CHARISMA

Cultural Heritage Advanced Research Infrastructures: Synergy for a Multidisciplinary Approach to conservation/restoration

REPORT ON RESEARCH ACTIVITY FROM BOTANICAL SOURCE TO ANALYTICAL RESULT

D10.2-1 Key

Document identifier	**D10.2-1 – ver. 0.3**
Due Date of Delivery to EC	**M12 (September 2010)**
Actual Date of Delivery	**M12**
Document date	**22/10/2010**
Deliverable Title	**Report on research activity from botanical source to analytical result**
Work package:	**WP 10: Innovative methodologies and instrumentation for laboratory research**
Lead Beneficiary:	**ICN**
Other Beneficiaries	**NGL, BM, Of-ADC, KIK-IRPA, DI-BS**
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Document status:	**Final**
Document link:	http://www.charismaproject.eu

Changes

Version	Date	Comment	Authors
0.1	28.09.2010	First version of the report	M. van Bommel, A. N. Proano Giabor
0.2	15.10.2010	WP6 Leader review	A. Sgamellotti
0.3	22.10.2010	Final version	M. van Bommel, A. N. Proano Giabor

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CHARISMA (Cultural Heritage Advanced Research Infrastructures: Synergy for a Multidisciplinary Approach to conservation/restoration) is a project co-funded by the European Commission in its 7th Framework Programme under the Grant Agreement nº 228330 began in October 2009.

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1. EXECUTIVE SUMMARY

Within the first twelve months of the project, task 2a of WP10 - *Innovative methodologies and instrumentation for laboratory research*, was focused on two aspects:

1- Collection of botanical sources

2- Development and evaluation of extraction protocols

After contacting almost 60 botanical gardens and commercial suppliers all over Europe, a total number of 76 plants sources from 7 different species were collected, dried and stored for laboratory research. During the collection, a broad general interest was raised among the contacted experts and suppliers. It is expected that in the forthcoming years, some other plants will be collected and some of them will be grown, especially for this project.

For both plants, containing flavonoids and containing anthraquinones, extraction protocols have been developed to ensure qualitative and quantitative extraction of organic colorants in a reproducible way. To evaluate the protocols, a round robin was performed using *weld* and *madder* as models. Based on the satisfactory results, further experimental steps have been planned, at the first annual meeting in Florence, IT, on September 15-16, 2010.

2. INTRODUCTION

Innovative methodologies and instrumentation for laboratory research

Main objective work package 10 is the development of:

- new methodologies for the characterisation and study of a range of materials used in paintings and heritage objects, with particular interest in organic colorants, using non-invasive and/or micro-destructive approaches;

- new research strategies dedicated to improve the understanding of the behaviour of certain systems (e.g. dye complexes), using existing advanced analytical techniques or newly developed instrumentation.

Within this general framework, during the period M1-M12, the first part of task 2, fully dedicated to organic colorants and labelled as subtask 10.2a - *From botanical source to analytical result* was carried out.

The task aims to determine what factors actually affect the composition of organic colorants in art objects and how modifications of this composition can alter the overall colour of the specific material. The climate conditions in which a botanical colorant source grows, the procedure of extraction of the colorant from the source, the preparation of lakes for paintings or the procedures of dyeing textiles are all significantly influencing the final dyestuff components in the artwork or historical object. The intrinsic variation in the colorant composition together with the possible alterations due to the interaction of the colorant with the environment, make the identification and characterisation of colorants in heritage objects one of the most significant challenges in diagnostics for conservation.

In the attempt to better characterise how the various parameters can influence the final composition, the effects of each separate contribution (botanical origin, modality of extraction, lake preparation, etc.) are separately investigated. In the subtask 10.2a, the different steps in the preparation procedure of colorants of botanical origin are studied by spectroscopic and chromatographic techniques, focussing the attention on three related anthraquinonic and three related flavonoid dyes.

The dye sources investigated are:

Madder	*Rubia tinctorum* L.
Wild Madder	*Rubia peregrina* L.
Galium species	*Galium verum* L.
	Galium mollugo L.
Weld	*Reseda luteola* L.
Dyer's broom	*Genista tinctoria* L.
Sawwort	*Serratula tinctoria* L.

At the start of the project, at the 28th meeting of Dyes in History and Archaeology held on October 21-23 2009 in Poznan, PL, a poster was presented on this activity, entitled:

CHARISMA, Joint research activity: From botanical source to analytical result

by Maarten van Bommel, Marjolein Groot Wassink, Suzan de Groot, Art Néss Proano Giabor, Jo Kirby, David Peggie, Sophia Sotiropoulou, Ioannis Karapanagiotis, Costanza Miliani, Catia Clementi, Ina Vanden Berghe, Heike Stege.

The poster met a relevant interest by the conference participants. It gave an overview of the complete WP10, task 2 activities to be developed within the framework of CHARISMA with a special focus on extraction protocols from plants. For the next meeting of Dyes in History and Archaeology, to be held in Lisbon, PT, next November 11-13, 2010, the presentation of a poster especially dedicated to the results of task 2a has been planned. An abstract has been already accepted.

3. COLLECTING BOTANICAL SOURCES

As a first approach to the research, the difference in organic colorant composition in plant sources collected from different areas in Europe have been examined. To investigate these possible differences, several European botanical gardens and commercial suppliers were contacted to ask for the supply of different dye sources.

Through an accurate survey carried out by all the task participants, a table was produced consisting of a list of botanical gardens, commercial plant suppliers, and relative contact addresses, for a large number of European countries.

For the purpose, standard letters were prepared, one for the botanical gardens and another for the commercial suppliers, in which the aim of the CHARISMA project was explained with emphasis on the specific interest on the botanical sources. The letters also included a request for materials to be used for the laboratory research. Using the collected list, a total of 57 gardens and suppliers were approached by mail, leading to a global final collection of 76 plant sources (13 madder, 10 wild madder, 8 *Galium verum* L., 14 *Galium mollugo* L., 10 weld, 13 Dyer's broom and 8 sawwort).

The species were collected from United Kingdom, France, Italy, Germany, Greece, Turkey, Austria, Belgium and the Netherlands, generating a good distribution of plants from all over Europe, as shown in Figure 1.

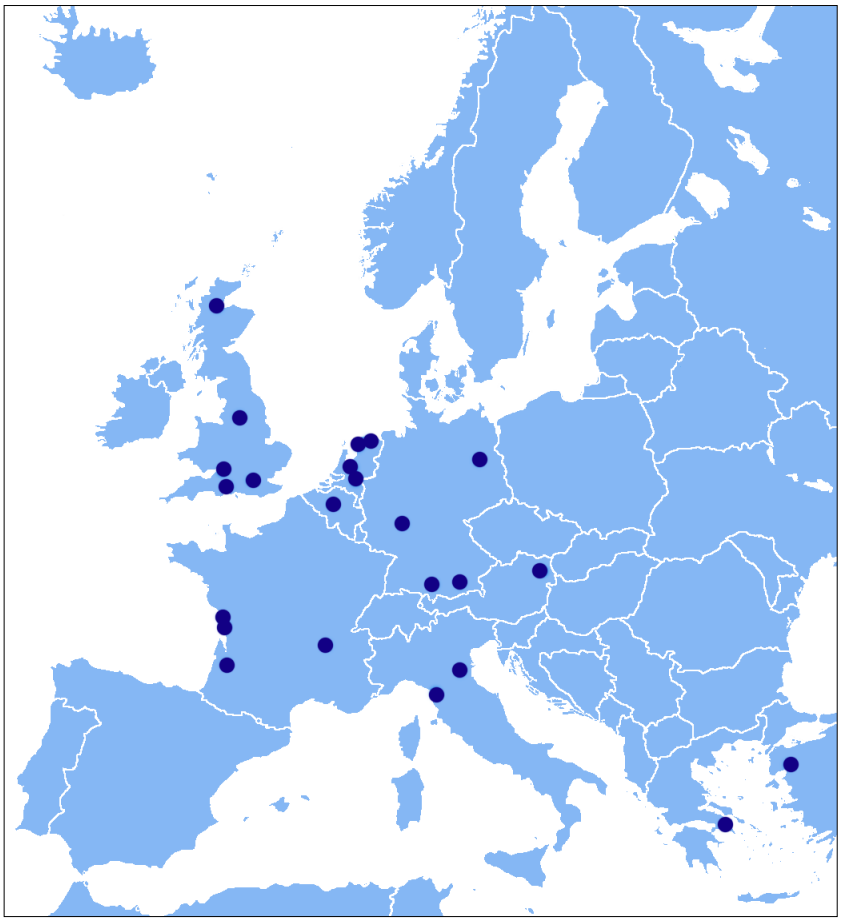


Figure 1: *provenance of the species collected*

The botanical gardens and suppliers who delivered the requested dye plants are listed in the following.

Germany: Kremer pigmente, Aichstetten; Botanische Staatssammlung, München; Botanischer Garten und Botanisches Museum Berlin-Dahlem, Berlin; Botanischer Garten der J. W. Goethe-Universität, Frankfurt am Main.

United Kingdom: Royal Botanic Gardens, Kew; Allan Hall, University of York, York; Royal Botanic Garden, Edinburgh; Really Wild Flowers, Dorset; Poyntzfield Herb Nursery, Dingwall; Arne Herbs Somerset, Bristol.

France: Jardin Botanique de la ville de Bordeaux, Bordeaux; Jardin Botanique de Lyon, Lyon; CRITT Horticole at Rochefort, La Rochelle.

Belgium: National Botanical Garden of Belgium, Meise.

Italy: Orto Botanico dell'Università di Ferrara, Ferrara; Orto Botanico dell'Università degli Studi di Pisa, Pisa.

The Netherlands: Universiteit Utrecht, Botanic Gardens Fort Hoofddijk, Utrecht; De Kruidhof, Botanische Tuin Fryslan, Buitenpost; Kwekerij de Keltenhof, Bergharen; Rubia pigmenta naturala, Steenbergen; Claudy Jongstra Studio, Spannum.

Austria: University of Applied Arts, Vienna.

Greece: National and Kapodistrian University of Athens, Athens.

It is expected that more species will be collected in the years to follow, as some of the gardens and suppliers still need to grow the plants especially requested.

The plants collected so far have been accurately labelled, dried and stored for the laboratory research.

4. DEVELOPMENT AND EVALUATION OF EXTRACTION PROTOCOL FOR FLAVONOIDS

As second step, the plants sources have been examined by extraction of the organic colorants. To compare the plants obtained from the different regions, it is necessary that the participants to the joint research extract the colorants in the same reproducible way. Therefore, extraction protocols have been developed in which the complete extraction process is accurately described. During the development of the extraction protocol, several experiments have been done to optimize the extraction yield and to avoid the precipitation or breakdown of specific colorants.

For plants containing flavonoids, using weld as a model plant, the following protocol has been developed:

WELD QUANTITATIVE EXTRACTION PROTOCOL

Grind around 1,60 g of the plant material; make sure that the sample is representative for the whole plant; note that it is important, before weighing, that the plant material is as dry a possible.

After grinding, take 10 samples from the grinded material, each weighing exactly 150 mg, put them into 20 ml scintillation flasks (if it is allowed by the analytical balance, weigh directly into the scintillation flasks), add then 2,5 ml de-mineralised water at 60˚C and leave the flasks pre- soaking for 30 minutes at room temperature.

When the sample is pre-soaked, add 5,0 ml demineralised water, make sure this time the de-mineralised water is pre-heated at 100˚C. Immediately introduce all flasks in a pre-heated oven at 100˚C and leave them for 30 minutes. Label all shell vials, *eppendorf* safe-lock vials and micro-inserts if needed.

After the heating for 30 minutes at 100 °C, extract with a micro pipette 250 µl of the solution from each flask and put the taken samples into *eppendorf* vials. Try to pipette the extraction fluid as quick as possible, so the extract in the flasks does not cool off. Every flask has to be taken independently from the oven each time that pipette is used, so the others keep the right temperature in the oven. Try to use the pipette in the same manner and with the same velocity. It is not recommended to leave the extraction for a too long period of time in the *eppendorf* save-lock vial, because precipitation of glycosides could occur even there. Centrifuge the *eppendorf* safe-lock vials for 3 minutes and extract 150 µl of the fluid into a shell vial. Make sure the fluid is not cloudy or that floating pieces are present in it: if so, centrifuge again before pipetting into the shell vial.

When the extracted fluid is added to the micro insert, add 150 µl methanol. Homogenize the solution in the shell vial with a vortex.

Pipette 250 µl of the shell vial solution into a micro insert. When this is done the 100˚C extraction-set is ready for the analysis.

Analyses: the HPLC-PDA analytical protocol of ICN, already developed in a previous project, can be found at www.organic-colorants.org, in the section protocols: HPLC-PDA protocol ICN.

Picture guide

1) The Grinding

The dry plant material can be pre-grinded in a coffee grinder.

The grinding can also be achieved by cutting into smaller pieces and then using a mortar and pestle

(Supplier: MTC Haldenwanger)

Porcelain mortar 55/2 : Art. nr. 630305

Porcelain pestle 56/1 : Art. nr. 630505

2) Weighing the samples

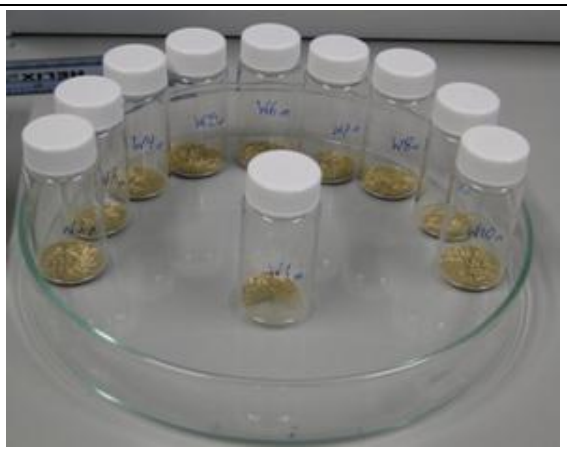
The samples were weight directly in the glass flasks to prevent material loss.

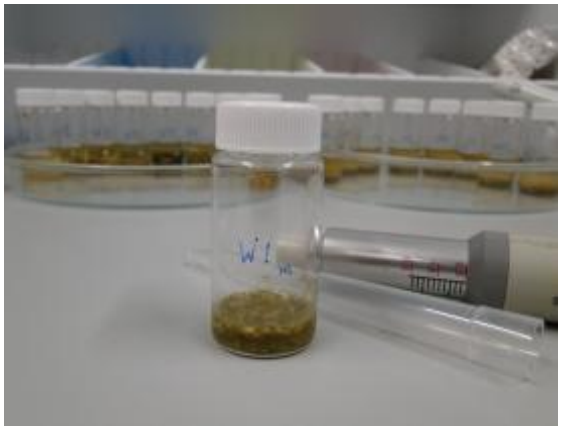
The scintillation flasks have a polypropylene screw cap with an inlay of cork/aluminium.

(Supplier: Fisher Emergo B.V. Landsmeer, Netherlands)

20ml glas flask: Art. nr. 281010

Plastic cap: Art. nr. 281012

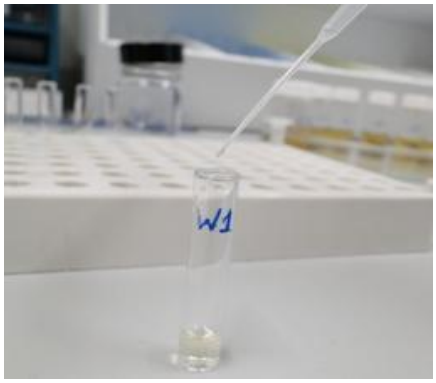




3) Soaking

2,5ml demiwater (60°C) is added to increase the osmotic dynamics of the sample.

It is observed that presoaking the sample stabilizes some glucosidal components. Try not to shake the flask at any time for all plant material should soak.

4) Ovens The oven is preheated 30 minutes before use at 100°C. Wilten Woltin B.V. de Bilt, the Netherlands Heraeus oven max. 70°C or Heraeus oven max. 150°C	
	**0,5ml Eppendorf safe-lock vials** The pipetting of the extraction is done quickly: Every flask that has to be extracted is taken independently from the oven, so the others keep the right temperature. It is not recommended to leave the extraction for too long in the eppendorf save-lock vial because precipitation of glucisides can occur even here. Fisher Emergo B.V. Landsmeer, the Netherlands Art. nr. 343421
Centrifuge Centrifuge the eppendorf vials for 3 minutes this can be longer or shorter, but try to do the same time for every eppendorf vial. Mini Centrifuge 50Hz National Labnet Co. Woodbridge	
	**Shell Vial** The Shell vial is used in order to have a good homogenization of the solution. Clear Shell Vials Micro tubes 1.0ml Art. Nr. 4102-840

Vortex This step takes only a few seconds Vortex 501 AP W. Krannich KG Göttingen, Germany	
	**Micro Insert vials** The use of this inserts depends on which autosampler is used. Micro-Insert Vials 0,3ml (20ul) Grace, Deefield USA MP/N:2109123/98118
Autosampler Supplier: Waters The 20 samples will be in the carrousel for a period of 20 hours.	

Time Guide		Time Table
1) Grind sample 1,6 g	60 minutes	
2) Take 10 samples each weighting 150 mg, put them into 20ml labeled flasks		
3a) Add 2,5 ml demineralised water (60°C)and leave soaking for 30 min	30 minutes	
3b) Add then 5,0ml demineralised water pre-heated at 100˚C	35 minutes	
4) Introduce flasks in a pre-heated oven at 100˚C and leave for 30 minutes		
5) Lable *eppendorfs,* inserts and vials if needed		
6) *Extract 250µl solution from each flask into an eppendorf tube	35 minutes	
7) Centrifuge for 3 minutes and extract 150µl of the solution into a micro tube		
8 Add 150µl methanol and homogenize with vortex		
9) Pipette 250µl of the solution into a micro vial		
- Analyses: (ICN) HPLC-PDA analytical protocol	20 hours	

*Do not let the flask cool down, pipette quickly

The weld quantitative extraction protocol was developed by ICN and then evaluated by Of-ADC, KIK-IRPA, BM and NGL. Weld from one botanical plant, delivered by ICN, was distributed to ensure that all partners used the same source for extraction. The relative standard deviations (RSD) of three relevant components in weld were determined and are presented in Table 1. The weld was analysed in ten-fold.

Table 1: RSD of the relevant components in weld.

	Weld		
Institute	Luteolin	Apigenin	Luteolin 3',7-diglucoside
ICN	2.8%	2.3%	2.4%
Of-ADC	7.1%	7.1%	9.2%
KIK-IRPA	4.9%	7.0%	9.7%*
BM	61.5%	64.3%	29.9%
NGL	38.1%	44.3%	45.4%

* based on 9 analyses, one outlier observed.

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5. DEVELOPMENT AND EVALUATION OF EXTRACTION PROTOCOL FOR ANTHRAQUINONES

For the anthraquinones, a similar protocol was developed although, due to the hydrophobicity, of the colorants some modifications were made. Several procedural methods prior to the definition of the protocol were tested in an experimental set of 4, 8 or 10 madder root samples, each weighing 300mg. For anthraquinone containing plants, using madder as a model plant, the following protocol has been developed.

MADDER QUANTITATIVE EXTRACTION PROTOCOL

Grind 3.2g of the plant material (it is important before weighing is that the plant material is as dry a possible). After grinding, take 10 samples from the grinded material, each weighing 300 mg, put them into 20 ml scintillation flasks (if allowed by your analytical balance, weigh directly into the scintillation flasks), add then 5,0 ml of 60°C demineralized water very slowly to the flasks (do not shake the flasks) and leave the flasks pre-soaking for 1 hour.

When the sample is pre-soaked, immediately introduce all flasks in a pre-heated oven at 60˚C and leave them there for 30 minutes. Lable all shell vials, *eppendorf* safe-lock vials and micro inserts, if needed.

Pipette into ten, 1,5ml eppendorf safe-lock vials, 250ul methanol.

After heating for 30 minutes at 60 °C, extract with a micro pipette 250 µl of the solution from each flask, take every time each flask from the oven so the others do not cool off, and insert it into the methanol containing 1,5ml eppendorf safe-lock vials (tips! for the extraction in the picture guide). Try to pipette the extraction fluid as quick as possible because of precipitation problems when cooling, but prevent shaking the flaks at all; migration of plant material to the flask wall can cause anomalies in the quantitative extraction. Return the flask every time to the 60°C oven after pipetting. Vortex the now locked 1,5ml eppendorf vials for some seconds and centrifuge them then for 30 minutes. Now that the first set is in the centrifuge carefully insert the flasks set from the 60°C oven into a 100°C pre-heated oven, leave them again for 30 minutes.

Extract 150 µl of the fluid from the centrifuge 1,5ml eppendorfs with a pipette into a micro insert. When all are done the 60˚C extraction-set is ready for analysis.

By now the 100˚C flasks in the oven are (almost) ready for extraction. Pipette into other ten, 1,5ml eppendorf safe-lock vials, 250ul methanol.

After heating for 30 minutes at 100 °C the flask set is ready for extraction.

Extract with a micro pipette 250 µl of the solution from each flask, take every time each flask from the oven, insert it into the methanol containing 1,5ml eppendorf safe-lock vials. The 100˚C extraction-set is ready for analysis.

Analyses: (ICN) HPLC-PDA analytical protocol can be found at www.organic-colorants.org, in the section protocols: HPLC-PDA protocol ICN

Picture guide quantitative extraction

1) The Grinding

The dry plant material can be pre-grinded in a coffee grinder.

The grinding can also be achieved by cutting into smaller pieces and then using a mortar and pestle

(Supplier: MTC Haldenwanger)

Porcelain mortar 55/2 | Art. nr. 630305

Porcelain pestle 56/1 | Art. nr. 630505

2) Weighing the samples

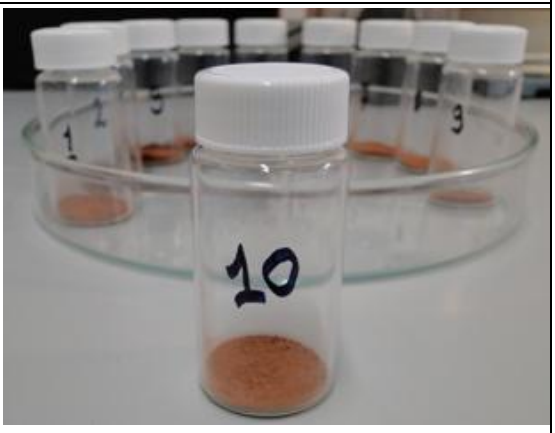
The samples were weight directly in the glass flasks to prevent material loss.

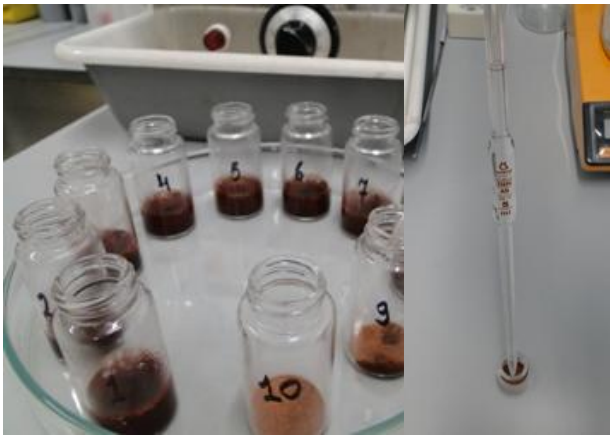
The scintillation flasks have a polypropylene screw cap with an inlay of cork/aluminium.

(Supplier: Fisher Emergo B.V. Landsmeer, Netherlands)

20ml glass flask: Art. nr. 281010

Plastic cap: Art. nr. 281012



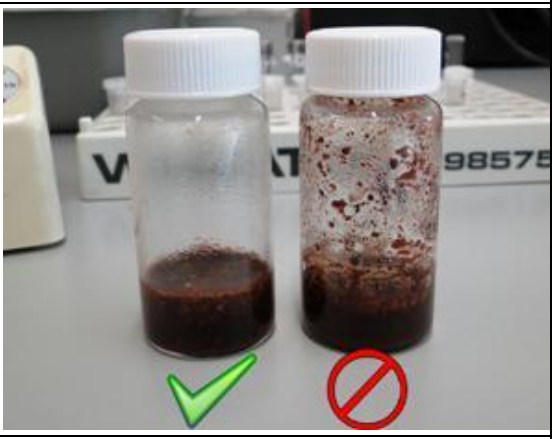


3) Soaking

5.0ml demineralised water at 60°C very precise and slowly. Try to not to wet the flask walls with the demineralised water too much. Use a 5ml glass volumetric pipette. Leave soaking for 30 minutes

4) No shaking

Try to avoid contact between the walls of the flask when pipetting. Do not shake or swing the flask, because some material can migrate to the flaks wall.



5) Oven

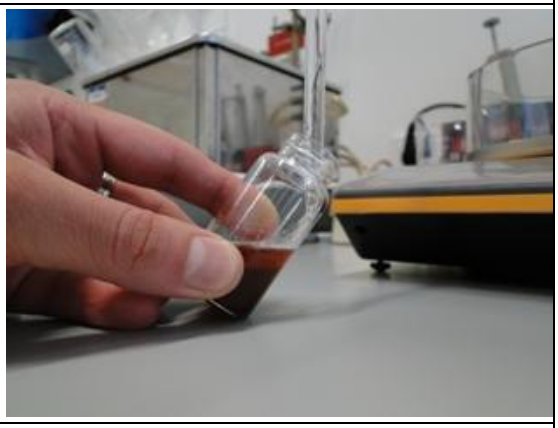
The ovens are preheated 30 minutes before use.

Wilten Woltin B.V. de Bilt, the Netherlands

Heraeus oven 150°C

6) Adding Methanol

5ml methanol is added to the flasks after cooling off 25 minutes. In this way components that are in the solution will not precipitate.

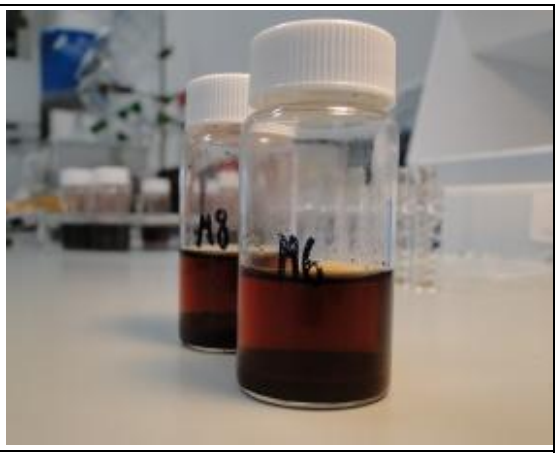


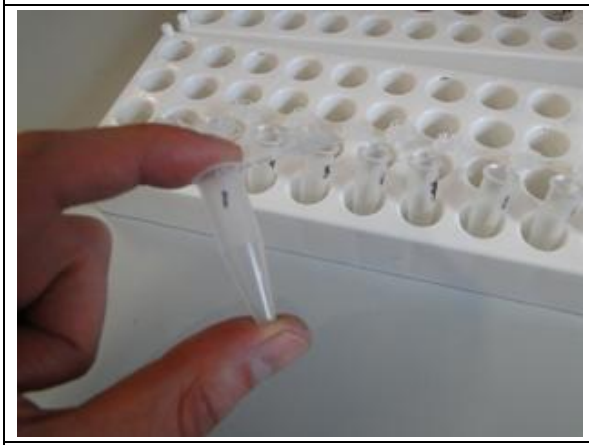
7) Vortexen

Using a vortex the flask shaken for a few seconds helps to homogenized the extraction

8) Sink in

Leave the flasks to sink in for approximately 45 minutes. In this way the clear extraction will be easier to pipette. Extract 300ul of this solution into 1.5 Eppendorf save-lock vials.





9) 1.5 Eppendorf safe-lock vials

The eppendorf safe-lock vials are this time bigger because methanol is added in an earlier stage of the extraction procedure.

Fisher Emergo B.V. Landsmeer, the Netherlands

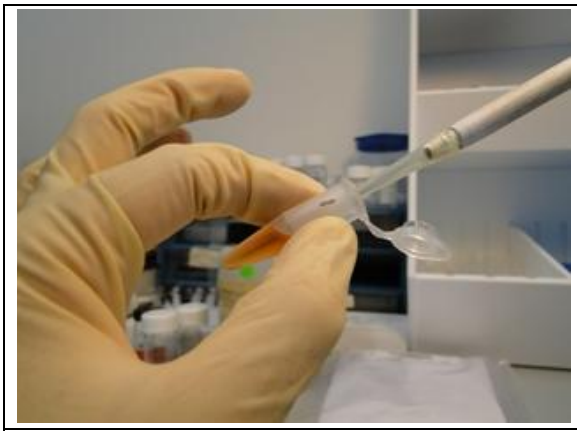
Art. nr. 343421

10) Centrifuge

Centrifuge the eppendorf vials for 5 minutes. Try to be precise with the timing if you have to split the set.

Mini Centrifuge 50Hz

National Labnet Co. Woodbridge

	**11) Pipette from Eppendorf vial** 250ul is pipetted from the eppendorf vials into a micro insert vial.
11) Micro Insert vials The use of this inserts depends on which auto sampler is used, the protocol can be adjusted. Micro-Insert Vials 0,3ml (20ul) Grace, Deefield USA MP/N:2109123/98118	
	**12) Auto sampler** Supplier: Waters The 20 samples will be in the carrousel for a period of 20 hours.

Time Guide Madder extraction protocol		
1) Grind sample 3.2 g		60 minutes
2) Take 10 samples each weighting 300 mg, put them into 20ml labelled flasks		
3a) Add gently 5,0 ml demineralised water preheated at 60°C leave soaking for 30 minutes		30 minutes
3b) Introduce flasks 30 minutes in pre-heated oven at 60˚C		35 minutes
4) Label all eppendorf vials and inserts, if needed		
5) add 250ul methanol to all 1,5 eppendorf vials		
6a) Extract 250µl solution from each flask into an eppendorf vial	6b) Return flasks set in a 100˚C pre-heated oven leave for 30 minutes	30 minutes
7) Centrifuge the eppendorf vials for 5 minutes		
8) Pipette 250µl of the solution into a micro vial		
	9) Extract 250µl solution from each flask into an eppendorf vial	20 minutes
	10) Centrifuge the eppendorf vials for 5 minutes	
	12) Pipette 250µl of the solution into a micro vial	
Analyses: (ICN) HPLC-PDA analytical protocol		20 hours

Time Table

The madder quantitative protocol was developed by ICN and then evaluated by Of-ADC, KIK-IRPA, BM and NGL which used their own HPLC-PDA analytical protocols.

Madder from one botanical plant, delivered by ICN, was distributed to ensure that all partners used the same sources for extraction. The relative standard deviations (RSD) of four relevant components in weld were determined and are presented in Table 2. The madder was analysed in ten-fold.

Table 2: RSD of the relevant components in madder.

	Madder			
Institute	Alizarin	Purpurin	Alizarin glycoside 1	Alizarin glycoside 2
ICN	6.1%	3.7%	5.1%	3.8%
Of-ADC	6.2%	6.3%	4.9%	5.2%
KIK-IRPA	7.9%	8.1%	3.7%	11.1%
BM	14.3%	11.9%	13.3%	5.3%
NGL	22.7%	20.5%	21.6%	16.3%

6. CONCLUSIONS

The RSD of ICN, Of-ADC and KIK-IRPA are acceptable, when it is taken into account that there is a natural variation of the components in the dye plants examined.

The RSD from the NGL are quite high for both weld and madder. This is easily explained by the fact that an HPLC system especially dedicated for small pigment samples was used in this case. Since the colorant present in the plant sources is much higher than usually present in paint samples, the system was overloaded and therefore was not suitable for the plant analysis of this work.

The RSD of the components analysed by BM were good for madder, but particularly high for weld. This is most probably due to precipitation of the weld components during the analysis. Additional measurements are planned to determine if this is the cause of the high deviation. If necessary, the extraction protocol for weld will be adapted.

Based on the analytical results obtained so far and the number of plants collected, the plant sources have been divided among partners according to the following schedule:

ICN	Analysis of wild madder (10) and dyer's broom (13)
Of-ADC	Analysis of madder (13) and weld (10)
KIK-IRPA	Analysis of *Galium verum* (14) and sawwort (8)
BM	Analysis of *Galium mollugo* (8)

All the analyses will be performed in three-fold and evaluated by qualitative and quantitative comparison. The results of the analyses are expected at M15.

This task is strongly connected to WP3.2: *Training on the technology of lake preparation and dyeing textiles from botanical/animal source dyestuffs*. Within the planned training workshop, the effect of different parameters in recipes will be demonstrated and further investigated.

In addition, this task is also connected to WP2.2a *Exchange of 2D and 3D analytical data*, devoted to techniques used for analysis of organic colorants.

After the first 12 months, all objectives are achieved.