the highest absorbance being 1.165 AU (results not shown), sample **H** is expected to be near the upper practically occurring limit of concentration of **chls** and breakdown products in weld samples. Because the absorbance of the

⁷ Dried weld samples:

A: Part of the plants: stems; cultivar: **G**; place of growth: Zeeland, SW of the Netherlands; harvested in: 2008; pore size of sieve used during grinding-sieving: 0.25 mm;

B: aerial parts; **H**; Groningen, N of the Netherlands; 2007; plants were placed outdoors during daytimes and indoors during night times for ~10 days; 0.25 mm;

C: stems; **G**; Zeeland; 2009; 0.25, 0.50, or 0.75 mm. Note: grown in another field as **A**;

D: reproductive parts, leaves, and upper parts of the stems; rest, as **B**;

E: leaves and reproductive parts; **G**; Zeeland; 2008; 0.25 mm;

F: leaves and reproductive parts; **G**; Noord Brabant, S of the Netherlands; 2010; 0.25 mm;

G: leaves and reproductive parts; **G**; Zeeland; 2010; 0.2 mm;

H: leaves; **G**; Noord Brabant; 2010; 0.2 mm. Note: harvested 3 weeks earlier than **F**.

Note: Samples **A, C, and E** though **H** were supplied by the industrial partner of the project. The general drying procedure used by them was: (root-removed) plants were placed indoors (roughly 30 °C; dark) for 2–2.5 weeks, on average.

extracts of sample **H** is ≤ 1.0 AU (entries 25 through 27),⁸ the method covers the entire range of concentrations of **chls** in weld samples.

Chls are situated inside the thylakoids of the chloroplasts of plant tissues, bound to apoproteins through different types of bonding, including Lewis acid–Lewis base interactions [2, 5]. DMF is a better solvent than absolute ethanol for the extraction of **chls** and their breakdown products (entries 9 and 12). Furthermore, there is only ~10% difference between the absorbance of the extracts of weld in acetone obtained by shaking overnight (entry 5), assumed to fully extract the **chls** and their breakdown products,⁹ and that of the extracts in DMF obtained by vortexing for 3 min (entry 12). Thus, to ascertain whether extraction with absolute ethanol was selective regarding the “types” of **chls** and plant parts being extracted, the extraction of different samples of weld using absolute ethanol was compared with that using DMF (Figure 3.1).

⁸ If sample **H** would be ground–sieved using a 0.25 mm sieve, the absorbance of its extracts would be lower than that seen in Table 3.1 (see also entries 10–12 of Table SI B.1, appendix B).

⁹ Extraction does not seem to continue upon steeping (2 h/4 °C) (entries 5 and 6). This is not the case for the other extraction procedures (entries 1 through 4). An extraction efficiency experiment would be needed to check this assumption.

Figure 3.1. Correlation between absorbance of ethanolic and DMF extracts of weld. Average absorbances are plotted; error bars = $1 \times$ standard deviation. Ethanolic extracts: 2 mm-pathlength plastic cuvette; $n = 8$ (A and F) and $n = 9$ (E, G, and H). DMF extracts: 1 mm-pathlength quartz cuvette; $n = 3$ (A, E, G, and H) and $n = 4$ (F).

The linear relation between the extracted amounts of **chls** and breakdown products by absolute ethanol and by DMF indicates that the extraction with absolute ethanol is not selective and, thus, that absolute ethanol can be suitably used as solvent for the comparative method described here. Additionally, it suggests that the ethanolic solution is not saturated throughout the possible range of concentration of **chls** and breakdown products in weld samples.

The method at hand rapidly compares the total absorbance of porphyrin ring-containing pigments (**chls** and their structurally similar breakdown products) in different weld samples. It is expected to minimally modify the composition of **chls** and breakdown products of samples and displays acceptable precision. Analysis of forty samples—including data processing—could be carried out in one working day, requiring a total amount of 200 mL of ethanol (5 mL per sample). Moreover, the method covers the entire range of occurring concentrations, uses ethanol as a mild, non-toxic extraction solvent—that is not selective regarding the “types” of **chls** and plant tissues being extracted—and is simple. Finally, although developed for the comparison of the chlorophyll content in weld, it is expected that—after additional validation—it could be applied on other plant materials too.

3.2. Supplementary material

Appendix B contains information supplementary to that in this chapter.

3.3. References

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Chapter 4

Photo-stability of the dye of weld in presence of aluminium ions

The content of this chapter is largely that of the following paper:

Villela A, van Vuuren MSA, Willemen HM, Derksen GCH, van Beek TA. Photo-stability of a flavonoid dye in presence of aluminium ions. *Dyes Pigments* 2019; 162: 222–31

The main colouring compounds of the dye plant weld (*Reseda luteola* L.) are the flavones luteolin (**lut**), **lut**-7-*O*-glucoside and **lut**-7,3'-*O*-diglucoside. Alum (an aluminium salt)-premordanted wool dyed with weld leads to yellow colours that are of low resistance to light. The photo-stability of **lut** in aerated methanol–water 8:2 (v/v) solution upon irradiation with light above 300 nm was studied at different **lut**–Al³⁺ ratios. Experiments using extracts of weld to dye wool premordanted with increasing quantities of aluminium salts were also carried out. The photo-stability of **lut** in the polar protic solvent and the photo-resistance (light-fastness) of the colour of weld-dyed wool decrease with increasing concentrations of aluminium ions. Thus, the lower the [Al³⁺] used for mordanting the wool, the more light-fast its colour. Lowering the [Al³⁺] appears to have no negative influence on the wash-fastness of the colour. As the gain in light-fastness by the use of low [Al³⁺] to premordant the wool is not extensive, however, this does not seem to be a way to meet today's requirement of light-fastness of the colours of dyed textiles by itself. Nevertheless, it may be part of a broader strategy to address the need for increased light-fastness of the colour of wool dyed with weld. Implementation of this approach by dyers is expected to clarify whether it results in benefits for textile dyeing practice.

4.1. Introduction

Varying the metal ion mordant changes the colour of the dyed textile and has a major influence on its light-fastness [1, 2]. Increasing concentrations of aluminium ion lead to intensified colours of solutions of **lut** and the flavonols—3-hydroxyflavones—kaempferol and quercetin, as seen by the change of their UV–vis absorption spectra [3–5]. However, members of the flavones and flavonols classes of flavonoids have been reported to display diminished photo-stability in the presence of Al^{3+} [6, 7]. Thus, the influence of increasing concentrations of aluminium ion on the photo-stability of the dye of weld was studied to address the issue of whether a compromise between the obtainable colours and their photo-resistance would be a way to meet today's requirement of light-fastness of the colours of textiles. The photo-stability of **lut** in presence of Al^{3+} at different **lut**– Al^{3+} ratios was studied in solution. Experiments using extracts of weld to dye wool premordanted with increasing quantities of aluminium salts were also carried out.

Glycosylation may confer stability to flavonoid aglycones [8], [9] (chapter 42). For example, the antioxidant activity of flavonoids is diminished by glycosylation [8], [10] (chapter 5). Weld contains an enzyme that breaks the glycosidic bonds of its flavone glucosides¹ [11]. **Lut**, **img** and **ldg** are obtained if this glycosidase is inactivated before extracting the flavonoids. In contrast, only **lut** is obtained if the enzyme is not inactivated. Thus, there is an issue of whether the glycosidase should be inactivated before extracting the flavonoids of weld in order to obtain the most photo-stable dye. Experiments were carried out in the context of such an issue. The relative photo-stability of **lut**, **img** and **ldg** in solution was studied, and results of the colours of alum-mordanted wool dyed with them are presented.

¹ Although there might be enzymes—and not only one enzyme—breaking the glycosidic bonds, the singular form of the word is used in this thesis.

4.2. Material and methods

The material and methods of the work is summarised in this section. Readers interested in further procedures and details are referred to section SI C.1 (appendix C).

4.2.1. Effect of different concentrations of Al^{3+} and glycosylation pattern of lut (aglycone, monoglucoside, and diglucoside) on its photo-stability in solution

The following chemical compounds were used for the preparation of stock and working solutions of the flavones, Al^{3+} , and HNO_3 used for construction of the calibration lines and preparation of the solutions used for the irradiation experiments described: **Lut** (96% by NMR; Indofine Chemical Company, Hillsborough/USA); **lmg** (93% by NMR; Extrasynthese, Genay/France); **ldg** (86% by NMR; Extrasynthese, Genay/France); aluminium nitrate nonahydrate (99.997%; Aldrich Chemistry, St. Louis/USA); nitric acid (65%, extra pure; Merck, Darmstadt/Germany).

Solutions of **lut**, **lmg**, and **ldg**—with and without Al^{3+} —were prepared in aerated methanol–water 8:2 (v/v). Nitric acid was added to all volumetric flasks, except to those containing **lut** and Al^{3+} in a ratio 1:10, because the lowering of the pH was due to the Lewis acid Al^{3+} in this case (rather than to HNO_3 or a combination of both). The apparent pH of all irradiated solutions was 3.6; the term apparent pH is used as the solvent of the solutions was not water, but the methanol–water mixture. Stirred solutions were irradiated at 32 °C in glass cuvettes using a phosphor-coated low pressure mercury vapour lamp, which led to irradiation only with light above 300 nm, most importantly in the 300–420 nm range. The photodecomposition of **lut**, **lmg**, and **ldg**—decrease of [flavone] over time—was monitored by RP-HPLC–UV.

Solutions used for the irradiation experiments were prepared on the day the experiments started, and transferred to the glass cuvettes (3.5 mL; 10 mm light path) in which they were irradiated. This was done without using Pasteur pipettes to prevent possible contamination. Slots for four cuvettes were made on the cover plate of a magnetic stirrer. The distance from the front window of the cuvettes to the centre of the lamp was 2.02 cm. Pictures of the set-up are seen in Figure SI C.1. Calibration solutions and solutions used for irradiation experiments were kept in the dark at room temperature not less than 60 min before use. This was motivated by the fact that flavonoid– Al^{3+} complexation does not reach equilibrium immediately.

Observations reported were made during five irradiation experiments. They are referred to as 1a and 1b (1st irradiation replicate), 2a and 2b (2nd irradiation replicate), and special irradiation experiment. During the first four irradiation experiments, the eight solutions—**lut**_{0.10}, **lut**_{0.10}–**Al**_{0.02}, **lut**_{0.10}–**Al**_{0.10}, **lut**_{0.10}–**Al**_{1.00}, **lut**_{0.05}, **lmg**_{0.05}, **ldg**_{0.05}, and **ldg**_{0.05}–**Al**_{0.05}—were irradiated in duplicate. Solutions **lut**_{0.20} and **lut**_{0.10}–**Al**_{0.99} were irradiated during the special irradiation experiment, with a filter blocking radiation of wavelengths shorter than ~420 nm (420 nm cut-off filter) being placed in front of the cuvette containing **lut**_{0.10}–**Al**_{0.99}. Notes:

- All solutions—flavones, Al^{3+} , and HNO_3 —were prepared anew for use in irradiation experiments 2a and 2b, and special irradiation experiment.
- Code of solutions used: $\text{flavone}_{[\text{flavone}]}-\text{Al}_{[\text{Al}]}$, in which $[\text{flavone}] = [\text{flavone}]_0$, $\text{Al} = \text{Al}^{3+}$, and concentrations are expressed in mM.

During the irradiation experiments, 0.05 mL aliquots of the solutions were collected at $t = 1.0$ h, 2.0 h, 3.0 h, 4.0 h, 8.0 h, and 24.0 h, as irradiations started on one day (**day 1**) and finished on the following one (**day 2**). During the special irradiation experiment, aliquots of the solutions were collected at $t = 2.0$ h, 4.0 h, 8.0 h, and 24.0 h. The photodecomposition of the flavones

over time was quantified by RP-HPLC–UV using peak areas of the 350 nm traces (**lut** and **lmg**) and of the 340 nm traces (**ldg**), and calibration lines. Except for solution **lut**_{0.20}, the [flavone] of all samples—*t*₀ (*t* = 0 h) through *t*₂₄ (*t* = 24 h)—of each irradiated solution was comprised within the range of concentrations used for construction of the calibration lines. A straight line mathematical model was fit through the experimental data {[flavone] (mM) vs. time (min)}. This regression analysis was carried out for each of the solutions irradiated during experiments 1a–2b and special irradiation experiment via the least-squares method.

The photodecomposition of the flavones was described as being of zero-order, in which the rate of reaction equals the rate constant ($d[\text{flavone}]/dt = k$). As the integrated form of the zero-order rate law is $[\text{flavone}] = kt + [\text{flavone}]_0$, the *k* values were obtained from the slope of the equations (*k* = –slope). The relative rates of photodecomposition were calculated through the formula k_j/k_i , in which *i* was **lut** in **lut**_{0.11}, **lut**_{0.10}, or **lut**_{0.05}, and *j* was **lut**, **lmg**, or **ldg** in other solutions. Notes:

- Solution **lut**_{0.20} was used for comparison of the rates of photodecomposition of **lut** in **lut**_{0.20}, **lut**_{0.11} and **lut**_{0.05}. For this purpose, a procedure similar to that outlined above was carried out. Peak areas vs. time were used instead of concentrations vs. time, however, and the slopes of the equations of the best-fit lines ($y = ax + b$) were compared;
- All irradiated solutions contained NO₃[–], originating from HNO₃, Al(NO₃)₃, or from both of them. As the oxidation state of the N-atom of the ion is +5, NO₃[–] may be reduced in different systems/under different conditions [12]. One such condition could be the irradiation with light in the 300–420 nm range used in the irradiation experiments reported here, even with the [NO₃[–]] being ≤3 mM. It does not mean that the ion has necessarily contributed to the photodecomposition of the flavones in the experiments of this study, however. Reasons for this are:
 - If reduction of NO₃[–] took place during the irradiation experiments, methanol may have acted as reducing agent instead of the flavones [12]; methanol was present in solution in quantities far larger than those of the flavones, as it was part of the solvent mixture;
 - The relative rates of decomposition of the flavones (Table 4.2) do not match the relative [NO₃[–]] of the solutions: 1.4 vs. 1.2 (**lut**_{0.10}–Al_{0.02}), 3.2 vs. 1.9 (**lut**_{0.10}–Al_{0.10}), 3.8 vs. 7.8 (**lut**_{0.10}–Al_{1.00}), 1.4 vs. 1.0 (**lmg**_{0.05}), 0.2 vs. 1.0 (**ldg**_{0.05}), and 0.6 vs. 1.4 (**ldg**_{0.05}–Al_{0.05});
 - The photo-stability of **lut** and **ldg** in solution (in presence of NO₃[–]) decreases with increasing [Al³⁺] (Table 4.2). This matches the results of the experiments in which wool was dyed with extracts of weld (experimental details in sections **SI C.1.13** and **SI C.1.15**): The light-fastness of the colours of dyed wool decreases with increasing quantities of Al³⁺ used to premordant the wool (Tables 4.4 and 4.5);
 - In preliminary experiments, the photodecomposition of **lut** in nitric acid-acidified aerated methanol–water 8:2 (v/v) solution was observed to be 35 to 55% slower than in non-acidified solution (section **SI C.2.2**).

Additionally, **lmg** and **ldg** appear to be stable upon overnight heating in presence of NO₃[–] at a concentration of 0.35 mM (sections **SI C.1.12** and **SI C.2.10**).

Spectrophotometric analyses were carried out using quartz cuvettes. On **day 1**, the remainders of the solutions used for irradiation were diluted 5× with aerated methanol–water 8:2 (v/v). On

day 2, t_{24} solutions were diluted and analysed in the same way. In the case of **lut**_{0.20}, t_0 and t_{24} solutions were diluted 10 $\times$.

Calibration solutions and samples used for irradiation experiments 1a—special irradiation experiment were analysed by RP-HPLC–UV undiluted. Analyses were carried out on a Waters system, equipped with C18 5 μ m-particle size column. Further details of the HPLC system and column used are described in publication by Vilella *et al.* [13] (p. 8545, method using the HPLC column). See the referred publication also for information on: Eluent, its flow rate and composition (solvents and gradient); volume of injection; and temperature of the column oven. The photodiode array detector scan range was 245–500 nm.

4.2.2. Effect of increasing quantities of Al^{3+} bound to mordanted wool on the photo-stability of the dye of weld

The following material/chemical compounds were used as wool, mordants, and source of dye: Ready-to-dye wool [Kova Wool Sateen White (part number W110); Whaleys (Bradford), Bradford/United Kingdom]; alum (puriss. *p.a.* grade; Sigma-Aldrich, Steinheim/Germany); aluminium sulphate tetradecahydrate (ViVoChem, Almelo/The Netherlands); tartaric acid (Brenntag, Dordrecht/The Netherlands); sample of the aerial parts of dried and ground weld {described by Vilella *et al.* [13]}; commercial extract of weld (Rubia Yellow; Rubia Pigmenta Naturalia, later Rubia Natural Colours, Steenbergen/The Netherlands).

4.2.2.1. Experiment using alum

Ready-to-dye wool was mordanted with a 10 g L⁻¹ aqueous (deionised water) solution of alum [$\text{KAl}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$] through heating to 90+ °C during 0.5–1 h, followed by a 1 h-period at ~95 °C [14]. Essentially the same procedure was also carried out with deionised water only (no alum; blank-mordanting), and with 2 g L⁻¹ aqueous solution of alum. In each case, four of the obtained ~5 $\times$ 5 cm-pieces of mordanted wool were dyed with a 96% ethanol–deionised water 3:1 (v/v) extract of the aerial parts of weld in deionised water at 80 °C for 15 min [14]. L^* , a^* and b^* quantities (CIELAB colour space) were measured for each piece of dyed wool, and their chroma [$C^*_{ab} = (a^{*2} + b^{*2})^{1/2}$] and hue angle [$h_{ab} = \arctan(b^*/a^*)$] were calculated [15]. The data were processed as follows: Standard deviation values were expressed with one significant digit, and average values were rounded up accordingly.

The light-fastness of the colours was determined according to the ISO 105–B02 norm. Two pieces of dyed wool were irradiated in a weathering tester in each of the three cases (dyed blank-mordanted wool, and dyed wool that had been mordanted with 2 g L⁻¹ and 10 g L⁻¹ aqueous solutions of alum). The scale 1 (poor)–8 (excellent)—with 3 being the acceptable lower limit—was used for reporting the results [16]. Colour difference $\{\Delta E^*_{ab} = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}\}$ values were calculated from the average values of the L^* , a^* , and b^* quantities (CIELAB colour space) measured before and after irradiation [15]. The wash-fastness of the colours of the pieces of dyed wool was determined according to the ISO 105–C06 norm at 40 °C for 30 min. Two pieces of wool were used—with only one of them being washed (thus, $n = 1$; one pair of samples)—in each of the three cases. Afterwards, both pieces were visually compared and L^* , a^* , and b^* quantities (CIELAB colour space) were measured. The scale 1 (poor)–5 (excellent)—with 3–4 being the acceptable lower limit—was used for reporting the results [16].

4.2.2.2. Experiment using aluminium sulphate and tartaric acid

Ready-to-dye wool was pretreated with an aqueous solution of a textile auxiliary agent aiming at protecting the fibre from fibre-to-fibre and fibre-to-metal action. The wool was then mordanted with an aqueous (tap water) solution of aluminium sulphate and tartaric acid at 95 °C during 1.75 h. This procedure was carried out with 0.2, 2, 10 and 25 g L⁻¹ solutions of aluminium sulphate tetradecahydrate, with the concentration of tartaric acid being 1.3 g L⁻¹ in all cases. Each piece of mordanted wool was dyed with an aqueous (tap water) solution of a commercial extract of weld [2.5% (w/w) extract-wool] at 100 °C during 1.75 h. The dyed wool was rinsed at 95°C using detergent and, then, rinsed further with tap water at the same temperature. L^* , a^* , and b^* quantities (CIELAB colour space) were measured for each piece of dyed wool, and their chroma (C^*_{ab}) and hue angle (h_{ab}) were calculated as above. The light-fastness of the colours was determined at TO2C (University College Ghent, Belgium) according to the ISO 105–B02 norm. Also in this case, the scale 1 (poor)–8 (excellent) was used for reporting the results.

Table 4.1. Conditions and material of the mordanting and dyeing procedures of the experiments described in section 4.2.2: Temperature, time, aluminium salt, and water.

Aluminium salt	Mordanting			Dyeing		Water		
	T (°C)	Time (h)		T (°C)	Time (h)	Mordanting	Rinsing after mordanting	Dyeing
$\text{KAl}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ (alum)	~95	~1.4	0.2	80		deionised	deionised	deionised
$\text{Al}_2(\text{SO}_4)_3 \cdot 14\text{H}_2\text{O}$ (aluminium sulphate tetradecahydrate) ^a	95	1.8	1.8	100		tap water ^b	tap water ^b	tap water ^{b,c}

^a Mordanting solution also contained tartaric acid.^b Water hardness classification: DH4.^c After the dyed wool had been rinsed using detergent.

4.2.3. Effect of the glycosylation pattern of lut (aglycone, monoglucoside, and diglucoside) on the colours of alum-mordanted wool dyed with it

The following chemical compounds and material were used for dyeing the alum-mordanted wool: **Lut** (96% by NMR; Indofine Chemical Company, Hillsborough/USA); **lmg** (95% by NMR; Extrasynthese, Genay/France); **ldg** (86% by NMR; Extrasynthese, Genay/France); sample of the aerial parts of dried and ground weld {described by Villela *et al.* [13]}.

Single pieces of the wool mordanted with a 10 g L^{-1} aqueous solution of alum described above were dyed at 80°C for 15 min—according to procedures adapted from that described by Villela *et al.* [14] (Supporting Information, page 2)—with:

- $11.5 \text{ }\mu\text{mol}$ of **lut**, **lmg**, or **ldg**;
- A 96% ethanol–water 3:1 (v/v) extract of 420 mg of a sample of the aerial parts of dried and ground weld.

A blank-without-wool was carried out similarly. The dyeing baths were sampled after removal of the pieces of dyed wool—while still warm and stirring—followed by $5\times$ dilution with DMSO. Later, these samples were analysed by RP-HPLC–UV (as above). The percentages of leftover flavones in the dyeing baths were calculated as follows:

- Individual flavones: Using calibration curves, with that of **lmg** having to be extrapolated due to low [**lmg**] in the sample;
- Extract of weld: The areas of **lut**, **lmg**, and **ldg** peaks of the wool-dyeing were compared with those of the blank-without-wool.

L^* , a^* , and b^* quantities (CIELAB colour space) were measured for the dyed wool, and their chroma (C^*_{ab}) and hue angle (h_{ab}) were calculated as above. For this, only a part of about $5 \times 1 \text{ cm}$ of the piece of wool was used in each of the four cases.

4.3. Theory

This section provides theoretical background for the discussion in the chapter. Its first paragraph consists of a brief overview of photophysical processes often taking place upon irradiation of organic molecules in solution, based on the book by Gilbert and Baggott [17].

Organic molecules may be excited from the electronic ground singlet state (S_0) to electronic excited states upon irradiation in the portion(s) of the UV–vis range of the electromagnetic spectrum in which they absorb light. This excitation may be to the lowest vibrational level of the first electronic excited singlet state (S_1). If the excitation is to a vibrationally excited level of S_1 , this excess vibrational energy is quickly dissipated through vibrational relaxation (VR) by collisions of the excited molecule with solvent molecules. This leads to heat generation. Molecules that are excited to higher electronic excited singlet states may reach the lowest vibrational level of S_1 through the nonradiative transition internal conversion (IC)—e.g., $S_2 \rightarrow S_1$ —followed by VR. Then, the molecules may return to S_0 with or without emission of light. If this transition is nonradiative, it takes place through $S_1 \rightarrow S_0$ IC followed by VR. If it is radiative, it is referred to as fluorescence. From S_1 , the organic molecules may also proceed to the first electronic excited triplet state (T_1) through intersystem crossing (ISC). Also from the vibrationless level of T_1 , they may return to S_0 with or without emission of light. If the transition is nonradiative, it takes place, again, through ISC—this time $T_1 \rightarrow S_0$ —followed by VR. If it is radiative, it is referred to as phosphorescence. These transitions can be schematically visualised in a Jablonski diagram.²

Depending on the structure, flavones and flavonols undergo tautomerism. In the case of the flavone **lut**, this takes place through proton transfer at the 5-hydroxyl and 4-carbonyl groups (pseudo-carboxyl group) (Fig. 4.1). Amat *et al.* studied photophysical processes taking place upon irradiation of **lut** [18]. In the electronic singlet ground state (S_0), the O5-bound tautomer was calculated to be energetically favourable over the O4-bound tautomer. In the first electronic singlet excited state (S_1), it is the opposite.

Figure 4.1. Tautomerism of **lut**, through proton transfer at the 5-hydroxyl and 4-carbonyl groups (pseudo-carboxyl group).

² Singlet (S) and triplet (T) refer to the electronic state of molecules, which relates to the spin of their electrons [17]. There is no need for further elaboration on them for the discussion in this thesis.

After photon absorption, **lut** loses some of the energy gained by planarization of its structure. Although the B-ring is 18° out of the plane of rings A–C in the S_0 , this decreases to only 1° in the S_1 [18]. Charge transfer from the B-ring towards rings C and A could be the reason for such planarization [19]. After that, **lut** is expected to undergo tautomerism, or excited state intramolecular proton transfer (ESIPT).

The main processes through which **lut** returns to S_0 are nonradiative, as its fluorescence quantum yield (ϕ_f) is $<10^{-4}$ in methanol [5]. The reason for this may be a high rate of $S_1 \rightarrow S_0$ IC. As the S_0 – S_1 energy gap of **lut** is $2.8 \cdot 10^4 \text{ cm}^{-1}$ —*i.e.*, larger than $2.5 \cdot 10^4 \text{ cm}^{-1}$ —the efficiency of the transition could be due to an acceleration by charge transfer from the B-ring, ESIPT, or both of them [18, 19]. After the IC, VR leads to the lowest vibrational level of S_0 [17].

Solvent participates in the proton transfer at the S_0 in methanol–water 8:2—the solvent used for evaluating the photo-stability of **lut** in the study reported here—as it is a polar protic solvent [20]. This may be the case at the S_1 too. Polar protic solvents lessen ESIPT [21, 22]. Thus, as the proton transfer at the S_1 might be mediated by the solvent—and possibly also by component partners of **lut** dimers/aggregates [21]—it is henceforth referred to as excited state proton transfer (ESPT).

4.4. Results and discussion

4.4.1. Effect of different concentrations of Al^{3+} on the photo-stability of the dye of weld

4.4.1.1. Photo-stability of **lut** in solution as a function of different concentrations of Al^{3+}

The photo-stability of **lut** in aerated methanol–water 8:2 (v/v) solution upon its irradiation with light above 300 nm at different t_0 concentrations—0.20 mM (**lut**_{0.20}), 0.11 mM (**lut**_{0.11}) and 0.05 mM (**lut**_{0.05})—was compared. Straight lines were fit through the experimental data; the slopes of the equations of the best-fit lines range from $-1.8 \cdot 10^{-14}$ to $-2.3 \cdot 10^{-14}$ AU. These, and the number of molecules decomposed in 24 h of irradiation—evidenced by the change in peak area—are very similar (Fig. SI C.11). This suggests that the rate of photodecomposition of **lut** is independent of its concentration, following a zero-order rate law under the conditions used in this study.

In the same set of experiments, the photo-stability of **lut** in presence of Al^{3+} at different **lut**– Al^{3+} ratios—5:1 (**lut**_{0.10}– Al _{0.02}), 1:1 (**lut**_{0.10}– Al _{0.10}), and 1:10 (**lut**_{0.10}– Al _{1.00})—was studied in duplicate. The experimental data were treated as above, with the photodecomposition of **lut** being described by the zero-order integrated rate law ($[\text{lut}] = kt + [\text{lut}]_0$). As the rate of photodecomposition is equal to the rate constant (k) in zero-order reactions ($d[\text{lut}]/dt = k$), the relative rates of the photodecomposition of **lut** could be obtained. The photo-stability of **lut** in solution decreased with increasing $[\text{Al}^{3+}]$, with the relative rates of its photodecomposition being 1.5 (**lut**_{0.10}– Al _{0.02}), 3.0 (**lut**_{0.10}– Al _{0.10}), and 4.0 (**lut**_{0.10}– Al _{1.00}) (Fig. 4.2 and Table 4.2).

Figure 4.2. Effect of increasing $[\text{Al}^{3+}]$ on the photo-stability of **lut** in solution. Note: Subscripts of code of solutions denote $[\text{lut}]_0$ and $[\text{Al}^{3+}]$, in mM; Solvent: aerated methanol–water 8:2 (v/v); r^2 = quality of description of the photodecomposition of **lut** by the zero-order integrated rate law ($[\text{lut}] = kt + [\text{lut}]_0$); Duration of irradiation of solutions = 24.0 h (1,440 min); Data from 1st irradiation replicate.

Table 4.2. Relative rate of photodecomposition of **lut** in solution as a function of different concentrations of Al^{3+} and glycosylation pattern (aglycone, monoglucoside, and diglucoside).

Solution ^a	Flavone decomposed in 24 h of irradiation (%)	r^2 ^b	Rate constant, k ($10^{-3} \text{ mol L}^{-1} \text{ min}^{-1}$)	Relative rate of photodecomposition ^c
1 st irradiation replicate				
lut _{0.11}	7	0.98	$4.9 \cdot 10^{-6}$	1
lut _{0.10} – Al _{0.02}	9	0.95	$6.4 \cdot 10^{-6}$	1.3
lut _{0.10} – Al _{0.10}	21	1.00	$1.5 \cdot 10^{-5}$	3.1
lut _{0.11} – Al _{1.05}	24	1.00	$1.7 \cdot 10^{-5}$	3.6
lut _{0.05}	12	0.99	$4.4 \cdot 10^{-6}$	1
lmg _{0.05}	16	0.99	$5.7 \cdot 10^{-6}$	1.3
ldg _{0.04} ^d	1	0.48	$3.5 \cdot 10^{-7}$	0.1
ldg _{0.05} – Al _{0.05} ^d	8	0.69	$2.9 \cdot 10^{-6}$	0.6
2 nd irradiation replicate				
lut _{0.10}	6	1.00	$4.3 \cdot 10^{-6}$	1
lut _{0.10} – Al _{0.02}	10	0.98	$6.7 \cdot 10^{-6}$	1.6
lut _{0.10} – Al _{0.10}	22	0.99	$1.4 \cdot 10^{-5}$	3.3
lut _{0.10} – Al _{0.99}	26	1.00	$1.8 \cdot 10^{-5}$	4.0
lut _{0.05}	11	0.97	$3.9 \cdot 10^{-6}$	1
lmg _{0.05}	17	0.99	$5.8 \cdot 10^{-6}$	1.5
ldg _{0.05} ^d	4	0.62	$1.3 \cdot 10^{-6}$	0.3
ldg _{0.05} – Al _{0.05} ^d	9	0.46	$2.3 \cdot 10^{-6}$	0.6

^a Subscripts of code of solutions denote [flavone]₀ and $[\text{Al}^{3+}]$, in mM; Solvent: aerated methanol–water 8:2 (v/v);

^b r^2 = quality of description of the photodecomposition of the flavones by the zero-order integrated rate law ($[\text{flavone}] = kt + [\text{flavone}]_0$); ^c Relative rates of photodecomposition = k_j/k_i —as $d[\text{flavone}]/dt = k$ in zero-order reactions—in which i is **lut** in **lut**_{0.11}, **lut**_{0.10}, or **lut**_{0.05}, and j is **lut**, **lmg**, or **ldg** in other solutions; ^d The photodecomposition of **ldg** over time—with and without Al^{3+} —is plotted in Figures SI C.12–15.

An explanation for the decrease of the photo-stability of **lut** with increasing $[\text{Al}^{3+}]$ is suggested. As discussed in the **Theory** section, the main processes through which electronic excited state **lut** returns to S_0 are nonradiative, possibly through an $S_1 \rightarrow S_0$ IC accelerated by charge transfer from the B-ring towards rings C and A, ESPT, or by both of them.³ In **lut**– Al^{3+} complexes, the charge transfer from the B-ring of **lut** after electronic excitation might diminish. Furthermore, the possibility of ESPT at the pseudo-carboxyl group decreases with increasing $[\text{Al}^{3+}]$, up to the point in which all **lut** molecules are forming a complex with Al^{3+} and ESPT becomes impossible. These two possibilities are elaborated upon in the remaining of this section.

The dihedral angle between the plane of ring B and that of rings A–C decreases upon complexation with Al^{3+} at the pseudo-carboxyl group [4, 23]. This planarization is linked to increased electronic delocalization [4, 23], which is consistent with the lowest UV–vis absorption bands of **lut**– Al^{3+} complexes being red-shifted relative to that of **lut** [Fig. SI C.7, and [5] (Fig. 7)]. Thus, the charge transfer from the B-ring of **lut** in **lut**– Al^{3+} complexes after electronic excitation might be less pronounced than that of free **lut**.

Complexation at the pseudo-carboxyl group is favoured over that at the 3'- and 4'-hydroxyl groups (catechol group) in a neutral environment [4]. In an acidic environment—methanol–water 9:1 acidified to an apparent pH of 2.5—flavonol– Al^{3+} complexation at the catechol group did not even take place [4]. The environment in which the experiments reported in this section were carried out was also acidic (methanol–water 8:2 at an apparent pH of 3.6). Consequently, complexation between **lut** and Al^{3+} is expected to have taken place primarily—if not solely—at **lut**'s pseudo-carboxyl group. Therefore, the decrease of the photo-stability of **lut** with increasing $[\text{Al}^{3+}]$ is also consistent with a corresponding decreasing possibility of ESPT at the pseudo-carboxyl group [6, 7].

This can be understood by knowing which complexes are formed at different **lut**– Al^{3+} ratios.⁴ Amat *et al.* studied it theoretically, with experimental data in methanol obtained by Favaro *et al.* as benchmark [5, 23]. The following—also represented in Figure 4.3—is based on their report:

- At a **lut**– Al^{3+} 3:1 ratio, **lut** is both free and as the **lut**– Al^{3+} 2:1 complex involving the pseudo-carboxyl group;
- At a **lut**– Al^{3+} 1:2 ratio, **lut** is largely present in the form of the **lut**– Al^{3+} 1:1 complex, also involving the pseudo-carboxyl group;
- At a **lut**– Al^{3+} 1:10 ratio, a mixture of the complexes **lut**– Al^{3+} 1:2 involving the pseudo-carboxyl group and the catechol group, and **lut**– Al^{3+} 1:1 (as in the previous bullet point) is likely.

³ Deprotonation at the 7-OH group of **lut** could also play a role in acceleration of the $S_1 \rightarrow S_0$ IC. This is further elaborated upon in section 4.4.2.1.

⁴ The rationale presented for the decreasing possibility of ESPT at the pseudo-carboxyl group with increasing $[\text{Al}^{3+}]$ is qualitative on the possible **lut**– Al^{3+} complexes present in solution, not involving the formation constants of the complexes.

Figure 4.3. lut-Al^{3+} complexes formed at different lut-Al^{3+} ratios in methanol [23].

Water in the solvent seems to be unfavourable for lut-Al^{3+} complexation. Because of this, the main species in methanol–water 8:2 solution at a lut-Al^{3+} 1:10 ratio is expected to be the same as that in methanol at a lut-Al^{3+} 1:2 ratio {Table SI C.1 and ensuing discussion, [5] (Fig. 7), and [23]}. Therefore, the decrease of the photo-stability of lut with increasing $[\text{Al}^{3+}]$ reported here is consistent with ESPT being:

- Possible at a lut-Al^{3+} 5:1 ratio, due to the main species in solution being the lut-Al^{3+} 2:1 complex and free lut , as represented in situation ii of Figure 4.3;
- Still possible at a lut-Al^{3+} 1:1 ratio—but to a much smaller extent than in the previous case—due to the main species in solution being the same as in the previous bullet point, but with an increased number of lut molecules forming a complex with Al^{3+} ;
- Impossible at a lut-Al^{3+} 1:10 ratio, due to the main species in solution being the lut-Al^{3+} 1:1 complex represented in situation iii of Figure 4.3.

The reported fluorescence in a situation in which all lut molecules are forming a complex with Al^{3+} evidences a process for the $\text{S}_1 \rightarrow \text{S}_0$ transition other than accelerated IC. Fluorescence is nearly the only process through which the lut-Al^{3+} complex(es) release(s) absorbed energy, as $\phi_f \approx 1$ in methanol [5]. Thus, there is a change of the preferred process through which lut releases absorbed energy at high $[\text{Al}^{3+}]$. As charge transfer from the B-ring of lut after

electronic excitation may diminish and the possibility of ESPT decreases, the possible accelerated $S_1 \rightarrow S_0$ transition via IC could be progressively replaced by an $S_1 \rightarrow S_0$ transition via fluorescence.

The rate constant of accelerated $S_1 \rightarrow S_0$ transition via IC (k_{IC}) of **lut** in methanol–water 8:2 might be of the order of magnitude of 10^{11} s^{-1} , by analogy to that of the O-3-glycosylated flavonol rutin in methanol, also a polar protic solvent [19]. Fluorescence lifetimes are typically of the order of magnitude of 10^{-9} s and the measured fluorescence lifetime (τ_f) is the reciprocal of the rate constant of fluorescence (k_f) if $\phi_f = 1$, *i.e.*, $\tau_f = k_f^{-1}$ [17] (chapter 4). Thus, the electronic excited state lifetime of free **lut** might be about two orders of magnitude shorter than that of **lut** molecules engaged in complex formation with Al^{3+} . Since molecules are more reactive in the electronic excited state than in the electronic ground state, this would account for the decrease of the photo-stability of the compound with increasing $[\text{Al}^{3+}]$.

4.4.1.2. Effect of mordanting wool with different quantities of Al^{3+} before its dyeing with extracts of weld on the colour and on its light-fastness

Two experiments using extracts of weld to dye wool premordanted with increasing quantities of Al^{3+} were carried out. In one of them wool was mordanted using alum [$\text{KAl}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$], and in the other, aluminium sulphate and tartaric acid. L^* , a^* , and b^* quantities (CIELAB colour space) were measured for the pieces of dyed wool, and their light-fastness was determined. As CIELAB chroma (C^*_{ab}) and hue angle (h_{ab}) approximately correlate to chroma and hue [15], the C^*_{ab} quantities are henceforth referred to as the chroma of the colours, and the h_{ab} quantities, as their hue.

The dyed wool becomes more colourful {saturated [24]}—displays higher chroma—with increasing $[\text{Al}^{3+}]$, with a plateau seeming to be reached at some point (Fig. 4.4 and Table 4.3). This takes place at nearly constant lightness (L^*) and hue (Table 4.3 and Table SI C.4). It is also worth noting that the colour of the dyed blank-mordanted wool—in which the wool had been pretreated as in the other cases, except that the mordanting bath had no alum—was pale, with a chroma value of only 15 (Fig. 4.4 and Table SI C.4). To some extent, the results of colourfulness contrast with those of an earlier report by Vinod *et al.* (2010), as the chroma of alum-premordanted silk—also a proteinaceous fibre—dyed with a flavonoid dye remained constant with increasing $[\text{Al}^{3+}]$ [25]. The K/S value increased, however, suggesting increased dye uptake [26]. Thus, the increase in colourfulness of the dyed wool with increasing $[\text{Al}^{3+}]$ reported here could be—at least in part—due to larger quantities of dye in the fibre.

Figure 4.4. Weld-dyed wool premordanted with varying quantities of Al^{3+} (experiment using alum). Pieces of dyed wool in columns, from right to left ($n = 4$, each case): Wool that had been premordanted with 10 g L^{-1} and 2 g L^{-1} aqueous solutions of alum, and blank-mordanted wool (wool that had been pretreated as in the other cases, except that the mordanting bath had no alum).

Table 4.3. Quantitation of the colours of weld-dyed wool samples premordanted with varying quantities of Al^{3+} (experiment using aluminium sulphate and tartaric acid): Measured (L^* , a^* and b^*) and calculated (C^*_{ab} and h_{ab}) quantities using the CIELAB colour space (each case, $n = 1$).

[Aluminium sulphate tetradecahydrate] in mordanting solution (g L^{-1}) ^a	L^*	a^*	b^*	Chroma (C^*_{ab})	Hue angle (h_{ab} ; degrees)
0.2	74.4	-5.6	31.4	31.9	100.1
2	76.8	-9.3	46.4	47.3	101.3
10	78.7	-11.1	53.9	55.0	101.6
25	78.0	-10.6	52.7	53.7	101.3

^a [Tartaric acid] = 1.3 g L^{-1} , all cases.

The light-fastness of the colours of the wool in both experiments was below 3, the acceptable lower limit using the scale 1 (poor)–8 (excellent) [16]. However, that of all samples decreased with increasing $[\text{Al}^{3+}]$. In the experiment in which alum was used, the colour difference values calculated from the L^* , a^* , and b^* quantities before and after irradiation increased at higher $[\text{Al}^{3+}]$ (Table 4.4). In the experiment in which aluminium sulphate and tartaric acid were used, the light-fastness of the colours of the samples themselves decreased with increasing $[\text{Al}^{3+}]$ (Table 4.5). This contrasts with earlier reports in which the colours of Al^{3+} -mordanted proteinaceous fibres dyed with flavonoid dyes have been reported as more or equally light-fast as those of non-mordanted fibres [16, 25, 27].

The wash-fastness of the colours of the samples of the experiment in which alum was used was also determined. On the scale 1 (poor)–5 (excellent), wash-fastness values of 3–4 are the acceptable lower limit [16]. The wash-fastness of all colours either matched or surpassed this limit (Table SI C.5). Although replicate samples would be needed for increased accuracy of the description of the phenomenon, the colour of dyed blank-mordanted wool (no alum) appears to be more wash-fast than that of dyed Al^{3+} -mordanted wool. This would contrast with earlier reports too. The colours of Al^{3+} -mordanted proteinaceous fibres dyed with flavonoid dyes have been reported as more wash- or water-fast than those of non-mordanted fibres [16, 25, 27].

Table 4.4. Light-fastness of the colours of weld-dyed wool premordanted with varying quantities of Al^{3+} (experiment using alum; samples of Figure 4.4), and CIELAB colour difference (ΔE^*_{ab}) values calculated from the L^* , a^* , and b^* quantities before and after irradiation.

[Alum] in mordanting solution (g L^{-1})	Light-fastness of the colour ^a	ΔE^*_{ab} ^b
Blank (no alum)	<1 / 1	4.78
2	<1	4.90
10	<1	6.25

^a Each case, $n = 2$; scale: 1 (poor)–8 (excellent) [16].

^b Calculated from average values; in each case, $n = 4$ (before irradiation) and $n = 2$ (after irradiation).

Table 4.5. Light-fastness of the colours of weld-dyed wool premordanted with varying quantities of Al^{3+} (experiment using aluminium sulphate and tartaric acid; samples of Table 4.3; each case, $n = 1$).

[Aluminium sulphate tetradecahydrate] in mordanting solution (g L^{-1}) ^a	Light-fastness of the colour ^b
0.2	1–2
2	<1 / 1
10	<1
25	<1

^a [Tartaric acid] = 1.3 g L^{-1} , all cases.

^b Scale: 1 (poor)–8 (excellent) [16].

The two experiments in which wool was mordanted with different quantities of Al^{3+} before its dyeing differed in a number of ways. Several of these differences are displayed in Table 4.1. The short heating time of the dyeing process of the alum-experiment is likely to have been positive for the wool in terms of its physical properties. This is due to the loss of soluble proteins from the wool upon high-temperature heating, which increases with the duration of the process [28]. Nevertheless, long, high-temperature heating is required for the dye molecules to arrive at the regions within the wool fibres where interaction of the dyes with the proteins is thermodynamically favoured [29]. Thus, the long, high-temperature heating of the dyeing process of the aluminium sulphate/tartaric acid-experiment is expected to have been positive in terms of the quality of the dyeing. A potential negative effect of the short, lower-temperature heating used in the alum-experiment would be low wash-fastness of the colour, as the dye molecules—not at the regions of thermodynamically favoured interactions yet—could have been extracted from the fibre with relative ease [29]. As mentioned above, however, the wash-fastness of the obtained colours either matched or surpassed the acceptable lower limit.

In spite of these and other differences between the two experiments, the light-fastness of the colours of weld-dyed wool decreased with increasing $[\text{Al}^{3+}]$ in both cases. As mentioned earlier in this section, more dye may have been taken up by wool mordanted with larger quantities of Al^{3+} . Light-fastness of the colour generally increases with the increase of the quantity of dye in the fibre [30, 31]. If this is the case also for the system dye of weld– Al^{3+} –wool, the detrimental effect of Al^{3+} on the light-fastness of the colours is even larger than that seen in Tables 4.4 and 4.5.

A similar explanation to that suggested in the previous section for the decrease of the photo-stability of **lut** with increasing $[\text{Al}^{3+}]$ is given here. That is, the decrease of the photo-stability of the flavones—main components of the dye of weld {[14] (p.7 of SI)}—with increasing $[\text{Al}^{3+}]$ may be due to a change of their preferred process for releasing absorbed energy. The $\text{S}_1 \rightarrow \text{S}_0$ transition could increasingly proceed via fluorescence rather than via IC accelerated by a photophysical process(es). This could lead to longer electronic excited state lifetimes of the flavones engaged in complex formation with Al^{3+} than those of the free flavones, accounting for the decrease of the photo-stability of the compounds. However, the picture here differs from

that of **lut** in solution due to additional geometrical constraints and changes in electronic distribution. These relate to the chemical structures of the flavones themselves—which differ from that of **lut**, with most of them containing sugar moieties—and to their interaction with the proteins of wool. Van der Waals forces are one of the important ways through which dye molecules interact with the proteins of wool [29]. Naturally, interactions such as this influence the electronic distribution of the flavones.

4.4.2. Effect of glycosylation pattern of lut (aglycone, monoglucoside, and diglucoside) on its photo-stability in solution and colours of alum-mordanted wool dyed with it

4.4.2.1. Photo-stability of lut in solution as a function of its glycosylation pattern

The photo-stability of **lut** in solution was studied in duplicate as a function of its glycosylation pattern: Aglycone, **lut**-7-*O*-glucoside (**lut** monoglucoside, **lmg**) and **lut**-7,3'-*O*-diglucoside (**ldg**). This was done in the same set of the experiments discussed in section 4.4.1.1, in aerated methanol–water 8:2 (v/v) solution with light above 300 nm. Also the experimental data were treated as done for **lut** in solution in the presence of Al^{3+} , with the relative rates of photodecomposition being obtained. Whereas **lmg** photodecomposed 1.4× faster than **lut**, the photodecomposition of **ldg** was much slower (Table 4.2 and Fig. 4.5). Although this increased reactivity of **lmg** upon irradiation relative to **lut** was also observed by Smith *et al.* [6], it seems counter-intuitive at first, due to the possibility of glycosylation to confer stability to flavonoid aglycones [8], [9] (chapter 42), [10] (chapter 5). Possible explanations for the effect of the glycosylation pattern on the photo-stability of **lut** in solution are elaborated upon in this section.

Figure 4.5. Effect of glycosylation pattern—aglycone, monoglucoside, and diglucoside—on the photo-stability of **lut** in solution. Note: Subscripts of code of solutions denote [flavone]₀, in mM; Solvent: aerated methanol–water 8:2 (v/v); r^2 = quality of description of the photodecomposition of the flavones by the zero-order integrated rate law ([flavone] = kt + [flavone]₀); Duration of irradiation of solutions = 24.0 h (1,440 min); Data from 2nd irradiation replicate.

The possibility of **lut** to increase the electronic density of the A-ring through deprotonation at the 7-OH group may account for the difference between its rate of photodecomposition and that of **lmg**. The electronic distributions of **lut** and apigenin—3'-deoxy **lut**—are similar [18]. The $S_0 \rightarrow S_1$ transition of apigenin is accompanied by a shift of electron density from ring A to ring C [18]. Upon irradiation with light above 300 nm, **lut** mainly undergoes an $S_0 \rightarrow S_1$ transition [18] (Table 4.2 and Fig. 4.3). Thus, in the experiments reported here, **lut** is expected to have undergone a change in electronic distribution equivalent to that of apigenin; importantly for this discussion, a lowering of the electron density of ring A.

With a $pK_a < 9$, the 7-OH is one of the two most acidic groups of **lut** [5, 32]. The tendency for deprotonation at this hydroxyl group should only increase in the S_1 because of the change in electronic distribution [22]. Thus, such a deprotonation could be a way for excited state **lut** to compensate part of the loss of electron density of ring A. This is impossible in the case of **lmg** because it has an *O*-glucose moiety instead of a hydroxyl group at C-7. Such an impossibility could lead to a change in the preferred process through which absorbed energy is released, causing the electronic excited state lifetime of **lmg** to be longer than that of **lut** and, in this way, account for the reduced photo-stability of **lmg**.

Stabilization of the transition state of the formation of the flavonoxyl radical of **lut** by intramolecular H-bond could explain the difference between the rates of photodecomposition of **lut** and **ldg**. The flavonoxyl radical of quercetin—a **lut**-like flavonoid having an additional hydroxyl group at C-3—is formed through high-energy irradiation (radiolysis) [33]. Flavonoxyl

radicals are formed under milder conditions too, since phenoxyl radicals are formed via irradiation of phenols in the UV–vis range of the electromagnetic spectrum [34]. Kozłowski *et al.* calculated the unpaired electron of the radical species to be delocalized, with C-2 bearing high spin density if the radical was to be formed at the 3-OH group [33]. Those authors expect molecular oxygen to react with the flavonoxyl radical of quercetin precisely at C-2, before the formed peroxy radical reacts as the decomposition of the flavonoid continues. Thus, the formation of the flavonoxyl radical is expected to be the first step of the photo-oxidative degradation—referred to as photodecomposition throughout this chapter—of **lut** and its glycosyl conjugates too.

In flavones that have a catechol group, the energy of the flavonoxyl radical is lowered by intramolecular H-bond involving the O-atom bearing the unpaired electron and the neighbouring hydroxyl group [33, 35], [10] (chapter 5). This is why the flavonoxyl radical of **lut** is expected to be formed at the catechol group; with that formed at the 4'-OH group having been calculated to be the most stable [35]. The H-bond at 3'-OH/4'-O• may stabilize not only the radical, but also the transition state of its formation. Such a stabilization could not take place in the case of **ldg** since it has an *O*-glucose group instead of a hydroxyl group at C-3'. A lower energy of the transition state of the formation of the flavonoxyl radical of **lut** would cause the activation energy barrier for the formation of the radical of **lut** to be smaller than that for the formation of the radical of **ldg**, resulting in a faster formation of the radical of **lut**.

The rate of photodecomposition of **ldg** in presence of Al^{3+} in aerated methanol–water 8:2 (v/v) solution was also obtained through the set of the experiments discussed in section 4.4.1.1; thus, with light above 300 nm. As seen in Table 4.2, **ldg** engaged in complex formation with Al^{3+} (**ldg**_{0.05}– Al _{0.05}) photodecomposed at least 2× faster than free **ldg**. By analogy to **lut**, the main species in solution at a **ldg**– Al^{3+} 1:1 ratio are expected to be the **ldg**– Al^{3+} 2:1 complex and free **ldg** (Fig. 4.3 and ensuing discussion). As previously discussed, **lut** in presence of Al^{3+} also photodecomposes faster than free **lut**. Similarly to **lut**, the faster photodecomposition of **ldg** in presence of Al^{3+} could have resulted from a smaller possibility of accelerated $\text{S}_1 \rightarrow \text{S}_0$ transition via IC due to diminished charge transfer from the B-ring towards rings C and A, diminished possibility of ESPT, or both of them being diminished. This possible change in the process of the $\text{S}_1 \rightarrow \text{S}_0$ transition of part of the **ldg** molecules could have led to their electronic excited state lifetime being longer, accounting for the decreased photo-stability of **ldg** in presence of Al^{3+} .

4.4.2.2. Colours of alum-mordanted wool dyed with **lut** as a function of the glycosylation pattern of the compound

Two experiments were carried out in which Al^{3+} -mordanted wool was dyed with different dyes. In one of them, alum-mordanted wool was dyed at 80 °C for 15 min. The percentages of leftover flavones in the dyeing baths were calculated after analysis by RP-HPLC–UV. This experiment consists of the following two parts:

- Wool was dyed with the individual flavones. In each case, a piece of mordanted wool was dyed with **lut**, **lmg**, or **ldg**;
- A piece of mordanted wool was dyed with an extract of weld (thus, with all three flavones simultaneously).

In the other experiment—not further described—wool mordanted with aluminium sulphate and tartaric acid was dyed at 100 °C for 1 h with the individual flavones. Thus, also in this case, pieces of mordanted wool were dyed with **lut**, **lmg**, or **ldg**. Then, the percentages of leftover

flavones in the dyeing baths and rinsing waters were calculated with RP-HPLC–UV calibration curves after their y-intercepts had been set to zero. A picture of the dyed pieces of wool of the first experiment is seen in Figure 4.6, and the percentages of leftover flavones after dyeing from both experiments are listed in Table 4.6.

Figure 4.6. Pieces of alum-mordanted wool dyed with the individual flavones—**lut**, **img**, or **ldg**—and with an extract of weld (from left to right).

Table 4.6. Leftover flavones after dyeing Al^{3+} -mordanted wool with **lut**, **img**, **ldg**, or an extract of weld (each case, $n = 1$).

Dye	Dyeing conditions	Leftover flavones in dyeing baths (%) ^a		
		Lut	Lmg	Ldg
Lut, img, or ldg	80 °C, 15 min ^b	5	16	63
Extract of weld	80 °C, 15 min ^b	6	18	66
Lut, img, or ldg	100 °C, 1 h	5	13	58

^a In dyeing baths and rinsing waters, in experiment in which dyeing processes were carried out at 100 °C for 1 h.

^b Dyeing processes of samples of Fig. 4.6.

The colours of alum-mordanted wool dyed with **lut** or **img** seem to be similar, but different from those in which either an extract of weld or **ldg** are used (Fig. 4.6 and Table SI C.6). The wool dyed with the latter two dyes was much less colourful—chroma 60 and 30, respectively—than that dyed with either **lut** or **img** (chroma 80–85).

In the case of **ldg**, this is possibly due to the quantity of flavone binding to the wool. Table 4.6 lists the percentages of leftover flavones in the dyeing baths. The uptake of **lut**, **img**, and **ldg** by the Al^{3+} -mordanted wool is estimated from them. The data suggest **lut** to be extensively taken up by the mordanted wool, as well as most **img**, but this to be the case only for one-third of **ldg** molecules. The lack of a free catechol group could be a reason for a limited binding of **ldg** to the mordanted wool. This could also be due to steric hindrance—bulkiness of the glucose moiety—and solubility in the dyeing bath (**ldg** is more hydrophilic than both **img** and **lut**),

equivalently to suggestion by Mouri *et al.* when reporting a preferential binding of flavonol aglycones to silk relative to that of the glycosides [36].

4.5. Conclusion

The photo-stability of **lut** in solution—polar protic solvent—and the light-fastness of the colour of weld-dyed wool decrease with increasing $[\text{Al}^{3+}]$. Thus, the lower the $[\text{Al}^{3+}]$ used for mordanting the wool, the more light-fast its colour. Lowering the $[\text{Al}^{3+}]$ appears to have no negative influence on the wash-fastness of the colour. As the gain in light-fastness by the use of low $[\text{Al}^{3+}]$ to premordant the wool is limited, however, this does not seem to be a way to meet today's requirement of light-fastness of the colours of dyed textiles by itself. Nevertheless, it may be part of a broader strategy to address the need for increased light-fastness of the colour of wool dyed with weld. Implementation of this approach by dyers is expected to clarify whether it results in benefits for textile dyeing practice. On one hand, lowering the $[\text{Al}^{3+}]$ to mordant the wool has the drawback of limiting the obtainable yellow colour, as its saturation increases with increasing $[\text{Al}^{3+}]$. On the other hand, reduction of the quantity of mordant should increase the needed environmental friendliness of the dyeing of textiles with natural dyes, and have financial benefits.

4.6. Supplementary material

Appendix C contains information supplementary to that in this chapter. Sections **material and methods**, **results and discussion**, **author contributions** and **references** are available. Reference is made in this chapter only to part of the information in appendix C. Thus, readers are referred to it for further details on the work.

4.7. References

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Chapter 5

Analysis of a natural dye: an experiment for analytical organic chemistry

The content of this chapter is largely that of the following paper:
Villela A, Derksen GCH, van Beek TA. Analysis of a natural yellow dye: an experiment for analytical organic chemistry. J Chem Educ 2014; 91(4): 566–9.

This experiment exposes second-year undergraduate students taking a course in analytical organic chemistry to high performance liquid chromatography (HPLC) and quantitative analysis using the internal standard method. This is accomplished using the real-world application of natural dyes for textiles. The extracted flavonoids of the plant weld are responsible for the yellow colour of the dyed wool. Dried and ground weld is extracted for dyeing wool and quantifying the plant's three main flavonoids. The students also mimic the work of chemists investigating historical textiles by carrying out a small scale extraction of the dyed wool. Twenty-one students carried out the experiment and their samples were analysed using either a traditional 5 μm -particle size HPLC column or a modern 1.8 μm -particle size ultrahigh-pressure liquid chromatography (UHPLC) column mounted in a conventional HPLC system.

5.1. Introduction

Undergraduate students of molecular life sciences and biotechnology programs of Wageningen University take the course Analytical Methods in Organic Chemistry (AMOC) at the beginning of the second year. AMOC has both theoretical and practical components. Its practical component is divided into spectroscopy and chromatography. During the latter, students perform experiments individually and become acquainted with a range of techniques used for the analysis of organic compounds. The experiment reported here was used in 2011, 2012, and 2013.

5.2. Description of the experiment

5.2.1. Introduction

The compounds of weld that are responsible for the yellow colour of dyed alum-treated wool belong to the class of flavonoids [1]. The three main compounds are 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4*H*-1-benzopyran-4-one (luteolin, **lut**), **lut**-7-*O*-glucoside (**lut** monoglucoside, **lmg**), and **lut**-7,3'-*O*-diglucoside (**lut** diglucoside, **ldg**) [2, 3]. The compound used as internal standard (**i.s.**) for their quantitation in weld is 5,7-dihydroxy-2-phenyl-4*H*-1-benzopyran-4-one (chrysin) [4]. The structures of these four compounds are depicted in Figure 5.1.

Figure 5.1. Structures of the three main flavonoids of weld and the compound used as internal standard (**i.s.**) for their quantitation in weld. Their identities are luteolin-7,3'-*O*-diglucoside (luteolin diglucoside, **ldg**), luteolin-7-*O*-glucoside (luteolin monoglucoside, **lmg**), luteolin (**lut**), and chrysin (**i.s.**).

Since the first use of the experiment, the protocol of the experiment has undergone modifications. Both student and teaching assistants' feedback contributed to this. Students who carried out the experiment in 2012 and 2013 were busy with its practical part for ~4 h. An updated version of the protocol of the experiment handed out to students is available in Appendix D.

5.2.2. Practical part

The students carry out two extractions of dried and ground weld simultaneously: One extract is used for dyeing wool and the other is used for quantifying the plant's three main flavonoids. For the wool dyeing, 1.5 g of the dried and ground weld is placed in an Erlenmeyer flask with 30 mL of 96% ethanol–water 3:1, and stoppered. The mixture is sonicated for 10 min. After filtration through a folded filter paper to a round-bottom flask, the ~20 mL of 96% alcohol is removed by a rotatory evaporator. The dyeing bath is prepared by transferring the alcohol-free extract from the round-bottom flask to a 150 mL beaker using 4×15 mL portions of deionized water. To quantify the plant's three main flavonoids, 200 mg of the dried and ground weld is placed in a 50 mL Erlenmeyer flask and is extracted and sonicated as described above, except for using only 20 mL of 96% ethanol–water 3:1. After sonication, a magnetic stir bar is added to the Erlenmeyer flask. This is followed by addition of 5.00 mL of a methanolic solution of the **i.s.** of known concentration using a volumetric pipette (see Appendix D for the possibility of replacing methanol by 96% alcohol), and 10 min-stirring. After filtration of the solution via a syringe filter, the sample is ready for HPLC analysis.

The students proceed by soaking four ready-made $\sim 5 \times 5$ cm pieces of wool (pretreated with aluminum potassium sulfate dodecahydrate, alum) in water at 50 °C. The dyeing bath is heated to 80 °C, and the four pieces of wool are added to it. The dyeing step lasts 15 min, after which the dyed wool is rinsed with water. A small piece of a single thread is removed from the dyed wool and the dye is extracted by placing it in 300 μ L of methanol–water–methanoic acid (formic acid) 80:15:5 in a microcentrifuge tube of 2 mL, and heating via a water bath at 60 °C for 30 min. Sample is ready for HPLC analysis after filtration of the cooled solution via a syringe filter. The samples from both parts of the experiment are given to one of the teaching assistants so that they can be analysed. This is done by reversed phase (RP)-HPLC–UV at 345 nm in either 80 min at 40 °C (Alltima 250 $\times$ 4.6 mm C18 5 μ m-particle size HPLC column) or 5 min at 35 °C [Eclipse XDB-C18 50 $\times$ 3.0 mm 1.8 μ m-particle size ultrahigh-pressure liquid chromatography (UHPLC) column]. In both cases columns are mounted in conventional HPLC systems. The gradient elution separation is carried out with an aqueous buffer pH 3 and either methanol (HPLC column) or methyl cyanide (acetonitrile) (UHPLC column). The chromatograms with the data needed are given to the students for data processing. Although this procedure was typically followed, involvement of the students in running the analyses would be preferable; *e.g.*, by accompanying the teaching assistant in preparing and starting the sequence of analyses and receiving a brief explanation of HPLC. Even though all students participate in a demonstration on HPLC at the beginning of the chromatography part of AMOC, the additional exposure to the instrument could be beneficial to the students' learning. In Appendix D, different parts of the protocol are discussed and material and methods for the preparation of the experiment are detailed.

5.2.3. Data processing

With the chromatogram of the weld sample containing peak areas and retention times, the students start by assigning the peaks corresponding to **ldg**, **lmg**, **lut**, and **i.s.** on the basis of the retention times. Then, using the equation that relates peak area to the quantity of compound in a sample and predetermined relative response factors (RRFs), the students calculate the concentration of the three main flavonoids in the weld sample. This is done using the following equation:

$$w_x = \frac{A_x}{A_{i.s.}} \cdot \frac{w_{i.s.}}{RRF}$$

in which A_x is the peak area of compound x ; $A_{i.s.}$ is the peak area of **i.s.**; $w_{i.s.}$ is the weight of **i.s.**; RRF is the relative response factor; and w_x is the weight of compound x . Because the peak areas are obtained from the chromatogram, the quantity (weight) of added **i.s.** is known, and the RRFs are given, students can calculate the quantity of compound x (**ldg**, **lmg**, or **lut**) in the sample. Then, the concentration of compound x in the weld sample can be calculated.

In addition, mimicking the work of chemists investigating historical textiles, the students identify the dye source of the freshly dyed piece of wool. This is done by assigning the peaks corresponding to **ldg**, **lmg**, and **lut** using chromatograms and UV–vis absorption spectra of authentic standards. They also compare the chromatogram of the dyed wool's extract sample with that of the weld's extract sample.

The work of the students is concluded by answering a few questions that include separation principles of RP-HPLC analysis and the relation between structure and UV (345 nm) absorbance of weld's three main flavonoids. Solutions to the assignments and answers to the questions of the protocol handed out to students, including derivation of the equation above, are available in Appendix D.

5.3. Hazards

Basic organic chemistry laboratory safety procedures [5] should be observed when carrying out the experiment. Erlenmeyer flasks should not be clamped during sonication, as they may break. There is the risk of implosion (glass breakage) during operation of rotatory evaporators. 96% alcohol is flammable [6]. Exposure to methanol (flammable, toxic) and formic acid (corrosive) must be minimized [6]. Sample filtration via a syringe filter has the risk of liquid spillage due to an improper connection between both parts. The microcentrifuge tube containing the methanol–water–formic acid extract should be cooled prior to opening because of the pressure build-up during the heating step. The support team should pay special attention:

- during the preparation of the methanol–water–formic acid solution and HPLC solvent A (aqueous buffer pH 3) because of handling of formic acid, when the use of protective gloves is recommended [7];
- so that vapours originating from HPLC solvent B (methanol or acetonitrile, toxic) bottle and HPLC waste container are properly exhausted [6].

5.4. Results and discussion

In 2011, 12 students carried out the experiment. Their samples were analysed using a traditional 25 cm-long, 5 μm -particle size RP-HPLC column. Five and four students carried out the experiment in 2012 and 2013, respectively.¹ Their samples were analysed using a modern 5 cm-long, 1.8 μm -particle size RP-UHPLC column mounted in a conventional HPLC system. Results obtained by a student in 2011 and another student in 2012 are depicted in Figure 5.2. The peaks of the main compounds in the chromatograms are assigned. The compounds responsible for the minor peaks also belong to the class of flavonoids. Their identities are listed in Appendix D. The signal-to-noise ratios of the chromatograms of the dyed wool's extract samples are much smaller than those of the chromatograms of the weld's extract samples because only small pieces of single threads of dyed wool are analysed, resulting in small quantities of flavonoids in the samples. This mimics of the work of chemists investigating historical textiles, in which it is crucial to minimize damage to artifacts.

Figure 5.2. Chromatograms of the extracts of weld and dyed wool prepared by two students. All cases: 345 nm traces. Peaks of the main compounds are assigned.

In 2011, 10 students submitted a report of the experiment. In 2012, this was done by the five students. On the basis of those reports, it was observed that nearly all students understood how separation by reversed phase chromatography works. Furthermore, half of the students knew how to use the internal standard method for quantitative analysis, and one-third of them had

¹ In addition to the 21 students who carried out the experiment in 2011–2013, the experiment was also carried out by 10 students in 2014.

difficulty with it. Additional observations on the students' learning regarding the didactic aims and background of the experiment are described in Appendix D.

5.5. Summary

Students are exposed to analysis by HPLC and quantitative analysis using the internal standard method by carrying out this experiment. This is accomplished using the real-world application of natural dyes for textiles, which is a current topic in chemistry.

5.6. Supplementary material

Appendix D contains information supplementary to that in this chapter. The following material is available:

- Updated version of the protocol of the experiment handed out to students;
- Discussion of different parts of the protocol;
- Material and methods for the preparation of the experiment;
- Instructor notes;
- Additional observations on the students' learning based on 2011 and 2012 reports;
- Updated version of the inventory of specialized material handled by students;
- Example data sets of the HPLC analysis of weld sample.

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Chapter 6

General discussion

6.1. Introduction

The work reported in this thesis describes concepts and analytical chemistry methods relevant for the use of natural dyes for textiles. The contents of **chapters 2, 3, and 4** refer to different aspects of textile dyeing technology. They all relate to the use of the dyestuff of weld (*Reseda luteola* L.). The analytical method for the analysis of the main colouring compounds of the plant—described in **chapter 2**—was applied to research (**chapter 4**) and education in chemistry (**chapter 5**). In this chapter, the works presented in **chapters 2–5** are summarised, elements of what they report are discussed, and future prospects of dyeing textiles with natural dyes are briefly elaborated upon.

6.2. Chapter 2

A validated RP-HPLC–UV method for the quantitation of **ldg**, **lmg**, and **lut** via internal standardisation in samples of weld is described in **chapter 2**. The method is simple, requiring very little manpower input per sample. With the exception of the 15-stirring point magnetic stirrer—that, less conveniently, could be replaced by individual magnetic stirrers—only standard laboratory equipment is needed. The analytical method proved fit-for-purpose based on its validation in terms of extraction efficiency, stability and losses of the compounds, accuracy, precision, and sensitivity.

As the method was developed using only one batch of weld (*R. luteola* H) plant material, it has the potential drawback of not being suitable for analysing samples of weld having a concentration of the three flavones much lower or much higher than that of *R. luteola* H. The industrial partner of the project analysed 120+ batches of weld plant material. No samples were encountered with such low concentrations of **ldg**, **lmg**, and **lut** that they could not be analysed using the method as described in **chapter 2** (that is, samples leading to injected quantities smaller than the smallest injected quantities of the calibration curves). However, there were samples having concentrations of the flavones that were too high. For the analyses of such samples, the industrial partner of the project modified the method by simply weighing 100 mg—instead of 200 mg—of sample. Such an adaptation of the method is expected to lead to:

- no major change in extraction efficiency even with the solvent–sample ratio increasing to 200, as it is already high (95%) with a solvent–sample ratio of 100;
- no change in accuracy; neither in terms of stability of the flavones or losses due to adsorption on glass and filters (the relative recovery experiments were also carried out using 100 mg of plant material), or in absolute terms (as there is even less plant material to which the flavones could bind);
- no major loss of precision as, if a 0.1 mg-least division balance is used, the weighing error remains <1%.

The validity of this approach would need testing. This could be done, for example, by comparing the results of the analyses of a small number of replicates of 100 and 200 mg of a few samples.

After the publication of a validated RP-HPLC–DAD method for the quantitation of **ldg**, **lmg**, apigenin-7-*O*-glucoside, luteolin-4'-*O*-glucoside, **lut** and apigenin in samples of weld by Gaspar *et al.* [1], the chromatographic separation using a 5 µm-particle size RP-HPLC column was speeded up by using a short sub-2 µm-particle size (RP-UHPLC) column. This

was done while still using a conventional HPLC system, after minor hardware adaptation. The method using the UHPLC column proved fit-for-purpose through comparison of accuracy and precision of the quantitation of **ldg**, **img**, and **lut** in *R. luteola* H with those achieved by using the HPLC column. Large gains in analysis time and eluent consumption were obtained. Thus, the use of short UHPLC columns on conventional HPLC systems is an economical way of modernising HPLC-based analyses.

6.3. Chapter 3

An analytical method for the comparison of the content of porphyrin ring-containing pigments—chlorophylls (**chls**) and their structurally similar breakdown products—in different samples of weld is described in **chapter 3**. The method:

- uses ethanol as a mild, non-toxic extraction solvent (that is not selective regarding the “types” of **chls** and plant tissues being extracted);
- covers the entire range of occurring concentrations;
- displays acceptable precision;
- is simple.

Nevertheless, the manpower input required by the method is not low. One working day was needed for the analyses and data processing of 40 samples. It is expected, however, that more samples can be analysed in the same time through implementation of the following:

- weighing accurately ~125 mg instead of (125.0 ± 0.9) mg, with a correction factor (125 divided by amount weighed) being used in the calculations. This would speed up the weighing of the samples;
- having 60 centrifuge tubes available instead of 20. This would speed up the analysis of the samples, as no tubes would need to be washed during the data-acquisition day.

Footnote 1 of **chapter 3** relates to a limitation of the method. Comparison of the content of porphyrin ring-containing pigments in samples of weld having different **chls**/chlorophyllides-to-“pheopigments” ratios is hampered, as the optical molar absorption coefficients of pheophytins and pheophorbides are ~40% smaller than those of the corresponding **chls** and chlorophyllides [2, 3]. Possibly, this can be fixed by conversion of **chls** and chlorophyllides to the corresponding “pheopigments” through addition of one drop of a 25% aqueous HCl solution to 5 mL of an extract of the pigments, as outlined by Lichtenthaler [4]. The conversion is expected to be rather fast, as **chls** are generally fully converted to pheophytins in ≤ 1 min [4]. Thus, one could test whether the same procedure described in **chapter 3** can be used with the extraction solvent being HCl-acidified ethanol instead of absolute ethanol.

One could test the usefulness of the method reported in **chapter 3** for the intended purposes and the hypothesis that chlorophylls *a* and *b* are the sources of the greenish hue that accompanies the yellow colour after dyeing alum pre-treated textiles with weld. This could be done by seeing whether there is a relation between the optical absorbance values—due to **chls** and their structurally similar breakdown products—of extracts of samples of weld and the chroma (saturation) of the greenish hue of alum-mordanted wool dyed with the same plant materials. However, although many samples would be needed, the presence of such a relation

would only be suggestive. This is because the chroma of the greenish hue could be due to something else. For example, it could be correlated to the total content of flavonoids.

6.4. Chapter 4

The effect of different concentrations of aluminium ion on the photo-stability of the dye of weld, and the relative photo-stability of **lut**, **img** and **ldg** in solution are reported in **chapter 4**. Results of the colours obtained by dyeing alum-mordanted wool with the three flavones are also presented.

The observed order of photo-stability of the flavones in solution was **ldg** >> **lut** > **img**. However, alum-mordanted wool dyed with **ldg** (or with an extract of weld) was much less colourful than that dyed with either **lut** or **img**. At least in part, this could be due to a reduced uptake of **ldg** by Al^{3+} -mordanted wool relative to that of **lut** or **img**. These observations should support answering the question whether it is preferable for the endogenous glycosidase of weld to be inactivated before extraction of the flavonoids in order to obtain the most photo-stable dye, but are not conclusive. A dye containing flavonoid glycosides and aglycones is obtained if this glycosidase is inactivated before extracting the flavonoids of the plant. Conversely, a dye containing only the aglycones is obtained if the enzyme is not inactivated before the extraction process. Comparison of the light-fastness of the colour of Al^{3+} -mordanted wool dyed with a glycosidase-hydrolysed extract of weld with that of Al^{3+} -mordanted wool dyed with an extract in which the glycosidase has been inactivated before extracting the flavonoids is expected to be decisive.

The light-fastness of the colour of weld-dyed wool decreased with increasing $[\text{Al}^{3+}]$ used for premordanting the wool. As the gain in light-fastness by the use of low $[\text{Al}^{3+}]$ was limited, this cannot be a way to meet today's requirement of light-fastness of the colours of dyed textiles by itself. In **chapter 4**, reference is made to a broader strategy of which using low $[\text{Al}^{3+}]$ for premordanting the wool is but one part in order to address the need for increased light-fastness of the colour of wool dyed with weld. Such a strategy—largely based on information in different publications [5-8]—could also include:

- Using slightly more dyestuff than one would normally use;
- Using the preferable approach regarding the use of a dyestuff that is flavonoid glycoside-rich or flavonoid glycoside-poor. This relates to the optional inactivation of the endogenous glycosidase before extracting the flavonoids of weld;
- Adding antioxidants—such as gallic acid—to the dyed textile, preferably, with the antioxidants being obtained from agricultural/food processing residues (as an example, gallic acid and many other polyhydroxyphenols can be obtained from pistachio green hull).¹

¹ The antioxidants could also be generated through the photodecomposition of part of the components of the dyestuff. For example, Al^{3+} -mordanted wool could be dyed with both outer scales of onions (onion skins) and weld. This could lead to a characteristic change in the yellow colour over time as, upon exposition to light, textiles dyed with onion skins appear to fade [9, 10] and Al^{3+} -mordanted wool dyed with weld darkens and becomes redder [11] (Fig. 6). Lightening of the fabric would be expected to precede its darkening/reddening since the flavonol quercetin is the main flavonoid aglycone of onion skins {[10, 12] and data not shown} and quercetin appears to be less stable upon exposition to light than luteolin, the main flavonoid aglycone of weld [9]. The photo-oxidative

The use of such a strategy limits the range of colours that can be obtained upon dyeing textiles with weld. However, each of its elements could lead to a small gain in light-fastness of the colour. Combined, such gains might be a way of meeting today's requirement of the property.

6.5. Chapter 5

Chapter 5 reports a work on education in chemistry at undergraduate level. Students of molecular life sciences and biotechnology of Wageningen University had the opportunity to deepen their knowledge on HPLC and quantitative analysis using the internal standard method while working on a “natural dyes for textiles”-themed experiment. Their assignment was to quantify **ldg**, **img**, and **lut** in a sample of weld, and to dye alum-mordanted wool with an extract of the same sample. Based on 15 reports by students, it was observed that nearly all of them understood how separation by reversed phase chromatography works. Furthermore, half of the students knew how to use the internal standard method for quantitative analysis, and one-third of them had difficulty with it.

Students also mimicked the work of chemists investigating historical textiles. For that, they had the assignment of performing a small-scale extraction of the dyed wool. After analysis of the sample by HPLC, each student was asked to compare the retention times and UV-absorption spectra of the three main flavonoids of the chromatogram of the wool sample with those of pre-analysed authentic standards of **ldg**, **img** and **lut**, and to compare the chromatographic profile of the wool sample with that of the weld sample.

The dyeing bath was prepared with deionised water in this experiment and in part of the experiments reported in **chapter 4**. Although it is expected that the extract of weld with which the bath was prepared contained ions, it could have been preferable to use a bath richer in ions. Osmotic pressure is developed as water enters the wool fibre, and electrolytes in the dyeing bath lead to a decrease in the difference between the pH inside the fibres and that of the bath [13]. The pH equilibration relate to the flow of ions through the dyeing bath–fibre interface. If the presence of electrolytes in the dyeing bath also influences the flow of the dye molecules through the dyeing bath–fibre interface, it might be preferable to prepare the dyeing bath with electrolytes-rich water.

A procedure for extracting dye molecules from the wool was developed for the experiment described in this chapter. A small piece of a single thread of dyed wool is extracted with methanol–water–formic acid 80:15:5 at 60 °C for 30 min. The development of this procedure was based on:

- the fact that methanol–water 8:2 is a good solvent for the extraction of the flavonoids of weld {Cristea *et al.* reported one of the works using this solvent for the purpose; they observed it to be the most efficient among five different solvents [14]};
- the work by Zhang *et al.*, in which samples of dyed textile fabrics were extracted with methanol–formic acid 95:5 at 60 °C for 30 min [9].

The extraction conditions are mild, with the chromatogram of the extract of the dyed wool being very similar to that of the extract of weld (Fig. 5.2). The larger ratios **img**–**ldg** and **lut**–

degradation of quercetin leads to at least one compound that displays antioxidant activity, 3,4-dihydroxybenzoic acid (protocatechuic acid) [10].

Img of the chromatogram of the extract of the dyed wool relative to that of the extract of weld are expected to be due to the following reasons:

- lower uptake of the diglycosides relative to the monoglycosides and of the monoglycosides relative to the aglycones by the Al^{3+} -mordanted wool (this is in agreement with the data discussed in section 4.4.2.2);
- worse extraction of the diglycosides relative to the monoglycosides and of the monoglycosides relative to the aglycones from the Al^{3+} -mordanted wool {the latter is in line with results published by Willemen *et al.* [11] (left panel of Fig. 5 and preceding discussion)}.

As the difference between the chromatograms in terms of relative quantities of the flavonoids is small, the reported procedure is suitable for the intended purposes. It may also be used for research purposes, as done—in an adapted form—by Willemen *et al.* [11].

6.6. Concluding remarks

The work reported in this thesis is focused on the use of dyestuff of weld (*R. luteola* L.) for textile dyeing. Obtaining dyes from crops cultivated as dye plants may not be environmentally friendly [15]. Advantageously from the environmental impact point of view, natural dyes for large-scale use can be obtained from sources such as residues of food production [16, 17]. Therefore, the large-scale use of weld as a dye plant might be something of the past. If that is the case, then the methods described in **chapters 2** and **3** could be used for analysing other sources of dyes after adaptation and additional validation. On what concerns the chromatographic separation of flavonoids, further improvement might be achieved by the use of a column packed with a sub-2 μm superficially porous stationary phase on UHPLC instrumentation [18].

More research on understanding the photodecomposition of natural dyes and on improving the light-fastness of the colours of textiles dyed with many such dyes is needed [19]. Therefore, the discussion in **chapter 4** and above is expected to contribute to ongoing or future works in the field of textile dyeing with natural dyes. The experiment for use in education in chemistry elaborated upon in **chapter 5** may be used as is, as well as the procedure for extracting dye molecules from wool described in the same chapter. The latter may also be used for research purposes. Finally, cultivation of dye plants—weld included—remains important for production of goods at a handicraft level in relation to biological diversity and cultural conservation.

6.7. References

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Summary

Coloured textiles have been used by mankind throughout times. The use of natural dyes experienced decline when synthetic dyes entered the market. Although dyeing textiles with natural dyes is not synonymous with environmentally friendly dyeing, there has been a renewed interest in natural dyes. Weld (*Reseda luteola* L.) used to be an important vegetable source of dye for textiles in Europe and the Mediterranean area. Alum (an aluminium salt)-pre mordanted wool dyed with weld leads to yellow colours, with the main colouring compounds of the plant belonging to the class of the flavonoids. The flavone luteolin (**lut**) and two of its glycosyl conjugates—**lut**-7-*O*-glucoside (**lut** monoglucoside, **lmg**) and **lut**-7,3'-*O*-diglucoside (**lut** diglucoside, **ldg**)—are the main flavonoids of the plant. The research described in this thesis is part of an academic and industrial project focusing on the use of dyestuff of weld for textile dyeing.

A reversed phase-high-performance liquid chromatography with UV detection (RP-HPLC–UV) analytical method was developed and validated for the quantitation of **ldg**, **lmg**, and **lut** via internal standardisation in weld plant material. This was done in connection with the expectation of having to analyse hundreds of samples in the course of the dye research of which this project is part. The method—described in **chapter 2**—is simple, requiring very little manpower input per sample. With the exception of the 15-stirring point magnetic stirrer—that, less conveniently, could be replaced by individual magnetic stirrers—only standard laboratory equipment is needed. The analytical method proved fit-for-purpose based on its validation in terms of extraction efficiency, stability and losses of the compounds, accuracy, precision, and sensitivity. Weld plant material having high concentrations of the flavones could be analysed through a slightly modified version of the method, using 100 mg instead of 200 mg of sample. Analyses of a small number of replicates of 100 and 200 mg of a few samples of the plant are expected to be sufficient to test the validity of this approach.

The analytical method was validated using a 5 µm-particle size RP-HPLC column. Then, the chromatographic separation was speeded up by using a sub-2 µm-particle size (RP-UHPLC) column. This was done while still using a conventional HPLC system, after minor hardware adaptation. The method using the UHPLC column proved fit-for-purpose through comparison of accuracy and precision of the quantitation of **ldg**, **lmg**, and **lut** with those achieved by using the HPLC column. Large gains in analysis time and eluent consumption were obtained in this way.

According to the industrial partner of the project, one of the problems to overcome was a greenish hue that accompanies the yellow colour after dyeing alum-pre mordanted textiles with weld. Such a greenish hue would be undesirable, and it was hypothesised that chlorophylls *a* and *b* are potential sources of it. In relation to this, an analytical method was developed for the comparison of the content of porphyrin ring-containing pigments—chlorophylls (**chls**) and their structurally similar breakdown products—in different samples of weld. The method, that is described in **chapter 3**:

- i. uses ethanol as a mild, non-toxic extraction solvent (that is not selective regarding the “types” of **chls** and plant tissues being extracted);
- ii. covers the entire range of occurring concentrations;
- iii. displays acceptable precision;
- iv. is simple.

The method has the drawback that the manpower input required is not low and displays a limitation. Comparison of the content of porphyrin ring-containing pigments in batches of weld

plant material having different **chls**/chlorophyllides-to-“pheopigments” ratios is hampered due to differences in optical molar absorption coefficients. One could test if this limitation can be overcome by conversion of **chls** and chlorophyllides to the corresponding “pheopigments” by using HCl-acidified ethanol—instead of absolute ethanol—as extraction solvent.

Alum-premordanted wool dyed with weld leads to yellow colours that are of low resistance to light. The work described in **chapter 4** was carried out in the context of such an issue. The effect of different concentrations of aluminium ion on the photo-stability of the dye of weld, and the relative photo-stability of **lut**, **img** and **ldg** in solution are reported. Results of the colours obtained by dyeing alum-mordanted wool with the three flavones are also presented.

On one hand, the observed order of photo-stability of the flavones in solution was **ldg** >> **lut** > **img**. On the other hand, alum-mordanted wool dyed with **ldg** (or with an extract of weld) was much less colourful than that dyed with either **lut** or **img**. The light-fastness of the colour of weld-dyed wool decreased with increasing $[Al^{3+}]$ used for premordanting the wool. As the gain in light-fastness by the use of low $[Al^{3+}]$ was limited, this cannot be a way to meet today’s requirement of light-fastness of the colours of dyed textiles by itself. Nevertheless, it could be part of a broader strategy to address the need for increased light-fastness of the colour of wool dyed with weld.

Undergraduate students of life sciences should be acquainted with HPLC and internal standard method for the quantitation of compounds. Students of molecular life sciences and biotechnology of Wageningen University had the opportunity to deepen their knowledge on both while working on a “natural dyes for textiles”-themed experiment. This experiment is reported in **chapter 5**.

Their assignment was to quantify **ldg**, **img**, and **lut** in a batch of weld plant material, and to dye alum-mordanted wool with an extract of the same batch. Based on 15 reports by students, it was observed that nearly all of them understood how separation by reversed phase chromatography works. Furthermore, half of the students knew how to use the internal standard method for quantitative analysis, and one-third of them had difficulty with it.

Students also mimicked the work of chemists investigating historical textiles. For that, they had the assignment of performing a small-scale extraction of the dyed wool. After analysis of the sample by HPLC, each student was asked to compare the retention times and UV-absorption spectra of the three main flavonoids of the chromatogram of the wool sample with those of pre-analysed authentic standards of **ldg**, **img** and **lut**, and to compare the chromatographic profile of the wool sample with that of the weld sample.

The procedure for extracting dye molecules from the wool used by the students was developed for the experiment described in this chapter. A small piece of a single thread of dyed wool is extracted with methanol–water–formic acid 80:15:5 at 60 °C for 30 min. These conditions are mild, with the chromatogram of the extract of the dyed wool being very similar to that of an extract of weld. Thus, the procedure is suitable for the intended purposes, and was used—in an adapted form—for research purposes.

The work reported in this thesis is focused on the use of dyestuff of weld (*R. luteola* L.) for textile dyeing. From an environmental impact point of view, obtaining natural dyes for large-scale use from sources such as residues of food production might be advantageous over obtaining dyes from crops cultivated as dye plants. Although the large-scale use of weld as a dye plant might be something of the past, what is reported in this thesis remains relevant either “as is” (**chapters 4 and 5**) or after adaptation/additional validation for analysing compounds in

other biological material (**chapters 2 and 3**). Finally, cultivation of dye plants—weld included—remains important for production of goods at a handicraft level in relation to biological diversity and cultural conservation.

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The future has a long history

Stadszegel, Zwolle/The Netherlands

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Different people contributed to the work of this thesis. I am grateful to all of them for the input. The political, conceptual and technical contribution to my PhD work of Eric, Monique, Hendra and Dorien (Rubia Natural Colours), and Elbert, Jacob, Barend, Han and Teris (Laboratory of Organic Chemistry) was of particular importance. I grew professionally considerably through working under the supervision of Han and Teris. Both of them are committed professionals, and the knowledge of Teris on analytical organic chemistry is vast.

My professional development during the large time span of working on this thesis also encompasses that which took place during activities that ran in parallel to it. Different people contributed to my growth as I worked as teaching assistant at Wageningen University and analytical chemist at Entomology. I am grateful to the input of all of them too. Working with Erik and Frank (Laboratory of Organic Chemistry), and Marcel (Laboratory of Entomology) was encouraging and inspiring.

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I was involved on a number of activities besides work. Two particularly important ones were the Dutch language course, and the tennis trainings and competitions. I am thankful for the time I had together with many people during those activities. Mates and those who served as organizers and teachers—professionally or as volunteers—contributed substantially to my development.

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God—my father in Heaven—did great things to me. All what He did allowed me to be where I now am, including being at this point of concluding the PhD work. I am very thankful to Him for the nearness, for everything.

During the whole process, I have seen many people much less often than I would have liked to. I would be glad to see many of them—both those I knew before this very journey started and others I got to know along the way—much more often in the future. With Fefo and Leka—my brother and sister—and many others, I look forward to—to varying degrees—share many adventures to come.

Publications and training activities

PhD work-related publications: full-text papers in peer-reviewed journals

Villela A, van Vuuren MSA, Willemsen HM, Derksen GCH, van Beek TA. Photo-stability of a flavonoid dye in presence of aluminium ions. *Dyes Pigments* 2019; 162: 222–31

Villela A, Derksen GCH, van Beek TA. Analysis of a natural yellow dye: an experiment for analytical organic chemistry. *J Chem Educ* 2014; 91(4): 566–9

Villela A, van der Klift EJC, Mattheussens ESGM, Derksen GCH, Zuilhof H, van Beek TA. Fast chromatographic separation for the quantitation of the main flavone dyes in *Reseda luteola* (weld). *J Chromatogr A* 2011; 1218(47): 8544–50

Villela A, Derksen GCH, Zuilhof H, van Beek TA. Spectrophotometric comparison of the content of chlorophylls in weld (*Reseda luteola*). *Anal Methods* 2011; 3(6): 1424–7

Overview of completed training activities

If no geographic reference is made, it is because the activity took place in Wageningen or its surroundings.

Discipline-specific activities

KNCV (Royal Netherlands Chemical Society) Annual Spring Meeting:

- Advances in hyphenated MS; 17 Apr 2008
- New developments in separation methods; 20 Apr 2012

7th Joint Meeting of AFERP, ASP, GA, PSE & SIF on Natural Products with pharmaceutical, nutraceutical, cosmetic and agrochemical interest (Athens/Greece); 04–07 Aug 2008

Annual NWO Meeting on Analytical Chemistry (Lunteren/The Netherlands):

- 2008 edition; 03–04 Nov 2008
- 2009 edition; 02–03 Nov 2009
- 2010 edition; 01–02 Nov 2010

Annual NWO Meeting on Organic Chemistry (Lunteren/The Netherlands); 20–21 Oct 2009

Advanced Organic Chemistry; 2009–2010 (VLAG and Laboratory of Organic Chemistry, WUR)

International Symposium and Exhibition on Natural Dyes (La Rochelle/France); 25–29 Apr 2011 (ARRDHOR CRITT HORTICOLE and CIHAM UMR 5648)

46th International Symposium on Essential Oils (Lublin/Poland); 13–16 Sep 2015 (Medical University of Lublin, Department of Pharmacognosy with Medicinal Plant Unit of Medical University of Lublin, and Polish Academy of Sciences)

19th Congress of the International Society for Ethnopharmacology (Dresden/Germany); 12–14 Jun 2019

Advances in Plant and Food Metabolomics (3rd WURomics Symposium); 12 Dec 2019 (EPS and WUR)

General courses

Information Literacy for PhD Including Introduction to EndNote; 27–28 May 2008 (Library, WUR)

20th VLAG PhD Week (Bergeijk/The Netherlands); 27–30 Oct 2008 (VLAG)

Training “Study Skills”; 10 Oct 2009 (Purple Monkey and WUR)

Techniques for Writing and Presenting a Scientific Paper; 29 Jun–02 Jul 2010 (Wageningen Graduate Schools, WUR)

Mini-Symposium “How to Write a World-Class Paper”; 26 Oct 2010 (Library, WUR)

Advanced Course Guide to Scientific Artwork; 07–08 Nov 2011 (Library, WUR)

Adobe InDesign; 09 Nov 2011 (Library, WUR)

Coaching of ACT (Academic Consultancy Training) Groups; 25 and 28 Mar 2013 (Educational Staff Development, WUR)

Workshop “Scientific Integrity”; 05 Jun 2013 (Wageningen Graduate Schools, WUR)

Teachers’ Lounge: (The Teacher’s Lounge, WUR)

- Kick-off event; 26 Apr 2016
- Café themed “Can we measure the quality of education?”; 15 Mar 2018

Workshop “Distinguishing Science and Metaphysics in Evolution and Religion” (Leiden/The Netherlands); 28–29 Aug 2018 (Lorentz Center)

Optional activities

Preparing PhD research proposal; submitted to VLAG on 29 May 2008

Spectroscopy (BIP 31306); Sep–Oct 2008 (Laboratory of Biophysics, WUR)

ORC PhD Study Trip (Beijing and Shanghai/China); 11–21 May 2009 (PhD students of the Laboratory of Organic Chemistry, WUR)

YELREM (Yearly Entomology Laboratory Research Exchange Meeting): (Laboratory of Entomology, WUR)

- 2016 edition; 01 Jun 2016
- 2018 edition; 24 May 2018
- 2019 edition; 07 Jun 2019

Plant–Soil–Microbe Interactions for Crop and Pest Management Workshop; 30 Jun 2016 (NIOO-KNAW, NWO, and PE&RC)

NEV (*Nederlandse Entomologische Vereniging*) Entomologendag:

- 28th edition; 16 Dec 2016
- 30th edition; 14 Dec 2018

12th Plant–Insect Interaction Workshop; 07 Nov 2017 (group of PhD students of the Laboratory of Entomology, WUR)

Appendix A

Chapter 2: Supplementary information

The content of this appendix is essentially that of the supplementary material of the following paper:

Villela A, van der Klift EJC, Mattheussens ESGM, Derksen GCH, Zuilhof H, van Beek TA. Fast chromatographic separation for the quantitation of the main flavone dyes in *Reseda luteola* (weld). J Chromatogr A 2011; 1218(47): 8544–50.

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SI A.1. Supplementary material and methods

SI A.1.1. HPLC

HPLC analyses carried out in Steenberg: Performed on a system from Dionex Corporation consisting of a low pressure gradient pump (P680 LPG-4), a DAD (UVD340U) and an autosampler (ASI-100), and equipped with a column oven (TCC-100). An Alltima 250 mm × 4.6 mm C18 5 µm-particle size column in combination with a C18 guard column was used. The volume of both, syringe and loop, was 250 µL. The draw rate of the autosampler was 10 µL sec⁻¹. The system was controlled by Chromeleon Client 6.70 SP6 build 1935 software.

SI A.1.2. Identification of the three main flavonoids of *R. luteola* and peak purity assessment

Through preliminary experiments, the identity of the three main flavonoids of *R. luteola* was verified by: (i) comparing the RP-LC–DAD retention times and UV–vis absorption spectra of the peaks of authentic standards to those of the main peaks of an extract; (ii) spiking that extract with **ldg**, **img**, and **lut** standards, followed by HPLC analysis; and (iii) MS and MS/MS data, via LC–(DAD)–MS analyses.

HPLC column: The purity of **ldg**, **img**, and **lut** peaks was assessed through: (i) DAD peak purity analysis (comparison of the purity and threshold angles of the chromatographic peaks by Empower 2 software); and (ii) LC–DAD–MS analysis of an extract of *R. luteola* (MS data and inspection of the UV–vis contour plots of the peaks). UHPLC column: Samples of *R. luteola* H were extracted and treated as usual, stored in freezer for six days, and analysed using the UHPLC column. One of the results was used for evaluation of the purity of **ldg**, **img**, and **lut** peaks. This was done using the system’s software (see section 2.2.4, Chapter 2) in two ways: (i) the peaks were integrated (discarding what was below 5% of their maximum heights) and all spectra within the limits of integration were automatically selected and overlaid, followed by their visual inspection; and (ii) inspection of their UV–vis contour plots.

Internal standard peak purity: *R. luteola* H samples (n = 8), as well as analytical blanks (n = 6)—i.e., samples lacking the plant material that were subjected to the whole analytical procedure—were extracted as usual. Each sample (ready for analysis) was placed in four vials. Part of them was analysed on the same day (overnight, HPLC column), part was stored at 4 °C, and the rest was stored at –20 °C. [Note: These were the same samples used for the evaluation of the precision of the analysis using the UHPLC column (see section 2.2.7)]. After five days, the samples stored at 4 °C were analysed using the UHPLC column. In both cases, HPLC

column and UHPLC column, the average area of the **i.s.** peak of samples was compared with that of the blanks.

SI A.1.3. Determination of the limits of detection (LOD) and quantitation (LOQ)

HPLC column: They were estimated based on the slopes of the calibration curves and the *s* of the baseline noise of analyses of blank samples [1]. The relation concentration vs. peak area was converted to concentration vs. peak height for **ldg**, **img**, and **lut** and the intercepts of the equations were set to zero. Analytical blanks (*n* = 5) were injected and the *s* of the baseline noise at 345 nm over the range 40.0–45.0 min was calculated, using all data points (ASCII files). The average of the *s* values was 0.043 ± 0.014 mAU. The LOD and LOQ values were equal to the concentration of **ldg**, **img**, and **lut** solutions that corresponded with 3.3 and 10× the *s* values of the baseline, respectively. The LOD values were additionally estimated based on the signal-to-noise ratio [1]. Using the *s* of the baseline noise value of 0.043 mAU, **ldg**, **img**, and **lut** standard solutions leading to signal-to-noise ratios of 2:1 or 3:1 were injected in triplicate. The stock solutions of **ldg** (90 $\mu\text{g mL}^{-1}$) and **img** (128 $\mu\text{g mL}^{-1}$) were prepared in methanol–dimethyl sulfoxide 7:3 (v/v), and that of **lut** (138 $\mu\text{g mL}^{-1}$) was prepared in methanol. Dilutions: In all cases, only methanol was used. UHPLC column: Generally, determined as described for the HPLC column, except that analytical blanks (*n* = 6) were injected and the *s* of the baseline noise was calculated at 345 nm (having 470 nm as reference λ) over the range 1.50–2.00 min, using all data points (CSV files). The average of the *s* values was 0.086 ± 0.014 mAU.

SI A.2. Supplementary results and discussion

SI A.2.1. Peak purity

For the chromatographic separation described here (HPLC column), it was observed by (LC–)MS analysis that no other compound of *R. luteola* co-elute with **lut**, whereas an unknown compound was observed to partially co-elute with **img** and **ldg**. Nevertheless, the contour plots of the **ldg** and **img** peaks indicated that there is no co-elution with any other UV–vis absorbing compounds with significantly different spectral profile at appreciable amounts. The same was observed for the peaks of all three flavones, **ldg**, **img**, and **lut**, via peak purity analysis. Moreover, there is no significant co-elution between **i.s.** and minor compounds of *R. luteola* as the change in the **i.s.** peak area due to such co-elution is <1%. Thus, the peak areas at 345 nm are representative for **ldg**, **img**, **lut**, and **i.s.**

UHPLC column: Evaluation of the spectral homogeneity of **ldg** and **img** peaks and inspection of their UV–vis contour plots indicated that there is no co-elution with any other compound with significantly different spectral profile at appreciable amounts. Although the same holds true regarding the UV–vis contour plot of the **lut** peak, the spectra at its front display different characteristics from the other ones recorded during the elution of the peak. Also using the UHPLC column, there is no significant co-elution between the **i.s.** and minor compounds of *R. luteola* as the change in the **i.s.** peak area due to such co-elution is <1%. The spectral inhomogeneity of the **lut** peak was observed not to interfere with its quantitation, as the quantitation of **ldg**, **img**, and **lut** using the UHPLC column was statistically the same as that carried out using the HPLC column (see section 2.3.7). Thus, also using the UHPLC column, the peak areas at 345 nm are representative for **ldg**, **img**, **lut**, and **i.s.**

Table SI A.1. Results of the estimation of the LOD and LOQ values of **ldg**, **img**, and **lut** using both columns (HPLC and UHPLC) based on the slopes of the calibration curves and the *s* of the baseline noise of analyses of analytical blanks.

Compound (z)	Relation [z] ($\mu\text{g mL}^{-1}$) vs. H_z ^a (R^2)	Relation [z] ($\mu\text{g mL}^{-1}$) vs. H_z ^a , with $b^b = 0$ (R^2)	LOD (ng mL^{-1}) (s)	LOQ (ng mL^{-1}) (s)
HPLC column (analytical blanks: n = 5)				
ldg	$y = 0.0028x - 0.004$ (0.9987)	$y = 0.0027x$ (0.9984)	50 (20)	160 (50)
img	$y = 0.004x - 0.0037$ (0.9998)	$y = 0.004x$ (0.9998)	40 (10)	110 (40)
lut	$y = 0.0113x + 0.0101$ (0.9998)	$y = 0.0114x$ (0.9996)	12 (4)	40 (10)
UHPLC column (analytical blanks: n = 6)				
ldg	$y = 1.9244x + 1.3292$ (0.9999)	$y = 1.9364x$ (0.9998)	150 (20)	440 (70)
img	$y = 2.5681x + 16.178$ (0.9878)	$y = 2.6656x$ (0.9854)	110 (20)	320 (50)
lut	$y = 4.1341x + 2.7314$ (0.9996)	$y = 4.1672x$ (0.9995)	70 (10)	210 (30)

^a Peak height.

^b Intercept of the [z] vs. H_z line.

SI A.3. Supplementary reference

[1] Validation of analytical procedures: text and methodology Q2(R1), comprising Q2A (1994) and Q2B (1996). International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; 2005.

Appendix B

Chapter 3: Supplementary information

The content of this appendix is essentially that of the supplementary material of the following paper:

Villela A, Derksen GCH, Zuilhof H, van Beek TA. Spectrophotometric comparison of the content of chlorophylls in weld (*Reseda luteola*). Anal Methods 2011; 3(6): 1424–7.

The absorption spectrum of an absolute ethanol extract of weld sample **B** (see footnote 7 of chapter 3) is presented in Figure SI B.1. The absorbance at the main red absorption band at ~665 nm was used to compare different samples.

Figure SI B.1. Absolute ethanol extract of weld sample **B** (see footnote 7 of chapter 3) in 10 mm-pathlength quartz cuvette. The wavelength of the main red absorption band is indicated.

Precision of the analytical method using absolute ethanol and 10 mm-pathlength plastic cuvettes (see footnote 6 of chapter 3) was evaluated. The dependence of the extraction efficiency on the particle size of the samples and the correlation between the absorbance of ethanolic and DMF extracts of different *R. luteola* samples using 10 mm-pathlength cuvettes were verified. Results are presented below.

Table SI B.1. Absorbance of extracts of weld samples due to chlorophylls and their structurally similar breakdown products obtained with ethanol and *N,N*-dimethylformamide, using two types of cuvettes. ^{a,b}

Entry	Plant material	n	Extraction procedure ^c and series of measurements	Solvent ^d	Absorbance (AU) and s_r (%) ^e
10 mm-pathlength plastic cuvette					
1	A	3	3-min vortexing	Abs EtOH	0.096 (4.4)
2	B	3	3-min vortexing; day <i>a</i>	Abs EtOH	0.159 (0.3)
3	B	3	3-min vortexing; day <i>a</i>	Abs EtOH	0.164 (2.5)
4	B	3	3-min vortexing; day <i>a</i>	Abs EtOH	0.160 (3.8)
5 ^f	B	9	3-min vortexing; day <i>a</i>	Abs EtOH	0.161 (2.7)
6	B	3	3-min vortexing; day (<i>a</i> + 3)	Abs EtOH	0.158 (3.1)
7	B	3	3-min vortexing; day (<i>a</i> + 3)	Abs EtOH	0.157 (1.1)
8	B	3	3-min vortexing; day (<i>a</i> + 3)	Abs EtOH	0.164 (1.7)
9 ^f	B	9	3-min vortexing; day (<i>a</i> + 3)	Abs EtOH	0.159 (2.9)
10	C 0.75	3	3-min vortexing	Abs EtOH	0.149 (6.1)
11	C 0.50	3	3-min vortexing	Abs EtOH	0.180 (4.6)
12	C 0.25	3	3-min vortexing	Abs EtOH	0.216 (1.5)
13	D	3	3-min vortexing; day <i>a</i> (n = 2) and day (<i>a</i> + 3) (n = 1)	Abs EtOH	0.271 (2.7)
14	E	3	3-min vortexing	Abs EtOH	0.643 (0.6)
10 mm-pathlength quartz cuvette					
15	B	3	3-min vortexing	DMF	0.215 (1.8)
16	C 0.25	3	3-min vortexing	DMF	0.287 (1.3)
17	D	3	3-min vortexing	DMF	0.390 (3.2)
18	E	3	3-min vortexing	DMF	1.026 (1.1)

^a λ of detection: entries 1 through 14, 665 nm – 750 nm (*i.e.*, absorbance at 750 nm subtracted from that at 665 nm); entries 15 through 18, 664 nm – 750 nm. *NB* Subtraction of absorbance at 750 nm was only done for consistency with data in chapter 3. In all cases the absorbance at 750 nm varied between –6 and 10 mAU.

^b **A** through **E** are the codes of (dried and ground-sieved) weld samples in order of increasing concentration of **chls** and breakdown products. Note: 1) Differences among samples include cultivar and plant parts used;²⁰ 2) Numbers behind **C**: Pore size of sieves used during the grinding–sieving process (in mm).

^c Always at room temperature and under reduced light.

^d Abs EtOH = absolute ethanol and DMF = *N,N*-dimethylformamide.

^e Average absorbance (s_r = relative standard deviation).

^f Combining results of previous 3 entries.

Precision: Repeatability was assessed by analysis of sample **B** (n = 9; entry 5 of Table SI B.1): s_r <3.0%. Sample **B** was analysed again on another day (n = 9; entry 9). The difference between both results was 1%. Based on the results seen in entries 15 and 18 of Table 3.1 (chapter 3), and 1 and 14 of Table SI B.1, samples with absorbance of 190 mAU using 2 mm-pathlength plastic cuvettes should display absorbance ≤ 1.0 AU using 10 mm-pathlength plastic cuvettes. Thus, users having many samples with absorbance ≤ 190 mAU (with 2 mm-pathlength cuvettes) may use the 10 mm-pathlength cuvettes for increased precision.

Dependence of extraction efficiency on particle size: An increase of absorbance with decreasing particle size is seen (entries 10 through 12). Additionally, as expected, s_r decreases with the decrease of the particle size. This suggests that it is harder to obtain representative samples from coarse plant materials than from fine ones.

Figure SI B.2. Correlation between absorbance of ethanolic and DMF extracts of *R. luteola*. Average absorbances are plotted; error bars = $1 \times$ standard deviation. Ethanolic extracts: 10 mm-pathlength plastic cuvette; $n = 3$ (C 0.25, D, and E) and $n = 9$ (B). DMF extracts: 10 mm-pathlength quartz cuvette; $n = 3$ (all).

As also seen previously, the absorbance of ethanolic and DMF extracts linearly correlate. Note: The slopes of the lines seen in Figure 3.1 (chapter 3) and in Figure SI B.2 are different due to different pathlengths of the cuvettes used.

A gradual increase in absorbance from sample 1 to 20 after simultaneous extraction of 20 samples (Vortex Genie-2; set to maximum speed) followed by sequential absorbance measurements was observed. The results (entries 1, 3, and 5 of Table SI B.2) indicate that the

extraction of **chls** and their breakdown products continues statically if the plant material is left in contact with the extraction solvent. Simultaneously, there is evidence that the five-minute centrifugation step at 13,000 rpm enhanced the extraction efficiency. In spite of the observed gradual increase in absorbance, the absorbance of the extracts seen in entry 2 is lower than those seen in entries 1 and 3, and the absorbance of the extracts seen in entry 4 is lower than those seen in entries 3 and 5.

Table SI B.2. Absorbance of acetone extracts of weld sample **B** obtained with two methods of extraction, after simultaneous extraction of 20 samples followed by sequential absorbance measurements. ^a

Entry	n	Extraction procedure and sample treatment ^b	Position of the samples within a series ^c	Absorbance (AU) and s_r (%) ^d
10 mm-pathlength quartz cuvette				
1	3	3-min vortex; 5-min centrifuge	1–3	0.158 (3.5)
2	4	3-min vortex	4–7	0.149 (5.3)
3	3	3-min vortex; 5-min centrifuge	8–10	0.170 (2.6)
4	4	3-min vortex	14–17	0.160 (4.4)
5	3	3-min vortex; 5-min centrifuge	18–20	0.184 (4.6)

^a λ of detection: 664 – 740 nm. Note: Range of readings at 740 nm: 1–4 mAU.

^b Room temperature.

^c Position in the analysed series of samples.

^d Average absorbance (s_r = relative standard deviation).

Appendix C

Chapter 4: Supplementary information

The content of this appendix is equal to that of the supplementary material of the following paper:

Villela A, van Vuuren MSA, Willemsen HM, Derksen GCH, van Beek TA. Photo-stability of a flavonoid dye in presence of aluminium ions. *Dyes Pigments* 2019; 162: 222–31

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SI C.1. Supplementary material and methods

SI C.1.1. General material

This section lists some of the compounds/material to which reference is made below (not necessarily explicitly). Additional compounds/material used are listed in the sections in which they are specifically referred to.

Solvents

- 96% ethanol (for general laboratory use grade; VWR International, Fontenay-sous-Bois/France)
- Distilled methanol: HPLC grade methanol was distilled
- Water was deionized using a Seradest SD 2000 ion-exchanger (Seral Erich Alhäuser, Ransbach-Baumbach/Germany), an EasyPure UV system (Barnstead/Thermolyne, USA), or a Milli-Q Integral 3 system (Millipore, Molsheim/France)

Compounds

- Alum $[\text{KAl}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}]$; puriss. *p.a.* grade; Sigma-Aldrich, Steinheim/Germany]
- Aluminium sulphate tetradecahydrate (ViVoChem, Almelo/The Netherlands)
- Tartaric acid (Brenntag, Dordrecht/The Netherlands)

Equipment

- Type K thermocouple PeakTech TF-50 connected to a digital thermometer PeakTech 3150 (PeakTech Prüf- und Messtechnik, Ahrensburg/Germany)
- 45 kHz ultrasonic cleaner (model USC 300 T; produced for VWR International in Malaysia)
- Balances: **i)** 0.1 µg-least division balance (type UM 3; Mettler-Toledo, Greifensee/Switzerland), **ii)** 5 µg-least division balance (Mettler Instrumente, Greifensee/Switzerland), **iii)** type AE 260 balance operating in 0.1 mg-least division mode (Mettler-Toledo, Greifensee/Switzerland), **iv)** 0.01 g-least division balance (type PM 600; Mettler-Toledo, Greifensee/Switzerland), and **v)** ABS 220-4 (Kern & Sohn, Balingen/Germany)
- Centrifuge (WS 235; Bauknecht Hausgeräte, Stuttgart/Germany)
- Incubator shakers: G24 Environmental and Innova 4080, both from New Brunswick Scientific (Edison/USA)
- Spectrophotometer used for measuring I^* , a^* , and b^* quantities (CIELAB colour space): Macbeth Color-Eye 7000 (X-Rite, Michigan/USA)

Miscellaneous

- Phosphor-coated low pressure mercury vapour lamp (84-2094-2 – Jelight, Irvine/USA) charged through a 220 V, 50 Hz power supply (PS-2004-20 – Jelight, Irvine/USA). Note: The emission profile of the lamp is depicted in Figure SI C.9
- Folded filter paper (597 ½, Ø 150 mm; Whatman, Dassel/Germany)

- Detergent SARABID PAW (CHT R. Beitlich, Tübingen/Germany)
- Ready-to-dye wool [Kova Wool Sateen White (part number W110); Whaleys (Bradford), Bradford/United Kingdom]
- Sample of the aerial parts of dried and ground weld {described by Villela *et al.* [1]}

SI C.1.2. Preparation of aerated methanol–water 8:2 (v/v)

Aerated methanol–water 8:2 (v/v) was prepared on the day of use, from methanol and water freshly aerated during 30 min. Such aerated solvents—methanol, water, or the mixture—were used for the preparation of stock and working solutions of the flavones, Al^{3+} and HNO_3 . The freshly prepared aerated methanol–water 8:2 (v/v) was also used for filling the volumetric flasks of calibration solutions and solutions used for the irradiation experiments described below up to the marks.

Distilled methanol and deionized water (EasyPure UV system) were used. These solvents were aerated simultaneously, but separately, each in its bottle. This was done by the bubbling of air filtered through active charcoal (coarse particles) for 30 min, on the day of use. The solvent-mixture was prepared using such aerated solvents. Quality of the aerated methanol–water 8:2 (v/v) was monitored by recording its UV–vis absorption spectrum against air at every irradiation experiment event described below, and comparing it with previous ones. Notes:

- The solvents were added to the aeration bottles—or replaced—from time to time.
- Details of the spectrophotometric analyses: See section SI C.1.8.

SI C.1.3. Determination of purity of lut, lmg, and ldg by NMR spectroscopy

Material and method of the determination of the purity by NMR spectroscopy of the flavones referred to in section SI C.1.4 are briefly reported in this section. The analyses were carried out by the use of the 0.1 μg -least division balance, maleic acid (grade: puriss., $\geq 99.0\%$ by HPLC; actual purity: 100.0%, according to certificate of analysis; Fluka) [2], DMSO- d_6 (99.5+ atom % D, Aldrich), and a 400 MHz NMR spectrometer (Avance III; Bruker, Fällanden/Switzerland). Notes:

- All cases: Flavones in vials with lids partially unscrewed were placed in a desiccator under reduced pressure on the day before use, or earlier. Exposure of the vials to light during the process was minimised.
- The following quantities were accurately weighed:
 - **Lut**: ~ 0.7 mg (**lut**) and ~ 0.6 mg (maleic acid)
 - **Lmg**: ~ 0.4 mg (**lmg**) and ~ 0.2 mg (maleic acid)
 - **Ldg**: 0.1 – 0.4 mg (**ldg**) and ~ 0.1 mg (maleic acid); carried out twice, using separate portions of **ldg** of the same batch
- ^1H -NMR experiments (30° pulse, 2 s relaxation delay) were used. The number of scans was either 512 (**lut**) or 1,024 (**lmg** and **ldg**).

SI C.1.4. Preparation of stock and working solutions of the flavones, Al^{3+} , and HNO_3 used for construction of the calibration lines and preparation of the solutions used for the described irradiation experiments

Lut stock solutions ($292 \mu\text{g mL}^{-1}$; 1.02 mM), **lmg** stock solutions ($192 \mu\text{g mL}^{-1}$; 0.42 mM), and **ldg** stock solutions ($47 \mu\text{g mL}^{-1}$; 0.08 mM) were prepared using either the 0.1 μg -least division balance or the 5 μg -least division balance. In each case, the flavone was transferred to a volumetric flask using a syringe and aerated methanol–water 8:2 (v/v). For completion of the dissolution of **lmg** and **ldg**, the partially filled flasks were wrapped in aluminium foil and placed in an incubator shaker (130–160 rpm) at room temperature for 1–1.5 day. Working solutions were prepared by dilution of stock solutions using volumetric pipettes, and volumetric flasks. In all cases:

- Aerated methanol–water 8:2 (v/v) was added to the marks.
- Solutions were stored in a refrigerator.
- Flavones in vials with lids partially unscrewed were placed in a desiccator under reduced pressure on the day before use, or earlier. Exposure of the vials to light during the process was minimised.

The following chemical compounds were used: **Lut** (96% by NMR; Indofine Chemical Company, Hillsborough/USA); **lmg** (93% by NMR; Extrasynthese, Genay/France); **ldg** (86% by NMR; Extrasynthese, Genay/France).

Aluminium nitrate nonahydrate (99.997%; Aldrich Chemistry, St. Louis/USA) was used (Note: Although the crystals of the salt are deliquescent[3] and seemed to have taken up some water, no special handling procedure was adopted; the salt was easy to handle throughout the work). Stock solutions were prepared by weighing 124 mg of the salt in a 25 mL volumetric flask using the balance operating in 0.1 mg-least division mode. Working solutions were prepared by subsequent dilutions of Al^{3+} stock solution using volumetric pipettes and volumetric flasks. In all cases, aerated methanol–water 8:2 (v/v) was added to the marks. Solutions were stored in a refrigerator.

HNO_3 (65%, extra pure; Merck, Darmstadt/Germany) stock solutions in water were prepared using aerated water (as described above), a 1 mL volumetric pipette, and a 100 mL volumetric flask. HNO_3 stock solutions in methanol–water 8:2 (v/v) were prepared by dilution of the HNO_3 stock solution in water using a 2 mL volumetric pipette, aerated methanol (as described above), and a 10 mL volumetric flask. After cooling down the flask, aerated methanol was added to the mark. Working solutions were prepared by dilution of the HNO_3 stock solution in methanol–water 8:2 (v/v) using volumetric pipettes and volumetric flasks. Aerated methanol–water 8:2 (v/v) was added to the marks. Solutions were stored in a refrigerator.

General note: All solutions (flavones, Al^{3+} , and HNO_3) were prepared anew for use in irradiation experiments 2a and 2b, and special irradiation experiment (see section SI C.1.7).

SI C.1.5. Calibration lines

Stock and working solutions of the flavones, Al^{3+} , and HNO_3 were used for preparing the calibration solutions with volumetric pipettes and 5 mL volumetric flasks. In all cases, freshly prepared aerated methanol–water 8:2 (v/v) was added to the marks of those flasks. After homogenization, solutions were kept in the dark at room temperature for more than 60 min prior to transferring aliquots to HPLC vials. Leftovers of the calibration solutions were stored in a

refrigerator. Vials with calibration solutions were placed in an HPLC autosampler for RP-HPLC–UV analysis (see section **SI C.1.9** below) on the same day of their preparation. In all cases, they were analysed ≤ 18 h after the start of the HPLC sample sequences.

The calibration lines were constructed using peak areas of the 350 nm traces (**lut** and **lmg**) and of the 340 nm traces (**ldg**). A straight line mathematical model with intercept set to zero was fit through the experimental data. Equations of the best-fit lines were obtained together with coefficient of determination (r^2) values. These regression analyses were carried out via the least-squares method using Microsoft Excel.

SI C.1.6. Solutions used for the irradiation experiments

This section reports the preparation of the solutions irradiated in experiments 1a, 1b, 2a, 2b, and special irradiation experiment (see section **SI C.1.7**). These solutions were prepared on the day each irradiation experiment started (**day 1**; see section **SI C.1.7**). After being let come to room temperature in the dark, stock and working solutions of the flavones, Al^{3+} , and HNO_3 were used for preparing the solutions with volumetric pipettes and volumetric flasks. The concentration of HNO_3 of the irradiated solutions was 0.35 mM. This was achieved by adding working solution of HNO_3 to all flasks, except to those containing **lut** and Al^{3+} in a ratio 1:10. In this case, the lowering of the pH was due to the Lewis acid Al^{3+} , rather than to the HNO_3 or to a combination of both. Aerated methanol–water 8:2 (v/v) was added to the marks in all cases. After homogenization, solutions were kept in the dark at room temperature not less than 60 min before the cuvettes in which they were irradiated were filled (not using Pasteur pipettes to prevent possible contamination), and aliquots were transferred to HPLC vials. Furthermore, aliquots of these solutions were used for the spectrophotometric analyses (see section **SI C.1.8**). Unnecessary exposure of solutions to light was avoided at all times. Notes:

- The 60 min-interval between the preparation of the solutions and their use was an arbitrary choice motivated by the fact that flavonoid– Al^{3+} complexation does not reach equilibrium immediately. This has been monitored spectrophotometrically by Surowiec *et al.* [4] and visually observed during this work. The intensity of the colour of HNO_3 -containing solutions of **lut** and Al^{3+} (ratios 5:1 and 1:1) seemed to increase over time in a preliminary experiment.
- Code of solutions used: $\text{flavone}_{[\text{flavone}]}-\text{Al}_{[\text{Al}]}$, in which $[\text{flavone}] = [\text{flavone}]_0$, $\text{Al} = \text{Al}^{3+}$, and concentrations are expressed in mM.

SI C.1.7. Irradiation experiments

The solutions prepared as described above were irradiated using a set-up consisting of a magnetic stirrer (RCT basic, IKA Labortechnik; setting of stirring rate: 5), a phosphor-coated low pressure mercury vapour lamp, and an air blower (220 V, 700 W) for cooling down the system. All this was placed inside a box prepared from cardboard boxes. The system was generally well closed, apart from a large opening at the front, and two smaller openings at the back and top. The front opening, used for sampling, had a removable cardboard cover. The two other openings had the functions of: **i.** making room for all cables and pole of the retort stand, and **ii.** dissipation of heat. The surroundings of the set-up were further protected from radiation originating from the lamp by covering the box with cloth of black colour. Adhesive tapes and

small pieces of cardboard were used for making slots for the cuvettes on the cover plate of the magnetic stirrer. The distance from the front window of the cuvettes to the centre of the lamp was 2.02 ± 0.04 cm (3/4-inch). Considering the diameter of the lamp, the distance from the front window of the cuvettes to the closest edge of the lamp was 1/2-inch. Pictures of the set-up are seen in Figure SI C.1.

The lamp was aligned equidistantly from the cuvettes prior to irradiations. The lamp, air blower, and magnetic stirrer were switched on 1.0–1.3 h before the beginning of each irradiation experiment for warm-up of the lamp and system. The observations reported were chiefly made during five irradiation experiments. They are referred to as 1a and 1b (1st irradiation replicate), 2a and 2b (2nd irradiation replicate), and special irradiation experiment. During the first four irradiation experiments, the eight solutions—**lut**_{0.10}, **lut**_{0.10}–Al_{0.02}, **lut**_{0.10}–Al_{0.10}, **lut**_{0.10}–Al_{1.00}, **lut**_{0.05}, **lmg**_{0.05}, **ldg**_{0.05}, and **ldg**_{0.05}–Al_{0.05}—were irradiated in duplicate. Solutions **lut**_{0.20} and **lut**_{0.10}–Al_{0.99} were irradiated during the special irradiation experiment, with a filter blocking radiation of wavelengths shorter than ~420 nm (420 nm cut-off filter) being placed in front of the cuvette containing **lut**_{0.10}–Al_{0.99} (Figs. SI C.1 and SI C.5).

The temperature inside the cardboard box—next to the irradiation site—was measured at different time-points during irradiation experiments 1a–2b using a thermocouple. The recorded temperatures ranged from 29 to 34 °C. Note: Due to fluctuation of the temperature readings (~0.5 °C), a small Erlenmeyer with water was placed inside the cardboard box—also next to the irradiation site—for use during the special irradiation experiment. The Erlenmeyer was closed with a rubber stopper having a hole in it, through which a mercury thermometer was passed. The temperature at which the irradiation took place was measured at five time-points using both devices, with the outcome being 30–34 °C (thermocouple, with the fluctuation of the readings being only 0.1 °C this time) and 31–35 °C (mercury thermometer).

Four solutions were irradiated simultaneously during irradiation experiments 1a–2b, as there were places for four cuvettes on the magnetic stirrer. Four glass cuvettes of 3.5 mL—with round PTFE tight-fit stoppers—having light paths of 10.00 mm and nearly identical UV–vis absorption spectra were used (Fig. SI C.9). The place in the set-up and the cuvette in which each solution was going to be irradiated were assigned randomly. A small magnetic stir bar was placed in each cuvette. As described in section SI C.1.6, no Pasteur pipettes were used to transfer the solutions from the volumetric flasks to the cuvettes. Notes:

- As the cuvettes were not at the centre of the cover plate, stirring of the solutions was not optimal.
- The ISO 105–B02 norm contributed to the ideas of having an air blower in the set-up and no radiation of wavelengths shorter than ~300 nm.
- In a preliminary experiment, cuvettes with square openings—and the corresponding disposable plastic caps—were observed to be unsuitable for use due to loss of solvent during the irradiation.

The photodecomposition of **lut**, **lmg**, and **ldg** was monitored by RP-HPLC–UV (see section SI C.1.9). Prior to the beginning of the irradiation experiments, aliquots of the solutions were transferred from the volumetric flasks to HPLC vials. These aliquots were referred to as having been collected at t_0 ($t = 0$ h). Furthermore, 0.05 mL aliquots of the solutions were collected at $t = 1.0$ h, 2.0 h, 3.0 h, 4.0 h, 8.0 h, and 24.0 h, as irradiations started on one day (**day 1**) and finished on the following one (**day 2**). Samplings were carried out as follows: Cuvettes were removed from the set-up, and aliquots of the solutions were transferred to HPLC vials of

maximal residual volume of 6 μL using Pasteur pipettes. Once closed, the cuvettes were placed back to their positions in the set-up. Each sampling took 4–6 min. For increased stability of the irradiation, the system was kept running during each sampling, *i.e.*, lamp, air blower, and magnetic stirrer remained on. For safety reasons, special safety glasses (Laser 2 000; nearly no transmission of light in the range 190–534 nm) were worn—and the removable cardboard cover was placed at the front opening of the box—during sampling. Notes:

- Special irradiation experiment: Aliquots of the solutions were collected at $t = 2.0$ h, 4.0 h, 8.0 h, and 24.0 h, *i.e.*, as irradiation experiments 1a–2b, except for $t = 1.0$ h, and 3.0 h.
- In all cases, samples were analysed by HPLC undiluted.
- Irradiation experiments 1a–2b and special irradiation experiment: All solutions— t_0 through t_{24} —were injected in the HPLC 24 h or less after sampling.

The photodecomposition of the flavones over time was quantified by RP-HPLC–UV (see section **SI C.1.9**) using peak areas of the 350 nm traces (**lut** and **img**) and of the 340 nm traces (**ldg**), and the calibration lines described above. Except for solution **lut**_{0.20}, the [flavone] of all samples— t_0 ($t = 0$ h) through t_{24} ($t = 24$ h)—of each irradiated solution was comprised within the range of concentrations used for construction of the calibration lines. A straight line mathematical model was fit through the experimental data {[flavone] (mM) *vs.* time (min)}. This regression analysis was carried out for each of the solutions irradiated during experiments 1a–2b and special irradiation experiment via the least-squares method using Microsoft Excel. Alike for the calibration lines, equations of the best-fit lines were obtained together with coefficient of determination (r^2) values.

The photodecomposition of the flavones was described as being of zero-order, in which the rate of reaction equals the rate constant ($d[\text{flavone}]/dt = k$). As the integrated form of the zero-order rate law is $[\text{flavone}] = kt + [\text{flavone}]_0$, the k values were obtained from the slope of the equations ($k = -\text{slope}$). The relative rates of photodecomposition were calculated through the formula k_j/k_i , in which i was **lut** in **lut**_{0.11}, **lut**_{0.10}, or **lut**_{0.05}, and j was **lut**, **img**, or **ldg** in other solutions.

The comparison of the rates of photodecomposition of **lut** in **lut**_{0.20}, **lut**_{0.11} and **lut**_{0.05} was carried out similarly (see section **SI C.2.7**). As the highest [**lut**] used to construct its calibration line in presence of HNO_3 was 0.13 mM, however, peak areas (10^{-2} AU s) *vs.* time (h) were used instead of concentrations *vs.* time. The slopes of the equations of the best-fit lines ($y = ax + b$)—as well as the changes in peak areas after 24.0 h of irradiation, relative to those at t_0 —were compared. Notes:

- Data used for this purpose were acquired during irradiation experiments 1a (**lut**_{0.11}) and 1b (**lut**_{0.05}), and special irradiation experiment (**lut**_{0.20}).
- The label **lut**_{0.20} was based on the weighed quantity of **lut**, and not on its concentration measured by RP-HPLC–UV using the calibration line, as the case of **lut**—as well as that of **img** and **ldg**—in all other solutions.

Figure SI C.1. Irradiation set-up: a) open, without the thermocouple; b) as used in the special irradiation experiment, with both temperature-measuring devices—mercury thermometer and thermocouple (attached to a glass rod using a rubber band)—visible; c) close-up of the 420 nm cut-off filter (special irradiation experiment); d) closed.

SI C.1.8. Spectrophotometric analyses

Spectrophotometric analyses were carried out using a Cary 100 Scan UV–vis spectrophotometer (Varian). Conditions: Double-beam mode, 1.0 nm spectral bandwidth, 800 nm to 200 nm in 1 min. Lamp warm-up prior to first measurement: Not less than 1 h. Quartz cuvettes of 3.5 mL having a light path of 10.00 mm were used. On **day 1**, the remainders of the solutions used for irradiation—temporarily kept at room temperature in the dark—were diluted 5× using volumetric pipettes and volumetric flasks. Volumes were completed with aerated methanol–water 8:2 (v/v). The UV–vis absorption spectra of these diluted samples were recorded against aerated methanol–water 8:2 (v/v). On **day 2**, t_{24} solutions were diluted and analysed in the same way. Notes:

- In the case of **lut**_{0.20}, t_0 and t_{24} solutions were diluted 10×.
- The UV–vis absorption spectra of the glass cuvettes mentioned in section **SI C.1.7** were measured against air.
- The UV–vis absorption spectrum of the 420 nm cut-off filter was measured against air. The filter was not washed before measurement; only nitrogen gas was blown onto it.

SI C.1.9. HPLC analyses

Calibration solutions and samples used for irradiation experiments 1a–special irradiation experiment were analysed by RP-HPLC–UV undiluted. Analyses were carried out on a Waters system, equipped with C18 5 μm -particle size column. Further details of the HPLC system and column used are described in publication by Villela *et al.* [1] (p. 8545, method using the HPLC column). See the referred publication also for information on: Eluent, its flow rate and composition (solvents and gradient); volume of injection; and temperature of the column oven. The photodiode array detector scan range was 245–500 nm. Peak areas of **lut** and **lmg** were obtained using 350 nm traces, and for those of **ldg**, 340 nm traces were used. Chemicals used for preparing solvent A of the HPLC eluent were: Formic acid (98+%; Acros Organics); ammonium formate ($\geq 99.0\%$; Fluka Analytical); EDTA 4Na·2H₂O (98%; Aldrich-Chemie).

Valid “positive” sample(s) were analysed during every sample sequence for monitoring the performance of the HPLC system (quality of the column and functioning of the lamp). They were prepared using the sample of aerial parts of dried and ground weld described by Villela *et al.* [1]. Such samples were prepared according to the procedure described in section 2.2 of that publication. “Positive” samples had been observed to be stable (remain valid) upon storage for three days in the refrigerator or 15 days in the freezer [1]. Solvent was analysed after a “positive” sample, for evaluation of the magnitude of sample carry-over. Notes:

- Measured quantities of **lut**, **lmg**, and **ldg** in the plant material remained similar (agreement $\geq 87\%$; exception were the results of one sample, which is regarded as an outlier) to those obtained more than one year earlier, referred to in the publication by Villela *et al.* [1] (Table 4, footnote d).
- Sample carryovers were $< 0.1\%$.
- Retention times of the four compounds (incl. **i.s.**) differed $\leq 1.2\%$ from those observed in analyses carried out more than one year earlier.

SI C.1.10. Recording of the emission spectrum of the lamp used for the irradiation experiments

The emission spectrum of the phosphor-coated low pressure mercury vapour lamp used was recorded using an absorption spectrometer (LP920 – Edinburgh Instruments, Livingston/United Kingdom), equipped with a flashlamp pumped Q-switched Nd:Yag laser (Brilliant – Quantel, Les Ulis/France). The instrument was calibrated with radiation of 355 nm, and 710 nm (second harmonic). The lamp was charged through the 220 V, 50 Hz power supply. In this way, the emission spectra of two lamps (same model; one old and one new) were recorded. This was done using two power supplies (same model; one old and one new).

SI C.1.11. Apparent pH measurements

Measurements were carried out by the use of an InoLab pH 720 (WTW; Weilheim/Germany) pH meter and a SenTix 20 (WTW; Weilheim/Germany) pH combination electrode. Technical buffers pH 4.01, 7.00 and 10.00 (WTW; Weilheim/Germany) were used for calibration of the electrode prior to measurements, and for checking the calibration afterwards. In the case of buffer solutions pH 4.01 and 7.00, the difference between the pH values measured after the

apparent pH measurements and the nominal pH values was ≤ 0.03 units. The apparent pH of the following solutions are reported:

- Calibration solutions. These solutions were stored in the refrigerator, and the measurements were carried out the day after their preparation. Note: **Lut** calibration solutions were not kept refrigerated as the other ones. They were at room temperature part of the time. As expected, however, this was of no influence on the measurements (Tables SI C.1 and SI C.2).
- Solutions used for the irradiation experiments at t_0 (2nd irradiation replicate, and **lut**_{0,20}). Also these solutions were stored in the refrigerator, and the measurements were carried out the day after their preparation (**day 2**).

Notes:

- The term apparent pH is used as the solvent of these solutions was not water, but methanol–water 8:2 (v/v).
- Remarks concerning the measurements (someone from WTW's technical personnel, personal communication; March 31, 2014):
 - Due to the low percentage of water in the samples, the readings fluctuated considerably. Although this could be improved by the use of another type of electrode, it is assumed that measured apparent pH values can be reliably compared. Comparison of the measured apparent pH values of the calibration solutions with those of the solutions used for the irradiation experiments at t_0 supports this assumption (Tables SI C.1 and SI C.2).
 - Although the use of the technical buffer pH 10.00 for calibration of the electrode was incorrect, this was of no influence on the calibration in the range of 3–7 pH units. All reported apparent pH values ranged between 3.4 and 3.7.

SI C.1.12. Assessment of the stability of **lmg** and **ldg** in presence of HNO_3

Aliquots of ~1 mL of each of the five **lmg** calibration solutions were placed in 25 mL screw-cap test-tubes on the day of their preparation, and heated at 60–65 °C overnight using an oil bath. The concentration of HNO_3 of the solutions was 0.35 mM (Table SI C.2). Vials with the heat-treated solutions were placed in an HPLC autosampler for RP-HPLC–UV analysis—as described in section SI C.1.9—on the day the experiment ended. They were analysed within 11 h from the start of the HPLC sample sequence. The same was done with the **ldg** (without Al^{3+}) calibration solutions. All described regarding **lmg** calibration solutions holds true also for those of **ldg**, with the exception that the solutions were kept at ~65 °C. Percentages of the areas of the peaks of the flavones after the heating treatment relative to the original areas of the peaks (section SI C.1.5) were calculated.

SI C.1.13. Experiment on the effect of increasing quantities of Al^{3+} (alum) bound to mordanted wool on the photo-stability of the dye of weld – part A (carried out in Wageningen)

Mordanting step

Ready-to-dye wool was mordanted with a 10 g L^{-1} aqueous (deionised water) solution of alum—liquid—wool quotient: 17 mL g^{-1} —through heating to $90+^{\circ}\text{C}$ during 0.5–1 h, followed by a 1 h-period at $\sim 95^{\circ}\text{C}$ [5] (Supporting Information, page 9). The average of the weights of the obtained $\sim 5 \times 6 \text{ cm}$ pieces of mordanted wool was 0.9 g.

Essentially the same procedure was carried out three more times: With deionised water only (no alum, blank-mordanting), and with 2 g L^{-1} and 15 g L^{-1} aqueous solutions of alum. In each case, the average of the weights of the obtained $\sim 5 \times 5 \text{ cm}$ pieces of mordanted wool was 0.8 g. Note:

- In the case of the 2 g L^{-1} aqueous solution of alum, there was poor stirring of the wool during the mordanting process. However, this did not influence the measurements of L^* , a^* , and b^* quantities (CIELAB colour space) for the pieces of weld-dyed wool (see part B below, and the relative standard deviation values in Table SI C.4).
- Differently from the published procedure, the rinsing of the mordanted wool was split in two: A minor rinsing was carried out after the mordanting step, and an additional one was carried out at a later stage.

Dyeing step

Four $\sim 5 \times 6 \text{ cm}$ pieces of wool mordanted with the 10 g L^{-1} aqueous solution of alum were dyed with a 96% ethanol–deionised water 3:1 (v/v) extract of the sample of the aerial parts of dried and ground weld at 80°C for 15 min. This was done according to the procedure described by Villela *et al.* [5] (Supporting Information, page 2), with the following relevant exceptions: After filtration of the extract into a 250 mL round-bottom flask and removal of the 96% ethanol, 60 mL of deionised water were added to the flask, with the dyeing process taking place in the round-bottom flask itself. A reflux condenser was connected to this flask, which was heated using a water-bath. Deionised water was used to rinse the pieces of dyed wool, which were, then, hung to dry. The dried pieces of dyed wool were handled with gloves, and stored in the dark. Essentially the same procedure was carried out three more times: For dyeing pieces of blank-mordanted wool, and of wool mordanted with 2 g L^{-1} and 15 g L^{-1} aqueous solutions of alum. Notes:

- All cases: Differently from the published procedure, no aluminium foil was used to cover the dyeing baths.
- In the case of the dyeing of the wool mordanted with 10 g L^{-1} aqueous solution of alum, the ratio of volume of extraction solvent to weight of weld sample was 18 mL g^{-1} , whereas in all other cases this ratio was 20 mL g^{-1} , as in the published procedure.
- Dyeing of the blank-mordanted wool: One of the pieces fell outside the 250 mL round-bottom flask while being transferred to the dyeing bath. Although it was immediately resoaked in the deionised water used for the pre-treatment of the mordanted wool, this could have led to inhomogeneity of the colour, as part of the piece of wool touched the tap water of the water-bath. In spite of the relative standard deviation of the measured b^*

quantities of the pieces of dyed wool (CIELAB colour space; part B below) being 6.3%, those of the calculated chroma (C^*_{ab}) and hue angle (h_{ab}) were $\leq 6.0\%$ (Table SI C.4).

- No proper dyeing took place in the case of the dyeing of the wool mordanted with 15 g L⁻¹ aqueous solution of alum. The pieces of “dyed wool” looked similar to those of the dyed blank-mordanted wool. The reason for this could neither be traced back to the mordanting process nor to the dyeing process. Thus, these pieces of wool were discarded.

SI C.1.14. Experiment on the effect of increasing quantities of Al³⁺ (alum) bound to mordanted wool on the photo-stability of the dye of weld – part B (carried out in Steenbergen)

The following specific compounds/material were used: Detergent solution used for determining the wash-fastness of colours contained ECE phosphate detergent B (SDC, Bradford/UK) and sodium perborate (NaBO₂·H₂O₂·3H₂O; BDH Laboratory, Poole/England); weathering tester used for determining the light-fastness of the colours [QUV Accelerated Weathering Tester (Q-Panel Company, Cleveland/USA)].

Colours of the weld-dyed samples of wool

L^* , a^* and b^* quantities (CIELAB colour space) were measured. Each of the three cases (dyed blank-mordanted wool, and dyed wool that had been mordanted with 2 g L⁻¹ and 10 g L⁻¹ aqueous solutions of alum): Four pieces of wool ($n = 4$). The light-beam of the spectrophotometer was used in "one large spot"-mode. Afterwards, chroma (C^*_{ab}) and hue angle (h_{ab}) were calculated for each of the pieces via the equations $C^*_{ab} = (a^{*2} + b^{*2})^{1/2}$ and $h_{ab} = \arctan(b^*/a^*)$ [6]. Data were processed as follows: Standard deviation values were expressed with one significant digit, and average values were rounded up accordingly.

Light-fastness of the colours, including measurement of L^* , a^* , and b^* quantities (CIELAB colour space) for the irradiated samples

The light-fastness of the colours of the pieces of dyed wool was determined according to the ISO 105–B02 norm. Each of the three cases: Two pieces of wool were irradiated in the weathering tester ($n = 2$). The scale 1 (poor)–8 (excellent)—with 3 being the acceptable lower limit—was used for reporting the results [7]. L^* , a^* , and b^* quantities (CIELAB colour space) were measured for the irradiated samples as described above, except that the light-beam of the spectrophotometer was used in "one small spot"-mode (measured at five places of each piece of wool). Afterwards, the CIELAB colour difference (ΔE^*_{ab}) values were calculated from the average values of the measured L^* , a^* , and b^* quantities before (Table SI C.4) and after irradiation (not shown) via the equation $\Delta E^*_{ab} = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$ [6].

Wash-fastness of the colours

The wash-fastness of the colours of the pieces of dyed wool was determined according to the ISO 105–C06 norm. The assay was carried out at 40 °C for 30 min. Each of the three cases: Two pieces of wool were used — with only one of them being washed (thus, $n = 1$; one pair of samples). Afterwards, both pieces were visually compared and L^* , a^* and b^* quantities (CIELAB colour space) were measured. The scale 1 (poor)–5 (excellent)—with 3–4 being the acceptable lower limit—was used for reporting the results [7].

SI C.1.15. Another experiment on the effect of increasing quantities of Al^{3+} (using aluminium sulphate and tartaric acid this time) bound to mordanted wool on the photo-stability of the dye of weld (carried out in Steenberg)

The following specific material was used: Commercial extract of weld (Rubia Yellow; Rubia Pigmenta Naturalia, later Rubia Natural Colours, Steenberg/The Netherlands); textile auxiliary agent Biavin 109 (mixture described as emulsified fatty compound, containing benzyl alcohol; aims at protecting the fibre from fibre-to-fibre and fibre-to-metal action; CHT R. Beitlich, Tübingen/Germany); apparatus that can be used for wool dyeing, mordanting, washing and rinsing (Linitest Original Hanau; Heraeus, Hanau/Germany).

Pieces of ready-to-dye wool weighing 40 g each were pretreated at room temperature with a 0.3 g L^{-1} aqueous solution of the textile auxiliary agent. The excess solution was removed by centrifugation. The primary function of this step was to wet the wool prior to its mordanting.

The pretreated wool was mordanted with an aqueous (tap water; water hardness classification: DH4) solution of aluminium sulphate and tartaric acid (liquid-wool quotient: 10 mL g^{-1}). This was done at 95°C using the Linitest Original Hanau apparatus operating at 2 kW during 1.75 h. The system was left to cool down to below 50°C . Then, the mordanted wool was rinsed twice using tap water at room temperature. The excess water was removed by centrifugation. This procedure was carried out with 0.2 , 2 , 10 and 25 g L^{-1} solutions of aluminium sulphate tetradecahydrate. The concentration of tartaric acid was 1.3 g L^{-1} in all cases.

Each piece of mordanted wool was dyed with an aqueous (tap water) solution of the commercial extract of weld [2.5% (w/w) extract-wool; liquid-wool quotient: 10 mL g^{-1}]. This was done at 100°C using the Linitest Original Hanau apparatus operating at 2 kW during 1.75 h. The dyed wool was rinsed with a 0.5 g L^{-1} aqueous solution of the SARABID PAW detergent at 95°C using the same machine. Tap water at 95°C was used to rinse the dyed wool further, which was, then, centrifuged for removal of the excess water.

L^* , a^* and b^* quantities (CIELAB colour space) were measured. The light-beam of the spectrophotometer was used in "one large spot"-mode. In each case, chroma (C^*_{ab}) and hue angle (h_{ab}) were calculated as above. The light-fastness of the colours was determined at TO₂C (Ghent/Belgium) according to the ISO 105-B02 norm. Also here, the scale 1 (poor)–8 (excellent) was used for reporting the results.

SI C.1.16. Colours of alum-mordanted wool dyed with lut, lmg, and ldg

The following specific compounds/material were used: **Lut** (used for dyeing the wool; 96% by NMR; Indofine Chemical Company, Hillsborough/USA); **lmg** (used for dyeing the wool; 95% by NMR; Extrasynthese, Genay/France); **ldg** (used for dyeing the wool; 86% by NMR; Extrasynthese, Genay/France); $\sim 5 \times 6 \text{ cm}$ -pieces of wool mordanted with a 10 g L^{-1} aqueous solution of alum weighing 0.9 g each (described above); DMSO (99.9% for spectroscopy; Acros Organics, Geel/Belgium).

Stock solutions of **lut** and of **lmg** were brought to room temperature in the dark. Separately, aliquots of the solutions were transferred to 50 mL round-bottom flasks using volumetric pipettes. In this way, 3.25 mg ($11.4 \mu\text{mol}$) of **lut**, and 5.11 mg ($11.4 \mu\text{mol}$) of **lmg** were transferred to the flasks. Solvents were removed by the use of a rotatory evaporator. Finally,

flasks were closed with a stopper and stored in refrigerator. Note: Stock solutions of **lut** and of **lmg** were prepared as described above, with the following relevant exceptions:

- **Lut** stock solution:
 - Concentration: $232\ \mu\text{g mL}^{-1}$ (0.81 mM).
- **Lmg** stock solution:
 - All **lmg** dissolved after storing the partially filled volumetric flask in refrigerator; *i.e.*, there was no need for using the incubator shaker.
 - Concentration: $341\ \mu\text{g mL}^{-1}$ (0.76 mM).
- Both **lut** and **lmg** stock solutions:
 - Non-aerated methanol–water 8:2 (v/v) was used. This solvent mixture was prepared using deionized water (EasyPure UV system), and was both used on the day of preparation and stored in refrigerator prior to use.

A different procedure was carried out for **ldg**. The flavone was weighed directly in a 50 mL round-bottom flask using the balance operating in 0.1 mg-least division mode. In this way, 7.1 mg (11.6 μmol) of **ldg** was transferred to the flask. Also in this case, the flask was closed with a stopper and stored in refrigerator. Note: The large weighing error is of little relevance in view of the nature of the experiment.

Wool was dyed with the individual flavones. In each case, one piece of the mordanted wool was dyed in the round-bottom flask containing **lut**, **lmg**, or **ldg**. This was done at 80 °C for 15 min, generally according to the procedure described by Villela *et al.* [5] (Supporting Information, page 2). Note: The procedure was further adapted as follows:

- 17 mL of deionized water (EasyPure UV system) were added to each round-bottom flask. Thus, the liquid–wool ratio remained $\sim 20\ \text{mL g}^{-1}$.
- The dyeing baths were heated by the use of a water-bath.

The dyeing baths were sampled after removal of the pieces of dyed wool, while still warm and stirring. Five times dilution of each 1.0 mL (measuring pipette) dyeing bath sample was done with DMSO (automatic pipette). Vials containing the diluted dyeing bath solutions were stored in the refrigerator. Once at room temperature, these samples were filtered via a syringe filter prior to RP-HPLC–UV analysis (see section **SI C.1.9**).

Another piece of the mordanted wool was dyed with an extract of weld in a similar way, with 420 mg of the sample of the aerial parts of dried and ground weld being extracted with 96% ethanol–water 3:1 (v/v) for this [5] (Supporting Information, page 2). Volumes of solvents and glassware were downscaled according to the weight of weld sample used. Finally, a blank-without-wool was carried out similarly, also with 420 mg of the weld sample. However, as besides the 96% ethanol, also the water was completely removed by the use of the rotatory evaporator, 17 mL of deionized water (Seradest SD 2000 ion-exchanger) were added to the round-bottom flask. As in the cases of **lut**, **lmg**, and **ldg**, the dyeing took place in a round-bottom flask. In both cases—wool- and blank-without-wool—samples of the dyeing baths were prepared for HPLC analysis as described in the previous paragraph.

All five dyeing bath samples described in this section were analysed in one HPLC analysis sample sequence, which was completed in <12 h. Note: Although the sample of the dyeing bath of the wool dyed with weld was in the refrigerator for four weeks, no precipitate was seen. This was also the case for the other four samples, that were in the refrigerator less than one week.

The percentages of leftover flavones in the dyeing baths were calculated. This was done using the following calibration curves:

- [**Lut**] ($\mu\text{g mL}^{-1}$) vs. peak area at 345 nm ($\mu\text{AU s}$): $y = 98\,814x + 49\,918$ ($R^2 = 0.9999$)
- [**Lmg**] ($\mu\text{g mL}^{-1}$) vs. peak area at 345 nm ($\mu\text{AU s}$): $y = 64\,571x + 49\,805$ ($R^2 = 0.9999$)
- [**Ldg**] ($\mu\text{g mL}^{-1}$) vs. peak area at 345 nm ($\mu\text{AU s}$): $y = 46\,221x + 26\,160$ ($R^2 = 0.9999$)

Although these curves were constructed two years earlier, the HPLC system—checked as described in section **SI C.1.9**—was performing well. Areas of **lut**, **lmg**, **ldg**, and **i.s.** peaks of the “positive” sample agreed >90% with those of an identical sample prepared and analysed 10–11 months earlier, and used as a reference. No sample carryover was observed and, compared with this reference sample, retention times of the four compounds varied ≤ 0.1 min. Whereas the areas of **lut** and **ldg** peaks fell within the ranges of the calibration curves, **lmg**’s calibration curve had to be extrapolated. The concentration of **lmg** in the sample of the dyeing bath was determined to be $10\,\mu\text{g mL}^{-1}$, whereas the concentration of the lowest calibration solution was $14\,\mu\text{g mL}^{-1}$.

The percentages of leftover flavones in the dyeing baths were also calculated from the dyeing processes with the extracts of weld. The 345 nm-areas of **lut**, **lmg**, and **ldg** peaks of the wool-dyeing were compared with those of the blank-without-wool.

L^* , a^* and b^* quantities (CIELAB colour space) were measured for the wool dyed with the individual flavones and with the extract of weld, and for the non-dyed alum-premordanted wool. Chroma (C^*_{ab}) and hue angle (h_{ab}) were calculated as described above. In each of the four cases of dyed wool, only a part of about 5×1 cm of the piece of wool was used. The light-beam of the spectrophotometer was used in “one small spot”-mode.

SI C.2. Supplementary results and discussion

SI C.2.1. Choice of solvent

The rate of photodecomposition of flavone has been observed to be solvent-dependent, being higher in methanol than in cyclohexane[8]. Kaneta and Sugiyama[9] and Smith *et al.*[10] studied the photodecomposition of flavones and flavonols in aerated ethanol and methanol–water 1:1. In the work reported here, aerated methanol–water 8:2 (v/v) was used. This solvent mixture has been observed to be the most efficient for the extraction of the main flavonoids of weld, among alcohols, water and mixtures thereof [11].

SI C.2.2 pH adjustment

The apparent pH of a $0.12\,\text{mM}$ solution of **lut** in aerated methanol–water 8:2 (v/v) was 6.8. Upon addition of the Lewis acid Al^{3+} , at a **lut**– Al^{3+} 1:10 ratio, it decreased to 3.5. In order to irradiate solutions having approximately the same concentration of protons, the other irradiated solutions reported here were acidified to an apparent pH of 3.6 ± 0.2 (see Table SI C.1). This was done with nitric acid, as nitrate was the counter-ion of the aluminium salt used. **Lmg** and **ldg** were observed to be stable in nitric acid-acidified solution at $\sim 65\,^\circ\text{C}$ overnight. In preliminary experiments, the photodecomposition of **lut** in nitric acid-acidified aerated methanol–water 8:2 (v/v) solution was observed to be 35 to 55% slower than in non-acidified solution. This is consistent with an increase of the energy releasing tautomerism by an increased concentration of protons in solution. The observed influence of pH on the photodecomposition of **lut** is also consistent with the observation that the triplet excited state of flavone—longer-

lived than the singlet excited state and, thus, having increased chances of reacting—is quenched by the hydrogen donor *p*-methoxyphenol[12, 13].

SI C.2.3. Purity of lut, lmg, and ldg, as determined by NMR spectroscopy

The outcomes of the determination by NMR spectroscopy of the purity of the flavones used for the preparation of solutions used for construction of the calibration lines and for the irradiation experiments (described in section SI C.1.4) are presented in this section. The origin of the signals seen in the spectra are also briefly elaborated upon.

Figure SI C.2. ^1H -NMR spectrum used for the determination of the purity of **lut** (Indofine Chemical Company) with maleic acid (m.a.). Signals relative to the non-exchangeable H-atoms of the flavone and m.a. are assigned.

In addition to the signals assigned in Figure SI C.2, signals relative to the H-atoms of the following moieties and molecules are also visible: Phenolic and carboxyl groups (9.2–13.0 ppm) [14], water (2.7–4.0 ppm), DMSO and its ^{13}C -satellite signals (2.2–2.6 ppm), and unknown compound(s) (0.9–1.2 ppm, 3.8 ppm, and signals of low intensity at 5.9–7.9 ppm). Signals at 1.8–2.2 ppm are likely due to H-atoms of residual acetone and its ^{13}C -satellite signals [15]. Purity was determined to be 96%.

Figure SI C.3. ¹H-NMR spectrum used for the determination of the purity of **lmg** (Extrasynthese) with maleic acid (m.a.). Signals relative to the non-exchangeable H-atoms of the aglycone of the flavone and m.a.—as well as the anomeric H-atom of the glucose moiety—are assigned.

In addition to the signals assigned in Figure SI C.3, signals relative to the H-atoms of the following moieties and molecules are also visible: Phenolic and carboxyl groups (9.2–13.0 ppm), water and glucose unit (2.7–3.7 ppm), DMSO and its ¹³C-satellite signals (2.2–2.7 ppm), and unknown compound(s) (0.7–1.2 ppm, 3.8 ppm, and signals of low intensity at 5.9–8.2 ppm). Signals at 1.8–2.1 ppm are possibly due to H-atoms of residual acetone and one of its ¹³C-satellite signals. Purity was determined to be 93%.

Figure SI C.4. One of the ^1H -NMR spectra used for the determination of the purity of **1dg** (Extrasynthese) with maleic acid (m.a.). Signals relative to the non-exchangeable H-atoms of the aglycone of the flavone and m.a.—as well as the anomeric H-atoms of the glucose moieties—are assigned.

Figure SI C.5. Other ^1H -NMR spectrum used for the determination of the purity of **1dg** (Extrasynthese) with maleic acid (m.a.).

In addition to the signals assigned in Figure SI C.4, signals relative to the H-atoms of the following moieties and molecules are also visible in Figures SI C.4 and SI C.5: Phenolic and carboxyl groups (9.5–13.0 ppm), water and glucose units (2.8–4.0 ppm), DMSO and its ^{13}C -satellite signals (2.3–2.7 ppm), and unknown compound(s) (0.7–1.3 ppm, and signals of low intensity at 5.7–8.2 ppm). Signals at 1.9–2.3 ppm are likely due to H-atoms of residual acetone and its ^{13}C -satellite signals. Signals at 3.0 and 3.2 ppm are likely to be due to H-atoms of an external contaminant (a residual compound in the NMR tube, for example), as they are seen in Figure SI C.4 but not in Figure SI C.5. Purity was determined to be 86%.

SI C.2.4. Effect of Al^{3+} on the absorption of light by solutions of **lut** and **ldg**

Figure SI C.6. Effect of increasing $[\text{Al}^{3+}]$ on the colour of solutions of **lut** and **ldg**. Solutions at t_0 from left to right: **lut**_{0.10}, **lut**_{0.10}- $\text{Al}_{0.02}$, **lut**_{0.10}- $\text{Al}_{0.10}$, **lut**_{0.10}- $\text{Al}_{0.99}$, **ldg**_{0.05}, and **ldg**_{0.05}- $\text{Al}_{0.05}$. Pictures were taken on **day 1** of irradiation experiments 2a (panel **a**) and 2b (panel **b**).

Figure SI C.7. Effect of complexation of **lut** with Al^{3+} at increasing $[\text{Al}^{3+}]$ on the UV-vis absorption spectrum of **lut**. Note: Subscripts of code of solutions denote $[\text{lut}]_0$ and $[\text{Al}^{3+}]$, in mM; Solvent: aerated methanol-water 8:2 (v/v); All cases: t_0 -solutions prepared for the 2nd irradiation replicate, diluted 5 $\times$.

Figure SI C.8. Effects of glycosylation pattern—aglycone, monoglucoside, and diglucoside—on the UV–vis absorption spectrum of **lut**, and of complexation of **ldg** with Al^{3+} at a 1:1 ratio on the spectrum of **ldg**. Note: Subscripts of code of solutions denote $[\text{flavone}]_0$ and $[\text{Al}^{3+}]$, in mM; Solvent: aerated methanol–water 8:2 (v/v); All cases: t_0 -solutions prepared for the 2nd irradiation replicate, diluted 5 $\times$.

SI C.2.5. Irradiation experiments: Light with which solutions were irradiated

The UV–vis spectra of the four glass cuvettes used in the irradiation experiments reported are depicted in Figure SI C.9. The UV–vis absorption spectrum of the 420 nm cut-off filter recorded is also depicted. The intensity of irradiation varies with the time of use of lamp and power supply; it seems that the newer the lamp and power supply, the higher the intensity of irradiation. The same lamp and power supply were used in the reported irradiation experiments. The emission spectrum resulting of this combination is displayed in Figure SI C.9. Under these conditions, **lut** mainly undergoes $S_0 \rightarrow S_1$ transition [16].

Figure SI C.9. UV-vis absorption spectra: Four glass cuvettes used in the reported irradiation experiments, and 420 nm cut-off filter (left axis); emission of the lamp (right axis).

SI C.2.6. Effect of different concentrations of Al^{3+} and glycosylation pattern of **lut** (aglycone, monoglucoside, and diglucoside) on its photo-stability in solution

Figure SI C.10. Effect of 24 h of irradiation on the UV-vis absorption spectrum of **lut** complexed with Al^{3+} in **lut**_{0.10}- Al _{0.99}. Note: Subscripts of code of solution denote **[lut]**₀ and $[\text{Al}^{3+}]$, in mM; Irradiation experiment 2a; Solvent: aerated methanol-water 8:2 (v/v); t_0 - and t_{24} -solutions were diluted 5×.

Table SI C.1. Apparent pH of solutions used for the irradiation experiments and change in absorbance at λ_{max} of the lowest absorption bands of **lut**, **img**, **ldg**—and complexes of **lut** and **ldg** with Al^{3+} —after 24 h of irradiation.

Solution ^a	Apparent pH	λ_{max} of lowest absorption band at t_0 (nm) ^b	Decrease in absorbance at λ_{max} of lowest absorption band (%) ^c	Relative decrease in absorbance ^d
1st irradiation replicate				
lut _{0.11}	—	350	5	1
lut _{0.10} – Al _{0.02}	—	350	6	1.2
lut _{0.10} – Al _{0.10}	—	354	16	3.2
lut _{0.11} – Al _{1.05}	—	410	20	4.0
lut _{0.05}	—	350	9	1
img _{0.05}	—	350	12	1.3
ldg _{0.04}	—	342	2	0.2
ldg _{0.05} – Al _{0.05}	—	344	6	0.7
2nd irradiation replicate				
lut _{0.10}	3.6	350	4	1
lut _{0.10} – Al _{0.02}	3.6	350	5	1.2
lut _{0.10} – Al _{0.10}	3.5	355	13	3.2
lut _{0.10} – Al _{0.99}	3.6	410	20	5.0
lut _{0.05}	3.6	350	8	1
img _{0.05}	3.6	350	11	1.4
ldg _{0.05}	3.7	342	2	0.2
ldg _{0.05} – Al _{0.05}	3.5	344	8	1.0
Special irradiation experiment				

lut _{0.20}	3.7	350	2	—
lut _{0.10} – Al _{0.99} (with the 420 nm cut-off filter) ^c	—	409	1	0.2 0.2

^a Subscripts of code of solutions denote [flavone]₀ and [Al³⁺], in mM; Solvent: aerated methanol–water 8:2 (v/v). t₀- and t₂₄-solutions were diluted 5× for the spectrophotometric analyses, except in case of **lut**_{0.20}, in which they were diluted 10×.

^b $\lambda_{\max}$ of lowest absorption bands: Unchanged after 24 h of irradiation, except in few cases in which there were spectral blue-shifts of 1 nm (**ldg**_{0.05}–**Al**_{0.05}, only 1st irradiation replicate), 1 nm (**lut**_{0.10}–**Al**_{0.10}) and 4 nm (**lut**_{0.10}–**Al**_{1.00}).

^c Absorbance at $\lambda_{\max}$ of the lowest absorption bands ranged from 203 mAU (**ldg**_{0.05}–**Al**_{0.05}, t₂₄; 2nd irradiation replicate) to 519 mAU (**lut**_{0.11}–**Al**_{1.05}, t₀).

^d Relative decrease in absorbance = decrease in absorbance at $\lambda_{\max}$ of lowest absorption band_j (%) / decrease in absorbance at $\lambda_{\max}$ of lowest absorption band_i (%), in which i refers to solutions **lut**_{0.11}, **lut**_{0.10}, or **lut**_{0.05}, and j refers to the other solutions.

^e Relative decrease of absorbance values are relative to solutions **lut**_{0.11} (1st irradiation replicate)—top—and **lut**_{0.10} (2nd irradiation replicate)—bottom.

— = not measured or applicable.

Results obtained by analysing the t₀- and t₂₄-solutions by UV–vis spectrophotometry are displayed in Table SI C.1. In the table—and text below—reference is made to the lowest absorption bands of the flavones and their Al³⁺-complexes. However, there are cases in which two bands next to each other were considered the “lowest absorption band”. This can be seen in Figures SI C.7 and SI C.8, in which the UV–vis absorption spectra of the compounds of the 2nd irradiation replicate t₀-solutions are depicted. This is a simplification. For elaboration on the electronic transitions giving rise to the absorption bands seen in the spectra **lut**–Al³⁺ complexes, the reader is referred to the work of Amat *et al.* [17].

The values of the relative decrease in absorbance at $\lambda_{\max}$ of the lowest absorption bands after 24 h of irradiation (Table SI C.1) follow the same trend of the relative rates of photodecomposition obtained through RP-HPLC–UV (Table 4.2). However, the values of the percentage of decrease in absorbance at $\lambda_{\max}$ of those bands are—with only one exception—lower than those of the percentage of flavone decomposed determined by HPLC. This is an indication that using spectrophotometry without prior separation by LC underestimates the extent of the photodecomposition of the flavones, due to the influence of other UV–vis absorbing-compounds.

Upon increasing [Al³⁺], the lowest UV–vis absorption band of the spectrum of **lut** and Al³⁺ in solution shifts towards longer λ and—after an initial decrease—displays larger intensity (Fig. SI C.7 and Table SI C.1). **Lut**—as well as the flavonol quercetin—has already been reported to behave in this way in methanolic solution [18, 19]. However, there is a quantitative difference between this phenomenon as reported here and that reported by Favaro *et al.* [18]. The ratio between the absorbance at the $\lambda_{\max}$ of the lowest absorption band of the spectrum of **lut** and Al³⁺ in solution, with **lut** and Al³⁺ present at a 1:10 ratio—considering **lut**_{0.11}–**Al**_{1.05} (1st irradiation replicate, t₀) and **lut**_{0.10}–**Al**_{0.99} (2nd irradiation replicate, t₀)—and the absorbance at the $\lambda_{\max}$ of

the lowest absorption band of free **lut** (without Al^{3+})—considering **lut**_{0.11} (1st irradiation replicate, t_0) and **lut**_{0.10} (2nd irradiation replicate, t_0)—was 1.04, whereas that observed by Favaro *et al.* was 1.5 [18] (Fig. 7).¹ Moreover, the ratio between the absorbance at 350 nm of the spectrum of **lut** and Al^{3+} in solution, with **lut** and Al^{3+} present at a 1:10 ratio—considering **lut**_{0.11}– $\text{Al}_{1.05}$ (1st irradiation replicate, t_0) and **lut**_{0.10}– $\text{Al}_{0.99}$ (2nd irradiation replicate, t_0)—and the absorbance at the same wavelength of free **lut**—considering **lut**_{0.11} (1st irradiation replicate, t_0) and **lut**_{0.10} (2nd irradiation replicate, t_0)—was 0.44, whereas that observed by Favaro *et al.* was 0.3 [18] (Fig. 7). In addition, the ratio between the absorbance at the λ_{max} of the lowest absorption band of the spectrum of **lut** and Al^{3+} in solution, with **lut** and Al^{3+} present at a 1:2 ratio—which lay at 405 nm—and that of free **lut** was 1.0 [18] (Fig. 7).

This suggests that a decreasing percentage of water in the solvent favours **lut**– Al^{3+} complexation, as methanol–water 8:2 was used here and non-anhydrous methanol was used by Favaro *et al.* [18].² Information on the influence of other solvents on flavonoid– Al^{3+} complexation is also available. Jurd and Geissman reported the lowest absorption band of the spectrum of **lut** and Al^{3+} in solution, with **lut** and Al^{3+} present at a 1:10 ratio—relative to the lowest absorption band of free **lut**—to be less shifted towards longer λ and of lower intensity than that reported by Favaro *et al.* [18, 20]. These differences were even more pronounced than those between the data reported here and the data reported by Favaro *et al.*. Thus, this suggests that methanol is more favourable for **lut**– Al^{3+} complexation than ethanol, as 96% ethanol was used by Jurd and Geissman [20]. Deng and van Berkel studied the influence of the solvent on the spectrum of the flavonol quercetin—and that of kaempferol—and Al^{3+} [21]. Methanol was observed to be the most favourable solvent for quercetin– Al^{3+} complexation, among acetonitrile, isopropanol and a methanol–acetonitrile mixture.

¹ The λ_{max} of the lowest absorption band of **lut** complexed with Al^{3+} , with **lut** and Al^{3+} present at a 1:10 ratio was 410 nm in the work reported here, but 425 nm in that reported by Favaro *et al.*, whereas the λ_{max} of the lowest absorption band of free **lut** (without Al^{3+}) was 350 nm in both works.

² There might be difference in acidity between the solutions used in the work reported here and those used by Favaro *et al.*. Whereas the solutions used for the irradiation experiments were acidified to an apparent pH of 3.6 (Table SI C.1), those used by Favaro *et al.* for evaluating the influence of increasing $[\text{Al}^{3+}]$ on the UV–vis absorption spectrum of free **lut** and **lut**– Al^{3+} complexes were not pH-adjusted. In addition, although the solution of **lut** and Al^{3+} in which **lut** and Al^{3+} were present at a 1:10 ratio was not pH-adjusted, the percentage of water in the solvent was still different—methanol–water 8:2 vs. non-anhydrous methanol.

Table SI C.2. RP-HPLC–UV calibration lines for the quantitative monitoring of the photodecomposition of **lut**, **lmg**, and **ldg** in solution.

Curve	Calibration solutions			Apparent pH range	[flavone] (mM) vs. peak area ($\mu\text{AU s}$)	r^2
	[flavone] (mM)	[Al ³⁺] (mM)	[HNO ₃] (mM)			
lut	0.01; 0.04; 0.08; 0.11; 0.13	—	0.35	3.7–3.6	$y = 2.80 \cdot 10^7 x$	1.00
	0.01; 0.04; 0.08; 0.10; 0.13	0.02	0.35	3.6	$y = 2.80 \cdot 10^7 x$	1.00
	0.01; 0.04; 0.08; 0.10; 0.13	0.10	0.35	3.5–3.4	$y = 2.79 \cdot 10^7 x$	1.00
lut –Al _{0.02}	0.01; 0.04; 0.08; 0.10; 0.13	0.02	0.35	3.6	$y = 2.80 \cdot 10^7 x$	1.00
	0.01; 0.04; 0.08; 0.10; 0.13	0.10	0.35	3.5–3.4	$y = 2.79 \cdot 10^7 x$	1.00
	0.01; 0.04; 0.08; 0.10; 0.13	1.05	—	3.6–3.5	$y = 2.79 \cdot 10^7 x$	1.00
lmg	0.01; 0.04; 0.08; 0.10; 0.13	—	0.35	3.6–3.5	$y = 2.86 \cdot 10^7 x$	1.00
	0.01; 0.02; 0.03; 0.04; 0.06	—	0.35	3.6	$y = 2.89 \cdot 10^7 x$	1.00
	0.01; 0.02; 0.03; 0.04; 0.06	0.05	0.35	3.6–3.5	$y = 2.76 \cdot 10^7 x$	1.00

Detection: 350 nm (**lut** and **lmg**) and 340 nm (**ldg**).

— = absent.

The unit of the [flavone] of some of the calibration curves of the table above was converted from mM to $\mu\text{g mL}^{-1}$. This was done for comparison with the curves listed earlier, used for calculating the percentages of leftover flavones in dyeing baths. The calibration curves of **lut**, **lmg**, and **ldg**—with HNO₃, without Al³⁺—then became:

- [**Lut**] ($\mu\text{g mL}^{-1}$) vs. peak area at 350 nm ($\mu\text{AU s}$): $y = 9.78 \cdot 10^4 x$ ($r^2 = 1.00$)
- [**Lmg**] ($\mu\text{g mL}^{-1}$) vs. peak area at 350 nm ($\mu\text{AU s}$): $y = 6.39 \cdot 10^4 x$ ($r^2 = 1.00$)
- [**Ldg**] ($\mu\text{g mL}^{-1}$) vs. peak area at 340 nm ($\mu\text{AU s}$): $y = 4.73 \cdot 10^4 x$ ($r^2 = 1.00$)

SI C.2.7. Effect of concentration of lut on its photo-stability in solution

Figure SI C.11. Effect of varying [lut] on its photo-stability in solution. Note: Subscripts of code of solutions denote [lut]₀, in mM; Solvent: aerated methanol–water 8:2 (v/v); r^2 = quality of description of the decrease in peak area of **lut** over time as a straight line; Decrease in **lut** peak areas reported: After 24 h of irradiation, relative to those at t_0 ; Duration of irradiation of solutions = 24.0 h (1,440 min).

The highest [lut] used to construct its calibration line in presence of HNO₃ was 0.13 mM (Table SI C.2). Thus, the photodecomposition of **lut** in **lut**_{0.20}, **lut**_{0.11} and **lut**_{0.05} is depicted in Figure SI C.11 as peak area vs. time, and not concentration vs. time. However, the relation between the peak area of **lut** and its concentration is expected to be linear throughout the range of [lut] of the study, as the peak height at t_0 ($t = 0$ h) in **lut**_{0.20} was 586 mAU; well below 1,000 mAU.

SI C.2.8. Evaluation of the contribution of light above 420 nm to the photodecomposition processes studied

Table SI C.3. Relative rate of photodecomposition of lut in solution in presence of Al^{3+} at a 1:10 **lut**- Al^{3+} ratio, with the use of a filter blocking radiation of wavelengths shorter than ~420 nm.

Solution	Flavone decomposed in 24 h of irradiation (%)	r^2 ^a	Rate constant, k ($10^{-3} \text{ mol L}^{-1} \text{ min}^{-1}$)	Relative rate of photodecomposition ^b
lut _{0.11} (1 st irradiation replicate) ^c	7	0.98	$4.9 \cdot 10^{-6}$	—
lut _{0.10} (2 nd irradiation replicate) ^c	6	1.00	$4.3 \cdot 10^{-6}$	—
lut _{0.10} - $\text{Al}_{0.99}$ (with the 420 nm cut-off filter) ^d	1	0.79	$8.0 \cdot 10^{-7}$	0.2 0.2

^a r^2 = quality of description of photodecomposition of the flavone by the zero-order integrated rate law ($[\text{flavone}] = kt + [\text{flavone}]_0$).

^b Relative rates of photodecomposition = k_j/k_i , in which i is **lut** in **lut**_{0.11} (1st irradiation replicate)—top—and **lut**_{0.10} (2nd irradiation replicate)—bottom—and j is **lut** in **lut**_{0.10}- $\text{Al}_{0.99}$ (with the 420 nm cut-off filter).
— = not applicable.

^c Copied from entries 1 and 9 of Table 4.2 for comparative purposes.

^d The light absorbance profile of the 420 nm cut-off filter is depicted in Figure SI C.9; The photodecomposition of **lut** over time is plotted in Figure SI C.16.

Taking into consideration the UV–vis absorption spectra depicted in Figures SI C.7 and SI C.8, the emission profile of the lamp depicted in Figure SI C.9, and the relative rates of decomposition displayed in Table SI C.3, the contribution of light above 420 nm to the studied photodecomposition of **lut** in solution—as a function of different $[\text{Al}^{3+}]$ and glycosylation pattern—is expected to have been very limited. Thus, the solutions are said to have been irradiated with light, most importantly, in the 300–420 nm range.

SI C.2.9. Photodecomposition of **ldg—with and without Al^{3+} —and lut in lut_{0.10}–Al_{0.99}—only with light >420 nm—over time**

Figure SI C.12. Photodecomposition of **ldg** in solution upon irradiation of **ldg**_{0.04} over 24 h (1st irradiation replicate). Note: Subscript of code of solution denotes **[ldg]**₀; Solvent: aerated methanol–water 8:2 (v/v); Equation of the best-fit line of the description of the photodecomposition of **ldg** by the zero-order integrated rate law (**[ldg]** = $kt + [\text{ldg}]_0$) is displayed, in which $y = [\text{ldg}]$, slope = $-k$, and $x = t$; r^2 = quality of this description; Duration of irradiation of solution = 24.0 h (1,440 min).

Figure SI C.13. Photodecomposition of **ldg** in solution upon irradiation of **ldg**_{0.05}-Al_{0.05} over 24 h (1st irradiation replicate). Note: Subscripts of code of solution denote $[Ldg]_0$ and $[Al^{3+}]$, in mM; All rest as for Fig. SI C.12.

Figure SI C.14. Photodecomposition of **ldg** in solution upon irradiation of **ldg**_{0.05} over 24 h (2nd irradiation replicate). Note: All as for Fig. SI C.12.

Figure SI C.15. Photodecomposition of **ldg** in solution upon irradiation of **ldg**_{0.05}–Al_{0.05} over 24 h (2nd irradiation replicate). Note: Subscripts of code of solution denote [**ldg**]₀ and [Al³⁺], in mM; All rest as for Fig. SI C.12.

Figure SI C.16. Photodecomposition of **lut** in solution upon irradiation of **lut**_{0.10}–Al_{0.99} (<420 nm-filtered light) over 24 h (special irradiation experiment). Note: Subscripts of code of solution denote [**lut**]₀ and [Al³⁺], in mM; Equation of the best-fit line of the description of the photodecomposition of **lut** by the zero-order integrated rate law ($[\text{lut}] = kt + [\text{lut}]_0$) is displayed, in which $y = [\text{lut}]$, slope = $-k$, and $x = t$; All rest as for Fig. SI C.12.

SI C.2.10. Stability of **lmg** and **ldg** in presence of HNO_3

The stability of the glycosyl conjugates of **lut**—**lmg** and **ldg**—in presence of HNO_3 was assessed. Solutions of each of the flavones at five concentrations containing HNO_3 at a concentration of 0.35 mM—the same $[\text{HNO}_3]$ of all HNO_3 -containing solutions that were used for the irradiation experiments—were heated overnight. Areas of the peaks obtained from the chromatographic analyses of the solutions before and after the heating treatment were compared. The average percentage of **lmg** peak areas after the heating treatment relative to the original peak areas—at five **lmg** concentrations—was 103% (range: 98–106%). The average percentage of **ldg** peak areas after the heating treatment relative to the original peak areas—at five **ldg** concentrations—was 101% (range: 98–105%). These figures—alongside inspection of the corresponding chromatograms—suggest that **lmg** and **ldg** are stable in presence of HNO_3 under the experimental conditions.

SI C.2.11. Effect of increasing quantities of Al^{3+} of mordanted wool—using alum—on the photo-stability of the dye of weld

Table SI C.4. Colours of weld-dyed wool premordanted with varying quantities of Al^{3+} (experiment using alum; samples of Figure 4.4): Measured (L^* , a^* and b^*) and calculated (C^*_{ab} and h_{ab}) quantities using the CIELAB colour space [each case, $n = 4$; data expressed as (average $\pm s$), s_r (%), in which s = standard deviation and s_r = relative standard deviation].

L^*	a^*	b^*	Chroma (C^*_{ab})	Hue angle (h_{ab} ; degrees)
Blank-mordanted wool (wool pretreated as cases below, except that there was no alum in the mordanting bath)				
(82.9 $\pm$ 0.7), 0.8	–(3.26 $\pm$ 0.08), 2.4	(14.4 $\pm$ 0.9), 6.3	(14.7 $\pm$ 0.9), 6.0	(102.8 $\pm$ 0.7), 0.7
Wool premordanted with 2 g L^{–1} aqueous solutions of alum ^a				
(82.2 $\pm$ 0.3), 0.4	–(10.8 $\pm$ 0.4), 3.4	(43.2 $\pm$ 0.6), 1.4	(44.6 $\pm$ 0.7), 1.5	(104.0 $\pm$ 0.3), 0.3
Wool premordanted with 10 g L^{–1} aqueous solutions of alum				
(82.0 $\pm$ 0.5), 0.6	–(12.9 $\pm$ 0.5), 3.9	(60 $\pm$ 2), 3.7	(61 $\pm$ 2), 3.6	(102.1 $\pm$ 0.6), 0.6

^a The colour of one of the pieces of dyed wool was not homogeneous; brief visual inspection before colorimetric measurements. Based on these results, this is of no problem.

According to Goodman [6], the axes of the CIELAB colour space represent approximately:

- L^* lightness
- a^* amount of red (positive) or green (negative)
- b^* amount of yellow (positive) or blue (negative)

CIELAB chroma (C^*_{ab}) and hue angle (h_{ab}) approximately correlate to chroma and hue, and lightness, chroma and hue together can describe the perception of a colour [6]. Thus, the L^* , C^*_{ab} and h_{ab} quantities of the pieces of dyed wool are used in this work as a description of their colours.

Table SI C.5. Wash-fastness of the colours of weld-dyed wool premordanted with varying quantities of Al^{3+} (experiment using alum; samples of Figure 4.4) based on visual comparison and change in lightness (ΔL^* , CIELAB colour space) — each case: $n = 1$ (*i.e.*, one pair of samples, with only one of the two of them being washed).^a

Concentration of alum in mordanting solution	Visual	Based on ΔL^*
Blank (no alum)	4 / 4–5	4–5
2 g L⁻¹	3–4 / 4	4
10 g L⁻¹	3–4 / 4	4

^a Scale: 1 (poor)–5 (excellent); wash-fastness values of 3–4 are the acceptable lower limit [7].

The colours of the pieces of weld-dyed wool that were premordanted with 2 g L⁻¹ and 10 g L⁻¹ aqueous solutions of alum darkened after washing (assay on the wash-fastness of the colours). There are three hypotheses on what could have led to this darkening:

- The anion of the salt could have complexed with an available site—catechol or pseudo-carboxyl group—of the flavones bound to the mordanted wool. Reasoning: **i.** the detergent solution used contained sodium perborate, and **ii.** boric acid complexes with the free catechol group of rutin—a 3-*O*-glycoside of the flavonol quercetin—leading to a red-shift of the lowest energy absorption band in presence of sodium acetate [22].
- A pH effect. The detergent solution used for washing the dyed wool is basic, and there might have not been full neutralization by the end of the washing procedure. Reasoning: there is a red-shift of the lowest energy absorption band of **lut** in 95% ethanol in basic conditions (sodium acetate) [20].
- Possible incomplete removal of flavone diglycosides not bound to the wool through the after-dyeing rinsing, followed by their removal through the more aggressive conditions of the wash fastness assay. Reasoning: alum-mordanted wool dyed with **ldg** is pale yellow (Fig. 4.6).

SI C.2.12. Effect of the glycosylation pattern of lut (aglycone, monoglucoside, and diglucoside) on the colour of alum-mordanted wool dyed with the individual flavones

Table SI C.6. Colours of alum-mordanted wool samples—non-dyed, and dyed at 80 °C for 15 min with different dyes (samples of Figure 4.6): Measured (L^* , a^* and b^*) and calculated (C^*_{ab} and h_{ab}) quantities using the CIELAB colour space (each case, $n = 1$).

Dye	L^*	a^*	b^*	Chroma (C^*_{ab})	Hue angle (h_{ab} ; degrees)
None ^a	85.4	−0.9	3.4	3.5	104.2
Lut	82.6	−14.9	76.6	78.1	101.0
Lmg	80.4	−6.7	83.9	84.2	94.5
Ldg	85.4	−10.4	27.1	29.0	110.9
Extract of weld	82.0	−13.3	58.8	60.3	102.7

^a Non-dyed alum-mordanted wool.

The relative photo-stability of the flavones in solution was **lmg** < **lut** < **ldg** (section 4.4.2.1). Interestingly, the hue angle values of alum-mordanted wool dyed with these flavones individually follow the same trend, but the chroma values follow the opposite trend (Table SI C.6).

SI C.3. Author contributions

Alexandre Villela: Formulated the research questions, designed/performed the experiments on the photo-stability of the flavones in solution, designed/performed the experiments on the dyeing of alum-mordanted wool with the flavones/extract of weld, analysed/interpreted all data, and wrote the chapter/supplementary material.

Monique S. A. van Vuuren: Designed/performed the experiments on the dyeing of aluminium sulphate/tartaric acid-mordanted wool with the flavones/extract of weld, carried out the measurement of all L^* , a^* , and b^* quantities (CIELAB colour space), performed most assays on the light/wash-fastness of the colours of the dyed wool, and analysed/interpreted the resulting data.

Hendra M. Willemsen: Contributed to interpretation of data of the part of the research in which MSAvV was involved, and had input on the writing of the chapter.

Goverdina C. H. Derksen: Coordinated the part of the research in which MSAvV was involved.

Teris A. van Beek: Coordinated the part of the research in which AV was involved, and had extensive input on the writing of the chapter/supplementary material.

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Appendix D

Chapter 5: Supplementary information

The content of this appendix is equal to that of the supplementary material of the following paper:

Villela A, Derksen GCH, van Beek TA. Analysis of a natural yellow dye: an experiment for analytical organic chemistry. J Chem Educ 2014;91(4):566–9.

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SI D.1. Updated version of the protocol of the experiment handed out to students

ANALYSIS OF A NATURAL YELLOW DYE

Coloured textiles have been used by mankind throughout times. The dyes have been obtained from different natural sources, including plants, molluscs and insects and were expensive in the past. After the first chemical synthesis of a dye (1856), natural dyes rapidly lost importance to synthetic ones. In the past decades, however, there has been a renewed interest in natural dyes.

Weld (*Reseda luteola* L.) was a popular source of yellow dye for textiles in Europe. A bright yellow is obtained when it is used for dyeing alum-treated wool (alum = aluminum potassium sulphate dodecahydrate). Flavonoids (phenolic compounds) are the main compounds responsible for the colour in this plant. Its three main flavonoids are luteolin-7,3'-*O*-diglucoside (luteolin diglucoside, **ldg**), luteolin-7-*O*-glucoside (luteolin monoglucoside, **img**) and luteolin (**lut**), whose structures are seen below. Chrysin is used as internal standard (**i.s.**) in this experiment.

From a historical point of view, for conservation purposes or for forensics, it is of interest to know the dye-source(s) used in historical clothing and objects of art. Small sample sizes, *i.e.* < 1 mg of textile, are crucial to minimize damage to the artifact. To avoid decomposition during the extraction, mild extraction conditions are needed.

Didactic aims

(1) Gaining experience in HPLC analysis and (2) getting acquainted with quantitative analysis using the internal standard method. The field of natural dyes for textiles serves as background.

Experimental part (for specialized material, see basket)

Part 1: Preparation of the dyeing bath and extraction of weld for HPLC analysis

The extractions described below (a and b) are carried out simultaneously:

a) Extraction of weld for wool dyeing:

Weigh 1.5 g of dried and ground weld in a 100 mL Erlenmeyer. Add 30 mL of 96% alcohol-water 3:1 (v/v), close it with a rubber stopper and place it during 10 min in an ultrasonic bath (no heat applied) by means of the adapted test-tube rack. Filter the solution through a folded filter paper into a 100 mL round-bottom flask. Remove only the alcohol using a rotary evaporator, *i.e.* some water remains. Transfer the extract into a 150 mL beaker containing a magnetic stir bar with 4× 15 mL of deionized water. This is the dyeing bath.

b) Extraction of weld for HPLC analysis and HPLC-sample preparation:

Weigh accurately ~200 mg of dried and ground weld in a 50 mL Erlenmeyer. Then, carry out the extraction as described above, except for using only 20 mL of 96% alcohol-water 3:1 (v/v). After sonication, a magnetic stir bar is added to the Erlenmeyer, followed by addition of 5.00 mL of *i.s.* solution in methanol (let it come to room temperature prior to use) and stirring for 10 min at 300 rpm. About 1.5 mL of the solution is filtered by means of a 2 mL disposable syringe through a 13 mm/Ø 0.45 µm syringe filter into a standard HPLC vial, discarding the first drops and filling ~ $\frac{2}{3}$ of the vial.

Part 2: Wool dyeing and extraction of dyed wool for HPLC analysis

c.1) Pre-treatment of the wool:

Place a beaker containing a magnetic stir bar and deionized water on a magnetic stirrer-electric hot plate and place a thermo-sensor—first cleaned with acetone—in the water. Add 4 pieces of alum-treated wool of ~5 × 5 cm by means of tweezers to the water and heat it to 50 °C. The volume of water should be enough for the wool to be soaked. There is no time requirement for this step, *i.e.* once the wool is thoroughly wet and the water reaches 50 °C, you can start the next step.

c.2) Dyeing procedure:

Replace the beaker containing the wool by the beaker containing the dyeing bath. Set temperature to 80 °C. Start the stirring and cover the beaker with aluminum foil. When 80 °C is reached, add the pre-soaked pieces of wool. The dyeing step lasts 15 min. Transfer the wool to a beaker with warm tap water and rinse the pieces of wool. Repeat this with cold tap water. Finally, dry the pieces of dyed wool with a towel. You are welcome to take the pieces of dyed wool home. There are small plastic bags available for that. If you do not want them, remove one thread of wool from one of the pieces (for step d) and hand in the remaining pieces to one of the assistants.

d) Extraction of dyed wool for HPLC analysis and HPLC-sample preparation:

Place 50 mL of tap water in a 50 mL beaker and heat it to 60 °C on an electric hot plate. This is the water bath. Place a small piece (~0.5 cm) of a single thread of dyed wool in a microcentrifuge tube of 2 mL and add 300 µL of methanol–water–formic acid 80:15:5 (v/v/v) using an automatic pipette. Cap the tube and place it in the water bath for 30 min using the

floating tube rack. Afterwards, cool the tube with tap water; then, open it. Filter the solution with a 1 mL disposable syringe through a 4 mm/ $\varnothing$ 0.45 μ m syringe filter into an HPLC vial, discarding the first drop. Use a standard HPLC vial with an insert; and fill $\sim 2/3$ of the insert.

*Part 3: HPLC analysis: quantitation of **ldg**, **lmg** and **lut** in the weld extract using the internal standard method and qualitative analysis of the flavonoids extracted from the dyed wool*

e.1) HPLC analysis:

Analyses are carried out on an RP C18 column (RP = reversed phase; non-polar octadecyl chains chemically bound to silica) in duplicate, with a solvent gradient, as follows:

- Mobile phase: aqueous buffer pH 3 (solvent A) and acetonitrile (B);
- UHPLC column: 1.8 μ m-particle size, 50 $\times$ 3.0 mm at 35 $^{\circ}$ C;
- The flow is 0.90 mL min $^{-1}$ and the injection volume, 2 μ L;
- Detector continuously scanning from 245 to 500 nm, with chromatograms at 345 nm being printed;
- The retention times are 1.0 min (**ldg**), 1.3 min (**lmg**), 2.2 min (**lut**) and 3.7 min (**i.s.**).

e.2) Determination of the concentration of **ldg**, **lmg** and **lut** in the weld sample:

Ask the assistant for the chromatograms containing the retention times and areas of **ldg**, **lmg**, **lut** and **i.s.** peaks. Use the following predetermined relative response factors (RRFs): 1.13 (**ldg**), 1.58 (**lmg**) and 2.29 (**lut**). Calculate the concentration of **ldg**, **lmg**, and **lut** in the weld sample, in mg g $^{-1}$. Assume 100% extraction efficiency and 100% recovery. Check the **i.s.** solution bottle for the concentration of **i.s.** and the written material of the course, for slides on the internal standard method.

e.3) Qualitative analysis of the flavonoids extracted from the dyed wool:

Ask the assistant for the chromatograms of the wool sample containing the retention times and UV–vis absorption spectra of **ldg**, **lmg** and **lut** peaks. Ask also for chromatograms of pre-analysed authentic standards, along with the same data. Compare them. Furthermore, compare the chromatograms of the wool sample with those of the weld sample. *NB* This is related to questions 3 and 4.

Questions

1. Explain the observed order of elution of **ldg**, **lmg** and **lut** (by RP-HPLC).
2. Considering the structures seen on page 1, does it make sense that the RRFs decrease in the order **lut** > **lmg** > **ldg**, whereas the molecular weights decrease in the order **ldg** (611 g mol $^{-1}$) > **lmg** (448 g mol $^{-1}$) > **lut** (286 g mol $^{-1}$)? *NB* Tip: which part of the molecules is responsible for the absorbance at 345 nm?
3. Do you think the analytical method used for analysing the dyed wool would be suitable for identifying weld as the dye-source of a yellow historical woolen artifact? Why/Why not?
4. Prior to the HPLC analysis, the flavonoids fixed to the alum-treated fibre were extracted using a 5% (v/v) solution of formic acid in methanol–water, at 60 $^{\circ}$ C (step d). Even under these mild conditions, glycosidic bonds (= chemical bonds between the sugar part and the main flavonoid structure of the sugar-containing flavonoids) are possibly partially broken.

This is much worsened by the harsh conditions (hydrochloric acid; 100 °C) of a traditional procedure as, then, the sugar-containing flavonoids are no longer seen in the chromatograms. What would a chromatogram of the extract look like if the harsh conditions of extraction had been used in step d? Draw it, considering only **ldg**, **lmg** and **lut**, and the scale of the y-axis. Which of the two extraction procedures is preferable for identifying weld as the dye-source of a yellow historical woolen artifact? Why?

The protocol ends here. Below, alternative versions of items (i) e.1 through e.3 and (ii) e.1 and e.2 are described. This is done in case of using (i) a traditional 250 × 4.6 mm 5 µm-particle size HPLC column and (ii) retention (or capacity) factors instead of retention times (exemplified when using the UHPLC column).

i. In case of using an HPLC column:

e.1) HPLC analysis:

Analyses are carried out on an RP C18 column (RP = reversed phase; non-polar octadecyl chains chemically bound to silica), with a solvent gradient, as follows:

- Mobile phase: aqueous buffer pH 3 (solvent A) and methanol (B);
- HPLC column: 5 µm-particle size, 250 × 4.6 mm at 40 °C;
- The flow is 1.00 mL min⁻¹ and the injection volume, 20 µL;
- Detector continuously scanning from 245 to 500 nm, with chromatograms at 345 nm being printed;
- The retention times are 34 min (**ldg**), 38 min (**lmg**), 49 min (**lut**) and 57 min (**i.s.**).

e.2) Determination of the concentration of **ldg**, **lmg**, **lut** in the weld sample:

Ask the assistant for the chromatogram containing the retention times and areas of **ldg**, **lmg**, **lut** and **i.s.** peaks. Use the following predetermined relative response factors (RRFs): 1.09 (**ldg**), 1.51 (**lmg**) and 2.28 (**lut**). Calculate the concentration of **ldg**, **lmg**, and **lut** in the weld sample, in mg g⁻¹. Assume 100% extraction efficiency and 100% recovery. Check the **i.s.** solution bottle for the concentration of **i.s.** and the written material of the course, for slides on the internal standard method.

e.3) Qualitative analysis of the flavonoids extracted from the dyed wool:

Ask the assistant for the chromatogram of the wool sample containing the retention times and UV-vis absorption spectra of the **ldg**, **lmg** and **lut** peaks. Ask also for chromatograms of pre-analysed authentic standards, along with the same data. Compare them. Furthermore, compare the chromatogram of the wool sample with that of the weld sample. NB This is related to questions 3 and 4.

NB If needed, change also description of HPLC vials in items b and d (see Figures SI D.1a and SI D.1b).

ii. In case of using capacity factors instead of retention times (exemplified when using the UHPLC column):

e.1) HPLC analysis:

Analyses are carried out on an RP C18 column (RP = reversed phase; non-polar octadecyl chains chemically bound to silica) in duplicate, with a solvent gradient, as follows:

- Mobile phase: aqueous buffer pH 3 (solvent A) and acetonitrile (B);
- UHPLC column: 1.8 μm -particle size, 50×3.0 mm at 35 °C;
- The flow is 0.90 mL min⁻¹ and the injection volume, 2 μL ;
- Detector continuously scanning from 245 to 500 nm, with chromatograms at 345 nm being printed;
- The capacity factors (k') are 3 (**ldg**), 4 (**lmg**), 8 (**lut**) and 14 (**i.s.**).

e.2) Determination of the concentration of **ldg**, **lmg** and **lut** in the weld sample:

Ask the assistant for the chromatograms of:

- solution of uracil in water, for obtaining the void time (t_m), the time unretained compounds or the eluent take to move through the column. *NB* Such a solution was analysed under the same conditions as those of your sample;
- weld sample containing the retention times and areas of **ldg**, **lmg**, **lut** and **i.s.** peaks.

Use the following predetermined relative response factors (RRFs): 1.13 (**ldg**), 1.58 (**lmg**) and 2.29 (**lut**) [1]. Calculate the concentration of **ldg**, **lmg**, and **lut** in the weld sample, in mg g⁻¹. Assume 100% extraction efficiency and 100% recovery [1, 2]. Check the **i.s.** solution bottle for the concentration of **i.s.**. Additionally, see the written material of the course, for slides on k' and on the internal standard method.

SI D.2. Discussion of different parts of the protocol

Below, different parts of the protocol are discussed. This is done for mentioning references used and indicating changes and developments carried out (when applicable), and commenting steps and questions. The procedures related to wool pre-treatment and dyeing used have elements of the procedures described by Colombini *et al.*, Cerrato *et al.*, and Cardon [3-5], as well as those adopted by the company Rubia Natural Colours for wool dyeing with dye obtained from madder roots.

Steps and questions of the protocol of the experiment:

Experimental part

a) Extraction of weld for wool dyeing

- Extraction of weld by sonication for 10 min was reported by Gaspar *et al.* [2]. Use of 96% alcohol–water 3:1 instead of the widely used methanol–water 8:2 aimed at lowering the toxicity of the extraction solvent.
- Assuming 6% extraction yield, use of 1.5 g of weld leads to dyeing using 3% (w/w) of extract (total extract relative to wool, as the weight of each piece of alum-treated wool of $\sim 5 \times 5$ cm is ~ 0.8 g and four of them are dyed in this experiment).
- Use of 60 mL of water (in four times, for a more efficient transfer of the extract to the beaker) leads to dyeing using a ratio volume of water–weight of wool of ~ 20 . *NB* It is

important not to have too much wool in too little dyeing bath fluid for obtaining an evenly dyed wool [3].

- If needed or wanted, the deionized water may be replaced by rain water (preferably), soft water, or hard water made soft by the addition of acetic acid (or vinegar) [6].
- b) Extraction of weld for HPLC analysis and HPLC-sample preparation
- Choice of **i.s.**, its concentration, addition to the sample, homogenization and sample filtration: mostly as described by Villela *et al.* [1].

NB

- The outcome of a preliminary experiment ($n = 1$) suggests that replacing methanol as the solvent of the **i.s.** solution by 96% alcohol is feasible. Additionally, four students carried out the experiment in the 2013 edition of AMOC already using the solution of the **i.s.** in 96% alcohol (data not presented). The shapes of the peaks of the main flavonoids in the chromatograms of the weld samples were fine. Thus, due to the same toxicity considerations as above, this replacement is encouraged.
 - Details of sample filtration via disposable syringe and syringe filter: the syringe filter is connected to the syringe after removal of the plunger. Then, the sample is poured into the syringe. Finally, the filtration proceeds by reinsertion of the plunger into the syringe's barrel. Such instruction might be a helpful complement to the information on filtration via syringe filter available in the protocol of the experiment handed out to students.
 - Waste handling: the remaining of the content of the Erlenmeyer can be discarded after filtration. The filtrate is disposed in the laboratory's waste container. Finally, the wet paper and plant material are disposed in the trash bin after evaporation of the organic solvents in the fume hood and recovery of the magnetic stir bar.
- c.1) Pre-treatment of the wool
- Soaking the alum-pre-treated wool in warm water has the double function of wetting the wool prior to its dyeing—apparently useful for obtaining an even colour—and preventing the wool to go from room temperature to 80 °C (temperature of the dyeing step) too fast, avoiding possible shrinking and harshening [3], Wulansari D, 2011 (personal communication), and Willemen HM, 2012 (personal communication).
 - The pieces of alum-treated wool should be handled by means of tweezers to prevent or minimize touching them with bare hands, which may lead to uneven colour of the dyed wool. This is done at this stage to be on the safe side, as this is the case when handling the wool before the treatment with alum (see section **SI D.3**).

c.2) Dyeing procedure

- Dyeing at 80 °C for 15 min: 20 pieces of wool pretreated with a textile auxiliary agent (that aims at minimizing effects of fibre–fibre and fibre–metal contact, Biavin 109; CHT R. Beitlich GMBH) and Al^{3+} were dyed with an extract of weld at 75–80 °C. After different periods of time (15, 30, 45 and 60 min), five pieces of dyed wool were removed from the dyeing bath. After their cooling down and rinsing with water, four of them were washed using conventional laundry soap. Finally, all pieces were hung to dry in the fume hood. The duration of the dyeing step did not influence the outcome. Thus, it is carried out for 15 min. Although it might be that periods of time shorter than 15 min would lead to equally satisfactory results, this was not investigated.

- In another preliminary experiment, also using wool pretreated with a textile auxiliary agent (as previous bullet point) and Al^{3+} , the resistance of the yellow colour to washing using conventional laundry soap was checked. The pieces of wool that were dyed at 60–65 °C remained coloured after washing. Thus, there should be no problem if the dyeing step takes place at a temperature somewhat lower than 80 °C (although the colour of wool dyed at 60–65 °C might be less intense than that of wool dyed at ~75 °C).
 - Rinsing of the dyed wool with (1) warm tap water and (2) cold tap water: an even more gradual temperature transition is described in *Dye Plants and Dyeing – a Handbook* [6]. This aims at preventing the wool to be subjected to a fast temperature change [3].
 - Suggested waste disposal: cooled down dyeing bath and first rinsing water are disposed in the laboratory's waste container.
- d) Extraction of dyed wool for HPLC analysis and HPLC-sample preparation
- A piece of ~0.5 cm of a single thread of dyed wool weighs ~0.2 mg.
 - The procedure for extracting the dyed wool is based on that described by Zhang *et al.* [7]. Using Al^{3+} -treated weld-dyed wool, it was observed that methanol–water–formic acid 80:15:5 is better for analysing the flavonoid glycosides of weld than methanol–formic acid 95:5. Its use leads to a chromatogram similar to that of weld.
- e.1) HPLC analysis
- Samples of students who carried out the experiment in 2011 were analysed using a traditional 25 cm-long, 5 μm -particle size RP-HPLC column. In contrast, 2012 and 2013 samples were analysed using a modern 5 cm-long, 1.8 μm -particle size RP-UHPLC column, but also mounted in a conventional HPLC system. Details of the chromatographic methods using both columns were described by Villela *et al.* [1].
 - The identities of the main compounds of the extract of weld are indicated in Figure 5.2. Largely based on the works by Marques *et al.* and Peggie *et al.* [8, 9], in decreasing order of retention times, the identities of the compounds responsible for the minor peaks seen in the same chromatograms are:
 - 5,7-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-4*H*-1-benzopyran-4-one (**lut**-3'-methyl ether, chrysoeriol);
 - 5,7-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one (apigenin);
 - an isomer of **lmg**;
 - chrysoeriol glycoside (possibly);
 - apigenin-7-*O*-glucoside;
 - an isomer of **ldg**;
 - apigenin-6,8-*C*-diglucoside.

These assignments were based on chromatographic retention times, mass spectral data and UV–vis absorption spectral data, using information from the literature and reference compounds [8]. Additionally, acid hydrolysed-extracts of wool dyed with weld were spiked with reference chrysoeriol and diosmetin (**lut**-4'-methyl ether) for assignment of the position of the methyl group of the **lut** methyl ether using HPLC–photodiode array detector [9]. Finally, this information was combined with that obtained through preliminary observations carried out at the Laboratory of Organic Chemistry of Wageningen University by means of HPLC–mass spectrometry, also using information from the literature.

- The use of 96% alcohol–water 3:1 for the extraction of the flavonoids of weld leads to deformation of the peaks of the most polar compounds. This is most obvious when the HPLC column is used. The peak corresponding to apigenin-6,8-*C*-diglucoside is split in two and the one corresponding to the isomer of **ldg** is partially split. The effect is noticeable when compared with Figure 1A of the work by Villela *et al.* [1], in which the flavonoids of weld were extracted with methanol–water 8:2. Deformation of the peaks is also seen when the UHPLC column is used, although possibly to a smaller extent than when the HPLC column is used. However, this does not hamper the use of the method, as the peaks of interest—*i.e.*, **ldg**, **lmg**, and **lut**—do not display shoulder(s) with either column (Figures SI D.2 and SI D.3).
- e.2) Determination of the concentration of **ldg**, **lmg**, and **lut** in the weld sample:
- The RRFs listed were determined in an earlier stage of the project and reported by Villela *et al.* in 2011 (see text in page 8549 and footnote *d* of Table 4 of that article) [1]. The HPLC analytical conditions used in this laboratory experiment are the same as those reported then (see section SI D.3).
 - Assumptions of 100% extraction efficiency and 100% recovery are made as they are not known. *NB* As described above, the analytical method used here is an adapted combination of the methods reported by Gaspar *et al.* and Villela *et al.* [1, 2].

Questions

4. Zhang and Laursen, and Valianou *et al.* described the observations and information on which the following was based:
- Even under these mild conditions, glycosidic bonds (= chemical bonds between the sugar part and the main flavonoid structure of the sugar-containing flavonoids) are possibly partially broken [10].
 - This is much worsened by the harsh conditions (hydrochloric acid; 100 °C) of a traditional procedure as, then, the sugar-containing flavonoids are no longer seen in the chromatograms [10, 11].

SI D.3. Material and methods for the preparation of the experiment

Part 1: Preparation of the dyeing bath and extraction of weld for HPLC analysis

a) Extraction of weld for wool dyeing:

- Dried and ground weld: dried aerial parts of weld (*Reseda luteola* L.) plants are ground and sieved using a mill, *e.g.*, cutting mill from Retsch (type SM1; 0.25 mm sieve). The plant material is stored in the dark, except for what is placed in the basket in an amber glass bottle;
- 1× 100 mL Erlenmeyer with a rubber stopper;
- 96% alcohol–water 3:1 (v/v): 375 mL of 96% alcohol (CAS RN: 64-17-5; *e.g.*, for general laboratory use) and 125 mL of deionized water, for 500 mL;
- Adapted test-tube rack: this is a test-tube rack that had parts of its grid removed using a pair of pliers in order to accommodate two Erlenmeyers (for placement in the sonication bath).

NB

- Alternatively to weld, the easy to obtain and widely available, outer scales of onions could be used as the source of a flavonoid dye [12–14]. There is the need for adaptation of the

experiment though. The following is either needed or might be important: use of different authentic standards, adaptation of the chromatographic gradient, determination of new RRFs, use of 1% (w/w) of extract (total extract relative to wool), simultaneous addition of all pre-soaked pieces of wool, and increase of the ratio volume of water–weight of wool to ~50.

- Grinding of weld: in the absence of a laboratory mill, although not tried during this work, a grinder for coffee beans could be tried.
- Alternatively to the use of one's own cultivated plants, weld can be bought. Below, two suppliers of chopped dried weld are listed:
 - Kremer Pigmente [15];
 - Brush Creek Wool Works [16].

b) Extraction of weld for HPLC analysis and HPLC-sample preparation:

- 1 × 50 mL Erlenmeyer with a rubber stopper;
- 0.5 mg mL⁻¹ i.s. solution in methanol: 50 mg of chrysin (480-40-0; *e.g.*, 97% grade, Aldrich) are weighed in a 100 mL volumetric flask. Methanol (67-56-1; *e.g.*, *p.a.* grade) is added up to ~¾ of the volume of the flask, which is then wrapped in aluminum-foil and placed in a shaker (150 rpm; room temperature) overnight. Finally, methanol is added to the mark. The solution is kept in a refrigerator. *NB* In case the 0.5 mg mL⁻¹ i.s. solution is prepared in 96% alcohol: all as described for its preparation in methanol, except for the use of 96% alcohol (as above) instead of methanol;
- Syringe filters: 0.45 µm, *e.g.*, PTFE membrane, 13 mm (catalogue 2165, Grace Davison Discovery Science);
- Disposable polypropylene (PP) syringes of 2 mL. *NB* There must be a rubber seal at the tip of the plunger for the solution to be properly pushed through the syringe filter;
- Standard HPLC vials with caps. *NB* Vials and caps may vary, depending on the HPLC system being used (see item e.1 below).
- If *k'* is used, 0.02 mg mL⁻¹ uracil solution in water: 2 mg of 2,4(1*H*,3*H*)-pyrimidinedione (uracil; 66-22-8; *e.g.*, 99+%, Acros Organics) are weighed in a 100 mL volumetric flask. Deionized water is added up to ~¾ of the volume of the flask, which is then placed during 10 min in a ultrasonic bath (neither heating nor cooling applied). Finally, deionized water is added to the mark.

NB

- If a 0.1 mg-least division scale is used, a large weighing error results, being the uncertainty of the weighing estimated to be 0.3 mg. This is of no relevance for the purpose of use of this solution, *i.e.*, determination of *t_m*. The nominal concentration of the solution remains unchanged, *i.e.*, 0.02 mg mL⁻¹;
- No toxicity of uracil is reported in the Merck Index *Online* [17].

Part 2: Wool dyeing and extraction of dyed wool for HPLC analysis

c.1) Pre-treatment of the wool:

- 2 L of aqueous 10 g L⁻¹ alum solution: 20 g of aluminum potassium sulfate dodecahydrate (alum) [KAl(SO₄)₂·12H₂O] (10043-67-1; *e.g.*, puriss. *p.a.* grade, Sigma-Aldrich) are dissolved in 2 L of deionized water;

- Wool treatment: 15 pieces of wool prepared for dyeing [e.g., Kova Wool Sateen White (part number W110); Whaleys (Bradford) Limited], of $\sim 17 \times 17$ cm (120 g overall) are individually rolled and placed in a 3 L round-bottom flask. The 2 L of alum solution are transferred to it. The flask is heated over a 0.5–1 h period until $T > 90$ °C, using the motor of a rotatory evaporator for stirring the wool (*NB* The water bath of the rotatory evaporator is suitable for that). It is kept stirring at ~ 95 °C for 1 h (*NB* If the wool is being stirred non-optimally, decrease of the stirring rate can be tried). Then, the flask is removed from the heating bath and left to cool down for some time. Next, the treated wool is thoroughly rinsed with deionized water and hung to dry. Finally, the wool is cut into pieces of $\sim 5 \times 5$ cm and the pieces of treated wool are placed in small plastic bags (4 pieces per bag). *NB*
 - Use tweezers or gloves to handle the wool at all times (as fat, protein and dirt from one's hand may lead to uneven colour of the dyed wool);
 - Safety warning: do not use any connecting part between the round-bottom flask and the rotatory evaporator, but connect the two directly (hazard of glassware breakage).

c.2) Dyeing procedure: nothing in particular.

d) Extraction of dyed wool for HPLC analysis and HPLC-sample preparation:

- Microcentrifuge tubes of 2 mL (e.g., Eppendorf);
- 20 mL of methanol–water–formic acid 80:15:5 (v/v/v): 16 mL of methanol (as above), 3 mL of deionized water, and 1 mL of formic acid (64-18-6; e.g., 98+%, Acros Organics);
- Syringe filters: 0.45 μ m, e.g., cellulose membrane, 4 mm (Minisart RC4, Sartorius Stedim Biotech);
- Disposable polypropylene (PP) syringes of 1 mL. *NB* Also here, there must be a rubber seal at the tip of the plunger;
- Standard HPLC vials with caps and inserts (due to the small volume of solution available).

*Part 3: HPLC analysis: quantitation of **ldg**, **img**, and **lut** in the weld extract using the internal standard method and qualitative analysis of the flavonoids extracted from the dyed wool*

e.1) HPLC analysis:

Analyses can be carried out using either an HPLC column or a UHPLC column (in a conventional HPLC system) as described by Villela *et al.* [1]. The preparation of HPLC solvent A and the conditions of HPLC analysis using both columns are detailed below.

1. Preparation of solvent A (aqueous buffer pH 3; 160 mM formic acid, 40 mM ammonium formate, and 0.04 mM of EDTA), that is used in both HPLC systems:
 - Add a magnetic stir bar to a 2.5 L bottle, followed by the addition of:
 - 2,100 mL of “ultrapure water”, that can either be prepared (e.g., with an EasyPure UV system, Barnstead/Thermolyne) or purchased (e.g., Lichrosolv water for chromatography, Merck);
 - 5.04 g of ammonium formate (540-69-2; e.g., puriss. *p.a.* grade, Fluka Analytical);
 - 12.9 mL of formic acid (as above);
 - 10 mL of 8 mM aqueous *N,N'*-1,2-ethanediylbis[*N*-(carboxymethyl)glycine] (EDTA) solution. *NB* Prepared by, e.g., the dissolution of 170 mg of EDTA·4Na·2H₂O (64-02-8; 98%, Aldrich-Chemie) in 50 mL of “ultrapure water”.

2. HPLC column:

- Solvent B: methanol (HPLC grade);
- Column: Alltima 250 × 4.6 mm C18 5 μm (with a C18 guard column) (Grace Davison Discovery Sciences);
- HPLC system used here consists of Waters 1525 μm binary pump, 996 photodiode array detector, 717 plus autosampler, and 5CH column oven;
- HPLC vials used:
 - 1 mL, 40 × 8 mm;
 - 40 × 8 mm, of maximal residual volume of 6 μL;
- Injection volume: 20 μL;
- Single injections;
- Needle wash solvent: methanol (HPLC grade; *NB* Efficient for keeping sample carryover below 1%);
- Flow: 1.00 mL min⁻¹;
- Linear solvent gradient: 85–60% A (0–35 min), 60–36% A (35–47 min), 36–23% A (47–60 min), 23–0% A (60–65 min), 0% A (65–70 min) (*NB* Subsequent injections are made after 10 min of re-equilibration);
- Column temperature: 40 °C;
- Detector: set to scan from 245 to 500 nm. *NB* Single wavelength detection (345 nm) is also an option. There is the need for an adaptation of the experiment though. In item e.3 of the protocol of the experiment (*qualitative analysis of the flavonoids extracted from the dyed wool*), together with retention times, UV–vis absorption spectra of peaks are used for the assignment of the peaks corresponding to **ldg**, **lmg**, and **lut**. Then, the assignment of these peaks would be carried out as done in item e.2, *i.e.*, solely based on the retention times of the compounds.

3. UHPLC column:

- Solvent B: methyl cyanide (acetonitrile) (75-05-8; HPLC grade);
- Column: Eclipse XDB-C18 50 × 3.0 mm 1.8 μm (without guard column) (Agilent Technologies);
- HPLC system used here consists of an Agilent 1200 Series binary pump, and Hewlett-Packard Series 1100 photodiode array detector, autosampler, and column oven;
- HPLC vials used:
 - 1.5 mL, 32 × 12 mm (standard);
 - 1.5 mL, 32 × 12 mm, with an insert;
- Injection volume: 2 μL;
- Analysis in duplicate;
- Flow: 0.90 mL min⁻¹;
- Linear gradient: 85–45% A (0–4.00 min), 45–85% A (4.00–4.01 min), 85% A (4.01–5.00 min) (*NB* Subsequent injections are made every 5 min);
- Actual column temperature: 35 °C (*NB* In the system used here, due to minimization of tubing—see below—the column lies across the column oven; the column oven temperature is set to 50 °C);

- Detector: set to scan from 245 to 500 nm. *NB* See note above (HPLC column) for the possibility of using single wavelength detection (345 nm).
- The following modifications are carried out to reduce the internal volume of the system:
 - The mixer is replaced by an Alltima 7.5 × 3.0 mm C18 5 μm guard column, which acts as the mixer and connects the damper and the injector;
 - Shortening of the tubing connecting the injector to the column and the column to the detector;
- Additionally, an in-line filter (*e.g.*, 0.3 μm, Agilent Technologies) can be placed before the column, aiming at extending its lifetime. *NB* Analyses of 2012 samples were carried out using such a filter.

NB

- If the HPLC to be used is not equipped with an autosampler, instructors may consider storing the samples in refrigerator prior to their manual injection. This was observed to be fine for samples in methanol–water 8:2 [1]. Then, the experiment could be adapted for being carried out, for instance, in two ~4 h periods with the students injecting the samples and starting processing the data.
- Injection of a solvent after the analysis of the last sample of a series can be considered for verifying whether there is sample carryover and, if so, to monitor its extent. A suggestion would be to consider sample carryovers below 1% (peak area) acceptable.

e.2) Determination of the concentration of **ldg**, **img**, and **lut** in the weld sample:

The chromatogram at 345 nm (Waters system) [Agilent system: 345 nm (bandwidth 4 nm), with 470 nm (bandwidth 40 nm) being the reference wavelength] of the weld sample, with the retention times and areas of **ldg**, **img**, **lut**, and **i.s.** peaks, is printed and given to the student. *NB* If k' is used, additionally, the chromatograms at 260 nm (bandwidth 4 nm)—with 470 nm (bandwidth 40 nm) being the reference wavelength (Agilent system)—of the uracil solution, with the retention time of uracil, are printed and given to the student.

e.3) Qualitative analysis of the flavonoids extracted from the dyed wool:

To be printed and given to the student:

- Chromatogram (as above, excluding peak areas) with UV–vis absorption spectra of **ldg**, **img**, and **lut** peaks;
- Chromatograms at 345 nm (Waters system) [Agilent system: 345 nm (bandwidth 4 nm), with 470 nm (bandwidth 40 nm) being the reference wavelength] of **ldg**, **img**, and **lut** standards, along with retention times and UV–vis absorption spectra of the peaks. *NB* Preparation of the standard solutions of the flavonoids was described by Villela *et al.* [1].

SI D.4. Instructor notes

For those assisting the students carrying out the experiment, in this section, there is a note on the title of the protocol handed out to students, solutions to its assignments, and answers to the questions.

Title

“*Analysis of a Natural Dye: ...*” (title of chapter 5 of this thesis) would be more accurate than “*Analysis of a Natural Yellow Dye: ...*”, as the colour of textiles dyed with weld depends on the metal used in the textile’s pre-treatment [18]. However, the latter title is thought to be fine, as weld was an important source in the past in Europe for dyeing textiles yellow [19], and it is more interesting than the former.

Item e.2

With the chromatogram of the weld sample containing peak areas and retention times, students can assign the peaks corresponding to **ldg**, **lmg**, **lut**, and **i.s.** based on retention times.* Calculation of concentration of **ldg**, **lmg**, and **lut** in the weld sample using the internal standard method can be explained as follows:

The relation between peak area and quantity (weight) of a compound can be expressed as:

$$A_x = R_x \cdot w_x \quad (1)$$

in which A_x is the peak area of compound x ; R_x is the response factor of compound x ; and w_x is the weight of compound x . As this is true for any compound, it is also the case for the **i.s.**. Thus, the following can be written:

$$A_{i.s.} = R_{i.s.} \cdot w_{i.s.} \quad (2)$$

division of *equation 1* by *equation 2* leads to:

$$\frac{A_x}{A_{i.s.}} = \frac{R_x}{R_{i.s.}} \cdot \frac{w_x}{w_{i.s.}} \quad (3)$$

as $R_x/R_{i.s.} = \text{RRF}$, *equation 3* can be rewritten as:

$$w_x = \frac{A_x}{A_{i.s.}} \cdot \frac{w_{i.s.}}{\text{RRF}} \quad (4)$$

As the peak areas are obtained from the chromatogram, the quantity (weight) of added **i.s.** is known, and the RRFs are given, students can calculate the quantity of compound x (**ldg**, **lmg**, or **lut**) in the sample using *equation 4*. As 100% extraction efficiency and recovery are assumed, no corrections need to be made to the obtained values. Finally, the concentration of compound x in the weld sample can be calculated. *NB*

- One of the strengths of the internal standard method can be highlighted to the students, *i.e.*, sample volumes do not need to be known as the ratio flavonoid to **i.s.** remains always constant.
- Additionally, it can be emphasized to students that there is no need for determination of individual response factors of analytes and **i.s.** via calibration curves. Instead, the RRFs are determined. This can be done through analyses of solutions containing variable weights of standards of the analytes and fixed weights of the **i.s.**. Finally, the slopes of the curves $w_x/w_{i.s.}$ vs. $A_x/A_{i.s.}$ provide the RRFs (see *equation 3*). This is described, *e.g.*, by Villela *et al.* [1].
- Concentrations of **ldg**, **img**, and **lut** in a sample consisting of the aerial parts of weld were determined to be 3.5 mg g^{-1} (**ldg**), 6.5 mg g^{-1} (**img**) and 1.0 mg g^{-1} (**lut**). [1] These values, however, are weld sample-dependent.
- * If k' is used, students carry out similar work, except that the k' need to be calculated first. As the retention by the column of the very polar compound uracil is small, its retention time (t_r) is considered to be the void time (t_m), the time unretained compounds (as well as the eluent) take to move through the column. Thus, the time a compound spends in the stationary phase (the adjusted retention time, t_r'), equals $t_r - t_m$ [20, 21]. Finally, the k' are calculated using the equation $k' = t_r'/t_m$ [20, 21].

Item e.3, and questions 3 and 4

Retention times and UV–vis absorption spectra of peaks in the chromatogram of the wool sample are compared with those in the chromatograms of authentic standards. This leads to assignment of the peaks corresponding to **ldg**, **img**, and **lut**. Then, comparison of the chromatograms of the wool sample and of the weld sample leads to the observation that the peaks corresponding to **ldg**, **img**, and **lut**—as well as other minor peaks—are present in both of them. Such observation of weld’s “fingerprint” in the chromatogram of the wool sample indicates the suitability of the analytical method for identifying weld as the dye-source of a yellow historical woolen artifact.

Use of the harsh conditions to extract the flavonoids from the dyed wool would lead to disappearance of the peaks of the glycosides, due to hydrolysis of glycosidic bonds in **ldg** and **img**. As **lut** is formed in the process, a **lut** peak of increased height would be seen in the chromatogram. As the procedure that employs mild extraction conditions is informative on the composition of flavonoid glycosides, it is preferred over the procedure that employs harsh conditions of extraction for identifying weld as the dye-source of a yellow historical woolen artifact. The latter yield only one peak for the three main constituents; namely, **lut**.

Question 1

In reversed phase chromatography the mobile phase is polar and the stationary phase is non-polar. Thus, polar compounds elute earlier than non-polar compounds. As polarity increases in the order **lut** < **img** < **ldg**, the observed order of elution is **ldg**–**img**–**lut**.

Question 2

As seen on page 1 of the protocol of the experiment, flavonoids **ldg** and **img** consist of **lut** with sugar (glucose) moieties linked to OH groups. The sugar moieties do not absorb light at 345 nm, the wavelength used for determining the concentration of **ldg**, **img**, and **lut** in the weld

sample. Only the **lut** part of **ldg** and **img** structures is responsible for the absorbance at 345 nm. Thus, for equal weights of **ldg**, **img**, and **lut**, peak areas will decrease in the order **lut** > **img** > **ldg**.

Equation 4 above can be rewritten as:

$$\text{RRF} = \frac{A_x}{A_{\text{i.s.}}} \cdot \frac{w_{\text{i.s.}}}{w_x} \quad (5)$$

As RRF and A_x are directly proportional, RRFs will also decrease in the order **lut** > **img** > **ldg**. This is the inverse of the decrease of molecular weights (MWs), as MW_{ldg} (611 g mol⁻¹) > MW_{img} (448 g mol⁻¹) > MW_{lut} (286 g mol⁻¹).

SI D.5. Additional observations on the students' learning based on 2011 and 2012 reports

Observations on the students' learning regarding the didactic aims and background of the experiment based on 2011 and 2012 reports, in addition to those described in chapter 5:

- None of the students related the increase in molecular weight of the flavonoids due to non-345 nm-absorbing glucose moiety or moieties ($\text{MW}_{\text{lut}} < \text{MW}_{\text{img}} < \text{MW}_{\text{ldg}}$) to the decrease in RRFs ($\text{RRF}_{\text{lut-i.s.}} > \text{RRF}_{\text{img-i.s.}} > \text{RRF}_{\text{ldg-i.s.}}$) (*question 2* of the experiment's protocol);
- In 2011, nearly all students observed the presence of peaks corresponding to **ldg**, **img**, and **lut** in the chromatogram of the wool sample, but only two of them answered satisfactorily the question on the suitability of the analytical method for identifying the dye-source(s) of a historical woolen artifact (item *e.3* and *question 3*);
- In 2012, three of the five students answered that mild extraction conditions are preferable to the harsh conditions of extraction for ascertaining weld as the dye-source of a yellow historical woolen artifact (*question 4*, introduced in 2012).

SI D.6. Updated version of the inventory of specialized material handled by students

Below, an updated version of the inventory of specialized material handled by students that is kept in the experiment's basket. *NB* In AMOC, experiments have baskets containing specialized material.

INVENTORY

- Dried and ground weld;
- 1 × 100 mL Erlenmeyer with a rubber stopper;
- 1 × 50 mL Erlenmeyer with a rubber stopper;
- 150 mL beaker;
- 96% alcohol–water 3:1 (v/v) (solvent cabinet);
- Adapted test-tube rack (Figure below);
- 0.5 mg mL⁻¹ **i.s.** solution in methanol or in 96% alcohol (refrigerator);
- Box with syringe filters (disposable):
 - 13 mm/Ø 0.45 µm;
 - 4 mm/Ø 0.45 µm;

- Disposable syringes (with a rubber seal at the tip of the plunger):
 - 1 mL;
 - 2 mL;
- Microcentrifuge tubes of 2 mL;
- Methanol–water–formic acid 80:15:5 (v/v/v) (solvent cabinet);
- Pieces of alum-treated wool of $\sim 5 \times 5$ cm (4 pieces per bag);
- Small plastic bags for storing the dyed wool;
- Floating tube rack (Figure below);
- Towel.

Figure SI D.1a. Adapted test-tube rack, floating tube rack, standard HPLC vial, and standard HPLC vial with insert.

The inventory ends here. Below, an alternative version of the figure is presented in case of using the HPLC column (if this is accompanied by the need to use 40×8 mm HPLC vials and 40×8 mm HPLC vials of maximal residual volume of 6 μL , as it has been the case at Wageningen University):

Figure SI D.1b. Adapted test-tube rack, floating tube rack, 40×8 mm HPLC vial, and 40×8 mm HPLC vial of maximal residual volume of 6 μL .

SI D.7. Example data sets of the HPLC analysis of weld sample

Below, two example data sets of the HPLC analysis of weld sample. They might be of interest, *e.g.*, for use as lecture examples (chromatograms and information in tables) and when HPLC systems equipped with single wavelength detectors are used (UV-vis absorption spectra of flavonoids).

Figure SI D.2. Chromatogram of weld sample (345 nm-trace; same as top left panel of Figure 5.2).

Table SI D.1. HPLC data of chromatogram depicted in Figure SI D.2.			
Flavonoids	Retention time (min)	Peak area (mAU s)	Peak height (mAU)
Idg	33.6	1292	55
Img	37.8	3323	173
lut	49.0	912	93
i.s.	57.2	3319	297

Figure SI D.3. Chromatogram of weld sample (345 nm-trace; same as top right panel of Figure 5.2).

Table SI D.2. HPLC data of chromatogram depicted in Figure SI D.3.

Flavonoids	Retention time (min)	Peak area (mAU s)	Peak height (mAU)
ldg	1.02	124	60
lmg	1.31	314	176
lut	2.26	82	46
i.s.	3.78	439	222

Finally, the following figures can be considered to be used for the calculations:

- quantity of dried and ground weld: 199.7 mg or 201.2 mg;
- concentration of the **i.s.** solution: 0.492 mg mL⁻¹ or 0.496 mg mL⁻¹.

Figure SI D.4. UV-vis absorption spectra acquired during chromatographic separations depicted in Figures SI D.2 and SI D.3, at peak apices. Solvent mixtures: aqueous buffer pH 3–methanol (solid lines) and aqueous buffer pH 3–acetonitrile (dashed lines).

SI D.8. Supplementary references

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