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Development of a process for obtaining non-mutagenic madder root (*Rubia tinctorum*) extract for textile dyeing

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ABSTRACT

Madder, used as a red dye, contains anthraquinones, and some of these can react with DNA possibly causing mutagenic effects. This is especially true for 1,3-dihydroxyanthraquinones with a hydroxymethyl (e.g., lucidin **6**, ibericin **10**) or methyl group (e.g., rubiadin **9**) at C-2. In this research, a new process was developed through which the concentration of mutagenic compounds is minimized by adapting extraction and fractionation parameters. The process was tested on lab scale but also on 5000 L industrial scale. The first step is the key biotechnological step. Roots are stirred in water and the concentration of lucidin **6** is reduced to (near) zero by endogenous enzymes. When lucidin **6** is absent, the formation of mutagenic ibericin **10** by a reaction with ethanol as extraction solvent, is not possible. Mutagenicity can be further reduced by heat treatment, which is common in industrial downstream processing, e.g., in spray drying. Removal of rubiadin **9** is possible by flash chromatography. All madder root fractions were tested in the *Salmonella* microsome assay (Ames test, TA100) for mutagenicity, which was correlated with the anthraquinone concentration.

1. Introduction

Rubia tinctorum L., also known under its synonym madder, has a long history of use as a red dye (Bechtold and Mussak, 2009; Blackburn, 2017; Schweppe, 1993). The genus *Rubia* belongs to the family Rubiaceae and comprises about 70 species (Eltamany et al., 2020). The herbaceous plant madder consists of small leaves and grows by clinging into each other like bedstraw. The plant is cultivated for the extensive long red colored roots, which are harvested in the second or third year of growth (Fig. 1) (Derksen and van Beek, 2002). The earliest evidence to date of the use of madder lake is in a 4000 years old ancient Egyptian quiver (Leona, 2009).

At the end of the 19th century the madder culture rapidly declined, due to the cheaper production of synthetic alizarin (Schweppe, 1993). Since about the turn of this century, madder extract is being made again and offered on a commercial scale in South America, Asia and Europe. More and more studies are published on extraction, purification and modification processes of natural dyes and their subsequent application to textiles (Ali et al., 2009; Cuoco et al., 2009; Duval et al., 2016; Elsahida et al., 2020; Farizadeh et al., 2009; Gupta et al., 2001a, 2001b; Hosseinnezhad et al., 2020; Islam and Mohammad, 2015; Kasiri and Safapour, 2014; Mansour et al., 2020; Montazer et al., 2007; Rather et al., 2019; Rossi et al., 2016; Siva et al., 2012). Due to the increasing public awareness of the disadvantages of synthetic fibers, the development of sustainable materials is increasing, such as the use of polylactic acid (PLA) as a fiber or the reuse of natural fibers such as hemp. This demand will increase exponentially in the coming years (Gedik and Avinc, 2020; Koenig et al., 2020; Reichert et al., 2020) as nowadays people desire fully sustainable fibers (Bechtold et al., 2007; Islam and Mohammad, 2015; Khattak et al., 2019). Due to this increasing demand for biobased yarns, there is also an increasing demand for natural textile dyes as every 100 g of fabric consists of 10−20 gram additives, of which 1−10 gram are dyes.

Madder extract is a complex mixture of compounds like anthraquinones (e.g., alizarin **7**, the main dyeing compound), sugars (e.g., sucrose), enzymes (e.g., primeverosidase), carboxylic acids, coumarins, flavonoids, iridoids and others (Blackburn, 2017; Derksen and van Beek, 2002; Duval et al., 2016; Eichinger et al., 1999). However, publications

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have casted doubt on the safety of madder.

Some anthraquinones were reported to react with deoxyribonucleic acid (DNA) indicating possible mutagenic effects (Ghodke et al., 2015; Ishii et al., 2012, 2010; Swanbeck, 1966; Yockey et al., 2017). Brown and Brown were the first to identify lucidin **6** and lucidin ethyl ether (ibericin **10**) as the main mutagenic anthraquinones in *Morinda* and *Coprosma* in the family Rubiaceae (Brown and Brown, 1976). In later articles it was reported that lucidin **6** is also present in *Rubia tinctorum*. A comprehensive study of madder constituents was performed by Kawasaki and Goda who found that especially 1,3-dihydroxyanthraquinones substituted with a methyl or hydroxymethyl group at C-2 (i.e., lucidin **6**, ibericin **10**, rubiadin **9** and lucidin methyl ether **12**) showed mutagenic effects. Anthraquinones with other functional groups at C-2 such as the carboxylic acids pseudopurpurin **4** and munjistin **5**, the aldehyde nordamnacanthal **11**, and purpurin **8** have no reported mutagenic properties. Some anthraquinones, like rubiadin **9**, display mutagenicity after metabolic activation by S9 liver cells (Inoue et al., 2008; Kawasaki et al., 1992; Lajkó et al., 2015; Marczylo et al., 2003; Räisänen et al., 2020; Yasui and Takeda, 1983). Similarly, conversion of ibericin **10** to lucidin **6** by S9 may be the cause of a markedly increased mutagenicity (Westendorf et al., 1988).

Madder is primarily used as a textile dye. The skin is in direct contact with the dyed textile. Possible human uptake of harmful anthraquinones by the skin is therefore relevant (Jäger et al., 2006). Jäger et al. investigated the mutagenicity (Ames test) of two madder root samples of different origin along the entire process of dyeing. Ethyl acetate extracts from roots boiled in water, aqueous dyebath samples and artificial sweat extracts from the dyed wool were analytically investigated by high pressure liquid chromatography - diode array detection (HPLC-DAD). They concluded that the mutagenic response of the different samples was positively correlated with the concentration of the lucidin **6** content and recommended that extracts of *Rubia tinctorum* should no longer be used for textile dyeing (Jäger et al., 2006).

However, the phenolic profile of a plant can be chemically and enzymatically changed by adaptation of the extraction conditions (Duval et al., 2016; Ford et al., 2018; Hachicha Hbaieb et al., 2015; Hostetler et al., 2012; Ramírez et al., 2014; Siva et al., 2012; Zhang and Laursen, 2009). Earlier Derksen et al. showed that madder root extract contains at least two endogenous enzymes, a primeverosidase and an oxidase (Fig. S1). In the presence of water and air, these two enzymes can convert any mutagenic lucidin **6** present to non-mutagenic nordamnacanthal **11** (Derksen et al., 2003).

This study examines the possibility of developing a new extraction procedure for madder root in which the concentration of mutagenic compounds is brought to zero or near-zero. This was investigated both on lab scale and 5000 L industrial scale. The composition and mutagenic potential of madder root extracts and fractions thereof obtained via different processing steps and having different anthraquinone compositions, were determined by HPLC and the *Salmonella* microsome assay (Ames test) respectively. A correlation between anthraquinone profiles and mutagenicity allowed to pinpoint key steps to reduce potential mutagenicity of dye extracts.

2. Material and methods

2.1. Chemicals

For sample preparation, extraction and analysis, all chemicals and solvents were at least analytical grade and were purchased from local commercial suppliers. Sodium azide, 2-aminoanthracene, dimethyl sulfoxide and dimethyl sulfoxide-d_6 were obtained from Sigma-Aldrich. Ethanol (96 % v/v) was obtained from Prolabo, 1-propanol from Merck and 1-butanol from Labscan. Ammonium formate, formic acid, aluminum sulphate, tartaric acid, formic acid, non-stabilized tetrahydrofuran, acetonitrile (HPLC grade), ethyl acetate, methanol and sodium diethylenediaminetetraacetic acid (EDTA), were supplied by VWR (Amsterdam, The Netherlands). Ethanol (96 % Pharmaceutical Agricultural Ethanol) for large scale madder root extraction was obtained

no.	compound name	R_1	R_2	R_3	R_4
1	lucidin primeveroside	OH	CH_2OH	primeveroside	H
2	ruberythric acid	OH	primeveroside	H	H
3	rubiadin primeveroside	OH	CH_3	primeveroside	H
4	pseudopurpurin	OH	COOH	OH	OH
5	munjistin	OH	COOH	OH	H
6	lucidin	OH	CH_2OH	OH	H
7	alizarin	OH	OH	H	H
8	purpurin	OH	OH	H	OH
9	rubiadin	OH	CH_3	OH	H
10	ibericin	OH	$CH_2OC_2H_5$	OH	H
11	nordamnacanthal	OH	CHO	OH	H
12	lucidin methyl ether	OH	CH_2OCH_3	OH	H
13	lucidin propyl ether	OH	$CH_2OC_3H_7$	OH	H
14	lucidin butyl ether	OH	$CH_2OC_4H_9$	OH	H

primeveroside = 6-O-ß-D-xylopyranosyl-ß-D-glucose

Fig. 1. Photo of *Rubia tinctorum* L. aerial plant and roots plus structural enumeration of anthraquinones reported in this article.

from Cargill b.v. (Bergen op Zoom, The Netherlands).

Different qualities of water were used. HPLC quality water was either prepared with an ultrapure water purification system (Easypure UV, Barnstead) with a resistivity of at least 18.3 MΩ cm or purchased from VWR International b.v. as HiPerSolv Chromanorm water for HPLC. All other water used was process water of °dH = 4. For process water, tap water was demineralized with a Lubron Compact C-110, filled with acidic cation exchange resin Lewatit S1567, with a capacity of 1500 L/h (Lubron, Oosterhout, The Netherlands).

For the Ames test the following chemicals were used: nicotinamide adenine dinucleotide phosphate disodium salt (NADP) (Roche Diagnostics, Woerden, The Netherlands), D-glucose-6-phosphate, disodium salt, 9-aminoacridine, N-ethyl-N-nitrosourea, dimethyl sulfoxide, mitomycin C and benzo(a)pyrene (Sigma Chemical Company, St. Louis, USA), 2-nitrofluorene, 2-amino anthracene and sodium azide (Aldrich, Brussels, Belgium). The strains used for the assays were the histidine-requiring *Salmonella typhimurium* TA1535, TA1537, TA98, TA100 purchased from dr. B.N. Ames (University of California Berkeley, USA) and the tryptophan-requiring *Escherichia coli* WP2*uvr*A strain purchased from the National Institute of Public Health (Bilthoven, The Netherlands). All were tested in the absence and presence of a liver fraction of Aroclor 1254-induced rats for metabolic activation (S9-mix). S9 mix was produced in-house by TNO (Netherlands organization for applied scientific research).

2.2. Reference and pure compounds

Alizarin **7** and purpurin **8** were purchased from Sigma-Aldrich. Lucidin primeveroside **1**, ruberythric acid **2,** pseudopurpurin **4** and munjistin **5** were obtained as described in a former publication (Willemen et al., 2019). Ten mg of rubiadin **9** was obtained from AnalytiCon Discovery (Potsdam, Germany). The latter batch was used for the first calibration curves and Ames test on pure rubiadin **9**. Later rubiadin **9** was synthesized according to the procedure by Dhananjeyan et al. starting from phthalic anhydride and 2-methylresorcinol. Purification was performed by chromatography on silica using ethyl acetate − hexane (3:1), followed by preparative HPLC (Dhananjeyan et al., 2005). Lucidin **6** was synthesized in two steps according to the method of Murti et al. and purified by chromatography on silica (Derksen et al., 1998; Murti et al., 1970). Ibericin **10** was synthesized by refluxing lucidin **6** (47.3 mg) for 2 days in ethanol at 60 °C. After drying, the sample was reconstituted in 1 mL tetrahydrofuran-water-formic acid (100:100:0.5) and purified using preparative HPLC by injecting four times 250 µL. Five fractions were collected, evaporated and lyophilized. Fraction 2 contained ibericin **10**. The compounds were analyzed by HPLC-DAD (diode array detection), proton nuclear magnetic resonance spectroscopy (^{1}H-NMR), carbon nuclear magnetic resonance spectroscopy (^{13}C-NMR) and liquid chromatography-mass spectrometry (LC–MS). Quantification was done on the HPLC-DAD system based on external calibration curves. Minor impurities observed by ^{1}H-NMR were invisible in the ultraviolet–visible spectroscopy (UV–vis) spectra from 200−600 nm and in LC–MS profiles and did not interfere with the anthraquinone analyses. Structures of the anthraquinones described in this article are depicted in Fig. 1.

2.3. HPLC-DAD, preparative-HPLC, HPLC-MS

When samples were too concentrated, they were diluted with tetrahydrofuran–water–formic acid (100:100:0.5) prior to injection. The obtained (diluted) samples were centrifuged (Herolab Microcen 13, IKA, Leerdam, The Netherlands) at 8515 g for 5 min or the samples were filtered through a 0.45 µm Spartan 13 filter (RC Whatman GmbH, Dassel, Germany). All samples were analyzed by HPLC. Prior to use, HPLC eluent was filtered through a 0.45 µm 50 mm diameter membrane filter (type RC; Dassel, Whatman GmbH, Germany) and sonicated for 5 min in a Retsch Transsonic 570 bath (Emergo, Landsmeer, The Netherlands).

The LC system consisted of a Dionex P680 HPLC pump and a Dionex UVD340U diode array detector. Injections were made by a Dionex ASI-100 automated sample injector. The system was connected to a computer via a Chromeleon Client 6.70SP10 system controller (Fisher Scientific, Landsmeer, The Netherlands). Two different elution programs were used: i) a linear gradient program for identification of components, (Derksen et al., 2002) or ii) an isocratic program (0–17 min acetonitrile-water (68:32) for quantification of alizarin and rubiadin. Detection of lucidin **6**, rubiadin **9** and ibericin **10** was performed at 280 nm.

For fractionation of samples or purification of standards a preparative HPLC-DAD system was used consisting of an LC-8A binary gradient LC pump and an SPD-M10 diode array detector. Injections were made by a SIL-10AP auto injector. The system was connected to a computer with LC Solutions software (Shimadzu, 's-Hertogenbosch, The Netherlands). The system was equipped with an Alltima end-capped C_{18} column, 250 × 22 mm i.d.; 100 Å pore size, 5 µm particle size (Alltech, Breda, The Netherlands) and a 10 × 20 mm Alltima C_{18} guard column. The same programs and solvents were used as for the analytical identification as described under i) and ii). The flow rate was 20 mL/min. Pure fractions were pooled and concentrated under reduced pressure in a rotary evaporator.

For regular anthraquinone analyses in madder root extracts, LC–MS with the same set-up and method as described earlier was used (Derksen et al., 2002). The ion trap mass spectrometer was operated in negative ion mode. For confirmation of the ether artefacts high-resolution mass spectrometry (HRMS) was used: a ThermoFinnigan Surveyor HPLC system coupled to an Orbi trap mass spectrometer (Exactive™, Thermo Scientific, San Jose, CA, USA), equipped with a ThermoFinnigan orthogonal electrospray interface both operating under Xcalibur® software (v1.3).

2.4. Plant material

The roots of *Rubia tinctorum* L. (madder root) used in this study were provided by Rubia Natural Colours (Steenbergen, Netherlands). The two-year-old roots were harvested in the south of The Netherlands. After harvest, the roots were washed with water, dried in potato cases in front of a drying wall with a forced air flow of 30 °C. The dried roots were ground twice in a Cross Beater Mill, 600 W, 600 mm rotor (Machinefabriek G.R. Veerman B.V., Olst, The Netherlands), first with a sieve of 15 mm and secondly with a sieve of 5 mm. The dried and ground roots were stored in airtight big bags at ambient temperature.

2.5. Preparation of direct (DE) and indirect (IE) madder root extracts

Direct extraction in refluxing liquid (DE): 25 g of madder root was suspended in 250 mL of refluxing ethanol-water 1:1 w/w. The extraction set-up consisted of a three-neck round-bottom flask connected with a condenser. After 30 min the suspension was filtered under reduced pressure. Ethanol (and part of the water) was removed from the extract by evaporation (~200 mL) under reduced pressure in a rotary evaporator (Büchi, R-200). The obtained concentrated extract was used for the Ames test (DE_{lab}liquid). In case of extraction on pilot scale, 450 kg of madder root was suspended in 4500 kg of ethanol-water 1:1 w/w. The set-up consisted of a stainless extraction vessel of 5 m^3 equipped with baffles, a propeller stirrer and reflux condenser cooled with tap water. Before heating, the solvent was blanketed with nitrogen. After refluxing the suspension was decanted, under a continuous flow of nitrogen, with a Flottweg decanter Z23−4 (Flottweg, Heemstede, The Netherlands) at 1050 L/h (flow 70 %, peel drive 135 and bowl drive 55 %). The obtained liquid was concentrated under reduced pressure (600 mbar) in a triple-effect falling film evaporator; overall the concentration increased 7-fold. The concentrated extract was dried in a spray-dryer, with 20 mass-% maltodextrin as support substrate, to obtain a powder. From both the

concentrate (DE_{pilot}liquid) and the powder (DE_{pilot}spraydried) a sample was taken.

For the other method (indirect extraction (IE) at lab scale), 12.5 g of madder root was suspended in 125 mL of water and intensely stirred at room temperature. Stirring was performed under nitrogen atmosphere ($IE_{lab}N_2$–liquid) or under ambient air conditions ($IE_{lab}air$–liquid). After 30 min, 125 mL ethanol (96 % v/v) was added and after 30 min of refluxing the suspension was filtered and evaporated in the same way as DE. Also, an IE extract on pilot-scale was made, with the same equipment and procedure as described for the DE pilot-scale samples except that before refluxing, madder root was stirred in water. Madder root (250 kg) was suspended in 2500 L of water and intensely stirred for 30 min at room temperature before addition of 2500 L of ethanol (96 % v/v) for further extraction. Samples $IE_{pilot}air$–liquid and $IE_{pilot}air$–-spraydried were taken.

2.6. Further and different treatment of madder root extracts

A DE_{pilot}liquid extract was fractionated by preparative-HPLC into three fractions (t_r <19 min, 19−22 min, > 22 min) using elution program i. Beside the starting extract (DE_{pilot}liquid) and the three obtained fractions (DE_{pilot}liquid–frac. < 19, DE_{pilot}liquid–frac.19−22, DE_{pilot}liquid–frac. > 22), also a pooled sample of all three fractions was tested for mutagenicity (DE_{pilot}liquid–frac.pooled). The samples were submitted to the Ames test and tested in two dilutions, i.e., 2500 and 5000 µg/plate.

To test the effect of a heat treatment on the mutagenicity of the anthraquinones, 0.500 g DE_{lab}liquid was suspended in 100 mL of water and extracted three times with 50 mL of ethyl acetate. The ethyl acetate layers were pooled and divided in two. One half was dried in a vacuum oven at 30 °C, the other half was dried at 120 °C for 43 h. All samples were reconstituted in dimethyl sulfoxide and submitted to the Ames test (DE_{lab}ambientdried, DE_{lab}highdried).

Samples obtained after spray drying, DE_{pilot}spraydried and $IE_{pilot}air$–spraydried, were further fractionated by column chromatography on a Sepacore® flash system X10/X50 (Büchi Labortechnik, Hendrik Ido-Ambacht, the Netherlands) equipped with a Büchi glass column, d = 15 mm h = 230 mm filled with C_{18} sorbent (40 µm, Bond-Elut, Agilent, The Netherlands). DE_{pilot}spraydried (2.000 g) was suspended in 200 mL tetrahydrofuran-water (25:75) and $IE_{pilot}air$–spraydried (1.000 g) was suspended in 200 mL ethanol-water (39:61). Both were centrifugated. For DE the column was equilibrated with tetrahydrofuran-water (25:75), 100 mL supernatant was applied and eluted with a tetrahydrofuran-water gradient: 70 mL (25:75), 40 mL (50:50), 80 mL (100:0). For IE the column was equilibrated with ethanol-water (39:61), 100 mL supernatant was applied and eluted with an ethanol-water gradient: 40 mL (39:61), 40 mL (45–55), 70 mL (50:50), 100 mL (96:4). Fractions of 10 mL were collected and evaluated for the presence of rubiadin by HPLC analysis. The fractions without rubiadin were pooled and concentrated by means of a rotary evaporator. The dried samples, DE_{pilot}spraydried–C_{18} and $IE_{pilot}air$–spraydried–C_{18} were used for the Ames test. All samples are schematically depicted in Fig. 2.

To produce different alkoxy artefacts of lucidin **6**, beside refluxing in ethanol-water in the indirect extraction procedure, $IE_{lab}N_2$–liquid, also some other solvents were used in the same set-up. These solvents were methanol, ethanol, propanol, 1-butanol and acetonitrile, no water was added, $IE_{lab}N_{2\text{-various solvents}}$.

2.7. Extraction of madder root dyed wool with artificial sweat and saliva

Wool was dyed following a general two-step madder root dye procedure. Five g of ready-to-dye wool was soaked in water at room temperature for 10 min and centrifuged in a house-hold laundry spin dryer. The wool was pre-mordanted in a solution of 0.133 g of tartaric acid and 1 g of aluminum sulfate in 100 mL of water at 95 °C for 1 h. Afterwards the wool was twice rinsed with water and centrifuged. A dyeing bath was prepared by adding 0.5 g of DE_{pilot}spraydried or 0.5 g $IE_{pilot}air$–-spraydried to 100 mL of water. Wool was added to the dye-bath, which was heated and kept at 100 °C for 1 h. Afterwards the wool was rinsed in boiling water twice, centrifuged and further air dried.

For extraction with artificial sweat and saliva, the methods described in DIN 53160−1: color fastness with artificial saliva, DIN 53160−2: color fastness with artificial sweat and ISO 105-E04: color fastness against perspiration, were used (DIN Deutsches Institut für Normung, 2010a,b; ISO International Organization for Standardization, 2008). Instead of histidine, arginine was used because histidine influences the Ames test (Jäger et al., 2006). One artificial saliva solution and two artificial sweat solutions were prepared, one of pH 8.0 and one of pH 5.5. For every test 0.3 g of dyed wool was added to 3 mL of artificial saliva or sweat and shaken. After 24 h at 37 °C the wool was removed and the samples were analyzed by HPLC-UV. Prior to the analysis the samples were diluted once with tetrahydrofuran-water-formic acid 100:100:0.5

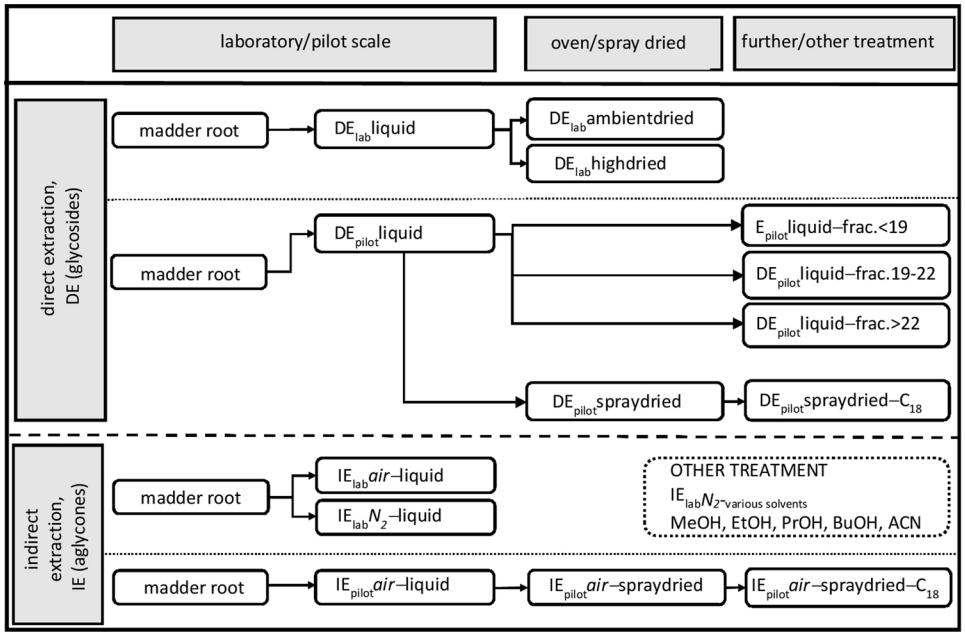

Fig. 2. Scheme of all samples made, tested in Ames test and analyzed by HPLC. Abbreviations; DE = direct extract (extract obtained with boiling ethanol-water 1:1 v/v), IE = indirect extract (extract obtained after pre-treatment with water followed by extraction with boiling ethanol-water 1:1 v/v), $_{lab}$ = laboratory-scale, $_{pilot}$ = pilot-scale, N_2 = sample prepared under nitrogen atmosphere, $_{\text{-various solvents}}$ = other solvents than ethanol were used for indirect extraction, *air* = sample prepared under ambient conditions, "ambientdried" = dried in an oven at room temperature, "highdried" = dried in oven at 120 °C, "spraydried" = drying droplets in a stream of gas, C18 = samples after rubiadin 9 removal by solid phase extraction with 40 µm reversed phase C_{18} material, frac. < 19, frac.19-22, frac. > 22 = retention times in minutes of collected fractions of a DE sample with preparative HPLC on end-capped reversed phase C_{18} material.

v/v/v.

2.8. Ames test

The microsome assay was performed at the Netherlands Organization for applied scientific research (TNO Triskelion, Zeist, The Netherlands) in accordance to the guideline no. 471 July 1998 of the Organization for Economic Co-operation and Development (OECD, Paris, France) (Mortelmans and Zeiger, 2000; Organisation for Economic Co-operation and Development, 1997). The first test was performed with strains *S. typhimurium* TA1535, TA1537, TA98, TA100 and *E coli* WP2*uvrA*. Further tests were performed with TA100. Prior to the actual testing, frozen stocks of each strain were checked for histidine or tryptophan requirement and for sensitivity to ampicilin, crystal violet and UV radiation. All strains were tested in the absence and presence of a liver fraction of Aroclor 1254-induced rats for metabolic activation (S9-mix). The S9 liver homogenate was prepared according to Ames et al. and Maron and Ames (Maron and Ames, 1983; Mccann et al., 1975). The S9 was checked for sterility. The protein and cytochrome P-450 content of the S9 fraction were determined according to the method published by Rutten et al. (Rutten et al., 1987). On the day of use, aliquots of S9 liver homogenate were thawed and mixed with a reduced nicotinamide adenine dinucleotide phosphate disodium salt (NADPH) generating system and kept on ice.

All madder root extracts were diluted based on the dry weight of the test substance. All wool extracts were diluted based on the amount of wool. Dimethyl sulfoxide was used as solvent. For the tests, preferably five concentrations were prepared (n = 3), ranging from 62 to 5000 μg/plate. However, sometimes sample volumes were too small and, in those cases, only two concentrations (n = 3) were prepared, 2500 and 5000 μg/plate. Negative (mitomycin C) and positive controls (benzo(a)pyrene 2-nitrofluorene, sodium azide, 2-aminoanthracene) were run simultaneously with the test substance. All determinations were carried out in triplicate (n = 3). A test substance was considered to be positive in the bacterial gene mutation test if the induction rate was >2 in comparison with a blank sample (0 μg/plate). This means that the number of revertant colonies on test plates increased two-fold or more relative to that on blank plates (Van den Wijngaard, 2007a, 2007b).

3. Results and discussion

The aim was to develop an extraction procedure for madder root in which the concentration of mutagenic compounds is strongly reduced or, preferably, brought to zero. Different samples were made and each one was analyzed by HPLC and the Ames test. Every madder sample was prepared by using one of the two earlier developed extraction methods (DE and IE), (Derksen et al., 2003) on laboratory and pilot scale ($_{lab}$ and $_{pilot}$), and from there on the effect of different downstream processing steps was investigated. First a suitable strain was selected for all Ames tests.

3.1. Selection of best dose-response mutation strain

The Ames test was performed with different strains. Data of strain TA1537 were not evaluated because the observed number of revertant colonies for both the blank sample and the positive controls was not within an acceptable range.

For strain TA98 and TA100 with and without S9 a positive dose response-relationship was observed for the tested extracts (Table S1) with a maximum of 11.8 for TA100 without S9 (Table S1: number of revertants DE lab TA100 –S9 divided by number of revertants blank = 1556/132 = 11.8). As strain TA100 gave the highest and clearest dose-response results for both types of extracts, it was decided to carry out all further mutagenic tests with this strain.

3.2. Different processing of DE and IE samples and effect on mutagenicity

From earlier research, we knew that if madder root is directly added to refluxing ethanol-water (DE), the anthraquinones are mainly present as glycosides while, if they are stirred in water prior to the same ethanol-water extraction (IE), they are present as aglycones (Derksen et al., 2003). Furthermore, if oxygen is present during the pre-treatment in water, most or all lucidin **6** is converted to nordamnacanthal **11** (Fig. S1) (Derksen et al., 2003). Based on these findings, various extracts from the same batch of madder roots, were prepared. Three parameters were varied: (1) presence or absence of oxygen, (2) scale of the extraction, (3) different evaporation and drying steps, see Fig. 2. Corresponding HPLC profiles are shown in Fig. 3. All madder root extracts were subsequently tested for mutagenicity (Table S2). For all DE extracts, both in the presence (+S9) and absence ϕ S9) of liver homogenate, a positive dose-response was found. In case of IE, an additional phenomenon was observed at higher doses (≥556 μg/plate), namely toxicity of the samples to the TA100 strain, as evidenced by a (slightly) less dense background lawn of bacterial growth ((S)LD) and presence of pinpoint non-revertant colonies (pp) (Table S2). The induction rate for 1667 μg/plate is separately visualized in Fig. 4 with a line at the positive threshold of 2. Not all extracts were mutagenic to the same extent, e.g., a direct extract produced at lab scale was much more mutagenic than for instance a spray dried sample on pilot scale.

3.3. Effect of presence or absence of oxygen

Indirect extraction under a nitrogen atmosphere gave an extract with a much higher mutagenicity than all other tested extracts (Fig. 4). Due to the absence of oxygen, $IE_{lab}N_2$—liquid contains much more lucidin **6**, see also the HPLC profile in Fig. 3. According to the literature lucidin **6** is the main mutagenic compound in madder root (Brown and Brown, 1976; Yasui and Takeda, 1983). Indeed, it could be confirmed that lucidin **6** is a mutagenic compound (Fig. 4, Table S2). Samples with less lucidin **6** gave a lower induction rate in the Ames test, in particular the samples that were pretreated with water. Stirring in water in the presence of air, as seen in IE_{lab}*air*—liquid, causes the conversion of lucidin **6** into the non-mutagenic nordamnacanthal **11** (Derksen et al., 2003).

3.4. Effect of scale of the extraction

One part of the tested samples was prepared at lab scale while another part was made on pilot scale. For the DE_{pilot}liquid samples the mutagenicity was lower than that of the corresponding laboratory samples, DE_{lab}liquid. This is probably caused by differences during downstream processing such as temperature, residence time and presence of oxygen. These parameters are the same in both laboratory and pilot extraction. During evaporation of the ethanol the pilot extract is exposed to higher temperatures and the vacuum is less than for the lab scale samples. Residence times are comparable. The spray dried sample, DE_{pilot}spraydried exhibited an even lower mutagenicity than the DE_{pilot}liquid sample, During spray drying, the madder root compounds are exposed for a short time to air of a high temperature. It could be that the temperature during downstream processing influences the mutagenicity, probably due to lowering the lucidin **6** content (Fig. 3). A separate experiment will be discussed below to show the influence of temperature, see paragraph 3.5 Fig. 7. It can be concluded that the mutagenicity is strongly dependent on the type of processing steps, such as presence of oxygen, extraction scale and circumstances of the selected drying method.

3.5. A closer look at the difference in mutagenic activity of different madder root extracts

The results in the former section show that lucidin **6** is a mutagenic compound in madder root. To clarify whether there are additional

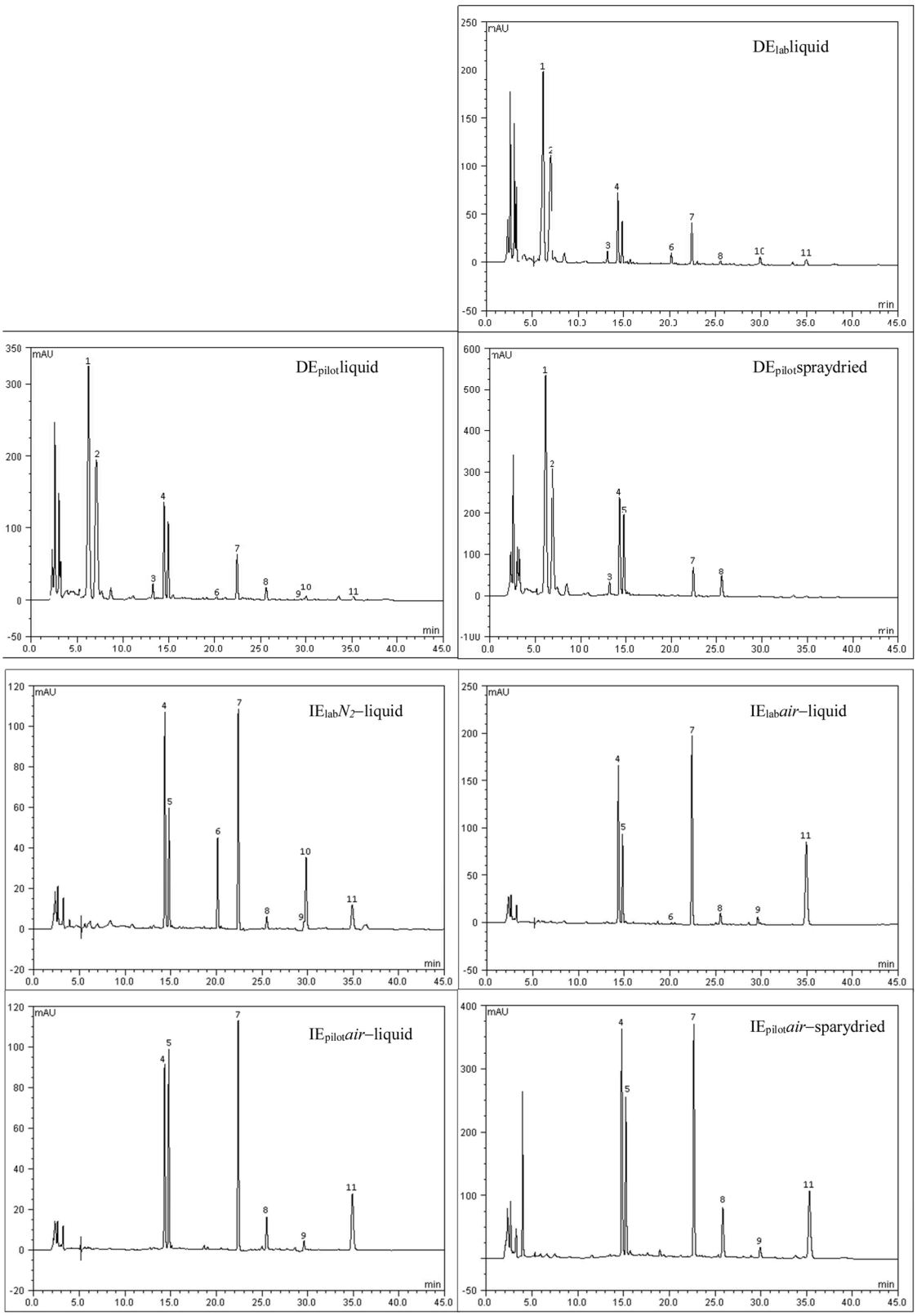

Fig. 3. HPLC profiles of *Rubia tinctorum* extracts, measured in the Ames test, 1 lucidin primeveroside, 2 ruberythric acid, 3 rubiadin primeveroside, 4 pseudopurpurin, 5 munjistin, 6 lucidin, 7 alizarin, 8 purpurin, 9 rubiadin, 10 ibericin, 11 nordamnacanthal, sample names see Fig. 2.

mutagenic compounds, the DE$_{pilot}$liquid extract with a very low concentration of lucidin **6** at a Retention time (Rt) of 20.2 min, was chosen and divided in three fractions by preparative reversed phase column chromatography: DE$_{pilot}$liquid–frac. < 19, DE$_{pilot}$liquid–frac.19−22, DE$_{pilot}$liquid–frac. > 22 with the numbers referring to the collection time in min. Because of the small sample amounts, only two dilutions were tested in Ames: 2500 μg/plate and 5000 μg/plate and only in combination with metabolic activation, +S9, see Table 1. The fraction

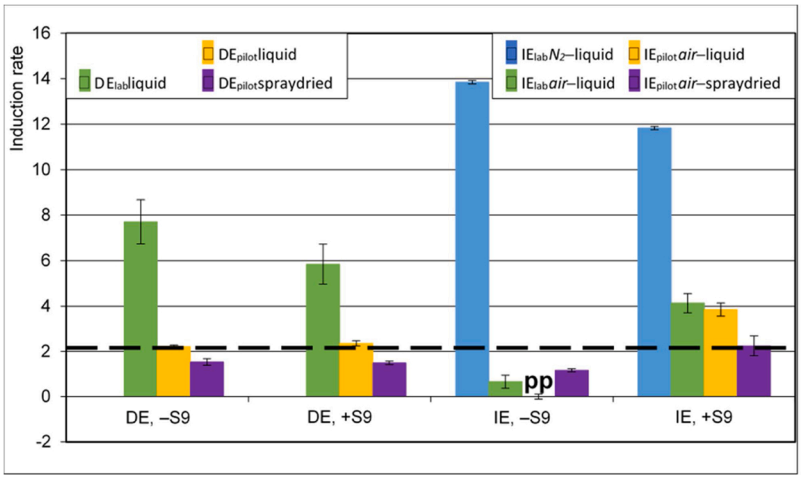

Fig. 4. Induction rate found in Ames test (TA100) of four madder root extracts (n = 3) at a dose of 1667 μg/plate, +S9, –S9 with and without liver homogenate from rats treated with arocolor. ——— threshold (induction rate = 2), pp = presence of pinpoint non-revertant colonies (pp), sample names see Fig. 2.

Table 1
Number of revertants in Ames test with S9-mix, and for C18 fractions without S9-mix (n = 3), produced at an extract dose of 2500 and 5000 μg/plate. Numbers in **bold** means sample was positive (induction rate ≥2).

further / other treatment on original extract	sample	2.5μg/plate	sdev	5.0μg/plate	sdev
		+S9		+S9	
fractionation of DE liquid extracts made on pilot scale	DE$_{pilot}$liquid–frac. < 19	118	29	100	5
	DE$_{pilot}$liquid–frac.19–22	113	6	*139*	*t 23*
	DE$_{pilot}$liquid–frac. > 22	154	6	**260**	44
		+S9		+S9	
EtOAc layer and ambient or high temperature oven dried	DE$_{lab}$ambientdried	**350**	21	**694**	34
	DE$_{lab}$highdried	143	21	**258**	56
		+S9		+S9	
C_{18} fractionation of DE and IE spray dried extracts both + S9 and –S9 tested	DE$_{pilot}$spraydried–C_{18}	194	15	238	12
	IE$_{pilot}$*air*–spraydried–C_{18}	255	45	220	60
		–S9		–S9	
	DE$_{pilot}$spraydried–C_{18}	213	32	201	8
	IE$_{pilot}$*air*–spraydried–C_{18}	262	24	–	*t*

Sample names see Fig. 2, EtOAc ethyl acetate, sdev standard deviation, +S9, –S9 with and without liver homogenate from rats treated with arocolor, *t* and *italic* is observation of (slightly) less dense background lawn of bacterial growth and presence of pinpoint non-revertant colonies.

between 19 and 22 min should include any lucidin **6,** if present. DE$_{pilot}$liquid–frac.19–22, did not give a positive Ames result, most likely due to the very low initial concentration of lucidin. However, the fraction DE$_{pilot}$liquid–frac. > 22 did, when metabolically activated by S9. So, it can be concluded that other compounds, which elute later, also have mutagenic activity.

Beside the fractions, also the initial extract and a pooled sample were investigated in the Ames test. Compared to the initial extract, DE$_{pilot}$liquid, the HPLC profile of the pooled sample DE$_{pilot}$liquid–frac.pooled (Fig. 3 and Fig. S2) showed small differences with regard to the pseudopurpurin **4** and purpurin **8** peaks. The amount of purpurin **8** had slightly increased, due to decarboxylation of pseudopurpurin **4** during the analytical work-up of the fractions. However, this did not influence the outcome of the Ames test (Table S2 and Table 1).

After scrutinization, a peak in DE$_{pilot}$liquid was pinpointed that correlated with the Ames test results. Using LC–MS, not one but two compounds with almost identical retention times could be distinguished, see Fig. 5. These compounds were identified as rubiadin **9** ([M−H]– at *m/z* 253, Rt 29.6 min) and ibericin **10** (([M−H]– at *m/z* 297, Rt 29.9 min) using retention time, UV spectrum and MS fragmentation information. Both compounds have been implicated in other studies as being mutagenic after metabolic activation with S9 (Blömeke et al., 1992; Ishii et al., 2010; Jäger et al., 2006; Kawasaki et al., 1992; Westendorf et al., 1988).

Several groups have indicated that ibericin **10** could be an artefact that is formed from lucidin **6** during extraction with ethanol, but no solid evidence has been presented so far (Chang and Lee, 1984; Kawasaki et al., 1992; Leistner, 1975; Westendorf et al., 1988). In this research madder root was extracted with several different alcohols, i.e., methanol, ethanol, propanol, 1-butanol (IE$_{lab}$*N*$_2$- various solvents). According to LC—MS analyses, all corresponding extracts contained the artefacts lucidin methyl ether **12** ([MH—]– at *m/z* 283), ibericin **10** (lucidin ethyl ether **10**), lucidin propyl ether **13** ([MH—]– at *m/z* 311) and lucidin butyl ether **14** ([MH—]– at *m/z* 325) (Fig. 6) and no ethers were detected when instead of an alcohol acetonitrile was used. This proves that ibericin **10** and lucidin methyl ether **12** do not occur naturally in *Rubia tinctorum* (Kawasaki et al., 1992). A structure search for lucidin propyl ether **13** yielded no results while a search for lucidin butyl ether **14** yielded three publications (Kitajima et al., 1998; Nguyen et al., 2017; Xiang et al., 2008). Interestingly Xiang et al. did not use butanol anywhere in their isolation process suggesting that lucidin butyl ether **14** might occur naturally in *Morinda angustifolia* (Xiang et al., 2008). In contrast, rubiadin **9** can be extracted from madder root with all alcohols as well as acetonitrile, so this compound does seem to be a genuine constituent of *Rubia tinctorum*. As expected the lucidin **6** content is inversely proportional to that of ibericin **10** as this can be quite easily synthesized from lucidin **6** by refluxing with ethanol (Derksen et al., 2003; Leistner, 1975). Therefore, an extract that contains no lucidin **6** will not produce ibericin **10**. Formation of a lucidin ether artefact seems more efficient when a higher alcohol is used. Lucidin **6** is almost completely converted to lucidin butyl ether **14** when madder root is extracted with 1-butanol while under the same conditions and methanol as extraction liquid conversion is far from complete (Fig. 6). The formation of the ethers can be explained by assuming that the OH-group at the CH_2OH substituent is easily lost because of the strongly electron-donating OH-groups at the *ortho* carbon atoms, resulting in a quinone methide as already proposed by Ishii et al. for lucidin sulphate (Ishii et al., 2010). Michael-type addition of water to the CH_2 group reverts the quinone methide to lucidin **6**, while addition of ethanol leads

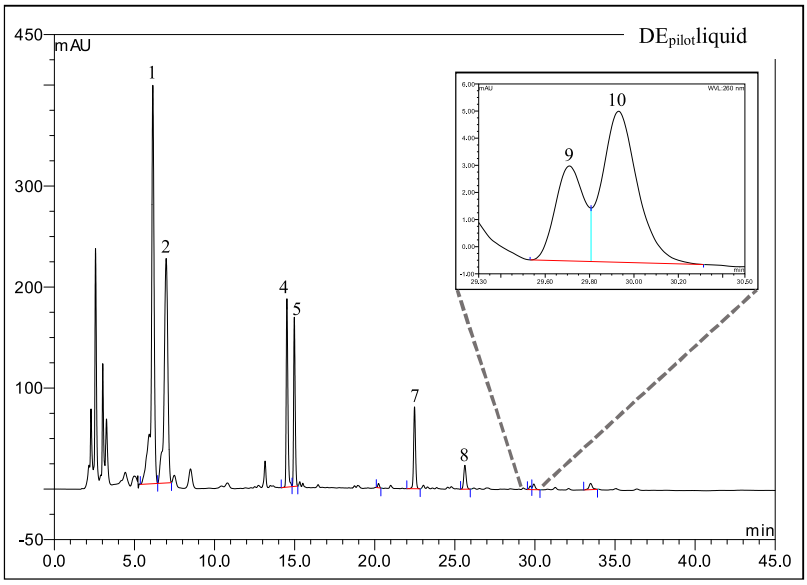

Fig. 5. HPLC profile of a DE_{pilot}liquid sample. The inset shows expanded section from Rt 29.3 – 30.5 min of the chromatogram. For sample names see Fig. 2; for compound names see Fig. 3.

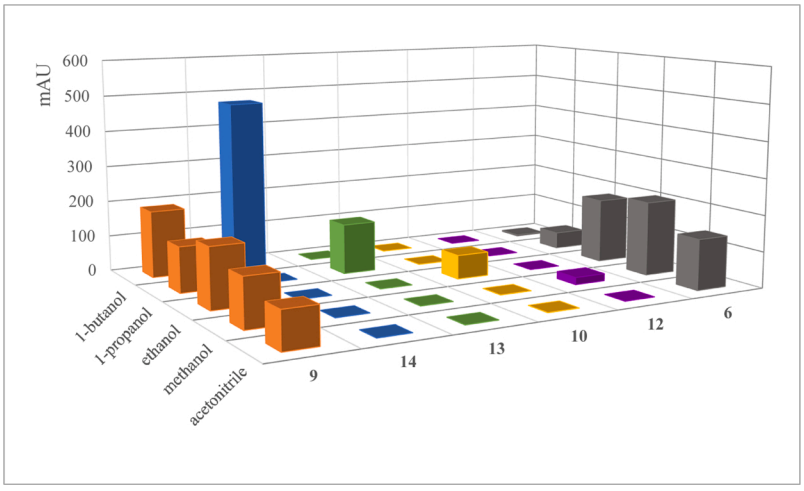

Fig. 6. Effect of extraction solvent on the formation of ether artefacts during extraction. On the z-axis the UV absorbance of the different ether peaks after HPLC separation is depicted. On the x-axis the extraction solvent is depicted, on the y-axis, the (formed) compound number. 6 lucidin, 9 rubiadin, 10 ibericin (lucidin ethyl ether), 12 lucidin methyl ether, 13 lucidin propyl ether, 14 lucidin butyl ether.

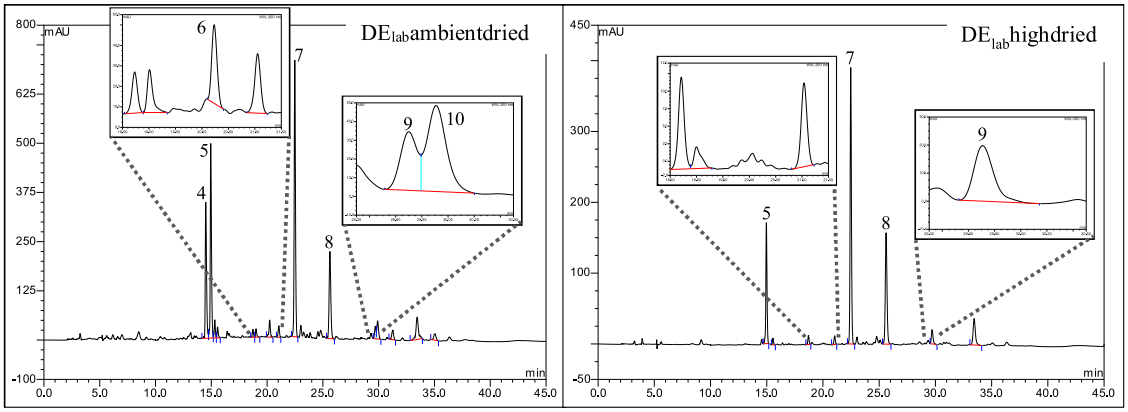

Fig. 7. HPLC profiles of the ethyl acetate fraction of a DE_{lab}liquid madder root extract. The ethyl acetate fraction was split in two and dried at two different temperatures, 30 °C (DE_{lab}ambientdried) and 120 °C (DE_{lab}highdried). In both profiles the left inset shows expanded section from Rt 18.5 – 21.5 min and the right inset from Rt 29.3 – 30.5 min. Compound names see Fig. 3.

to ibericin **10**. Other alcohols will react in a similar fashion.[24] Higher alcohols are stronger nucleophiles, as reflected by their higher pK_a values, which explains why conversion to the corresponding ethers is more efficient (Fig. S3).

To determine whether temperature indeed influences the mutagenicity of a madder root extract, the DE_{lab}liquid extract was separated in an aqueous and an ethyl acetate fraction. The ethyl acetate layer, which contained mainly the anthraquinone carboxylic acids and other aglycones including lucidin **6**, was divided in two. One part was dried under reduced pressure in a rotary evaporator, DE_{lab}ambientdried, while the other part was dried in an air-ventilated oven at 120 °C, DE_{lab}highdried. The HPLC profiles of the obtained fractions and the results of the + S9 mutagenic test are presented in Fig. 7 and Table 1. The 120 °C treatment mimics the temperature during pilot scale processing. During evaporation and during spray drying the components are exposed to temperatures of 79 °C and 100 °C respectively. Apparently lucidin **6** and ibericin **10** are temperature sensitive because both compounds were no longer detected after a heat treatment at 120 °C. Earlier Chang et al. reported on possible instability of ibericin **10** (Chang and Lee, 1984). Rubiadin **9** appears to be as stable as alizarin **7**, which is in line with the fact that the CH_3 substituent has no leaving group, in contrast to lucidin **6** and ibericin **10** (Fig. S3).

3.6. Mutagenic activity of anthraquinones as a function of their concentration and S-9 catalyzed interconversion

The mutagenic activity of lucidin **6**, ibericin **10** and rubiadin **9** was assessed at various concentrations with the TA100 strain with and without S9-mix (Table 2). The minimal mutagenic concentrations for lucidin are 0.087 and 0.56 μg/plate with and without metabolic activation, respectively. Rubiadin **9** is only mutagenic after metabolic activation (+S9) at a concentration of 2.8 μg/plate. The mutagenic concentrations for ibericin **10** are 13.5 and 3.9 μg/plate with and without metabolic activation.

Kawasaki and Goda and Westendorf et al. have mentioned that some anthraquinones only display mutagenicity after metabolic activation by S9 liver cells (Kawasaki et al., 1992; Westendorf et al., 1988). When rubiadin **9** was incubated with S9, several metabolites were formed including lucidin (Kawasaki et al., 1992). Similarly conversion of ibericin **10** to lucidin **6** may be the cause of the markedly increased mutagenicity when ibericin **10** is activated by S9 (Westendorf et al., 1988). A plausible explanation for this is the presence of cytochrome P450 monooxygenase activity in the S9-mix. Ishii et al. proposed a pathway for metabolic activation of lucidin primeveroside **1**, ibericin **10** and rubiadin **9** leading to lucidin **6**. Subsequently lucidin **6** forms an adduct with DNA by reaction with 2′-deoxyguanosine and 2′-deoxyadenosine (Ishii et al., 2014, 2012, 2010).

3.7. Preparation of a rubiadin-free madder root extract

From the previous results it can be concluded that madder root can be processed in such a way that in vitro mutagenicity is exclusively caused by rubiadin **9** present in the extract. To obtain an extract without rubiadin **9**, spray dried madder root extracts DE_{pilot}spraydried and IE_{pilot}*air*–spraydried were dissolved and fractionated on a C-18 column. As soon as there was breakthrough of rubiadin **9**, the elution was stopped. The rubiadin-free samples, DE_{pilot}spraydried–C_{18} and IE_{pilot}*air*–spraydried–C_{18} were investigated in the Ames test with and without S9-mix at 2500 and 5000 μg/plate (Table 1). The HPLC profiles of the rubiadin **9** free fractions are depicted in Fig. S4. After removing rubiadin **9**, both samples were no longer mutagenic in the Ames test.

3.8. Extraction of madder-dyed wool with artificial saliva and sweat

Pieces of wool dyed with DE_{pilot}spraydried and IE_{pilot}*air*–spraydried (HPLC profile in Fig. 3)were extracted artificial saliva and artificial sweat, at two pH values according to DIN-110. The obtained saliva and sweat solutions are representative for the general exposure of human skin to one piece of dyed clothing. The HPLC profiles of the saliva solution of DE dyed wool and of alkaline sweat solution of IE dyed wool are depicted in Fig. S5. The other four IE/DE saliva and sweat extracts gave comparable results in terms of type and amount of extracted anthraquinones. Only very low amounts of anthraquinones were found and the concentrations of mutagenic lucidin **6** or rubiadin **9** were below the detection limit. Thus, the exposure to mutagenic lucidin **6** or rubiadin **9** while wearing clothes dyed with madder root extract is zero.

4. Conclusion

In this study, it has been shown that it is possible to prepare a madder root extract free of any (in vitro) mutagenic components. Lucidin **6** and rubiadin **9** are the naturally occurring anthraquinones in madder root responsible for the mutagenicity in the *Salmonella* microsome assay (Ames test). Through proper processing steps, lucidin **6** formation can be avoided, and instead the non-mutagenic nordamnacanthal **11** is formed (Derksen et al., 2003). If there is no lucidin **6**, then by default no ether artefacts, like ibericin **10**, can be formed due to the use of an alcohol as extraction liquid. Rubiadin **9** can be completely removed from the product by a simple C_{18} flash-chromatographic step. Additionally, during processing the type of drying and drying temperature are important for the reduction of mutagenic activity. Dyeing tests (not shown) showed that the color of fabric dyed with the processed or mutagenic-free

Table 2
Number of revertants counted in Ames test (TA100) at increasing concentrations of 6 lucidin, 9 rubiadin and 10 ibericin, (n = 3).

	lucidin **6**					rubiadin **9**					ibericin **10**			
μg/plate	–S9	sdev	+S9	sdev	μg/plate	–S9	sdev	+S9	sdev	μg/plate	–S9	sdev	+S9	sdev
0.0069	142	7	159	13	0.0101	122	10	157	22	0.0194	105	12	164	21
0.0207	145	17	165	17	0.0302	114	10	117	10	0.0581	119	2	138	0
0.0621	189	11	161	33	0.0907	114	4	171	32	0.174	119	13	166	16
0.186	**455**	11	149	11	0.272	124	4	144	11	0.523	121	9	140	9
0.559	**1099**	11	**207**	20	0.816	124	4	144	11	1.57	135	14	149	21
1.68	**2172**	11	**667**	15	2.45	121	4	**327**	35	4.30	193	9	**241**	3
4.94			**1708**	14	7.21	157	12	**656**	6	12.9	**241**	9	**743**	24
					21.6	156	6	**2407**	33	38.7	**473**	5	**2241**	23
lucidin **6**	–S9	y = 1231x+185			rubiadin **9**	–S9	n/a			ibericin **10**	–S9	y = 9x+120		
	R^2	0.9783				R^2	n/a				R^2	0.9863		
	y = 2	0.048 μg/plate				y = 2	n/a				y = 2	13.5 μg/plate		
	+S9	y = 320x+118				+S9	y = 105x+76				+S9	y = 55x+86		
	R^2	0.9937				R^2	0.9849				R^2	0.9905		
	y = 2	0.56 μg/plate				y = 2	2.8 μg/plate				y = 2	3.9 μg/plate		

x = concentration of pure **10**, **9** or **6** in μg/plate, y = number of revertants on Ames plate. y = 2 means the concentration of pure compound (μg/plate) which gives a doubling of the amount of revertants according to the blank. n/a not applicable.

madder root dye is not different from the one dyed with the standard non-processed extract.

CRediT authorship contribution statement

Goverdina C.H. Derksen: Investigation, Writing - original draft, Conceptualization, Funding acquisition. **Frédérique L. van Holthoon:** Investigation, Validation, Visualization. **Hendra M. Willemen:** Investigation, Writing - review & editing. **Cyrille A.M. Krul:** Conceptualization, Investigation, Writing - review & editing. **Maurice C.R. Franssen:** Supervision, Writing - review & editing, Visualization. **Teris A. van Beek:** Supervision, Conceptualization, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.indcrop.2021.113344.

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