**ARISTOTLE UNIVERSITY OF THESSALONIKI**

FACULTY OF SCIENCE

SCHOOL OF CHEMISTRY

MASTER COURSE IN ARCHAEOLOGICAL MATERIALS SCIENCE - ARCHMAT

MASTER THESIS

# A COMPREHENSIVE STUDY OF NATURAL ORGANIC DYES IN HISTORICAL MEDITERRANEAN TEXTILES BY LIQUID CHROMATOGRAPHY WITH PHOTODIODE ARRAY AND MASS SPECTROMETRIC DETECTION

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**THESSALONIKI 2018**

**ΑΡΙΣΤΟΤΕΛΕΙΟ ΠΑΝΕΠΙΣΤΗΜΙΟ ΘΕΣΣΑΛΟΝΙΚΗΣ**

ΣΧΟΛΗ ΘΕΤΙΚΩΝ ΕΠΙΣΤΗΜΩΝ

ΤΜΗΜΑ ΧΗΜΕΙΑΣ

ΜΕΤΑΠΤΥΧΙΑΚΟ ΠΡΟΓΡΑΜΜΑ ΣΤΗΝ ΕΠΙΣΤΗΜΗ ΤΩΝ ΥΛΙΚΩΝ
ΑΡΧΑΙΟΛΟΓΙΚΟΥ ΕΝΔΙΑΦΕΡΟΝΤΟΣ

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## ΤΡΙΜΕΛΗΣ ΕΞΕΤΑΣΤΙΚΗ ΕΠΙΤΡΟΠΗ

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Η τριμελής εξεταστική επιτροπή που ορίστηκε σύμφωνα με την απόφαση της Γ.Σ.Ε.Σ. του Τμήματος στη συνεδρίασή της αριθμ. 664/21-05-2018, για την κρίση της Μεταπτυχιακής Διπλωματικής Εργασίας της Meihui Li, Αρχαιολόγου, συνήλθε σε συνεδρίαση στο Αριστοτέλειο Πανεπιστήμιο Θεσσαλονίκης την 13/11/2018, όπου παρακολούθησε την υποστήριξη της εργασίας με τίτλο «Συγκριτική Μελέτη Φυσικών Οργανικών Χρωστικών Σε Ιστορικά Μεσογειακά Υφάσματα Με Υγρή Χρωματογραφία-Φασματοσκοπία Μάζας» και την ενέκρινε με βαθμό δέκα (10).

## Abstract

Thesis involves an optimized chromatographic and spectroscopic study of textiles, dyed with natural organic dyestuffs, aiming at the identification of chromophore constituents and their possible degradation products. Liquid chromatography - mass spectrometry coupled with photodiode array detection has been applied for the samples' analyses after the selective extraction of chromophores from the substrate. The under investigation natural organic dyes are those with a particular cultural value since they have been widely used for textiles staining in the Mediterranean region since Antiquity. Improved "Mild Acid Hydrolysis" with methanol/formic acid enabled the observation of intact organic dyes from the silk textiles, while a water/ACN solvent system has also been employed for the pre-treatment of dyestuffs from both reference and silk samples, which has successfully preserved the chemical information of dye compounds in order to create a comprehensive database for the characterization of natural organic dyes in further studies. Moreover, three different chromatographic programs were applied and compared in LC-PDA-MS, and the best separation result from Program B has been employed to the analysis of the real samples from Turkish silk textiles created by a historical dyeing recipes, and a certain amount of effective information in terms of qualitative analysis has been clarified.

***Keywords:*** *Natural organic dyes; Mild extraction; LC-PDA-MS; Historical Mediterranean; Silk textiles*

## Περίληψη

Η παρούσα διπλωματική εργασία αφορά την βελτιστοποιημένη χρωματογραφική και φασματοσκοπική μελέτη κλωστοϋφαντουργικών προϊόντων βαμμένων με φυσικές οργανικές χρωστικές, με στόχο την ταυτοποίηση των έγχρωμων συστατικών τους καθώς και πιθανών προϊόντων αποικοδόμησης τους. Οι υπό διερεύνηση φυσικές οργανικές χρωστικές είναι ιδιαίτερης πολιτιστικής αξίας, δεδομένου ότι έχουν χρησιμοποιηθεί ευρέως για την βαφή υφασμάτων στην περιοχή της Μεσογείου από την Αρχαιότητα. Για την εκχύλιση των έγχρωμων ενώσεων των φυσικών οργανικών χρωστικών από τα δείγματα αναφοράς καθώς και από τα ιστορικά δείγματα μεταξιού, εφαρμόστηκε μία ήπια μέθοδος εκχύλισης με σύστημα διαλυτών νερού/ACN σε λουτρό υπερήχων. Επιπλέον, για την απόσπαση των χρωστικών από τα ιστορικά δείγματα προηγήθηκε στάδιο προκατεργασίας με μίγμα μεθανόλης/μυρμηκικού οξέος και θέρμανση σε ήπιες συνθήκες. Η εφαρμογή ήπιων συνθηκών εκχύλισης επέτρεψε την απόσπαση των έγχρωμων ενώσεων των φυσικών οργανικών χρωστικών, διατηρώντας επιτυχώς το σύνολο των χημικών πληροφορίων που θα μας επέτρεπαν τον απόλυτο χαρακτηρισμό των φυσικών χρωστικών. Για την ανάλυση των δειγμάτων μετά την εκλεκτική εκχύλιση των έγχρωμων ενώσεων από τα υποστρώματα, εφαρμόστηκε η υγρή χρωματογραφία – φασματοσκοπία μάζας, LCMS. Συνολικά εφαρμόστηκαν και συγκρίθηκαν τρία διαφορετικά προγράμματα LCMS, εκ των οποίων τα καλύτερα αποτελέσματα διαχωρισμού και ταυτοποίησης επιτεύχθηκαν με το Πρόγραμμα Β. Το πρόγραμμα αυτό εφαρμόστηκε και για την ανάλυση των ιστορικών δειγμάτων από τουρκικά μεταξωτά υφάσματα, βαμμένα βάσει ιστορικών συνταγών βαφής.

Οι πληροφορίες που συλλέχθηκαν, αξιοποιήθηκαν για την δημιουργία μιας ολοκληρωμένης βάσης δεδομένων για τον χαρακτηρισμό των έγχρωμων ενώσεων των φυσικών οργανικών χρωστικών που θα μπορέσει να χρησιμοποιηθεί σε περαιτέρω μελέτες.

***Λέξεις Κλειδιά:*** *Φυσικές Οργανικές Χρωστικές; Ήπια Εκχύλιση; LC-PDA-MS; Ιστορικά Μεσογειακά; Μεταξωτά υφάσματα*

# Contents

## 1. Introduction

When the first moment human perceived the world by their unique intelligence, the story of dyes and dyeing practices could be seen to start. As a physical property of matter, color gives a basic description to the world we live in, which is to define, to distinguish, and to develop this very fundamental knowledge of all existences. Additionally, the fact that people get to use colorants or dyestuffs from the resources they can conveniently reach is even earlier than what were found in historical records or archaeological remains. It is really hard to say at what exact time people participate in dyeing practices, but it may be easier to see what materials and how they utilize to dye. The source of dyestuffs can be divided into two major groups: plants and animals. As the most common dye of plants origin, madder, of which root can produce a brilliant red, grows in both Asia and Europe, and it is regarded as one of the indigenous dyes widely used by ancient people. Besides, mainly growing in India and far-East region, indigo is the oldest vat dye for blue color. As for the dyes from animals' sources, "lac" dyes from insects as well as the "Tyrian purple" from shellfish can be the most typical example. After 16$^{th}$ Century, due to the discovery of New World, increasing dyestuffs from new land, such as: American Cochineal and Brazilwood, were exported into Europe, which boosted the dyes trade as well as dramatically enlarged the availability of various dyes in the lower class of European society. With the advancement of dye industry in 19$^{th}$ Century, the first synthetic dye came into being, which is as the significant turning point of dye history that synthetic dyes were replacing the natural ones and widely applied by modern generations. This short overview of dye history highlights the relationship between the availability of nature resources, the choice of

dyestuffs, and the technology applied, which can help to understand the socioeconomic and cultural context of dyeing artefacts. Therefore, the study and characterization of natural dyes in the ancient textiles can lead to some significant insights regarding to the understanding of technological development through human civilizations and in revealing economic, social, and culturally historic aspects (Illaria Degano, Ribechini, Modugno, & Colombini, 2009).

## 1.1 Dyeing History in the Ancient Mediterranean World

The palette for natural dyes had already been established before pre-historic Mediterranean, including anthraquinone-based chromophores for the reds, and then indigo for the blues as well as 6,60-dibromoindigo for purple. These three types of dyes can be regarded as the most valued and oldest colorants in history, which were traded all over the ancient regions. In addition, since yellows were very easy to obtain from natural sources, it can be produced locally, which completed this original palette of ancient dyes that most of other colors in progress could be generated (Bechtold & Mussak, 2009).

Over centuries, this classical palette had been preserved and well enriched, and the earliest evidence of textile dyeing can be traced back to approximately 3000-2000 B.C., but there are few records in terms of dyeing methods to explain the production of textile and its color. By 1500 BC, the Phoenicians had discovered murex, which was known as “Tyrian purple”. During the era of Roman Empire, considerable trades of natural dyes flourished, and dyes were treated as valuable as gold. The priest and craftsmen kept the secrets of natural colorants and it was essential to have a sort of license to manage some

special dyestuffs. Wearing in a dyeing clothes could be a representation of high social status for Romans. For example, the Tyrian purple was just for the garments wore by Roman noble elites as a symbol of wealth and powder (Marras, 2015). Ancient dyes and the process of dyeing textile have also been recorded mainly by two famous historians in the first century. In Roman naturalist Pliny the Elder's writings, he recorded a common use of indigo and woad dyes among Gallic tribes. And the Greek physician Dioscorides described the dyes extracted from madder, dyers' bugloss for reds, and saffron, weld for yellows. Some plants that are rich in tannins, such as: barks of oaks, walnut hulls and oak galls, have been used to produce dye and mordant as well as to create colors from brown to black. By 1250s, most of the well-developed dyeing method had been recorded by medieval monks. While as the medieval unions of dye trade grew, those merchants developed the tradition of dye procedures but kept as the trade secret. Meanwhile, famous centers of dye production and trade appeared, such as: Venice, Florence, Genoa, Basle, Frankfort, Nuremberg, Antwerp, and London (Gürses, Açıkyıldız, Güneş, & Gürses, 2016).

The golden age of natural dyes in Mediterranean region and even the whole Europe has probably been the Renaissance period with an increasing demand of new dyestuffs for the dyeing industry. A lot of investigations in terms of dying practices appeared due to the needs of extended dye production, including documental sources that had shown Lac was the third most expensive color in Italy (after gold and Ultramarine) at that time (Marras, 2015). Just until the 15th Century, the first book on dyeing had been published, and its different translated versions spread out in the following years. Moreover, due to the discovery of New World, various plant spices for producing new natural dyes were

exploited, and the most famous dyestuffs were from brazilwood and American cochineal. However, within few centuries, the industrial revolution had led to a dramatic increase in dyeing and textile manufacturing. In the 19$^{th}$ Century, the period known as its flourishing scientific discoveries, chemists were driven by the high demands from industries to seek new dyes (Henderson, 2013). In 1856, Perkin synthesized a mauve dye and it was seen as the opening of new era without return, which just took 10 years (1870–1880) for synthetic dyes to completely replace the natural dyes (Degano, Ribechini, Modugno, & Colombini, 2009). Therefore, the classical palette for natural dyes was completed by the chemists with their cheaper synthetic dyes of a great commercial value.

Dying techniques is also an important aspect of historical development of natural dyes and textiles. Direct dyes have been used since pre-historic times with its advantage that no specific pre-treatments are needed when conducting a dyeing process, while it also reveals the disadvantages of limited color fastness and low water resistance. Therefore, dyeing remains produced from this type of pre-historic method have only been found under some extreme conditions, for example, in dry desert sand or oxygen-deficient environment. With the later development of more complicated and advanced dyeing methods, indigoid dye, appeared around 4000 years ago, is regarded as the oldest vat dye used by man. This type of dyeing method involves dipping the fibers into the colorless dye bath and drying them in the sun to get blue, insoluble dye in the surface of the fiber after the treatment with urine followed by the fermentation of the plant. Moreover, the indigoid dye was actually introduced by Phoenician traders and migrants into the Mediterranean area, of which use gradually spread to the whole Europe. Another similar blue dye in Europe was woad, which

was used since the Bronze Age (2500-800 BCE) but eventually replaced by indigo. As a famous vat dye in ancient Mediterranean world, Tyrian purple was traded by the ingenious and industrious Phoenician around 800 BCE. In order to strengthen chemical affinity for the textile fibers, especially for those red dyes extracted from plants root or insects, a mordant solution containing metal salt is required before dyeing textiles, which enables the metal ion to form the complexes between dyes and textiles for better dyeing effects. The common mordants being used includes: aluminum, iron, tin, chromium or copper ions and tannins as well. The majority of natural dyes belongs to the group of mordant dyes. Kermes and lac are seen as the known oldest mordant dyes produced by insects (Gürses et al., 2016).

Overall, the completion of palette for natural dyes, together with its advancement in dying method, has greatly contributed a colorful past of human civilization and also illuminates a way to more splendid future. In other words, the historical development of natural dyes and dying practices indicates a bond between “Nature” and “Culture”: a constant quest of human intelligence, and the power to exploit coloring resources of nature and fully apply in their art by the appropriate ways of dying.

## 1.2 Cultural Significance of Natural Dyes

As Jenny Balfour-Paul (2000) mentioned: “The colors used on textiles and artifacts, their social significance and the scope of their trade, are part and parcel of a people’s overall history.” In this sense, colorants, or mainly dyes, as the material substance created by nature, are transformed into the social products processed by human’s work, containing their wisdom, energy, imagination and social values, which can be generalized as “ Culture”.

To clarify the cultural significance of natural dyes, we firstly need to realize the purposes of dye use during the historical development of our civilization. For millennia, humans have employed colorants supplied from nature to adorn their bodies, to dye clothes, textiles and artefacts, to pay tribute to their gods and, above all, to express their thoughts and creativity by coloring practices. The very primitive use of colorants is proved by the evidence of cave paintings dated back to about 400.000 years ago. Anthropologists also claim that pre-historic men used to employ natural colorants to pain and to dye their body with rituals purposes during hunting or to scare enemies. This practice was also very common in many other ancient tribes. For example, it was said that the warriors from Iceni tribe that is the ancient inhabitants of Britannia, were used to paint their faces with the blue woad dye before going into battle. After that, with the beginning of the textile era, early humans learnt to exploit natural materials to dye fibers. From historical records as well as the results of archaeological investigations, it is effectively indicated that the use of natural dyes for tinctorial purpose, which was prevalent in various ancient cultures worldwide, such as: China, India, Mesopotamia, Egypt and Central South America. Similar to many other civilizations, ancient Egyptians were very familiar with the dyeing properties of some natural species, but they were regarded as the first generation to employ such dyes for artistic purposes. They dyed their clothes and other objects with a number of natural organic dyes which had been recorded in details as documentary and the techniques they used as well as the descriptions of dying procedures were also kept from one generation to another. In addition, organic dyestuffs were also proved to be used as the preparation for cosmetics (Marras, 2015).

Therefore, the first cultural significance in general refers to the discovery and use of natural dyes and pigments that contributed to the maintenance of the age-old bond between humankind and nature, and could now help to revive and enhance it. Natural dyes and colorants are an essential part of the world's ecological and cultural heritage, of which selection and uses to create colors are common to all civilizations. And throughout history as above, we can see that natural colorants have played a major part in economic and cultural exchanges between nations (Cardon, 2010).

Moreover, as a unique knowledge about the coloring properties of indigenous plant and animal species retained by a certain group of people through a long-term dyeing work, it contributes to various aspects of culture: its art, symbols and religious beliefs, and its medicine. However, nowadays, this traditional knowledge is fast vanishing due to the influences of modern life styles into some remote regions and the failure of passing through this intangible heritage to new generations (Cardon, 2010). Thus, the second significance of natural dyes is to record the traditional knowledge rather than losing it.

Last but not least, with the goal of sustainable development, natural dyes could shed light on the future development of modern dyeing practices. As we all known, after the invention of the first artificial and synthetic dyes during the 19th century, the industrialized European countries generally applied synthetic dyes extracted from fossil resources, coal-tar and oil for daily use, which caused a big cultural revolution and had a deeply influence until now. While in the new era of synthetic colorants, recent researches on natural dyes has shifted from economic issues to historical, archaeological and heritage aspects, with

the growing awareness of the threats on environment and of the need for a sustainable "green economy" (Cardon, 2010).

To be summarized, as the global heritage of colors, natural dyes don't represent only the past of human material life, but also their spirit world of beauty and accumulation of knowledge. Natural dyes also refer to various social practices including the production and trade of dyes and textiles, which is regarded as the big aspect of one's cultural context. Moreover, the focus on natural dyes and dyeing has preserved the tradition of a certain social group and will illuminated a more environment-friendly future of human world.

### 1.3 Scientific Development of Dyes Analysis in Ancient Textiles

The scientific analysis of natural dyes, combining with other historical references and the discoveries from archaeological excavations about ancient textiles, gives deep insights into the relationships between different cultures and their natural environment, early developments in the cultivation and breeding of selected coloring plant and animal species, international trade routes, ancient empiric knowledge of dyeing chemistry and intercultural technological influences in dyeing processes (Cardon, 2010). However, since the dyeing textile are one of the most degradable material, the chromophore analysis of natural organic dyes in historical textiles can be an extremely challenging work for researchers due to the following reasons: (1) A wide range of dye sources and complexity of their chemical properties and structures. (2) Very low amount of dye compounds extracted from true samples for analysis. (3) Degradation in textiles complicates their identification (Ilaria

Degano, Biesaga, Colombini, & Trojanowicz, 2011). In order to solve these problems, more advancing techniques for the analysis of natural organic dyes are required.

Basically, the characterization of natural organic dyes consists of three stages of analysis: extraction, separation and detection. And among all the analytical techniques suitable for the determination of organic dyes in historical textiles, the chromatographic techniques involving UV-Vis and mass spectrometric detection has proved to be an accurate and sensitive combination of scientific methods allowing very fine dye analysis, through the separation of all compounds present and their characterization. With the development of extraction and separation methods in chemistry, recent studies has put more emphasis on lowering detection limits and developing "mild" extraction of the colorants from the dyed textile fibers to be analyzed, leading to improved recognition of the molecular structures composing the dye (Cardon, 2010).

As an important approach in a preliminary stage, thin layer chromatography (TLC) has often been applied for the separation of chemical compounds of natural organic dyes. While in the last twenty years, due to its limitations on detection, identification and quantitative spot evaluation, TLC has been gradually replaced by high-performance liquid chromatography (HPLC) with ultraviolet-visible detection (Szostek, Orska-Gawrys, Surowiec, & Trojanowicz, 2003). In 1985, the pioneering work on historical textile done by Jan Wouters, has introduced the HPLC system for the analysis of dyes in textiles, which also has become the most widespread reference technique for dyestuffs analysis till today (Marras, 2015). The separation and reached levels of identification of dyes compound has been gradually improved by many excellent in the following years.

In Greece, a series of inspiring works on the characterization of natural dyes in historical Mediterranean textiles were published between 2006 and 2011. Karapanagiotis et al. recorded the organic dyes found in Byzantine and post-Byzantine icons, made in the Mount Athos area and in the adjacent area of Chalkidiki. The HPLC combined with spectrophotometric UV-Vis detection was employed to the dyestuff identification, proving that the reddish cochineal and a ‘‘soluble’’ redwood appear to be the most common organic dyes of the icons tested (Karapanagiotis, Valianou, Daniilia, & Chryssoulakis, 2007). Based on this chromatographic attempt, Karapanagiotis developed his study by applying LC-MS coupled to a negative electrospray ionization mode to investigate the colorants used in icons of the Cretan School of iconography, which successfully provided information on the identity of some unknown coloring components, of the aforementioned dyes, detected in the historical samples (Karapanagiotis, Minopoulou, Valianou, Daniilia, & Chryssoulakis, 2009). In 2010, organic colorants contained in 30 textiles (16th to early 20th century) from the monastery of Mount Athos have been investigated by HPLC–DAD–MS and compared by different detection modes. The LC–MS in negative atmospheric pressure chemical ionization (LC– MS-APCI−) mode was attempted to investigate cochineal (*Dactylopius coccus Costa*) samples, while positive electrospray ionization (LC–MS-ESI+) mode was used for identification of fuchsin components. Finally, detailed HPLC–DAD studies were also performed on young fustic (*Cotinus coggygria Scop.*) and Persian berries (Rhamnus trees) (Mantzouris, Karapanagiotis, Valianou, & Panayiotou, 2011).

Despite of the wide applications and the successful results of the UV-VIS detection, it also has some serious limitations on sensitivity, selectivity and inability to identify

unknown compounds without a proper spectral library. Thus, the published scientific works started to seek the potentiality of Mass Spectrometric detection applied to dyestuffs analysis (Marras, 2015). In 2008, Rosenberg published a comprehensive review on the characterization of natural organic dyestuffs of historical interest by LC-MS, which mainly discussed the structures of the most important natural organic dyestuffs traditionally used and their analytical determination focused on the mass spectral fragmentation patterns of the different classes of dyestuffs (Rosenberg, 2008). Almost in the same time, Zhang and Laursen succeeded to characterize dyestuffs in ancient textiles from Xinjiang, China, which confirmed the effectiveness of using HPLC with photodiode array and mass spectrometric detection to detect the blue, red and yellow dyes in pre-historic textile (Zhang, Good, & Laursen, 2008).

Even though the development of HPLC has been over 30 years, we are still facing a lot of difficulties as well as uncertainties when conducting a complete characterization of some organic dyestuffs occurred in historical and art objects. In Volodymyr Pauk's review article, he suggested that to use HPLC for the evaluation of authenticity of historical artifacts, especially to investigate degradation process of natural colorants. Additionally, It is also useful to search for new specific components, markers of authenticity and characteristics for synthesized or natural colorants. From the aspect of detection, taking advantage of new instruments, e.g. mass spectrometers with high resolution and accuracy, or those with ion mobility separation units, is expected since they can improve identification or overcome isobaric interferences and support isomeric discrimination (Pauk, Barták, & Lemr, 2014).

Despite the advancement of chromatographic techniques, extraction methods of natural organic dyes from textiles also play a vital role in dye analysis. In order to obtain better results of analysis, one may need to fully know about the chemical structure or properties of dyestuffs involved, and select the best extraction method of the dyes before the chromatographic work. Historically, the standard procedure of dye extraction was proposed by Wouters and Verhecken in 1985. It is an HCl-based method for dye extraction from wool dyed with several coccid insect dyes. Wool dyed fibers were hydrolysed in a mixture of 37% HCl/MeOH/H2O (2:1:1, v/v/v) placed in a boiling water bath for 10 min, and after dried in vacuum, the residue was re-dissolved in an appropriate volume of H2O/MeOH (1:1, v/v). Although losses during manipulations are avoided and good extraction yields can be obtained, the use of an HCl-based extraction procedures leads to information loss (fiber destruction and chromophore glycosidic bonds break), and thus some mild procedures have been developed (Manhita, Ferreira, Candeias, & Dias, 2011).

In the attempt to overcome these problems several investigations have been carried out in the following years. Even though each of them uses a different pathway to dissociate the colorants from their complexes, all the methods are based on a common approach: a complexing agent (e.g. F- ions, EDTA or HCOOH) competing with the dyes for the metallic ion (e.g. Al3+ in case of alum mordanted lakes). In 1995 Tiedemann and Yang presented a milder procedure consisted in heating the sample with 0.1% H2EDTA in H2O/DMF (1:1, v/v) at 100°C for 30 min in a boiling bath and then cooling it rapidly. This H2EDTA/DMF extraction was as powerful as the traditional HCl method and well preserve the sample structure for further investigation (Marras, 2015). Few years later, as Sanyova and Reisse

pointed out, milder extraction methods which would minimize unwanted effects such as acid hydrolysis, decarboxylation or methylation, but still extract alizarin, purpurin and other colorants as quantitatively as possible were desirable. The use of hydrofluoric acid solutions for the extraction of anthraquinones from their aluminum complexes in madder lakes fulfilled the hope of non-destructive extraction of pseudopurpurin, glycosides and other labile molecules, and it proved to be at least as efficient as HCl for alizarin and purpurin (Manhita et al., 2011).

In 2005, Zhang and Laursen developed and improved two mild textile extraction methods that used ethylenediaminetetraacetic acid (EDTA) and formic acid and were efficient in extracting dyes, with good preservation of glycosidic linkages. In the study, the methods were tested on silk dyed with yellow and red natural dyes. For the flavonoid dyes on silk, EDTA method seemed to be more efficient, while formic acid procedure was more adequate for the anthraquinone-type dyes. The formic acid and EDTA extraction methods not only gave higher extraction yields than did HCl procedure but also more information about the nature of the original dyestuff could be obtained (ZHAnG & Laursen, 2005).

In general, the main extraction methods can be concluded as HCl/MeOH/H2O method by Wouters, HCl+MeOH/DMF method by Surowiec, Formic acid and Na2EDTA/ACN/MeOH method by Zhang and Laursen, Na2EDTA in H2O/DMF by Tiedemann and Yang, Oxalic acid method by Guinot and Andary. Thus, a systematic comparative study of five different dye extraction procedures based on hydrochloric, citric, oxalic and trifluoroacetic acids (TFA) and an HCOOH/H2EDTA mixture was presented by Valianou et al.. All five extraction methods were tested on dyed wool with red and yellow

mordant dyes (madder, cochineal, weld and young fustic), a yellow direct dye (turmeric) and a blue vat dye (woad) and also on historical textile samples. Particular working conditions were established after a vast study, but in general, wool samples were treated in a boiling water bath with a mixture in the adequate proportions of the respective acid:MeOH:H2O, left to dry, and the residue reconstituted with DMSO. In the case of HCOOH/H2EDTA mixture, EDTA solution was added to the initial mixture after wool extraction of 5 min with formic acid. The upper solution was then isolated in a second tube and dried, and the procedure was repeated to treat the residue of the first tube. TFA method provided elevated extraction yields for most of the studied dye components and was selected to be used on historical samples, where it was successfully applied in the identification of several coloring compounds, some of which could not have been detected if the HCl method was used (Valianou, Karapanagiotis, & Chryssoulakis, 2009).

Therefore, well developing mild extraction of natural dyes in the pretreatment part, together with chromatographic works analyzed by High Performance Liquid Chromatography (HPLC) completed by the detection equipment, such as: UV-visible range and/or Mass Spectrometry (MS), has contributed to an improved characterization of compounds and the molecular structures of natural organic dyes and also their degradation processes.

## 2. General Introduction to the Thesis Work

As we have known from the introductory part above, natural organic dyes have been involved in human activities over centuries. Obtaining from natural resources, dyestuffs were directly employed to impart color to a variety of artifacts such as textiles, ointments, cosmetics and artworks. With the advancement of technology, people learned to produce dyes from these raw materials containing desirable chromatic characteristics. However, among all the possible topics in the field of historical art materials, one of the most interesting and fascinating is the study of natural organic dyes, with the main reason that it reveals the ancient connection between art and chemistry. The knowledge of "chemistry" we have nowadays was actually called "alchemy" in the ancient times. And the artists of the past were full professionals who must be provided, as well as of artistic talent, of a full mastery of the materials and their properties and of a very good practice in their manipulation. There are at least three significance to develop a study focusing on the advancement in the identification of natural organic dyes from historical textiles. The first is to help improving the conservative or restorative treatments of historical textiles. The second is related to the study of the chemical composition of dyes, which can help to better understand the degradation processes of organic dyes in textiles. Last but not least, the identification of dyes from different historical periods and regions may contribute to the determination of dating, provenance study or in some case confirm the attribution to a specific artist or school (Marras, 2015).

Apart from the practical reasons above, the studies and analysis of natural dyes are also of great significance in the collaboration of trans-disciplines conducting relevant artistic,

archaeological, historical and ethno-anthropological researches. The study of ancient dyes, together with the increasing knowledge on historical materials, could provide useful information for historians, who can know more about the ancient artist's and craftsman's techniques, the commercial routes for the trade and the distribution of these materials. Since dyes were extremely valuable goods, their identification in an artwork could also give information about the social status of the owner (Marras, 2015).

However, the identification of natural organic dyes in historical textiles is a real analytical challenge due to many factors that have already briefly mentioned in the previous chapter (*"1.3 Scientific Development of Dyes Analysis in Ancient Textiles"*). To be more specific, natural organic dyes are complex mixtures of a big variety of substances characterized by different structures (e.g. anthraquinones, flavonoids, etc.) and different properties (e.g. hydrophilic, hydrophobic), expected to be present also in the samples collected from historical textiles. Most of these compounds are barely distinguishable due to their chemical similarity and the fact that some of them are structural isomers differing on the position of a functional group (positional isomers). In addition, the limitation of collecting a small amount of samples causes the problem that the very low concentrations of dyes on the samples are difficult to be detected when analyzing. Moreover, the degradation process seriously complicates the identification of dyes by substitution of original compounds with their degradation products. Natural dyestuffs also include in their compositions very labile substances which are easily affected by hydrolytic and photo-oxidative degradation processes. Thus, the combination of an efficient extraction procedure,

chromatographic separation and the use of a high resolution detector, is required to obtain the selectivity and sensitivity necessary for the thesis work.

The aim of the research in this thesis is to test and select the method that can retain more information about the chemical composition of natural organic dyes in historical textiles relating to its plant and insect sources and to the dyeing process by analytical techniques. In the preparation stage of dye analysis, in order to preserve as much chemical information of dye compounds as possible, a water/organic (ACN) solvent system is employed to extract dyes from both raw materials and silk textiles as standards and real samples respectively. Since natural organic dyes are very complicated mixtures of compounds, three different chromatographic programs in Liquid Chromatography-Mass Spectrometry (LC-MS) are tested and compared for achieving the best separation result in reference samples of Madder, American Cochineal, Brazilwood, Weld and Rhamnus. The best chromatographic program is applied for the analysis of dyes on real sample (the silk textiles stained with Madder, American Cochineal and Rhamnus). Identification of all dye compounds is based on 3 parameters: retention time (elution sequence), absorption in UV-vis spectra and mass spectra. Additionally, by comparing the database created by the raw dyestuff samples as references, both relevant chemical and historical information of real samples may be obtained.

The innovative aspect of this study can be the use of a powerful analytical instrument: The Liquid Chromatography - Mass Spectrometry with Photodiode Array Detection, which provides the high resolution, selectivity and sensibility required to achieve such challenging analytical goal. In particular, this LC-PDA-MS method is also reported for the

characterization of the biomarkers in ancient samples of dyes. After extraction of dyes, different compounds such as anthraquinonoids and flavonoids can be identified through the chemical constituents present in the samples. And these compounds are identified through retention times and UV spectra and mass spectra in comparison with pure standards. Moreover, a mild extraction protocol has been used with the purpose of maximize the information obtainable from the micro-samples. The application of such pre-treatment procedure, which combines a soft de-complexation with an effective extraction of organic components, allowed the identification of both aglycones and corresponding glycosides characterizing the different dyeing species considered.

This work in general reports the methodology and the results of the analytical characterization of natural organic dyes. The planned approach basically involves three steps: (1) the extraction and preparation of dyes from both raw materials and silk textiles; (2) the analysis of the above mentioned dyes by means of Liquid Chromatography coupled with Photodiode Array and Mass Spectrometric Detection; (3) the validation of the methodology by application to real samples of dyes from historical Turkish and Mediterranean origins. Meanwhile, a complete characterization of dyes by means of optimized chromatographic and spectroscopic techniques has been carried out resulting in the creation of a comprehensive reference spectra database of colored compounds from the most common red and yellow natural organic dyes.

Most importantly, however, the structure of this thesis, which can be seen as the logic of the whole text, starts with an introductory section (Chapter 1) that briefly explains the history of natural dyes in the ancient Mediterranean world from a European perspective,

the cultural significance of dye studies and the scientific development of dye analysis. This will indicate what is natural dyes, how dyes and relevant techniques got developed through times, why we need to study them and by which methods people can analyze them. Thus, with these questions, the thesis work is created with very clear background information, challenges, aim, innovative points, and well-defined contents (Chapter 2). The theoretical part is largely developed into Chapter 3 where the color theory, classification of dyes according to their chemical structures and analytical chemistry in dye studies are presented to be as the basis of further analysis in the experimental part (Chapter 4). In experimental section, all chemicals and materials, techniques and procedures applied in the different phases of lab works, including the preparation of both standard and silk dyeing samples procced with specific preparation/extraction procedures and also the chromatographic analysis of samples. Chapter 5 is reserved to be the discussion of experimental results with a focus on qualitative studies. The identification of compounds in standard red and yellow dye allows to create the database for further characterization and comparison in real samples. The selection based on the analytical effectiveness of 3 chromatographic programs, however, is essential for the chromatographic analysis of real samples. And with the selective program applied in the silk textiles, the qualitative analysis of natural organic dyes can be conducted. The last chapter is the conclusion, followed by reference list at the end (Chapter 6& Reference).

# 3. Basic Chemistry of Natural Organic Dyes

## 3.1 Color Theory

The color is a very complex phenomenon that involves both human that perceive the chromatic sensation and the object that is perceived as colored. As an interpretation created by human's mind to describe a property of objects, the concept of color helps us acquire knowledge about the properties of the surfaces. To build the colors, our mind analyzes ratios of signals in retinal photoreceptors. The color and luminance play different roles in our perception of art and real life, because they are analyzed by different subdivisions of our visual system. The normal human eye has three different types of cones with photo-pigments that are sensitive to three different areas of the spectrum. Each cone is programmed to be sensitive either to long waves (red), medium (green) or short (blue) and are referred to as cones-L,-M, or-S. Light of any wavelength in the visible spectrum from 400 to 700 nm will excite one or more than one type of sensor. Our mind determines the color by comparing the signal of each type of cone. Furthermore, humans perceive color when the different wavelengths composing white light suffer interference by matter (absorbed, reflected, refracted light, scattered, or diffracted) or when non-white light is emitted from a source. Visible light is a small part of the electromagnetic spectrum, which extends from the high-energy cosmic rays passing through gamma rays, x-rays, ultraviolet, visible, infrared to the low energy microwaves and radio waves. (Details shown in Fig.1) Humans detect the range of the visible spectrum between 380 nm (violet) and 740 nm (red) viewing a continuum of color: the Rainbow. Our perception of color also depends on the

type of light source, i.e. the average distribution of photons with different wavelengths.

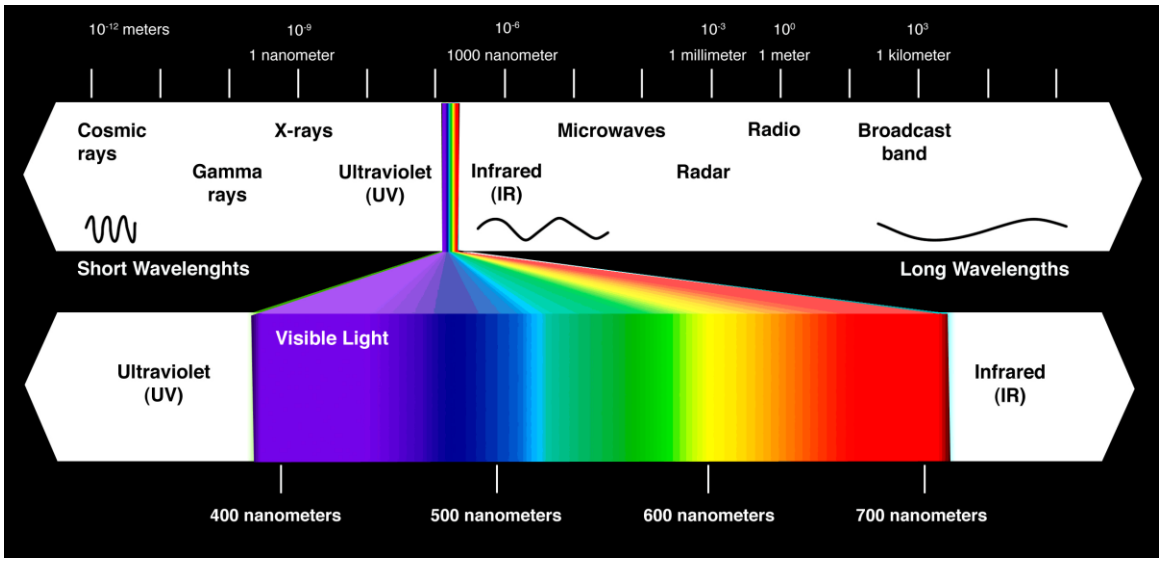



**Fig. 1** The Electromagnetic Spectrum

The color is also the part of perception that is carried to the eye from our surroundings by differences in the wavelengths of light. During this process, the absorption of electromagnetic radiations in the UV and visible regions by a molecule causes the electronic excitation, where an electron moves from a low electronic energy level to a higher one. (See **Fig. 2**) A covalently unsaturated group responsible for absorption in the UV or visible region is known as a chromophore. For example, C=C, C≡C, C=O, C≡N, N=N, NO2 etc. If a compound absorbs light in the visible region (400–800 nm), only then it appears colored. Thus, a chromophore may or may not impart color to a compound depending on whether the chromophore absorbs radiation in the visible or UV region. In addition, chromophores like C=C or C≡C having π electrons undergo π → π* transitions and those having both π and non-bonding electrons, e.g., C=O, C≡N or N=N, undergo π → π*, n → π* and n → σ* transitions. Since the wavelength and intensity of absorption depend

on a number of factors, there are no set rules for the identification of a chromophore (Gürses et al., 2016).

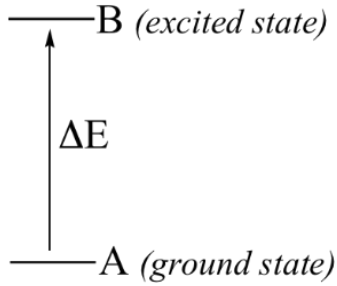



**Fig. 2** The Scheme of Electronic Excitation

However, a covalently saturated group, when attached to a chromophore (substituents), changes both the wavelength and the intensity of the absorption maximum, is known as auxochrome, e.g., NH2, OH, SH, halogens etc. Auxochromes generally increase the value of λmax as well as εmax by extending the conjugation through resonance. These are also called color enhancing groups. An auxochrome itself does not show absorption above 200 nm. Actually, the combination of chromophore and auxochrome behaves as a new chromophore having different values of λmax and εmax. (See in **Fig. 3**) Common auxochromes include alkyl (-R), hydroxyl (-OH), alkoxy (RO-) and amino (-NH2) groups and halogens (Cl, Br, I, F) as well. When two or more chromophoric groups are conjugated, the absorption maximum is shifted to a longer wavelength (lower energy) and usually to a greater intensity compared to the simple unconjugated chromophore. In general, the longer the conjugated system, the higher are the values of λmax and εmax. Thus, a compound with sufficient conjugation absorbs in the visible region (400–800 nm) and becomes colored (Gürses et al., 2016).

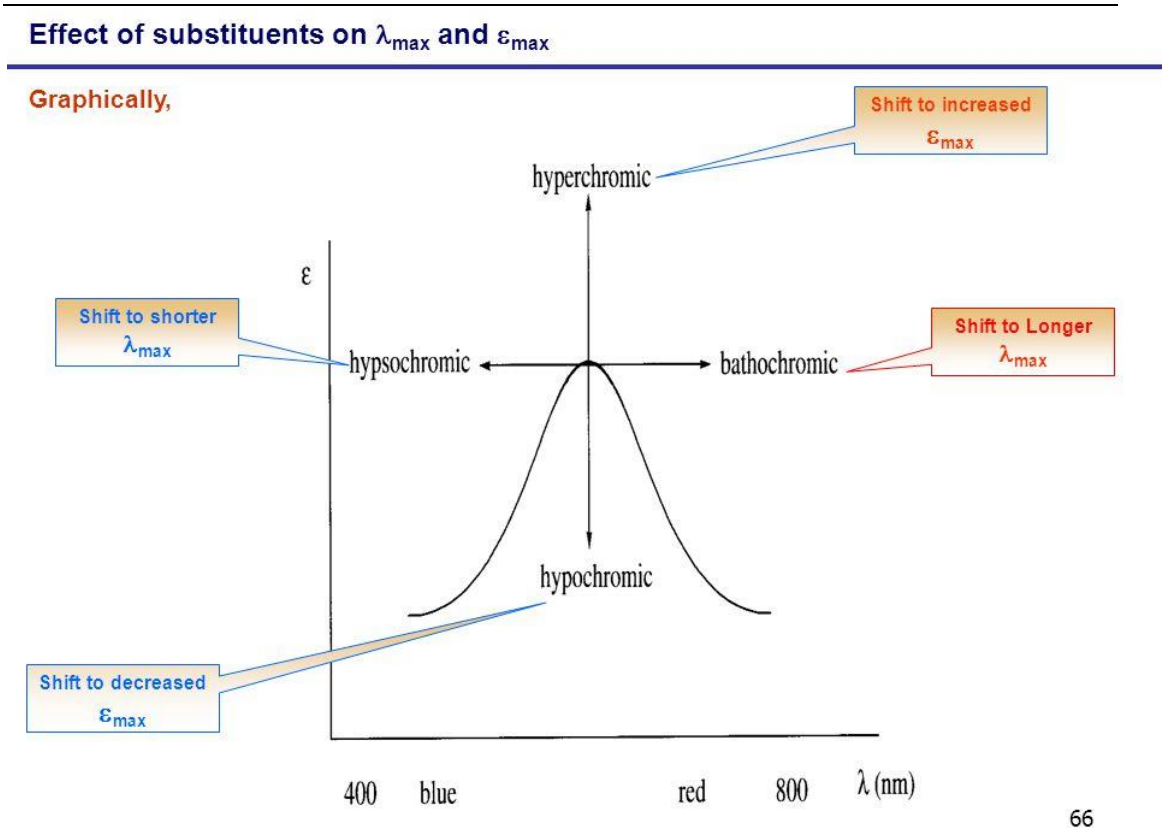



**Fig. 3** The Effect of Auxochromes

To be summarized, the organic dye molecules contained three main components refer to color, such as: chromogen, chromophore and auxochrome. (1) The chromogen is a chemical compound that is either colored or could be made colored by the attachment of suitable substituent. The chromophore and the auxochrome(s) are also part of the chromogen. (2) The chromophore (from the Greek words, “chroma” and “pherein” mean “color” and “to bear” respectively) is a chemical group that is responsible for the appearance of color in compounds (the chromogen) where it is located. The colorants are sometimes also classified according to their main chromophore. (3) The auxochrome (from the Greek word, “auxein” means “to increase”) is a function that intensify the color of the molecules increasing by shifting the absorbed wavelength value to a longer one value (bathochromic effect). The auxochromic groups also have the function to make the

molecule soluble (or to increase its solubility) and, as said before, to convert them in dye. Sometimes is directly responsible of the color because of the shifting of this absorption value from the ultraviolet area to the visible one and making colored a previously uncolored substance (Gürses et al., 2016) (Marras, 2015).

Therefore, based on this color theory, quantitative analysis of organic compounds that absorb UV/Vis radiation can be conducted. According to Beer-Lambert Law, the absorbance of a chromophore at a given wavelength varies in a linear fashion with its concentration: the higher the concentration of the molecule, the greater its absorbance, which is formulated as: $A = \varepsilon \cdot b \cdot c$, where "A" is measured absorbance, "ε" is wavelength-dependent absorptivity coefficient (M-1 cm-1), "b" is path length/width of the cuvette holding the sample (cm) and "c" is analyte molar concentration (M). When doing the analysis, we firstly need to prepare standard solutions of known concentration and measure the absorbance of standard solutions at λmax. Then, after plot "Absorbance vs Concentration" and obtain the slope, we can use slope to determine the concentration of the analyte in the sample solution. (See the scheme in **Fig. 4**) However, there are some limitation in terms of the application of Beer-Lambert Law: (1) deviations in absorptivity coefficients at high concentrations (>0.01M) due to electrostatic interactions between molecules in close proximity; (2) scattering of light due to particulates in the sample; (3) fluorescence or phosphorescence of the sample; (3) changes in refractive index at high analyte concentration; (4) shifts in chemical equilibria as a function of concentration; (5) non-monochromatic radiation, deviations can be minimized by using a relatively flat part of the absorption spectrum such as the maximum of an absorption band; (6) stray light.

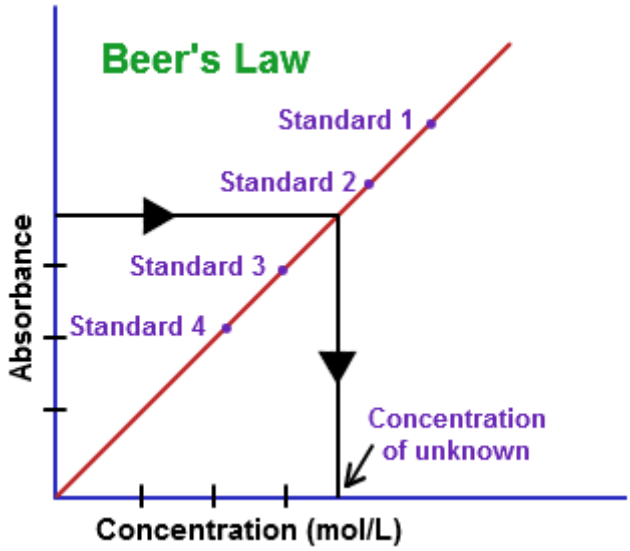


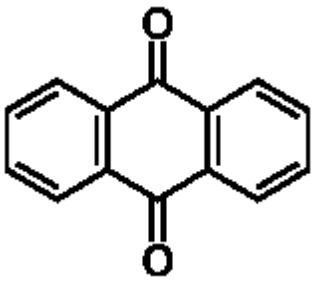

**Fig. 4** The Scheme of Beer-Lambert Law

Overall, color theory, combining with Beer-Lambert Law, is an essential foundation for the application of UV/Vis Spectrometry in cultural heritage, especially for both qualitative and quantitative analysis of natural organic colorants.

## 3.2 Dye Classification According to Chemical Structures

Classifying dyes according to the chemical class their chromophores belong to is useful for the analyst in order to choose the right sample extraction procedure to recover the chromophores from historical samples and to develop the right identification procedure. Four main and other dyes are described.

***Anthraquinoid Dyes***



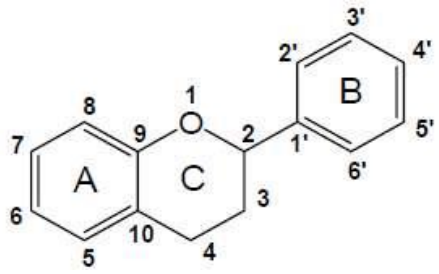
Antraquinones are derivatives of the 9,10-anthracenedione molecule (anthraquinone) which basic structure is characterized by an anthracene core which presents a quinonic group. The different anthraquinonoid compounds differ in the nature and positions of substituent groups. They can be divided into two wide classes, alizarin and emodin types, depending on their biosynthetic pathway and substitution pattern. The alizarin type anthraquinones have only one of the rings unsubstituted and are formed via chorismate/δ-succinylbenzoic acid pathway. They are mainly found in *Rubiaceae* plants (such as *Rubia, Morinda, Galium* and *Cinchona*). The emodin type anthraquinones have instead both rings substituted and are formed through the polyketide pathway (acetate- malonate pathway) (Marras, 2015).

From the technical point of view, anthraquinones can be mordant, acid or vat dyes although the great majority belongs to the first category. In the vat and acid applications of anthraquinonoid dyes, tends to predominate the violet, blue and green hues. The mordant ones use to be polygenetic, that means that the resulting lake color can vary considerably depending on the mordanting agent (in this case from red to purple or blue) (Marras, 2015).

According to the molecular structure, the mordant-dye complex is established by means of the carbonyl group and the adjacent phenolic group. The most famous anthraquinone dye is the alizarin which can form crimson red lakes with calcium, barium and strontium, pink-red lakes with alum, brown-violet lakes with chrome and copper, and black-violet lakes with mercury and iron III. All the main red colorants obtained from vegetal and animal sources belong to this class. Anthraquinone dyes are generally characterized by a good stability to light and to washing (Marras, 2015).

Anthraquinoid dyes have been used as reds since antiquity due to their abundance in nature (several insects and roots contain anthraquinones). A number of red organic dyes are extracted from the roots of different species of the *Rubiaceae* family. They contain anthraquinones along with their glycosides. The main species used for dyeing purposes in the West are madder (*Rubia tinctorium* L.), wild madder *(Rubia peregrina* L.), and Lady's bedstraw (*Galium verum* L.). Other species, such as munjeet (*Rubia cordifolia* L.) and different species of *Relbunium*, were used in the East and in America. Madder lake is one of the most common and ancient lakes and was found in archaeological objects as a coloring material. In addition to plants source, an important number of red anthraquinoid dyes can be extracted from insects belonging to the *Coccideae* family, which are common plant parasites. Their main constituent is carminic acid. Depending on the specie, they contain minor components whose detection can help in identifying the source of the dye. The main known species are kermes (*Kermes vermilio Planchon*); various kinds of cochineal, such as Polish cochineal (*Porphyrophora polonica* L.), Armenian cochineal (Porphyrophora haemli Brandt), American cochineal (Dactylopius coccus Costa); and Indian lac, or lac dye (*Kerria lacca Kerr*). However, it is worth noting that the anthraquinone structures found in plants are only substituted on one aromatic ring, whereas the insect-derived ones are substituted on both (Illaria Degano et al., 2009).

***Flavonoid Dyes***




The flavonoids are poliphenolic compounds derived from the 15-carbon-based flavonoid backbone. This basic structure is composed of three circle generally called A,B and C and specifically by an hexagonal heterocyclic ring (C) to which two aromatic rings (A, B) are attached. The A ring is condensed to C while the B ring is linked to C in position 2. Within this common structure the degree of oxidation and the chemical nature of the substituent on the three rings differentiate the different compounds (more specifically the degree of oxidation and substitution pattern of the phenyl ring (C-ring) identifies the different classes of flavonoids) (Marras, 2015).

Flavonoid dyes mainly contain flavones (a) and isoflavones (b) (See in Fig. 4) and yield yellow shades, but depending on their structures, red and dark flavonoid dyes can be obtained as well. Flavonoids are ubiquitous in the plant kingdom, especially in their glycosylated forms, and have been used since antiquity as mordant dyes. Although their aglycones form stable complexes with mordants through the phenolic moieties and the C3 carbonyl group, they are quite labile dyes, due to their sensitivity to photooxidative processes and bleaching. Hydroxybenzoic acids have been suggested as being the degradation products of flavonoid photooxidation. It should be noted that dyes containing

flavones are more resistant to fading than the ones containing flavonols. The amount of

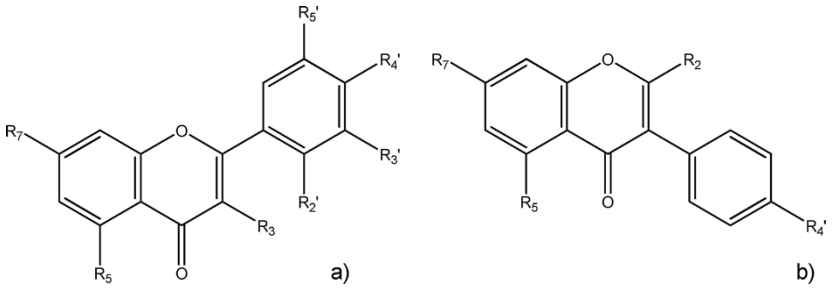



**Fig. 4** Structure of flavonoids

coloring material surviving in historical and archaeological materials is therefore quite low and there may be degradation products. For this reason, and due to the fact that the same flavonoid may be contained in a number of different species, assessing the dye source requires care and skill. The identification of their glycosides or secondary components may be a more reliable way to recognize the origin of the dyeing material (Illaria Degano et al., 2009).

Flavonoid Dyes mainly include both yellows and soluble/insoluble redwood dyes. From a European perspective, the main sources of flavonoid yellows are weld (*Reseda luteola* L.), young fustic (*Cotinus coggyria Scop.*), dyer's greenweld (*Genista tinctoria* L.), sawwort (*Serratula tinctoria* L. Gaud.), and the berries of some species of Rhamnus (Ferreira, Hulme, McNab, & Quye, 2004). Redwood dyes can be divided into those that are soluble and insoluble. Soluble redwood dyes contain omoisoflavonoids and are extracted from the bark of various species of the genus *Caesalpinia* (*Caesalpinia sappan, C. brasiliensis, C. crista, C. echinata*), which grows both in southern Asia and South America. While the main insoluble redwoods are sandalwood (*Pterocarpus santalinus* L.), imported from South India; narrawood (*Pterocarpus indicus* Wild.), native to the forests of

Burma and the Philippines; barwood (*Pterocarpus soyauxii* and *Pterocarpus erinaceus*) and camwood (*Baphia nitida*), which are West African species (Illaria Degano et al., 2009).

### *Indigoid Dyes*

Indigoid dyes are applied as vat dyes and give blue and purple shades. The most commonly used indigoid dyes are Tyrian purple and indigo, whose discovery dates back to several thousand years ago. They were also used as lakes, by absorbing the coloring material onto white chalk-containing clay or just by solving the chromophores into an appropriate binding media.

They consist of complex mixtures of indigoid chromophores, whose structures are depicted in Fig. 5. The chromophores are not present in the plant or the animal source but are produced during the fermentation of the dyeing vat.

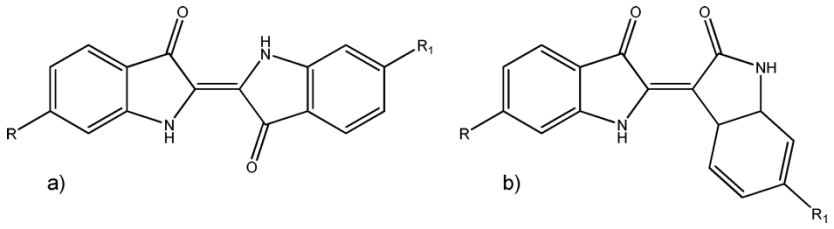
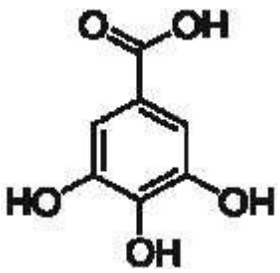



**Fig. 5** Indigoid chromophores: (a) R1, R2 = H, indigotin; R1 = H and R2 = Br, 6-monobromoindigotin; R1 , R2 = Br, 6,6′ -dibromoindigotin; (b) R1 , R2 = H, indirubin; R1 =H and R2 = Br, 6-monobromoindirubin; R1, R2 = Br, 6,6′ -dibromoindirubin.

### *Tannins*




Tannins are polyphenolic compounds very common in vegetal world. They are characterized by high molecular weight, solubility in water (with the exception of some high molecular weight structures) and ability to form with proteins insoluble or soluble complexes. Tannins have been largely used in history in the leather industries for the tanning process and in the artistic world for the production of pigments and inks such as the famous Iron gall ink. They are divided into two groups, hydrolysable tannins and proanthocyanidins, depending on their structure. Hydrolysable tannins have a carbohydrate (generally *D*-glucose) as central core, a 6 to 9 galloyl units skeleton, and presents partial or total esterification of hydroxyl groups by means of phenol molecules. On the base of the phenol involved in the process we can classify the hydrolysable tannins into two main groups: gallotannins (gallic acid), ellagitannins (ellagic acid). Their name derive from their tendency to easily be hydrolysed (forming carbohydrate and phenolic acids) by mild acids and bases, hot water and enzymes action (Marras, 2015).

Proanthocyanidins are much more widely spread in nature than hydrolysable tannins. The name derives from their capacity to produce anthocyanidins via acid catalyzed oxidation reaction. They are oligomers or polymers of flavonoid units (2 to 50) linked by carbon- carbon bonds. Unlike the hydrolysable tannins they are not susceptible to cleavage by hydrolysis. They are also known as condensed tannins due to their condensed chemical structure. As the flavonoid units can present different substituents and the position on the

interflavan bonds can vary in the molecules, proanthocyanidins are usually characterized by complex structures. They can be water soluble or insoluble depending on their chemical structure. Nowadays are preferentially included into the flavonoids class (Marras, 2015).

***Other Dyes***

Some violet and red dyes were extracted from lichens. The most famous one is orcein, occasionally used to obtain a fake purple color: dyeing with lichens was cheaper than using shellfish products. Depending on the applied mordant, red or purplish shades can be obtained. The dye consists of a complex mixture of chromophores; the main ones are trans- and cis- isomers of α-, β-, and γ -amino-hydroxy-orcein. Another important group of dyes, although of minor and local importance, are polymethine dyes, widely used as direct dyes, especially for cotton, wool, and silk in combination with other dyes. The main representatives of this class are saffron, turmeric, and annatto (or Orellana) (Illaria Degano et al., 2009).

## 3.3 Analytical Chemistry in Dye Studies

As a branch of Chemistry, analytical chemistry in dye studies is aiming at the study of the separation, identification and quantification of chemical components of both natural and artificial materials, which further promotes the qualitative analysis that identifies the chemical species in the sample and quantitative analysis that determines the concentration of one or more of these components. However, in order to proceed the analysis, the primary stage refers to the separation of chemical compounds. Thus, analytical chemistry requires

the study of the methods and their different techniques to solve analytical problems (Varella, 2012).

Chromatography is the most general separation method used for analysis of natural organic dyes. According to the definition from International Union of Pure and Applied Chemistry (IUPAC), 'chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (the stationary phase), while the other (the mobile phase) moves in a definite direction. A mobile phase is described as "a fluid which percolates through or along the stationary bed in a definite direction". It may be a liquid, a gas or a supercritical fluid, while the stationary phase may be a solid, a gel or a liquid. If a liquid, it may be distributed on a solid, which may or may not contribute to the separation process.' And a standard chromatographic system should basically consist of four component parts: (1) A device for sample introduction (2) A mobile phase (3) A stationary phase (4) A detector (Ardrey, 2003).

Thus, to be summarized, the principle of chromatography is based on the concentration equilibrium of the compounds between the stationary phase which is immobilized in a column, and the mobile one passing through the system. Then the differential separation of the several constituents or analytes all along the column leads to their separation. Sensitive detectors permit the analysis of very small quantities (a few nanograms). The signal is registered and leads to a chromatogram indicating the variation of the composition of the mobile phase with time at the end of the column (Varella, 2012).

A number of different chromatographic techniques are in use and these differ in the form of these four components and their relative importance. The two components which

are associated with the separation that occurs in a chromatographic system are the mobile and stationary phases (Ardrey, 2003). High Performance Liquid Chromatography (HPLC) is based on the selective partitioning of the molecules of interest between two different phases. The partition mechanisms between analytes, mobile phase and stationary phase are based on coefficients of adsorption or partition. The isocratic mode means that the mobile phase (solvent or combination of solvents) has a fixed composition. A gradient elution has the composition of the mobile phase varying with time. While travelling through the column under high pressure, analytes are separated selectively between the mobile phase and the stationary phase. If the stationary phase is polar (silica gel), then a less polar mobile phase is used and the technique is called normal phase chromatography. If the stationary phase is non polar (bonded silica), a polar mobile phase is then used, generally water with co-solvent (methanol or acetonitrile) and the technique is referred to as reversed phase chromatography (Varella, 2012).

In fact, a detector is usually placed at the end of the column to conduct either quantitative or qualitative analysis of samples. In fact, a type of analysis (quantitative or qualitative) closely determines the detectors that may be used in conjunction with HPLC. For qualitative (identification) applications, it depends upon the comparison of the retention characteristics of the unknown with those of reference materials. In the case of gas chromatography, this characteristic is known as the retention index and, although collections of data on ‘popular’ stationary phases exist, it is unlikely that any compound has a unique retention index and unequivocal identification can be effected. In liquid chromatography, the situation is more complex because there is a much larger number of

combinations of stationary and mobile phases in use, and large collections of retention characteristics on any single 'system' do not exist. In addition, HPLC is a less efficient separation. While quantitative accuracy and precision often depend upon the selectivity of the detector because of the presence of background and/or co-eluted materials (Ardrey, 2003). The most widely used detectors in HPLC are based on the optical properties of the analytes: refractive index, absorption and fluorescence. The refractive index detector is a universal detector, responding to any eluted compound. Detectors based on the absorption of light in the ultraviolet and visible ranges (UV/VIS detectors) are the most commonly used, responding to a wide variety of compounds with good to excellent sensitivity. However, the selectivity of UV/VIS detector is not enough in some applications as it normally gives rise to relatively broad signals, and if more than one component is present, these overlap and deconvolution is difficult. Besides, the photodiode array detector (PDA) not only permits to obtain the retention time but also the UV/VIS spectrum of each of the components being analyzed. It is essential of course that the mobile phase be transparent in such conditions. Fluorescence detectors has more selectivity, since both absorption and emission wavelengths are utilized, but is only applicable to a limited number of analytes, even when derivatization procedures are used. Finally, the mass spectrometer detector provides both quantitative information and in most cases a definitive identification of each component (qualitative information) (Ardrey, 2003).

Based on the HPLC system, Liquid Chromatography/Mass Spectrometry (LC/MS) is also applied as a powerful analytical technique that combines the resolving power of Liquid Chromatography (LC) with the detection specificity of Mass Spectrometry (MS). In general,

Liquid chromatography (LC) separates the sample components and then introduces them to the mass spectrometer (MS), where charged ions are created and detected. LC/MS data may be used to provide information about the molecular weight, structure, identity and quantity of specific sample components. During the process of chromatographic analysis in LCMS, solvent from the pumping system is forced through the HPLC column and dissolved sample is injected into the flowing stream. The material dissolved in the mobile phase interacts with the packing material and equilibration separation occurs as the material moves down the column. Then, the separated compounds elute off the column at different times enter the interface where solvent is evaporated and the compounds are ionized, and are then pulled into the evacuated mass spectrometer. Electrical lenses focus the charged beam of ions and carry them into the mass analyzer. They are swept down the analyzer by a scanning direct-current/ratio frequency signal that selects ions of particular mass/charge (m/z) value to strike the detector face and trigger a signal. Finally, the signal is combined in the computer with control information that it is sending to the mass spectrometer to create a three dimensional array of signal strength versus time versus m/z information for storage and processing. In fact, there are two types of elution methods, including isocratic and gradient elution. In isocratic elution, the mobile phase does not change in composition during the run, and it is usually preferred for separating simple mixtures of compounds. While in gradient elution, the mobile phase is changed in a regular and reproducible manner. It is usually preferred for separating complex mixtures of compounds and for washing out late-running peaks. They run slowly in order to be reproducible.

As for the interface of LCMS, its main functions refer to volatilize and remove the unwanted eluent solvent and its additives without removing the target compounds of interest, in order to reduce the total input to the mass spectrometer, if the sample concentration is high enough and allow the signal to be detected by the analyzer. In addition, the interface can also ionize the target compounds of interest (since most components in the HPLC eluent are uncharged) so they can be drawn into the high-vacuum environment of the mass spectrometer analyzer. In modern LC/MS systems solvent elimination and ionization steps are combined in the source and take place at atmospheric pressure (AP). API techniques are relatively "soft" ionization techniques. Common types of interfaces used in LCMS are Electrospray ionization (ESI) and Atmospheric pressure chemical ionization (APCI). ESI is usually applied to non-volatile and thermally unstable highly polar compounds, while APCI is applied on volatile and thermally stable compounds of low or medium polarity. (See in **Fig. 5**)

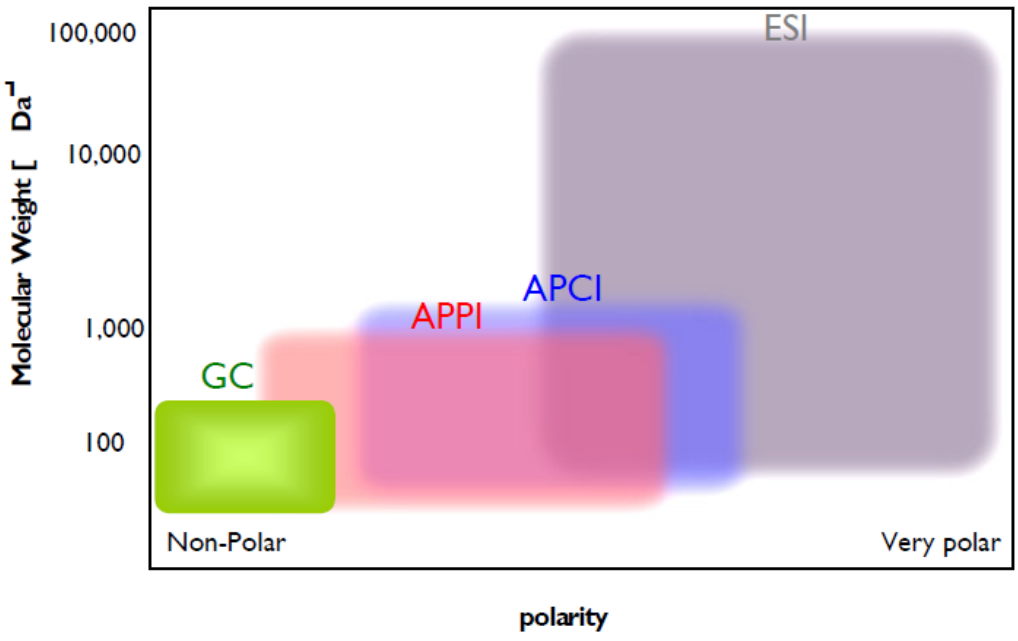



**Fig. 5** Application of the various LC/MS techniques

Therefore, Liquid chromatography - mass spectrometry has demonstrated its usefulness for the analysis of natural organic dyes in many examples reported in the literature. And with certain techniques in terms of detection and interface of LCMS, this analytical approach can be greatly applied into several chemical investigations, including: molecular weight determination, structure elucidation, qualitative analysis, quantitative analysis and impurities detection.

## 4. Experimental

### 4.1 Chemicals & Materials

All chemicals used were of HPLC LCMS grade purchased from HiPerSolv CHROMANORM (VWR International S.A.S., France). Raw dyestuff materials: Madder, American Cochineal, Brazilwood, Weld and Rhamnus were obtained from Merck (Darmstadt, Germany). The dyestuff materials were homogenized prior to analysis in dried form. Silk samples dyed with Madder, American Cochineal and Rhamnus by the operation of traditional textile dyeing recipes were obtained from Marmara University, Istanbul, Turkey.

### 4.2 Extraction of Dyes from Raw Materials as Reference Samples

Raw dyestuffs powder, was weighed in Eppendorf tubes (ranging from 0.4 mg to 0.7mg), and was dissolved in 1 mL of ACN/$H_2O$ (1:1 v/v) solvent system by using a SPIN-MICROPIPETTE. The treated solution was then put into an ultrasonic bath (Elma

Transonic T 310) for dyes' extraction for 20 mins at room temperature. The dye extracts were centrifuged at 12.000 rpm for 5 min, and the upper liquid was transferred into LCMS vials for analysis.

## 4.3 Extraction of Dyes from Silk Samples

Samples of silk dyed with Madder, American Cochineal and Rhamnus (2 reds, 1 yellow) were firstly weighed around 0.6 mg on Eppendorf tubes. The extraction method was mainly based on Zhang's (2005) pioneering work on "Mild Extraction", but a few changes have to be done according to specific conditions of our own samples and equipment. 0.4 mL of $CH_3OH$/HCOOH, 90/10 (v/v) were added into the tubes reacting with the silk samples inside, aiming at breaking the metal bond from mordant. The tubes were capped and kept in the water bath at 60°C for 30 min. After being cooled to room temperature, the tubes were uncapped and the solvent (formic acid and methanol) was evaporated under vacuum until dryness. The residues were then dissolved in 0.5 mL of ACN/$H_2O$ (1:1 v/v) solvent system and centrifuged at 12.000 rpm for 5 min. The upper liquid was transferred into LCMS vials for analysis.

## 4.4 Chromatographic Analysis

Analyses were performed using the Liquid Chromatograph – Mass Spectrometer LCMS-2010EV, Shimadzu. The chromatographic system was controlled with LabSolutions/ LCMS solution software. The components of the extracts were separated on an Altima™ C18 5u (250mm x 3.0mm) reversed-phase column, thermostated at 40ºC. The analytes

were monitored with a Photodiode Array Detector (PDA) and a Mass Spectrometer (MS) connected in-line and characterized by their retention times, UV-vis and mass spectra. In order to ensure universal elution conditions for chemically different compounds, three gradients of acetonitrile, methanol and water were used according to the literature. The chemical compositions of the identified dyes were confirmed by LC-PDA-MS, with Electrospray ionization operated in negative scan mode (ESI-) under the same chromatographic conditions. The UV signal was registered at 254nm, 350 nm (for yellow dyes), 430nm, 450nm and 490nm (for red dyes). The selective chromatographic programs for analysis as followed: (1) Program A is from Otlowska et al.'s (2017) newest work: "The mobile phase flow rate was 0.4 mL $min^{-1}$, and elution was performed using 0.1% (v/v) formic acid in water (solvent A) and ACN/MeOH (1:1; v/v) (solvent B) using composition gradient 10% B to 100% B in 20 min. The analysis was stopped after 30 minutes and the column was equilibrated for 10 minutes at 10% B. All mass-spectrometric data were recorded in negative ionization scan mode (m/z 50–1000)" (Otłowska, Ślebioda, Wachowiak, & Śliwka-Kaszyńska, 2017). (2) Program B is from Koliarmou (2015): The mobile phase consisted of 0.1% $H_2O$/HCOOH (A) and 0.1% ACN/HCOOH (B). A flow rate of 0.3 mL $min^{-1}$ was run in the following gradient: 95% A +5% B at the beginning, 70% A +30% B at 15 min, 40% A +60% B at 25 min, 5% A +95% B at 35 min, and 95% A +5% B at 42 min. (3) Program C is based on Manhita et al. 's (2011) work but with a change on the constitution of the mobile phase and the flow rate: the mobile phase consisted of acetonitrile (A) and 0.1% of aqueous HCOOH (v/v) (B). A flow of 0.5 mL $min^{-1}$ was used with the following gradient: 0–100% A from 0 to 20 min, 100% A from 20 to 30 min.

Between sample injections, a 5-min run of solvent B was used for column equilibration (Manhita et al., 2011).

## 5. Results& Discussion

In this chapter, the experimental results of 5 standard dye samples (Madder, American Cochineal, Brazilwood, Weld and Rhamnus) and 3 silk samples dyed with madder, american cochineal and rhamnus will be presented, including their chromatographic, mass spectrometric and UV-Vis properties. Details will be shown in tables, and further comparison on the results of 3 chromatographic programs will be made after the identification of compounds from each reference sample. Therefore, with this well-established database of the most common dyestuffs occurred in textiles through times, qualitative analysis can be done in the real samples. However, the results of this qualitative analysis will not just be used for the identification of dye compounds in silk samples treated by traditional textile dyeing recipes, but also for indicating the most comprehensive method regarding to the extraction and chromatographic separation suitable for the analysis of natural organic dyes.

### 5.1 Identification of Compounds in Standard Red Dyes

#### *Madder*

Although originated in India, Madder in particular was a very important red dye and in high demand that it was widely cultivated in Europe and the Middle East during historic periods.

Comparing to the anthraquinone insect dyes, it has less tinctorial power but with the advantage of producing shades from pinks to blacks, purples and reds with different mordants. As one of the most valued colors in history, Turkish red, a very bright red dye, was obtained from madder by a very complex method (involving about twenty individual steps) and was particularly suited for cotton dyeing (Ferreira et al., 2004).

Investigations on the chemical nature of the coloring principles in madder date back to the first half of the eighteenth century, leading to the identification of alizarin and its derivatives as important chromophores. It should be emphasized that alizarin is not always detected when extracting samples from historical artworks, whereas purpurin and/or other anthraquinoids typically are. This has prompted the interpretation that the absence of alizarin is an indication that the textile may have been dyed with *Rubia peregrina* (wild madder). It is therefore suggested that detailed measurements of the relative peak areas of the anthraquinoids be undertaken for the identification of the madder source, because alizarin may, even if being initially absent in yarn dyed with *Rubia peregrina*, occur due to the treatment of the sample with acidic alcoholic solution. Under such circumstances, the concentration of alizarin related to purpurin (the dominant chromophore in wild madder) would still be relatively low. This situation may also apply for other *Rubia* species. In addition, the invention of synthetic alizarin indicates that alizarin, as one representative of a large family of anthraquinone derivatives, is formed in madder roots and that is both characteristic of the actual species, as well as of the vegetative status of the plant: While from young (i.e. about 1-year-old) plants, only a few tenths of a percent of a relatively simple mixture of anthraquinoid dyes can be isolated (relative to the weight of the dried

root), mainly consisting of alizarin, purpurin, pseudopurpurin and rubiadin, both the number and the concentration of anthraquinoid compounds increases in mature plants, yielding up to 2% by weight of the root, where 19 anthraquinones have already been isolated and identified (Rosenberg, 2008).

In this investigation of madder extracts from standard raw plants, alizarin and purpurin were detected by all the chromatographic programs, which are the anthraquinone compounds for the characterization of *Rubiaceae* family of plants. In addition, anthraquinone aglycones and heterosidic dyes' compounds are also characterized by their retention time, UV and mass spectra, including: (1) Lucidin primeveroside; (2) Ruberythric acid; (3) Rubiadin primeveroside; (4) Pseudopurpurin; (5) Lucidin; (6) Alizarin; (7) Xanthopurpurin; (8) Purpurin; (9) Rubiadin; (10) Munjistin; (11) Nordamnacanthal; (12) Anthragallol. The details of each program are shown in the Figures 1a, 1b, 1c and Tables 1a, 1b and 1c.

**Program A**

In Program A, 8 compounds in sequence were observed in Figure 1a and analyzed in Table 1a, including: (1) Ruberythric acid at 533 m/z; (2) Pseudopurpurin at 299 m/z; (3) Lucidin at 269 m/z; (4) Alizarin at 239 m/z; (5) Xanthopurpurin at 239 m/z; (6) Rubiadin at 253 m/z; (7) Nordamnacanthal at 267 m/z; (8) Anthragallol at 255 m/z.

**Fig. 1a:** LC-PDA chromatogram (λ= 430nm) of Madder extract in Program A.

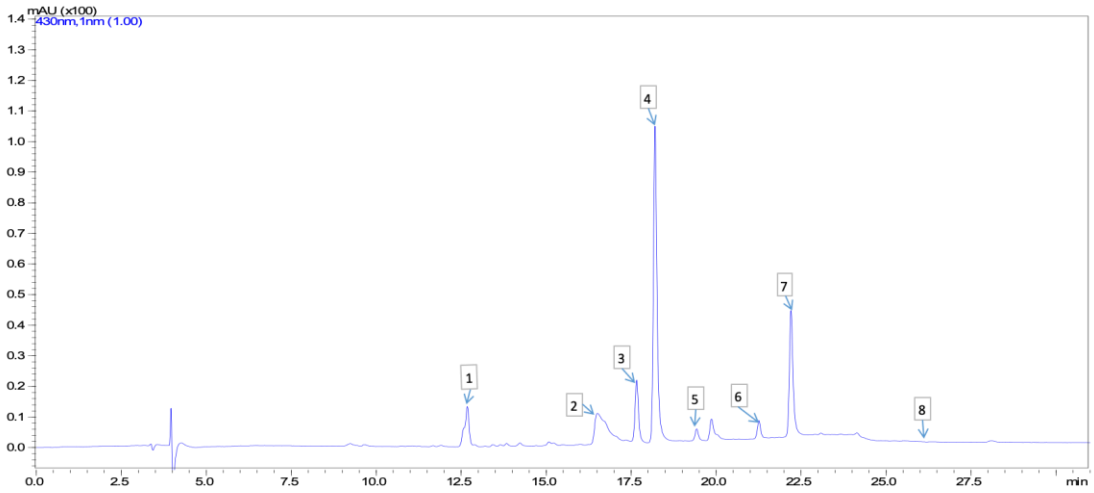



**Table 1a:** Spectro-chromatographic data of Madder compounds in Program A.

| Peak No. | Compound Name | $t_R$ (min) | Area | UV λmax (nm) | $[M-H]^-$ (m/z) |
|---|---|---|---|---|---|
| 1 | Ruberythric acid | 12.73 | 686.128 | 413/333/640 | 533 |
| 2 | Pseudopurpurin | 16.81 | 2.037.830 | 493/639/749 | 299 |
| 3 | Lucidin | 17.77 | 424.189 | 277/412/336/626/675 | 269 |
| 4 | Alizarin | 18.23 | 3.778.133 | 247/229/277/429/626 | 239 |
| 5 | Xanthopurpurin | 19.50 | 182.504 | 412/626/675/750 | 239 |
| 6 | Rubiadin | 21.30 | 97.188 | 413/626/676/754 | 253 |
| 7 | Nordamnacanthal | 22.27 | 1.875.114 | 255/294/421/626/754 | 267 |
| 8 | Anthragallol | 26.20 | 8.492 | 387/412/485/622/640 | 255 |

**Program B**

In Program B, 10 compounds in sequence were observed in Figure 1b and analyzed in Table 1b, including: (1) Lucidin primeveroside at 563 m/z; (2) Ruberythric acid at 533 m/z; (3) Rubiadin primeveroside at 547 m/z; (4) Pseudopurpurin at 299 m/z; (5) Lucidin at 269

m/z; (6) Alizarin at 239 m/z; (7) Xanthopurpurin at 239 m/z; (8) Purpurin at 255 m/z; (9) Rubiadin at 253 m/z; (10) Munjistin at 283 m/z.

**Fig. 1b:** LC-PDA chromatogram (λ= 430nm) of Madder extract in Program B.

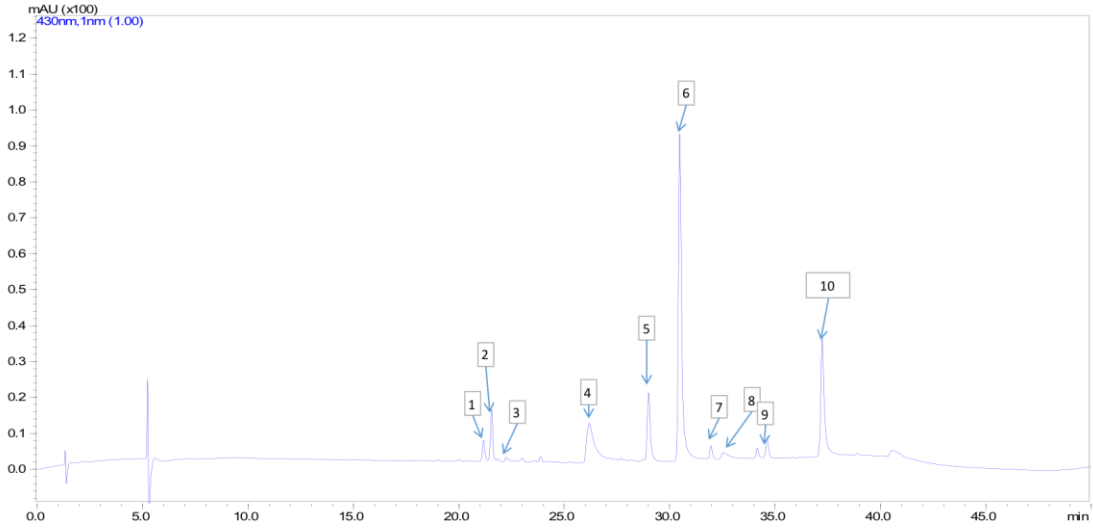



**Table 1b:** Spectro-chromatographic data of Madder compounds in Program B.

| Peak No. | Compound Name | $t_R$ (min) | Area | UV λmax (nm) | $[M-H]^-$ (m/z) |
|---|---|---|---|---|---|
| **1** | Lucidin primeveroside | 21.23 | 303.218 | 207/265/408/531/559 | 563 |
| **2** | Ruberythric acid | 21.60 | 663.343 | 208/259/419/332/525 | 533 |
| **3** | Rubiadin primeveroside | 23.13 | 159.471 | 209/525/559/645/662 | 547 |
| **4** | Pseudopurpurin | 26.17 | 2.742.985 | 209/284/495/619/661 | 299 |
| **5** | Lucidin | 29.10 | 1.077.829 | 208/279/411/508/619 | 269 |
| **6** | Alizarin | 30.50 | 10.610.916 | 204/247/277/429/618 | 239 |
| **7** | Xanthopurpurin | 32.03 | 107.107 | 211/279/419/471/525 | 239 |
| **8** | Purpurin | 32.60 | 300.909 | 211/471/419/619/591 | 255 |

| | | | | | |
|---|---|---|---|---|---|
| **9** | Rubiadin | 34.73 | 126.267 | 212/277/419/525/559 | 253 |
| **10** | Munjistin | 37.33 | 4.110.056 | 213/257/296/419/532 | 283 |

**Program C**

In Program C, 9 compounds in sequence were observed in Figure 1c and analyzed in Table 1c, including: (1) Lucidin primeveroside at 563 m/z; (2) Ruberythric acid at 533 m/z; (3) Pseudopurpurin at 299 m/z; (4) Lucidin at 269 m/z; (5) Alizarin at 239 m/z; (6) Xanthopurpurin at 239 m/z; (7) Purpurin at 255 m/z; (8) Rubiadin at 253 m/z; (9) Munjistin at 283 m/z.

**Fig. 1c:** LC-PDA chromatogram (λ= 430nm) of Madder extract in Program C.

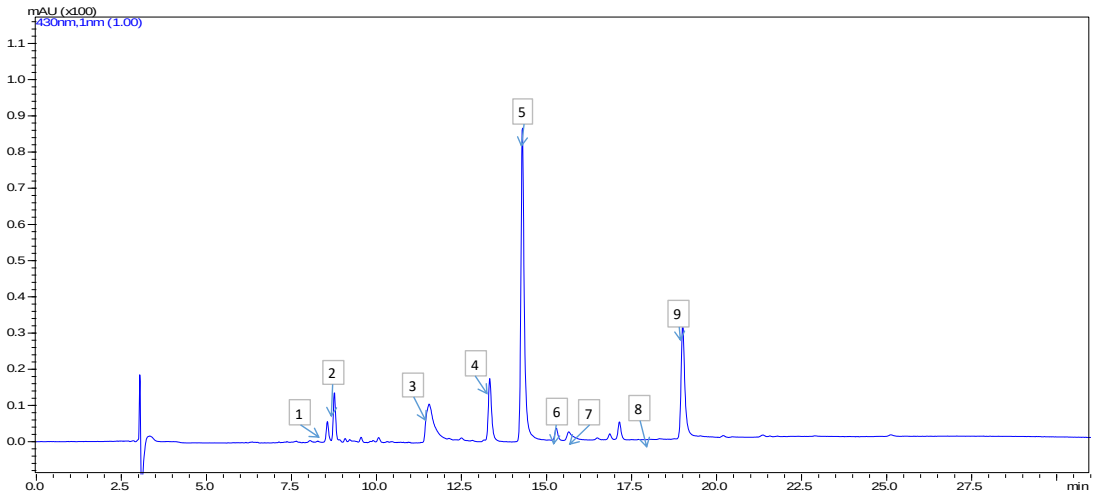



**Table 1c:** Spectro-chromatographic data of Madder compounds in Program C.

| Peak No. | Compound Name | $t_R$ (min) | Area | UV λmax (nm) | $[M-H]^-$ (m/z) |
|---|---|---|---|---|---|
| **1** | Lucidin primeveroside | 8.56 | 204.971 | 208/265/405/341/620 | 563 |

| | | | | | |
|---|---|---|---|---|---|
| **2** | Ruberythric acid | 8.83 | 477.319 | 208/259/415/326/618 | 533 |
| **3** | Pseudopurpurin | 11.57 | 1.698.677 | 210/283/493/620/664 | 299 |
| **4** | Lucidin | 13.40 | 687.564 | 209/279/412/339/621 | 269 |
| **5** | Alizarin | 14.33 | 5.832.909 | 203/247/277/428/621 | 239 |
| **6** | Xanthopurpurin | 15.37 | 64.384 | 212/279/412/326/621 | 239 |
| **7** | Purpurin | 15.73 | 240.087 | 212/478/619 | 255 |
| **8** | Rubiadin | 17.77 | 14.988 | 213/412/620/664 | 253 |
| **9** | Munjistin | 19.07 | 1.675.575 | 214/257/296/421/620 | 283 |

However, in addition to the characterization of these total 12 compounds detected in all programs, the formation of these compounds should be clarified. The primary anthraquinone components in madder (*R. tinctorum*) roots are the glycosides Ruberythric acid and Lucidin primeveroside. And Ruberythric acid could be hydrolyzed to Alizarin in planta, which is also catalyzed by endogenous enzymes in the plant. Similar to Ruberythric acid, Rubiadin primeveroside has glycoside substitution at the b-hydroxy in the 2- position, making it significantly more stable to hydrolysis. Lucidin can be oxidized to Nordamnacanthal due to the catalyzation by endogenous oxidase enzymes in the plant. Munjistin is also observed in *R. tinctorum*, but a glycoside (munjistin-*3*-*O*-glucoside) has only ever been detected in *R. cordifolia* and *R. akane*, suggesting that Munjistin is formed in planta (especially in other species) through a different mechanism. It is possible that Nordamnacanthal can be further oxidized to form Munjistin. Xanthopurpurin is formed through decarboxylation of Munjistin, and it may also be formed directly from Lucidin through an acid-(or base)-catalyzed loss of formaldehyde through a retro-aldol type process. Purpurin is identified in R. tinctorum. Pseudopurpurin glycosides galiosin and Pseudopurpurin glucoside occur in planta – their presence has been detected in low

concentrations in *R. tinctorum*, and are most probably the origin of Pseudopurpurin as a result of hydrolysis. Rubiadin primeveroside occurs at very low concentrations and is most likely the origin of trace amounts of Rubiadin detected, as a result of hydrolysis (Ford, Rayner, & Blackburn, 2018).

***American Cochineal***

Of great historical importance are the red dyes obtained from scale insects which mostly are plant parasites from the *Coccidea* family. American cochineal *(Dactylopius coccus Costa)* is one of the most important species, which main dye constituent is carminic acid. In fact, various species have characteristic fingerprints of other minor anthraquinone components (including kermesic acid and flavokermesic acid), as well as of some still unidentified anthraquinoid compounds commonly named dcII, dcIV and dcVI which allow one to distinguish them in historical samples. Although American cochineal was used in South and central America long before, it was introduced to Europe by the Spaniards only in the late sixteenth century. It is obtained from the egg-filled female insects of *Dactylopius coccus Costa*, a parasite of the *Opuntia cactus* family after drying in the sun or in an oven. The main constituent of American cochineal again is carminic acid, with minor amounts of flavokermesic acid and kermesic acid (Rosenberg, 2008).

As the main colorant of American cochineal, carminic acid is 7-*C*-*α*-*D*-glucopyranoside of kermesic acid. Its isomers, dcIV (7-*C*-*α*-*D*- glucofuranoside of kermesic acid) and dcVII (7-*C*-*β*-*D*-glucofuranoside), differ from it only in a sugar moiety. Their MS spectra obtained in the product ion mode are similar and the main observed losses are typical of

carboxylic acids and *C*-glycosides. According to bibliography, a relatively small collision energy (below a CE of 15 V) is sufficient to fragment the quasi-molecular ions (m/z 491) through the loss of $CO_2$ (observed at m/z 447), but much richer spectra are obtained with a higher CE (e.g., 20 V); careful interpretation of those spectra allows us to propose two fragmentation paths of the examined derivatives of carminic acid. dcII (7-*C*-*α*-*D*-glucopyranoside of flavokermesic acid) differs from carminic acid only in its lack of a hydroxyl group at the C-5 position (leading to a 16-Da lower molecular mass). Instead of this hydroxyl group, the molecule of dcIII (7-*C*-*α*-*D*-glucopyranoside of 5-aminokermesic acid) has a primary amine group (resulting in a 1-Da lower molecular mass). However, in the spectrum of dcIII, the signals from ions formed by the loss of a water molecule is much more intense than those due to the loss of carbon dioxide (contrary to what is seen for carminic acid). This means that the presence of the amino group in dcIII makes fragmentation via the loss of water energetically favored (Lech, Witkoś, Wileńska, & Jarosz, 2015).

Kermesic acid and flavokermesic acid differ in the presence of the hydroxyl substituent at the C-5 position, so their molecular masses differ by 16 Da. The former is an aglycone of carminic acid, and the latter an aglycone of dcII. The MS spectra of the quasi-molecular ions of kermesic acid (m/z 329) and flavokermesic acid (m/z 313) show only peaks attributed to the subsequent losses of $CO_2$ (44 Da) and CO (28 Da). The accurate mass of the $[M-H]^-$ quasi-molecular ion of dc9 is 611.1075 (elemental composition of $C_{29}H_{23}O_{15}$). The difference between the elemental composition of carminic acid and that of dc9 can be attributed to a benzoate moiety (Lech et al., 2015).

However, the quasi-molecular $[M-H]^-$ ion of dc7 is registered at m/z 611 (611.1037 in the high-resolution mass spectrum), corresponding to an elemental composition of $C_{29}H_{23}O_{15}$, identical to the composition of dc9. It can therefore be identified as an isomer of dc9, but these molecules show different MS spectra and that obtained for dc7 is substantially poorer. A similar phenomenon - a lack of detachment of $CO_2$ from the hydroxybenzoic carboxylic group (not engaged in bonding) - has already been reported for diesters of aliphatic dicarboxylic acids with hydroxybenzoic acid. Assuming such a structure for dc7, the formation of ions at m/z 429 (obs. m/z 429.0809, diff. for $C_{21}H_{18}O_{10}$ 4.19) can be explained by atypical detachment of the carbophenoxy ester group together with the neighboring hydroxyl group (182 Da, $C_8H_6O_5$), which probably originates from a charge-remote mechanism based on the elimination of $CO_2$, $H_2O$, and the hydroxybenzoic acid moiety (Lech et al., 2015).

Therefore, the total compounds observed from 3 programs include: (1) dcIII: *C*-glucopyranoside of 5-aminokermesic acid; (2) dcII: *C*-glucopyranoside of flavokermesic acid; (3) ca: Carminic acid (*C*-glucopyranoside of kermesic acid); (4) dcIV: *C*-glucofuranoside of kermesic acid (isomer of carminic acid); (5) dcVII: *C*-glucofuranoside of kermesic acid (isomer of carminic acid); (6) dc7: *C*-glucoside, carboxyphenyl ester of carminic acid ((carminyloxy)benzoic acid); (7) fa: Flavokermesic acid; (8) ka: Kermesic acid, actually followed the scheme of fragmentation above. Details are shown in Figures 2a, 2b, 2c and Tables 2a, 2b, 2c.

**Program A**

In Program A, 6 compounds were identified: The first known compound as the largest peak in Figure 2a is carminic acid at 491 m/z, followed by its isomer: dcIV at 491 m/z. The third peak is dc7 at 611 m/z, and again at 491 m/z should be another isomer of carminic acid: dcVII. The last two peaks refer to fa and ka respectively, at 313 m/z and 329 m/z. In this program, the large peak before carminic acid remained unknown.

**Fig. 2a:** LC-PDA chromatogram (λ= 490nm) of American Cochineal extract in Program A.

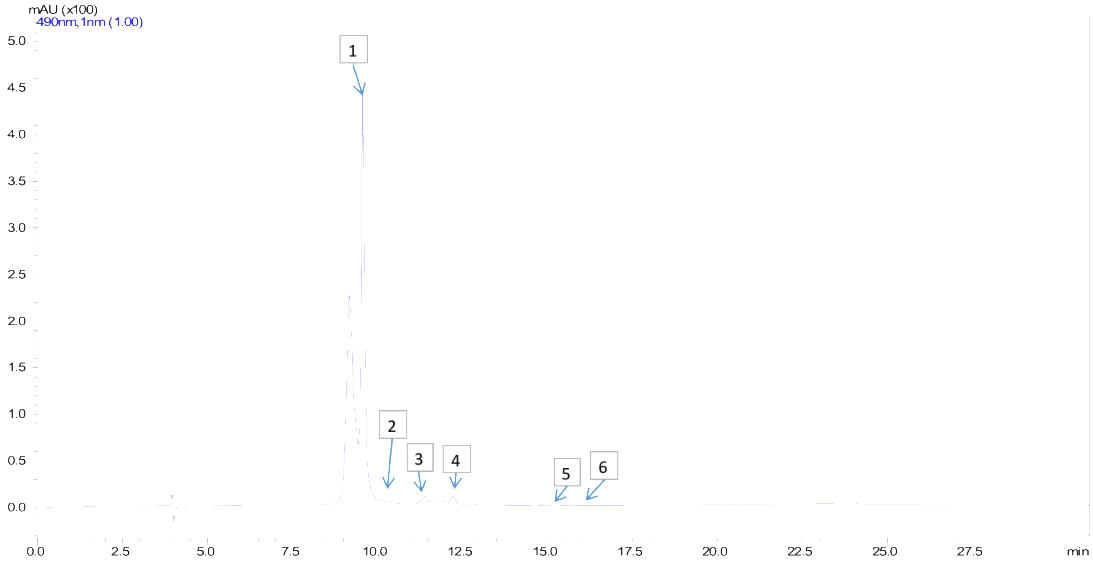



**Table 2a:** Spectro-chromatographic data of American Cochineal compounds in Program A.

| Peak No. | Compound Name | $t_R$ (min) | Area | UV λmax (nm) | $[M-H]^-$ (m/z) |
|---|---|---|---|---|---|
| 1 | ca: Carminic acid (*C*-glucopyranoside of kermesic acid) | 9.27 | 5.434.497 | 276/223/494/612/640 | 491 |

| | | | | | |
|---|---|---|---|---|---|
| **2** | dcIV: *C*-glucofuranoside of kermesic acid (isomer of carminic acid) | 10.67 | 61.317 | 497/482/471/636/664 | 491 |
| **3** | dc7: *C*-glucoside, carboxyphenyl ester of carminic acid ((carminyloxy)benzoic acid) | 11.97 | 129.650 | 496/483/635/677 | 611 |
| **4** | dcVII: *C*-glucofuranoside of kermesic acid (isomer of carminic acid) | 12.30 | 116.542 | 277/492/637/677/748 | 491 |
| **5** | fa: Flavokermesic acid | 15.20 | 52.367 | 433/636/677/745 | 313 |
| **6** | ka: Kermesic acid | 15.47 | 3.913 | 462/637/677/420 | 329 |

**Program B**

In Program B, 8 compounds were identified: The first peak in Figure 2b is dcIII at 490 m/z. While the largest peaks in the middle refer to dcII at 475 m/z and main compound: carminic acid, which has shown a good separation compared to Program A. Then, 2 isomers of carminic acid appeared in the 4$^{th}$ and 5$^{th}$ peak respectively. And the following is dc7 at 611 m/z. The last two peaks again are fa and ka at 313 m/z and 329 m/z. Program B actually showed a very good result for identifying both traces and compounds of American Cochineal.

**Fig. 2b:** LC-PDA chromatogram (λ= 490nm) of American Cochineal extract in Program B.

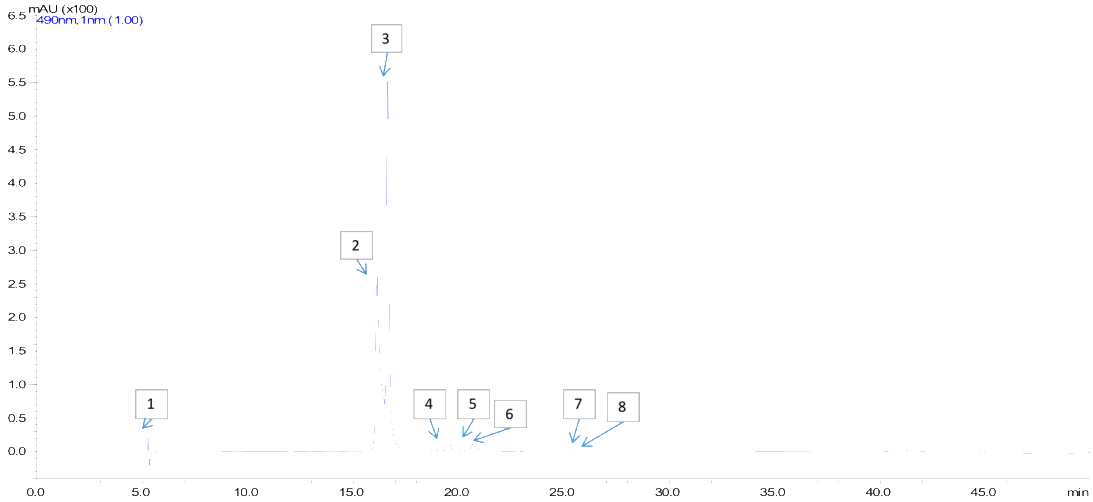



**Table 2b:** Spectro-chromatographic data of American Cochineal compounds in Program B.

| Peak No. | Compound Name | $t_R$ (min) | Area | UV λmax (nm) | $[M-H]^-$ (m/z) |
|---|---|---|---|---|---|
| **1** | dcIII: *C*-glucopyranoside of 5-aminokermesic acid | 5.03 | 1.817.901 | 255/501/641/719/776 | 490 |
| **2** | dcII: *C*-glucopyranoside of flavokermesic acid | 15.97 | 6.105.956 | 276/221/493/613/621 | 475 |
| **3** | ca: Carminic acid (*C*-glucopyranoside of kermesic acid) | 16.233 | 8.604.809 | 275/222/493/613/728 | 491 |

| | | | | | |
|---|---|---|---|---|---|
| **4** | dcIV: *C*-glucofuranoside of kermesic acid (isomer of carminic acid) | 19.67 | 239.844 | 208/276/487/621/727 | 491 |
| **5** | dcVII: *C*-glucofuranoside of kermesic acid (isomer of carminic acid) | 20.60 | 138.486 | 209/277/487/636/669 | 491 |
| **6** | dc7: *C*-glucoside, carboxyphenyl ester of carminic acid ((carminyloxy)benzoic acid) | 20.97 | 204.063 | 209/274/482/621/6699 | 611 |
| **7** | fa: Flavokermesic acid | 25.37 | 72.090 | 210/282/430/621/727 | 313 |
| **8** | ka: Kermesic acid | 25.93 | 11.769 | 210/276/475/621/727 | 329 |

**Program C**

In Program C, 7 compounds were identified: The first and second peak in Figure 2c are in fact of carminic acid at 491 m/z, but the main compound here broke into 2 peaks. While the 3$^{rd}$ and 4$^{th}$ refer to dcIV at 491m/z and dc7 at 611 m/z, followed by fa and ka at 313 m/z and 329 m/z. And the last peak could be dcVII at 491 m/z again. The problem of this program refers to the lack of separation of dcII at 475 m/z and carminic acid. The dcII was hidden in the first peak due to the breakage of carminic acid.

**Fig. 2c:** LC-PDA chromatogram (λ= 490nm) of American Cochineal extract in Program C.

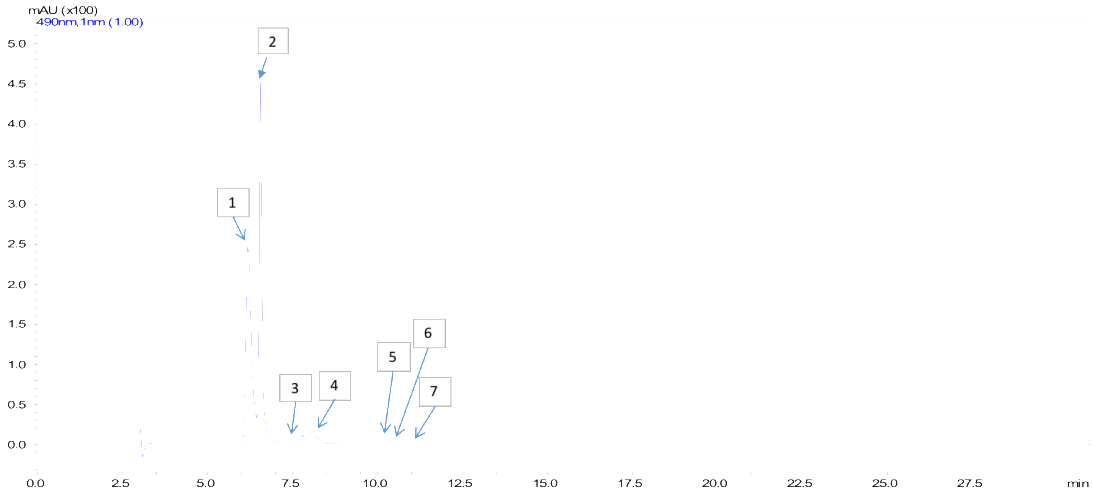



**Table 2c:** Spectro-chromatographic data of American Cochineal compounds in Program C.

| Peak No. | Compound Name | $t_R$ (min) | Area | UV λmax (nm) | $[M-H]^-$ (m/z) |
|---|---|---|---|---|---|
| 1 | ca: Carminic acid (*C*-glucopyranoside of kermesic acid) | 6.19 | 4.305.297 | 276/224/493/613/641 | 491 |
| 2 | ca: Carminic acid (*C*-glucopyranoside of kermesic acid) | 6.59 | 4.721.263 | 276/224/493/612/666 | 491 |
| 3 | dcIV: *C*-glucofuranoside of kermesic acid (isomer of carminic acid) | 7.50 | 49.647 | 223/277/482/657/726 | 491 |
| 4 | dc7: *C*-glucoside, carboxyphenyl ester of carminic acid | 8.43 | 69.609 | 276/225/486/657/726 | 611 |

| | | | | | |
|---|---|---|---|---|---|
| | ((carminyloxy)benzoic acid) | | | | |
| **5** | fa: Flavokermesic acid | 10.90 | 35687 | 228/283/430/346/618 | 313 |
| **6** | ka: Kermesic acid | 11.43 | 14.798 | 230/275/346/486/518 | 329 |
| **7** | dcVII: *C*-glucofuranoside of kermesic acid (isomer of carminic acid) | 12.30 | 1.176 | 210/283/493/620/664 | 491 |

Overall, the characterization of American Cochineal in these 3 different programs is generally based on the fragmentation paths of carminic acid and its isomers: dcIV and dcVII, while the minor compounds may contribute to distinguish the species from the Coccidea family.

***Brazilwood***

The species of red dyewood are all tropical and “brazilwood” was a name used in Europe for different soluble redwoods. Up to the beginning of the 16th century, “brazilwood” referred to *Caesalpinia sappan* L. (common name sappanwood) and it was originated in South East Asia. In 1500, with the discovery of Brazil, named after the redwood trees flourishing along the coastline, “brazilwood” became a different species of tree, the *Caesalpinia echinata Lam*. With the exploitation of the South America tropical rain forests in the subsequent centuries, “brazilwood” became a general name used to refer soluble redwoods from different geographical origins in South America and from different botanical species. The main coloring constituents of brazilwood derived from *Caesalpinia* spp. are the homoisoflavonoids brazilin and brazilein. Brazilin has been isolated, which by

oxidation gives brazilein, the main chromophore in brazilwood. The tree is indigenous to the East Indies, Central and South America and also to Africa. Brazilwood was known in Europe long before the discovery of South America because it was imported as a dyewood from the East Indies. Soon after the discovery of South America, large quantities of the valuable redwood were found in the forests along the Amazon and the newly discovered country was then given the name "Brazil" (Manhita et al., 2013).

The identification of Brazilwood is based on its homoisoflavonoid compound: Brazilin ($C_{16}H_{14}O_5$), whose molecular weight is 286.28. As already noted, it is readily oxidized by contact with atmospheric oxygen or other chemical oxidants to brazilein (MW 284.27) with a loss of two hydrogen atoms to form a carbonyl. In the results of all programs below, we have totally found: (1) Brazilin and (2) Brazilein, and relevant compounds include: (3) Adduct of Brazilin; (4) 9-*O*-methyl-Brazilin; (5) Dimer of Brazilin; (6) Compound Bra'. Details of each chromatographic program are shown in Figures 3a, 3b, 3c and Tables 3a, 3b, 3c.

**Program A**

In Program A, the first peak refers to the adduct of Brazilin at 303 m/z, followed by the main compound: Brazilin at 285 m/z. While Brazilein at 283 m/z and Dimer of Brazilin at 571 m/z are actually not well-separated. The 5$^{th}$ peak was identified as 9-*O*-methyl-Brazilin at 299 m/z, and the last one can be compound Bra' at 265 m/z.

**Fig. 3a** LC-PDA chromatogram (λ= 280nm) of Brazilwood extract in Program A.

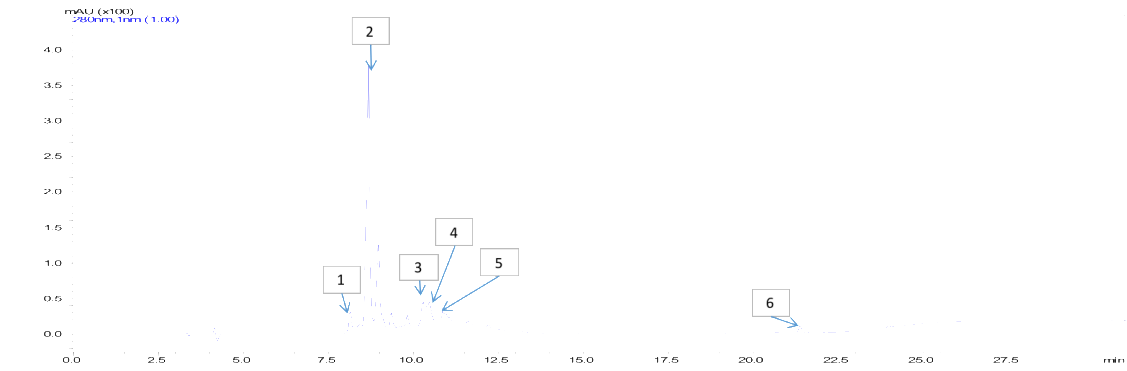



**Table 3a:** Spectro-chromatographic data of Brazilwood compounds in Program A.

| Peak No. | Compound Name | $t_R$ (min) | Area | UV λmax (nm) | $[M-H]^-$ (m/z) |
|---|---|---|---|---|---|
| **1** | Adduct of Brazilin | 8.70 | 5.173.079 | 207/253/286/727 | 303 |
| **2** | Brazilin | 9.03 | 434.693 | 287/446/666/727 | 285 |
| **3** | Brazilein | 10.40 | 585.914 | 204/445/279/617 | 283 |
| **4** | Dimer of Brazilin | 10.57 | 323.526 | 205/283/446/727 | 571 |
| **5** | 9-*O*-methyl-Brazilin | 10.90 | 237.967 | 206/285/441/591/618 | 299 |
| **6** | Compound Bra’ | 20.80 | 13.673 | 213/446/495/621/678 | 265 |

**Program B**

In Program B, adduct of Brazilin at 303 m/z is the first compound detected, closely followed by Brazilin at 285 m/z. The 9-*O*-methyl-Brazilin at 299 m/z and Dimer of Brazilin at 571 m/z are recorded as the 3$^{rd}$ and 4$^{th}$ respectively. Brazilein at 283 m/z and compound Bra’ were identified as the last two peaks.

**Fig. 3b** LC-PDA chromatogram (λ= 280nm) of Brazilwood extract in Program B.

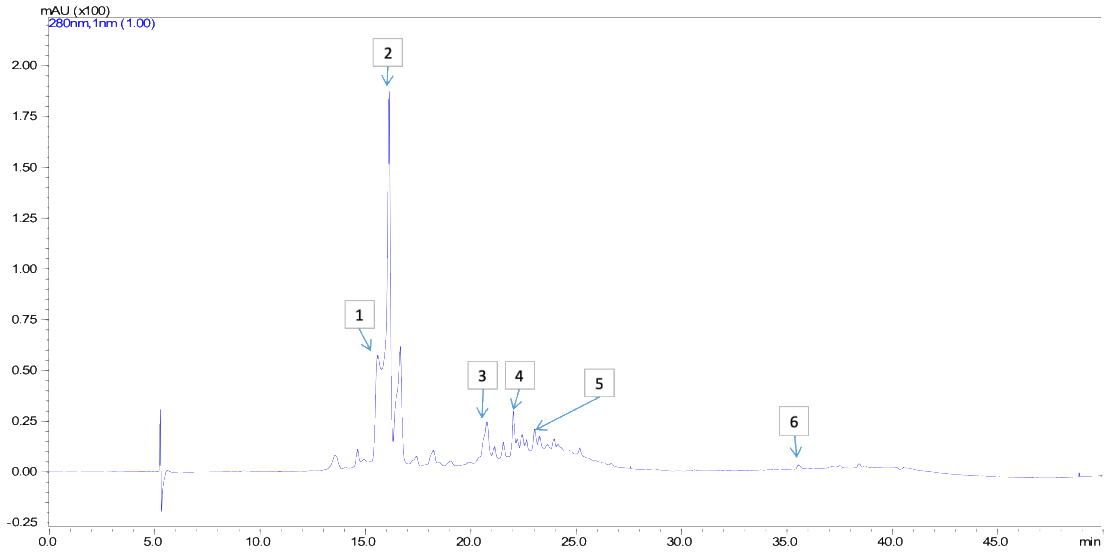



**Table 3b:** Spectro-chromatographic data of Brazilwood compounds in Program B.

| Peak No. | Compound Name | $t_R$ (min) | Area | UV λmax (nm) | $[M-H]^-$ (m/z) |
|---|---|---|---|---|---|
| **1** | Adduct of Brazilin | 16.23 | 5.180.165 | 207/253/286/619/591 | 303 |
| **2** | Brazilin | 16.73 | 29.524 | 287/442/470/388/619 | 285 |
| **3** | 9-*O*-methyl-Brazilin | 20.87 | 429.470 | 205/285/442/618/591 | 299 |
| **4** | Dimer of Brazilin | 22.07 | 113.788 | 205/284/442/591/618 | 571 |
| **5** | Brazilein | 23.03 | 307.695 | 207/284/442/591/618 | 283 |
| **6** | Compound Bra' | 35.40 | 296.727 | 213/283/470/591/618 | 265 |

**Program C**

The sequence of compounds identified in Program C is similar to that of Program A but with better separation: the first peak refers to the adduct of Brazilin at 303 m/z, followed by the main compound: Brazilin at 285 m/z. While Brazilein at 283 m/z and Dimer of Brazilin at 571 m/z. The 5$^{th}$ peak was identified as 9-*O*-methyl-Brazilin at 299 m/z, and the last one can be compound Bra' at 265 m/z.

**Fig. 3c** LC-PDA chromatogram (λ= 280nm) of Brazilwood extract in Program C.

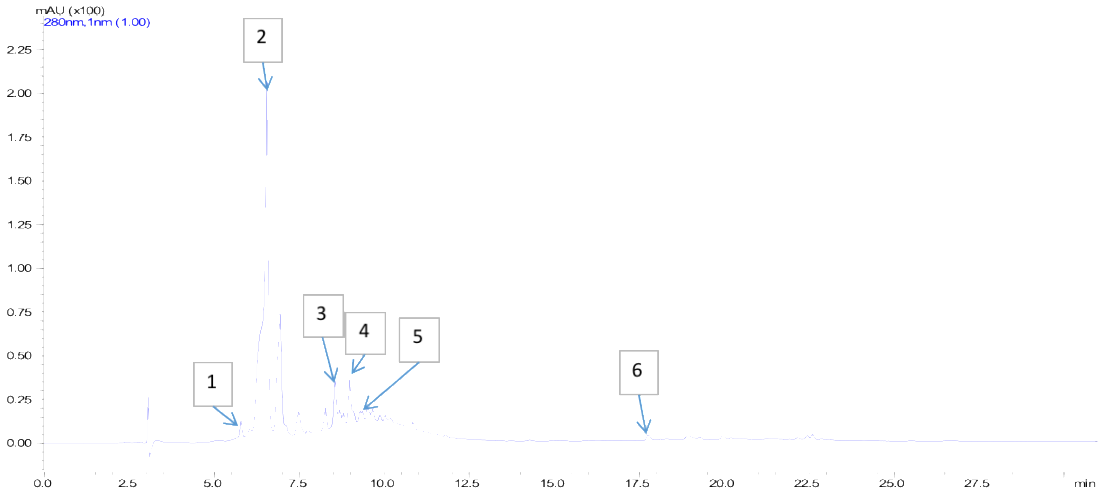



**Table 3c:** Spectro-chromatographic data of Brazilwood compounds in Program C.

| Peak No. | Compound Name | $t_R$ (min) | Area | UV λmax (nm) | $[M-H]^-$ (m/z) |
|---|---|---|---|---|---|
| **1** | Adduct of Brazilin | 6.57 | 5.042.863 | 207/253/286/658/621 | 303 |
| **2** | Brazilin | 6.93 | 381.070 | 287/442/658/728 | 285 |
| **3** | Brazilein | 8.60 | 579.868 | 205/444/280 | 283 |
| **4** | Dimer of Brazilin | 9.00 | 290.036 | 204/283/442/621/728 | 571 |

| | | | | | |
|---|---|---|---|---|---|
| **5** | 9-O-methyl-Brazilin | 9.33 | 385.786 | 208/287/442/621/727 | 299 |
| **6** | Compound Bra’ | 17.53 | 179.870 | 213/434/494/621/727 | 265 |

## 5.2 Identification of Compounds in Standard Yellow Dyes

### ***Weld***

Weld (*Reseda luteola* L) grows wild in most of Europe but was also cultivated. More plant material is required than for most other yellow dyes but it is mentioned in many textile dyeing recipes, suggesting that it was quite popular. When used with alum as a mordant it produces bright and fast yellow colors owing to the presence of the flavones luteolin and apigenin as the major constituents. Sugar derivatives are also present, but *O*-glycosides are usually hydrolyzed to the parent flavonoid in the dyebath. The flavonoid content of weld was shown to average 2% by weight. Weld was also frequently used with woad or indigo to give fast green dyes (Ferreira et al., 2004).

Since most of the plants used to produce the yellow dyestuff have unique patterns of flavonoid components, it is hard to reliably identify the plant source without the appropriate plant reference material because many plants contain other classes of colorants. For example, luteolin and apigenin were identified in all yellow paints. Luteolin is a major dyestuff in a few natural sources, such as *Reseda luteola* L., *Serratula tinctoria* L., *Genista tinctoria* L., etc. Unfortunately, the presence of major components of natural dye is not

sufficient to determine the origin of plant sources used to prepare dyes and the minor coloring substances in the sample are fingerprints, characteristic to a specific plant. In the case of yellow dyes investigated in our study, only those samples where chrysoeriol was identified together with apigenin and luteolin are likely to have been dyed using *Reseda luteola* L., which can also be proved in all our programs and indicates their validity (Otłowska et al., 2017).

Totally 9 compounds were identified in 3 programs, including: (1) Apigenin-6, 8-di-*C*-glucoside; (2) Luteolin-di-*O*-glucoside; (3) Luteolin-3 ,7-di-*O*-glucoside; (4) Luteolin-7-*O*-glucoside; (5) Apigenin-7-*O*-glucoside; (6) Luteolin-3'-*O*-glucoside; (7) Luteolin; (8) Apigenin; (9) Chrysoeriol. Generally, from the literature of standard Weld, Apigenin-*C*-di-glucoside showed a pseudo-molecular ion $[M-H]^-$ at m/z 593, and compound with a pseudo-molecular ion $[M-H]^-$ at m/z 609 was identified as Luteolin-*O*-di-glucoside, but the different UV λmax (nm) at 338nm and 340nm led to the classification of Luteolin-di-*O*-glucoside and Luteolin-3 ,7-di-*O*-glucoside respectively. The compound with a pseudo-molecular ion at m/z 447, which loses glucose residue (162 Da), leading to an aglycone ion at m/z 285, can be attributed to isomers of Luteolin-*O*-glucoside: Luteolin-7-*O*-glucoside. In fact, identification of luteolin-7-*O*-glucoside was straightforward, as this compound is available in pure form. The precursor ion $[M-H]^-$ at m/z 431 and 269, can be recognized as Apigenin-7-*O*-glucoside and Apigenin (Otłowska, Ślebioda, Kot-Wasik, Karczewski, & Śliwka-Kaszyńska, 2018). Details can be seen in Figures 4a, 4b, 4c and Tables 4a, 4b, 4c.

**Program A**

In Program A, Apigenin-6, 8-di-*C*-glucoside at 593 m/z and Luteolin-di-*O*-glucoside at 609 m/z were identified at the beginning as the first and second peaks respectively. The 3rd and 6th peaks refer to the main compounds: Luteolin-7-*O*-glucoside at 447 m/z and Luteolin at 285 m/z. Besides, Apigenin-7-*O*-glucoside at 431 m/z and Luteolin-3'-*O*-glucoside at 447 m/z almost appeared in the same time but still can be distinguished in peak no. 4 and 5. Apigenin at 269 m/z can be observed in peak no. 7 and Chrysoeriol at 299 m/z in peak no. 8.

**Fig. 4a** LC-PDA chromatogram (λ= 350nm) of Weld extract in Program A.

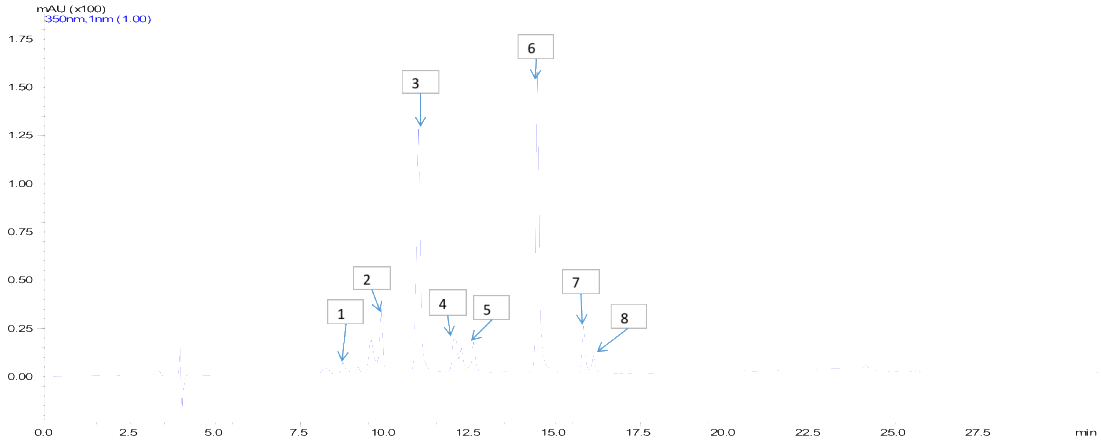



**Table 4a:** Spectro-chromatographic data of Weld compounds in Program A.

| Peak No. | Compound Name | $t_R$ (min) | Area | UV λmax (nm) | $[M-H]^-$ (m/z) |
|---|---|---|---|---|---|
| **1** | Apigenin-6, 8-di-*C*-glucoside | 8.83 | 137.140 | 332/667/643/522/471 | 593 |
| **2** | Luteolin-di-*O*-glucoside | 9.97 | 204.086 | 338/642/559/677/471 | 609 |

| | | | | | |
|---|---|---|---|---|---|
| **3** | Luteolin-7-*O*-glucoside | 11.07 | 924.369 | 251/348/522/643/471 | 447 |
| **4** | Apigenin-7-*O*-glucoside | 12.07 | 155.401 | 335/522/554/642/471 | 431 |
| **5** | Luteolin-3'-*O*-glucoside | 12.63 | 100.916 | 337/522/508/471/638 | 447 |
| **6** | Luteolin | 14.50 | 943.009 | 347/290/508/471/481 | 285 |
| **7** | Apigenin | 15.90 | 143.903 | 335/522/471/638/453 | 269 |
| **8** | Chrysoeriol | 16.20 | 56.526 | 343/529/495/471/642 | 299 |

**Program B**

In Program B, similar to Program A, Apigenin-6, 8-di-*C*-glucoside at 593 m/z and Luteolin-di-*O*-glucoside at 609 m/z were identified at the beginning as the first and second peaks respectively, and peaks no. 3 and 6 refer to the main compounds: Luteolin-7-*O*-glucoside at 447 m/z and Luteolin at 285 m/z as well. Besides, Apigenin-7-*O*-glucoside at 431 m/z and Luteolin-3'-*O*-glucoside 447 m/z almost appeared in the same time but still can be distinguished in peaks no. 4 and 5. Apigenin at 269 m/z can be observed in peak no. 7 and Chrysoeriol at 299 m/z in peak no. 8.

**Fig. 4b** LC-PDA chromatogram (λ= 350nm) of Weld extract in Program B.

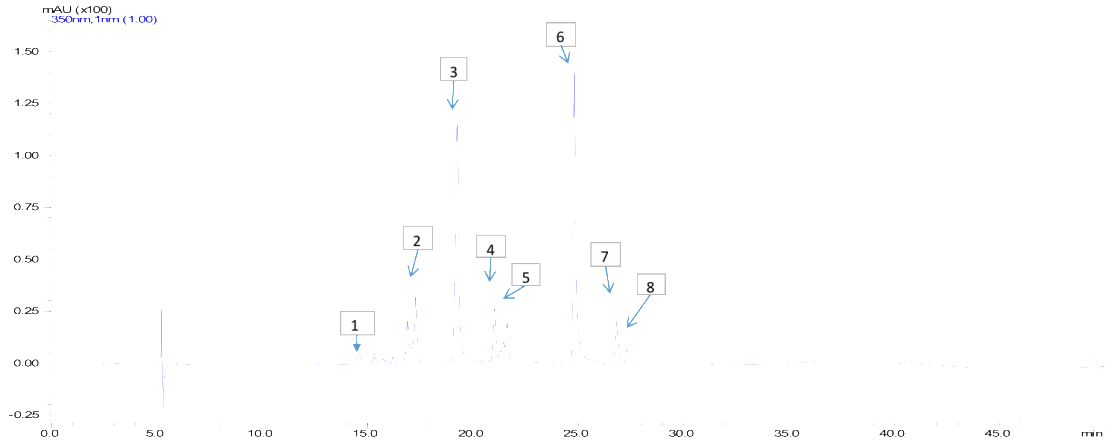



**Table 4b:** Spectro-chromatographic data of Weld compounds in Program B.

| Peak No. | Compound Name | $t_R$ (min) | Area | UV λmax (nm) | $[M-H]^-$ (m/z) |
|---|---|---|---|---|---|
| **1** | Apigenin-6, 8-di-*C*-glucoside | 14.70 | 121.857 | 208/271/333/552/491 | 593 |
| **2** | Luteolin-di-*O*-glucoside | 17.33 | 325.803 | 207/340/268/472/618 | 609 |
| **3** | Luteolin-7-*O*-glucoside | 19.30 | 1.636.955 | 206/347/253/543/618 | 447 |
| **4** | Apigenin-7-*O*-glucoside | 21.13 | 338.461 | 208/335/267/472/551 | 431 |
| **5** | Luteolin-3'-*O*-glucoside | 21.73 | 351.785 | 209/338/268/471/618 | 447 |
| **6** | Luteolin | 24.90 | 1.954.218 | 208/347/292/618/681 | 285 |
| **7** | Apigenin | 26.93 | 332.258 | 210/334/267/472/618 | 269 |
| **8** | Chrysoeriol | 27.33 | 359.923 | 211/341/472/618 | 299 |

**Program C**

Program C actually shows a better separation result comparing to Program A and B. Apigenin-6, 8-di-*C*-glucoside at 593 m/z appeared in a minor peak at the beginning, while Luteolin-di-*O*-glucoside and Luteolin-3 ,7-di-*O*-glucoside both at 609 m/z were identified clearly as the second and third peaks respectively. And peaks no. 4 and 7 refer to the main compounds: Luteolin-7-*O*-glucoside at 447 m/z and Luteolin at 285 m/z as well. Besides, Apigenin-7-*O*-glucoside at 431 m/z and Luteolin-3'-*O*-glucoside at 447 m/z almost appeared in the same time again but still can be distinguished in peaks no. 5 and 6. In the end, Apigenin at 269 m/z and Chrysoeriol at 299 m/z can be observed in peaks no. 8 and no. 9.

**Fig. 4c** LC-PDA chromatogram (λ= 350nm) of Weld extract in Program C.

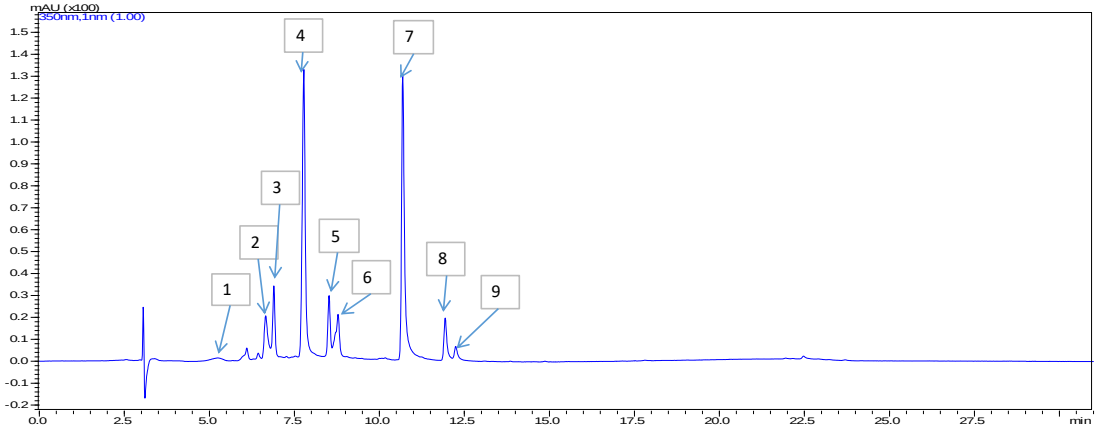



**Table 4c:** Spectro-chromatographic data of Weld compounds in Program C.

| Peak No. | Compound Name | $t_R$ (min) | Area | UV λmax (nm) | $[M-H]^-$ (m/z) |
|---|---|---|---|---|---|
| 1 | Apigenin-6, 8-di-*C*-glucoside | 5.30 | 333.642 | 217/271/329/497/621 | 593 |

| | | | | | |
|---|---|---|---|---|---|
| **2** | Luteolin-di-*O*-glucoside | 6.70 | 286.567 | 208/339/268/535/621 | 609 |
| **3** | Luteolin-3 ,7-di-*O*-glucoside | 6.90 | 249.706 | 207/340/268/621/646 | 609 |
| **4** | Luteolin-7-*O*-glucoside | 7.80 | 1.169.779 | 205/347/253/621/646 | 447 |
| **5** | Apigenin-7-*O*-glucoside | 8.60 | 260.317 | 210/337/267/535/621 | 431 |
| **6** | Luteolin-3'-*O*-glucoside | 8.83 | 348.823 | 214/338/268/621/671 | 447 |
| **7** | Luteolin | 10.77 | 1.113.734 | 209/347/252/266/292 | 285 |
| **8** | Apigenin | 12.00 | 164.688 | 225/337/267/484/455 | 269 |
| **9** | Chrysoeriol | 12.30 | 151.629 | 229/341/267/476/461 | 299 |

***Rhamnus***

Rhamnus is a botanical genus belonging to *Rhamnaceae* family and including approximatively 100 species. These shrubs can reach from 1 to 10 m and come from various areas in the world. The ripe fruits contain three different families of chemical compounds. In the ripe fruits, flavanols and anthraquinones are present and anthocyanin compounds are biosynthesized in the skin (Cuoco, Mathe, & Vieillescazes, 2014). However, in this investigation, the sample was reported to be from the specie of *Rhamnus petiolaris*. This Buckthorn dye plant plays a very important role in natural dyestuff sources. Buckthorn berries are an old Turkish dyestuff source. The natural dyestuffs (such as flavonoids) are effective metal ion chelators. Metal - flavonoid or metal - anthraquinone complexes are generated by the reaction of metals like aluminum, tin and iron with these dyestuffs. In alkaline solutions, natural pigments are precipitated as insoluble metal-dyestuff complexes.

The composition of natural organic dyes depends not only on the species and origin of dye plant, but also on the procedures used for the extraction from dye plants and on the method used for natural dye production. Moreover, their composition is influenced by ageing processes. The main dyestuff present in the aqueous extract from Rhamnus berries is reported to be a glycoside derivative of rhamnetin (3,5,3′,4′-tetrahydroxy-7-methoxyflavone), and its identification as well as dyestuff analysis is one of the most important targets aimed in the scientific examination of paintings, textiles, illuminated manuscripts and other historic and archaeological materials (Cuoco et al., 2014).

Persian berries, in general, contain a wide range of flavonoids. In addition to the common flavonoids quercetin and kaempferol, there are some derivatives more characteristic for this species, including rhamnetin, rhamnazin, rhamnocitrin, together with xanthorhamnin (a 3-*O*-glycoside of rhamnazin). Nonflavonoid coloring components, such as the anthraquinone derivative emodin, are also present (Rosenberg, 2008). However, from the results of 3 chromatographic programs below, the number of compounds reached to 20, including: (1) Kaempferol-3-*O*-acetyl-rhamnoside; (2) Kaempferol-3-*O*-*β*-*D*-xyloside; (3) Quercetin; (4) Kaempferol-3-*O*-rhamninoside; (5) Kaempferol-3-rutinoside; (6) Kaempferol-3-*O*-rhamnoside; (7) Emodin-8-glucoside; (8) Rhamnocitrin-3-*O*-acetyl-rhamninoside; (9) Kaempferol; (10) Aloemodin; (11) Rhamnetin-3-*O*-rhamninoside; (12) Emodin; (13) Rhamnetin/ Isoramnetin; (14) Kampferide; (15) Quercetin-3-*O*-rhamninoside; (16) Rhamnocitrin; (17) Isorhamnetin-3-*O*-rhamninoside; (18) Rhamnocitrin-3-*O*-rhamninoside; (19) Rhamnazine; (20) Rhamnazin-3-*O*-rhamninoside. Details were shown in Figures 5a, 5b, 5c and Tables 5a, 5b, 5c.

**Program A**

In Program A, only 7 compounds were identified, indicating a poor characterization potentiality of the program. Quercetin-3-*O*-rhamninoside at 755 m/z was shown in peak no. 1, while peak no. 2 can be identified as Kaempferol-3-rutinoside at 593 m/z. The largest peak observed is no. 3, which can corresponds to Kaempferol-3-*O*-rhamnoside at 431 m/z. Kaempferol at 285 m/z and Rhamnazin-3-*O*-rhamninoside at 783 m/z were shown in the minor peaks in no. 4 and 5. The last two peaks, however, can be Aloemodin and Emodin both at 269 m/z.

**Fig. 5a** LC-PDA chromatogram (λ= 350nm) of Rhamnus extract in Program A.

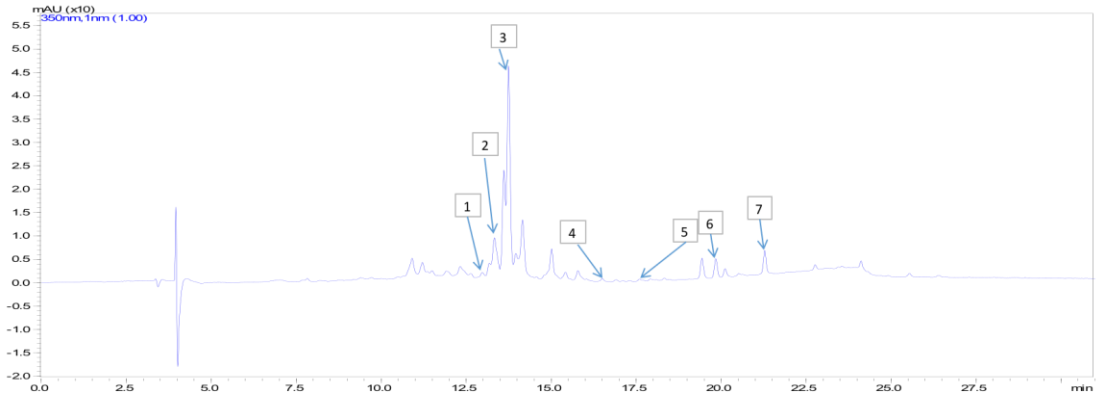



**Table 5a:** Spectro-chromatographic data of Rhamnus compounds in Program A.

| Peak No. | Compound Name | $t_R$ (min) | Area | UV λmax (nm) | $[M-H]^-$ (m/z) |
|---|---|---|---|---|---|
| **1** | Quercetin-3-*O*-rhamninoside | 12.97 | 164.112 | 358/440/620/681 | 755 |
| **2** | Kaempferol-3-rutinoside | 13.40 | 830.881 | 222/268/349/558/620 | 593 |

| | | | | | |
|---|---|---|---|---|---|
| **3** | Kaempferol-3-*O*-rhamnoside | 13.73 | 1.874.824 | 222/268/348/558/620 | 431 |
| **4** | Kaempferol | 16.53 | 65.027 | 440/354/620/681 | 285 |
| **5** | Rhamnazin-3-*O*-rhamninoside | 17.43 | 13.222 | 470/498/358/419/620 | 783 |
| **6** | Aloemodin | 19.87 | 338.904 | 223/433/551/625/677 | 269 |
| **7** | Emodin | 21.37 | 323.417 | 221/440/625/677/755 | 269 |

**Program B**

Program B has shown 11 compounds involved. The first three peaks were so close together but still can be identified. Peak no. 1 is Kaempferol-3-*O*-*β*-*D*-xyloside at 417 m/z, peak no. 2 is Rhamnocitrin at 299 m/z, and peak no. 3 is Isorhamnetin-3-*O*-rhamninoside at 769 m/z. Kaempferol-3-rutinoside at 593 m/z was observed in peak no. 4, followed by the main compound: Kaempferol-3-*O*-rhamnoside at 431 m/z in peak no. 5. Peak no. 6 was regarded as Emodin-8-glucoside at 431 m/z. Minor compounds were also identified in peaks no. 7, 8 and 9, which refer to Rhamnocitrin-3-*O*-rhamninoside at 753 m/z, Rhamnazine at 329 m/z and Kaempferol at 285 m/z. The last two peaks, again, correspond to Aloemodin and Emodin both at 269 m/z.

**Fig. 5b** LC-PDA chromatogram (λ= 350nm) of Rhamnus extract in Program B.

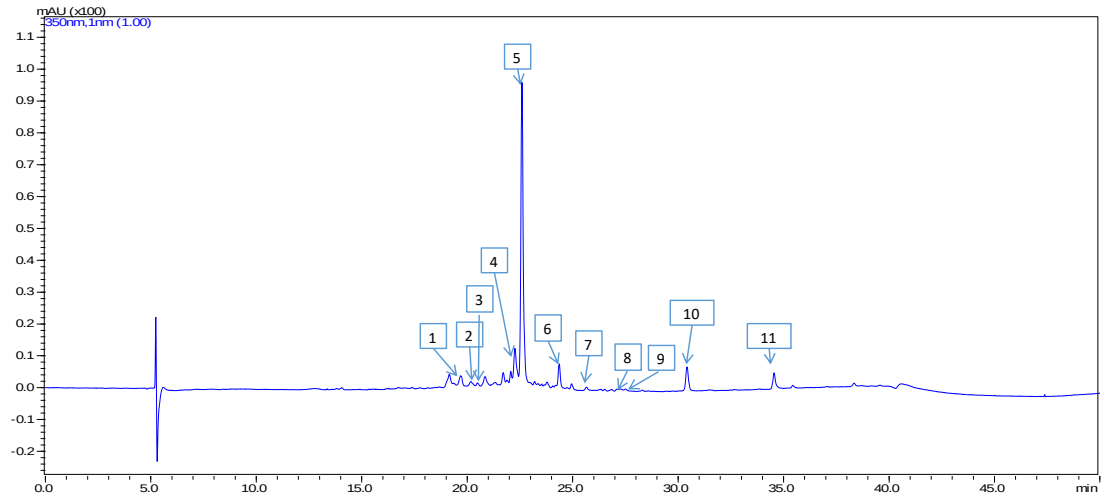



**Table 5b:** Spectro-chromatographic data of Rhamnus compounds in Program B.

| Peak No. | Compound Name | $t_R$ (min) | Area | UV λmax (nm) | $[M-H]^-$ (m/z) |
|---|---|---|---|---|---|
| **1** | Kaempferol-3-*O*-*β*-*D*-xyloside | 19.77 | 307.701 | 210/266/345/426/485 | 417 |
| **2** | Rhamnocitrin | 20.17 | 172.036 | 210/266/345/426/485 | 299 |
| **3** | Isorhamnetin-3-*O*-rhamninoside | 20.57 | 101.784 | 210/485/433/356/726 | 769 |
| **4** | Kaempferol-3-rutinoside | 22.33 | 652.093 | 214/269/349/618/681 | 593 |
| **5** | Kaempferol-3-*O*-rhamnoside | 22.67 | 6.206.877 | 221/268/348/546/620 | 431 |
| **6** | Emodin-8-glucoside | 24.47 | 252.541 | 214/280/421/618/681 | 431 |
| **7** | Rhamnocitrin-3-*O*-rhamninoside | 25.50 | 76.483 | 212/272/280/364/620 | 753 |
| **8** | Rhamnazine | 27.17 | 9.111 | 212/360/485/620/681 | 329 |
| **9** | Kaempferol | 27.53 | 14.300 | 213/350/433/618/681 | 285 |
| **10** | Aloemodin | 30.53 | 1.725.402 | 217/264/433/618/680 | 269 |

| | | | | | |
|---|---|---|---|---|---|
| **11** | Emodin | 34.60 | 1.127.352 | 216/288/266/440/592 | 269 |

**Program C**

Program C has shown the largest amount of compounds with very good separation. Peak no. 1 is Kaempferol-3-*O*-acetyl-rhamnoside at 781 m/z. Peak no. 2 is Kaempferol-3-*O*-*β*-*D*-xyloside at 417 m/z. Peak no. 3 is Quercetin at 301 m/z and peak no. 4 is Kaempferol-*3*-*O*-rhamninoside at 739 m/z. The main compound here was identified in peak no. 5 as Kaempferol-3-rutinoside at 593 m/z, closely followed by Kaempferol-3-*O*-rhamnoside at 431 m/z. Emodin-8-glucoside also at 431 m/z was then observed in peak no. 7. Rhamnocitrin-3-*O*-acetyl-rhamninoside at 795 m/z and Kaempferol at 285 m/z were identified in peaks no. 8 and 9 respectively but with minor absorption. Aloemodin and Emodin at 269 m/z can be found in peaks no. 10 and 12, and peak no. 11 is for Rhamnetin-3-*O*-rhamninoside at 769 m/z. Peaks no. 13 and 14 correspond to Rhamnetin/Isorhamnetin at 315 m/z and Kampferide at 299 m/z.

**Fig. 5c** LC-PDA chromatogram (λ= 350nm) of Rhamnus extract in Program C.

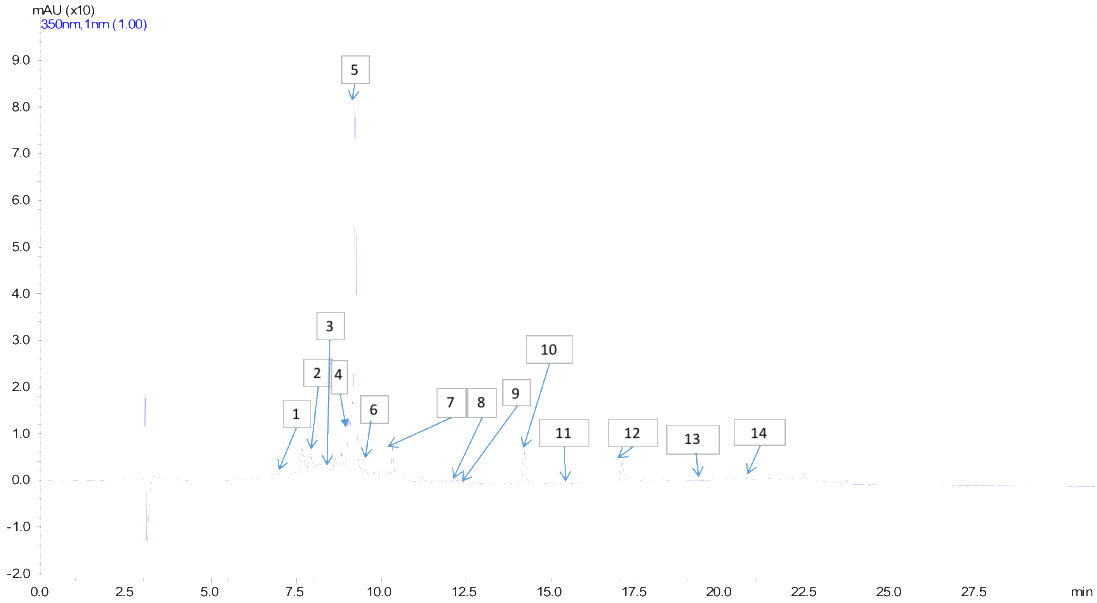



**Table 5c:** Spectro-chromatographic data of Rhamnus compounds in Program C.

| Peak No. | Compound Name | $t_R$ (min) | Area | UV λmax (nm) | $[M-H]^-$ (m/z) |
|---|---|---|---|---|---|
| **1** | Kaempferol-3-*O*-acetyl-rhamnoside | 7.20 | 159.521 | 226/351/728 | 781 |
| **2** | Kaempferol-3-*O*-*β*-*D*-xyloside | 8.00 | 299.342 | 230/269/342/727 | 417 |
| **3** | Quercetin | 8.40 | 224.910 | 228/276/366/727 | 301 |
| **4** | Kaempferol-3-*O*-rhamninoside | 8.87 | 213.538 | 228/274/347/619/727 | 739 |
| **5** | Kaempferol-3-rutinoside | 9.11 | 507.204 | 223/270/345/619/727 | 593 |
| **6** | Kaempferol-3-*O*-rhamnoside | 9.30 | 2.533.943 | 222/268/348/619/727 | 431 |
| **7** | Emodin-8-glucoside | 10.33 | 357.936 | 224/272/280/421/621 | 431 |

| | | | | | |
|---|---|---|---|---|---|
| **8** | Rhamnocitrin-3-*O*-acetyl-rhamninoside | 12.23 | 88.346 | 236/274/363/618/727 | 795 |
| **9** | Kaempferol | 12.40 | 209.356 | 236/350/619/666/727 | 285 |
| **10** | Aloemodin | 14.23 | 628.452 | 225/264/281/433/619 | 269 |
| **11** | Rhamnetin-3-*O*-rhamninoside | 15.53 | 113.237 | 239/484/358/621/680 | 769 |
| **12** | Emodin | 17.10 | 450.341 | 288/265/253/226/440 | 269 |
| **13** | Rhamnetin | 19.60 | 29.334 | 243/484/546/372/620 | 315 |
| **14** | Kampferide | 20.63 | 13.530 | 244/274/541/366/620 | 299 |

## 5.3 Comparison of the Chromatographic Programs

Comparison of Programs A, B and C is based on the total number of identified compounds, the area of the corresponding peak (indicative of concentration) as well as the effectiveness and validity of separation results in each program. For the characterization of compounds in Madder, 9 compounds were identified in both Programs A and C, while 10 compounds were separated and identified in Program B. (1) Ruberythric acid (alizarin primeveroside), (2) Pseudopurpurin, (3) Alizarin, (4) Xanthopurpurin, (5) Rubiadin, (6) Munjistin and (7) Lucidin were identified in all programs, with Alizarin to be observed as the main compound. However, the minor compounds appeared in each program led to the difference of the 3 programs. Nordamnacanthal and Anthragallol was only shown in Program A, and Rubiadin primeveroside only in Program B, while Lucidin primeveroside and Purpurin were identified in Programs B and C. Overall, the separation of 3 programs show good results,

and Program B has the largest amount of identified compounds. Details were showed in Table 6a.

***Madder***

**Table 6a:** Compounds of Madder Identified in Program A, B and C.

| *Program* | A | B | C |
|---|---|---|---|
| *1* | Ruberythric acid | Lucidin primeveroside | Lucidin primeveroside |
| *2* | Pseudopurpurin | Ruberythric acid | Ruberythric acid |
| *3* | Lucidin | Rubiadin primeveroside | Pseudopurpurin |
| *4* | Alizarin | Pseudopurpurin | Lucidin |
| *5* | Xanthopurpurin | Lucidin | Alizarin |
| *6* | Rubiadin | Alizarin | Xanthopurpurin |
| *7* | Nordamnacanthal | Xanthopurpurin | Purpurin |
| *8* | Anthragallol | Purpurin | Rubiadin |
| *9* | | Rubiadin | Munjistin |
| *10* | | Munjistin | |

***American Cochineal***

As for the characterization of American Cochineal, (1) ca, (2) dcIV, (3) dcVII, (4) dc7, (5) fa and (6) ka were separated and idetified in all programs. However, as a minor compounds of American Cochineal, dcIII: *C*-glucopyranoside of 5-aminokermesic acid was only

identified in Program B, while both Programs A and C haven't shown a good separation and clear identification of the minor compounds observed before the peak of ca: Carminic acid (*C*-glucopyranoside of kermesic acid). In addition, dcII: *C*-glucopyranoside of flavokermesic acid also identified by Program B. Thus, Program B showed the best results compared to Program A and C. Details can be seen in Table 6b.

**Table 6b:** Compounds of American Cochineal Identified in Program A, B and C.

| *Program* | A | B | C |
|---|---|---|---|
| *1* | ca: Carminic acid (*C*-glucopyranoside of kermesic acid) | dcIII: *C*-glucopyranoside of 5-aminokermesic acid | ca: Carminic acid (*C*-glucopyranoside of kermesic acid) |
| *2* | dcIV: *C*-glucofuranoside of kermesic acid (isomer of carminic acid) | dcII: *C*-glucopyranoside of flavokermesic acid | dcIV: *C*-glucofuranoside of kermesic acid (isomer of carminic acid) |
| *3* | dc7: *C*-glucoside, carboxyphenyl ester of carminic acid ((carminyloxy)benzoic acid) | ca: Carminic acid (*C*-glucopyranoside of kermesic acid) | dc7: *C*-glucoside, carboxyphenyl ester of carminic acid ((carminyloxy)benzoic acid) |
| *4* | dcVII: *C*-glucofuranoside of kermesic acid (isomer of carminic acid) | dcIV: *C*-glucofuranoside of kermesic acid (isomer of carminic acid) | dcVII: *C*-glucofuranoside of kermesic acid (isomer of carminic acid) |
| *5* | fa: Flavokermesic acid | dcVII: *C*-glucofuranoside of | fa: Flavokermesic acid |

| | | | |
|---|---|---|---|
| | | kermesic acid (isomer of carminic acid) | |
| *6* | ka: Kermesic acid | dc7: *C*-glucoside, carboxyphenyl ester of carminic acid ((carminyloxy)benzoic acid) | ka: Kermesic acid |
| *7* | | fa: Flavokermesic acid | |
| *8* | | ka: Kermesic acid | |

***Weld***

In the investigation of Weld extract in 3 chromatographic programs, there were totally 8 compounds identified in all programs, including: (1) Apigenin-6, 8-di-*C*-glucoside, (2) Luteolin-di-*O*-glucoside, (3) Luteolin-7-*O*-glucoside, (4) Apigenin-7-*O*-glucoside, (5) Luteolin-3'-*O*-glucoside, (6) Luteolin, (7) Apigenin and (8) Chrysoeriol. However, Luteolin-3 ,7-di-*O*-glucoside was only found in Program C. Thus, three programs all showed good separation result while Program C has seen to be better. Details are shown in Table 6c.

**Table 6c:** Compounds of Weld Identified in Program A, B and C.

| *Program* | A | B | C |
|---|---|---|---|
| *1* | Apigenin-6, 8-di-*C*-glucoside | Apigenin-6, 8-di-*C*-glucoside | Apigenin-6, 8-di-*C*-glucoside |

| | | | |
|---|---|---|---|
| *2* | Luteolin-di-*O*-glucoside | Luteolin-di-*O*-glucoside | Luteolin-di-*O*-glucoside |
| *3* | Luteolin-7-*O*-glucoside | Luteolin-7-*O*-glucoside | Luteolin-3 ,7-di-*O*-glucoside |
| *4* | Apigenin-7-*O*-glucoside | Apigenin-7-*O*-glucoside | Luteolin-7-*O*-glucoside |
| *5* | Luteolin-3'-*O*-glucoside | Luteolin-3'-*O*-glucoside | Apigenin-7-*O*-glucoside |
| *6* | Luteolin | Luteolin | Luteolin-3'-*O*-glucoside |
| *7* | Apigenin | Apigenin | Luteolin |
| *8* | Chrysoeriol | Chrysoeriol | Apigenin |
| *9* | | | Chrysoeriol |

***Rhamnus***

The characterization of Rhamnus can be extremely varied, due to the complexity of compounds. Only 5 compounds can be shown in all programs, including: (1) Kaempferol-3-rutinoside, (2) Kaempferol-3-*O*-rhamnoside, (3) Kaempferol and (4) Aloemodin and (5) Emodin. Program B has shown better results and Program A, with extra identification of Kaempferol-3-*O*-*β*-*D*-xyloside, Rhamnocitrin, Isorhamnetin-*3*-*O*-rhamninoside, Kaempferol-3-*O*-rhamnoside, Emodin-8-glucoside, Rhamnocitrin-3-*O*-rhamninoside and Rhamnazine. In addition to the compounds identified in Program B, Program C showed the best result in terms of separation and characterization with totally 14 compounds observed. Kaempferol-3-*O*-acetyl-rhamnoside, Quercetin, Kaempferol-3-*O*-rhamninoside,

Rhamnocitrin-3-*O*-acetyl-rhamninoside, Rhamnetin-3-*O*-rhamninoside and Rhamnetin/ Isorhamnetin were only found in Program C. Overall, Program C has the best results for identifying Rhamnus extract. Details were summarized in Table 6d.

**Table 6d:** Compounds of Rhamnus Identified in Program A, B and C

| *Program* | A | B | C |
|---|---|---|---|
| *1* | Quercetin-3-*O*-rhamninoside | Kaempferol-3-*O*-*β*-*D*-xyloside | Kaempferol-3-*O*-acetyl-rhamnoside |
| *2* | Kaempferol-3-rutinoside | Rhamnocitrin | Kaempferol-3-*O*-*β*-*D*-xyloside |
| *3* | Kaempferol-3-*O*-rhamnoside | Isorhamnetin-3-*O*-rhamninoside | Quercetin |
| *4* | Kaempferol | Kaempferol-3-rutinoside | Kaempferol-3-*O*-rhamninoside |
| *5* | Rhamnazin-3-*O*-rhamninoside | Kaempferol-3-*O*-rhamnoside | Kaempferol-3-rutinoside |
| *6* | Aloemodin | Emodin-8-glucoside | Kaempferol-3-*O*-rhamnoside |
| *7* | Emodin | Rhamnocitrin-3-*O*-rhamninoside | Emodin-8-glucoside |
| *8* | | Rhamnazine | Rhamnocitrin-3-*O*-acetyl-rhamninoside |
| *9* | | Kaempferol | Kaempferol |
| *10* | | Aloemodin | Aloemodin |
| *11* | | Emodin | Rhamnetin-3-*O*-rhamninoside |

| | |
|---|---|
| *12* | Emodin |
| *13* | Rhamnetin |
| *14* | Kampferide |

***Brazilwood***

The identification of Brazilwood extract in 3 chromatographic programs has shown similar results that 6 compounds were successfully analyzed in all programs, including: (1) Brazilin, (2) Brazilein, (3) Adduct of Brazilin, (4) 9-*O*-methyl-Brazilin, (5) Dimer of Brazilin and (6) Compound Bra'. Thus, there is no need to do further comparison of the programs in analyzing Brazilwood extract.

To be summarized, in terms of the separation and characterization results in Program A, B and C, Program B seems to be suitable at identifying red dyes, especially for American Cochineal, while Program C has done excellent work on identifying yellows, especially for the very complex characterization of Rhamnus extract. Since our silk samples was dyed with Madder, American Cochineal and Rhamnus followed by traditional textile dyeing recipes, and Program B has generally shown good separation and identification results, that allowed the characterization to all standard samples above, we selected Program B to proceed the work on the qualitative analysis of natural organic dyes in silk samples.

## 5.4 Qualitative Analysis of Natural Organic Dyes from the Silk Samples

The qualitative analysis of Madder, American Cochineal and Rhamnus in Silk Samples is initially achieved by the analysis of LC-MS from selective Program B. Further confirmation was also achieved by comparison of the UV–Vis and mass spectra of the unknown peaks and the retention time of standard samples above that have been investigated. Thus, this identification method can give the largest degree of certainty of identification for the techniques used here, especially to samples with relatively small concentrations of the investigated unknown compounds that result in high pseudo-molecular ion intensities, yielding a good quality daughter ion spectrum.

***Madder***

As for compound identification of the silk sample dyed with Madder, totally 9 compounds in sequence were confirmed, including: (1) Lucidin primeveroside at 563 m/z, (2) Ruberythric acid at 533 m/z, (3) Rubiadin primeveroside (trace) at 547 m/z, (4) Munjistin at 283 m/z, (5) Pseudopurpurin at 299 m/z, (6) Anthragallol at 255 m/z, (7) Lucidin at 269 m/z, (8) Alizarin at 239 m/z and (9) Rubiadin at 253 m/z. And Peak no.1: Lucidin primeveroside, Peak no.2: Ruberythric acid, Peak no.5: Pseudopurpurin and Peak no.8: Alizarin have shown the relevant large peak, which were identified as the major compounds in this sample. In addition, compounds referring to Alizarin, Anthragallol and Rubiadin are also enough for the characterization of species of *Rubiaceae* family of plants. Details were shown in Figure 7 and Table 7.

**Fig. 7:** LC-PDA chromatogram (λ= 420nm) of compounds in the silk sample dyed with Madder.

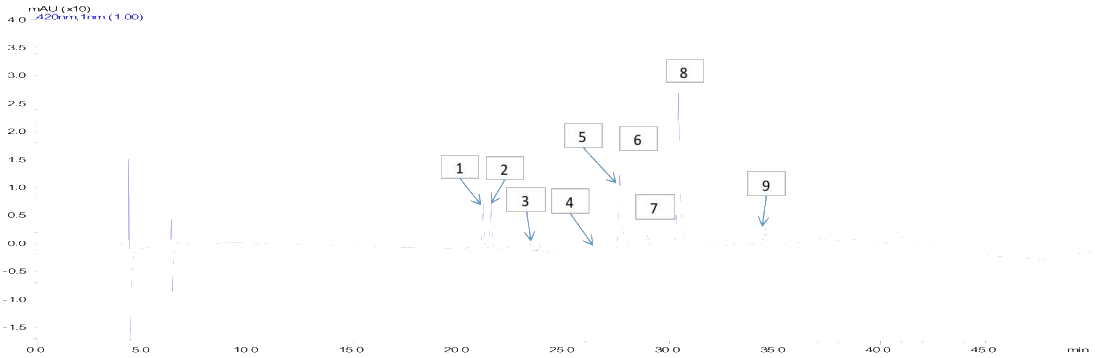
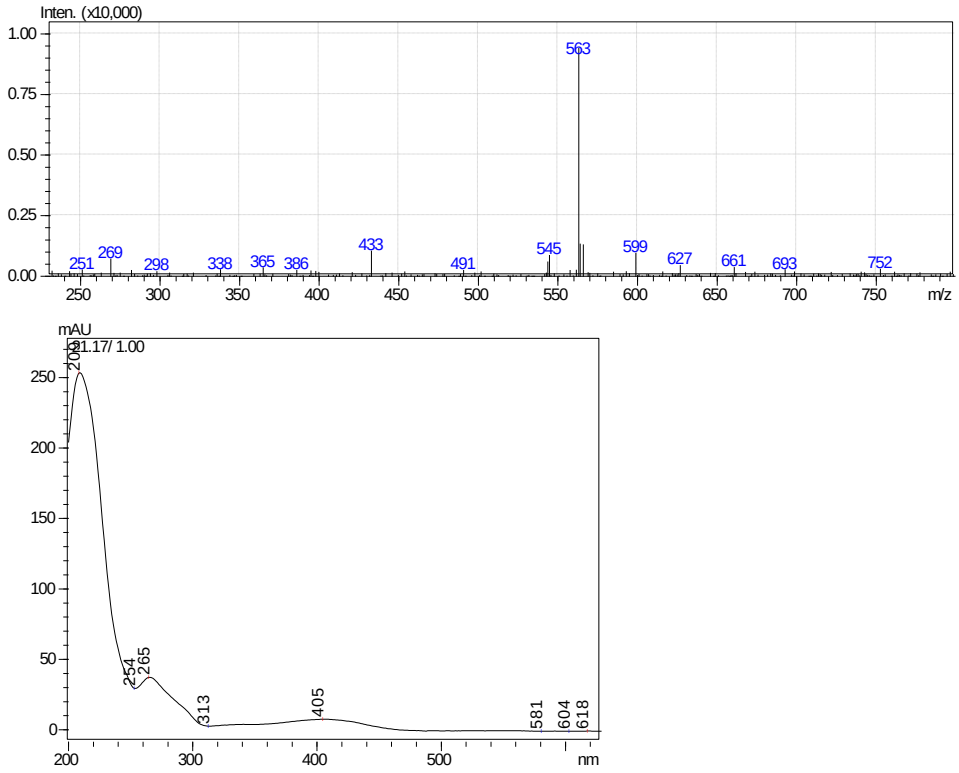



**Table 7:** Spectro-chromatographic data of compounds in the silk sample dyed with Madder.

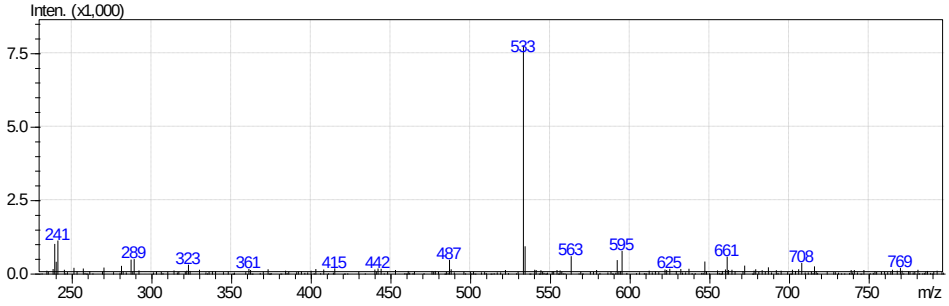
| Peak No. | Compound Name | $t_R$ (min) | UV λmax (nm) | $[M-H]^-$ (m/z) |
|---|---|---|---|---|
| **1** | Lucidin primeveroside | 21.17 | 209/265/405/618/638 | 563 |
| **2** | Ruberythric acid | 21.63 | 211/259/416/331/619 | 533 |
| **3** | Rubiadin primeveroside (trace) | 23.90 | 212/266/450/591/618 | 547 |
| **4** | Munjistin | 24.47 | 213/284/415/619 | 283 |
| **5** | Pseudopurpurin | 27.53 | 214/484/417/619/681 | 299 |
| **6** | Anthragallol | 27.70 | 212/282/410/619/682 | 255 |
| **7** | Lucidin | 29.03 | 214/279/403/619/681 | 269 |
| **8** | Alizarin | 30.50 | 215/278/427/618/681 | 239 |
| **9** | Rubiadin | 34.57 | 216/278/411/620/664 | 253 |

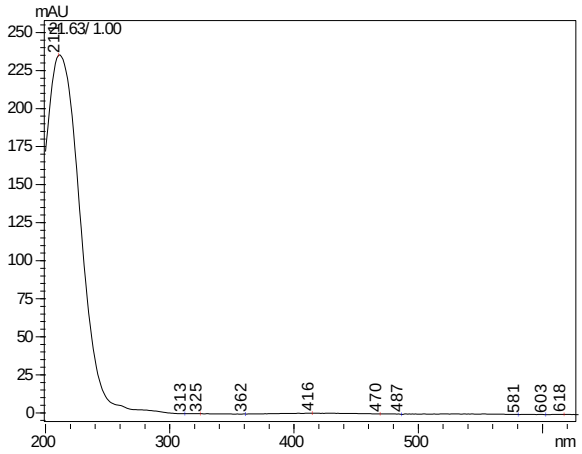
However, as for the details of each compound observed in the chromatographic program (Selective Program B), the qualitive analysis based on the retention time for each eluted peak, ESI(-) mass spectral data and UV-vis absorption is briefly clarified below:

(1) Lucidin primeveroside: Retention time of 21.17 min; $[M-H]^-$ at m/z 563.



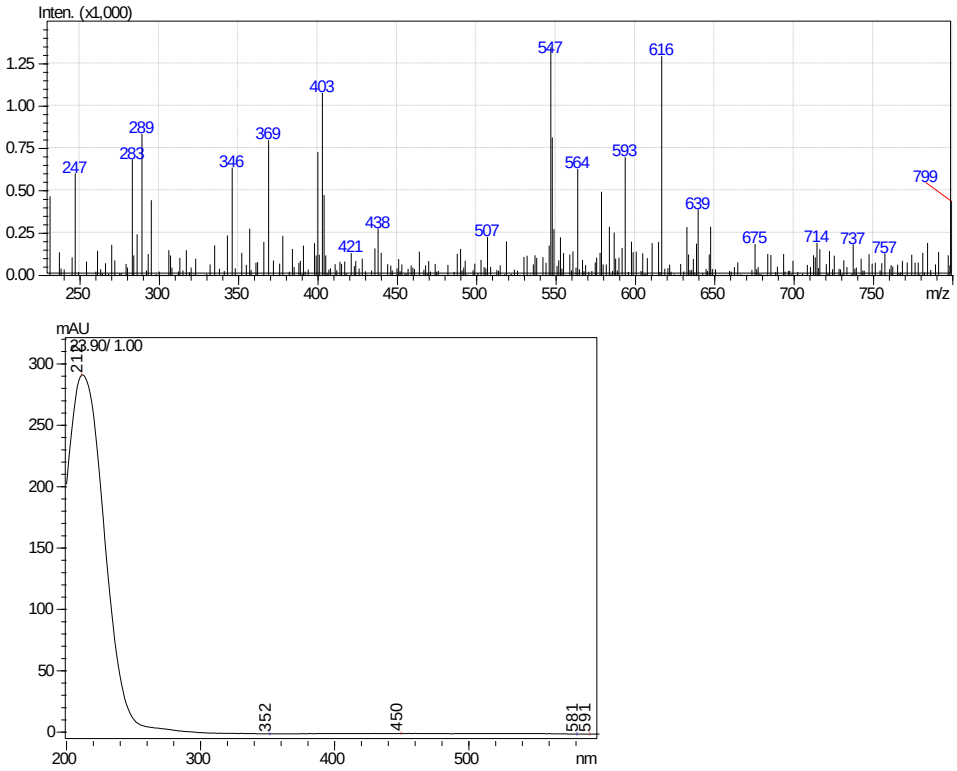

(2) Ruberythric acid: Retention time of 21.63 min; $[M-H]^-$ at m/z 533.



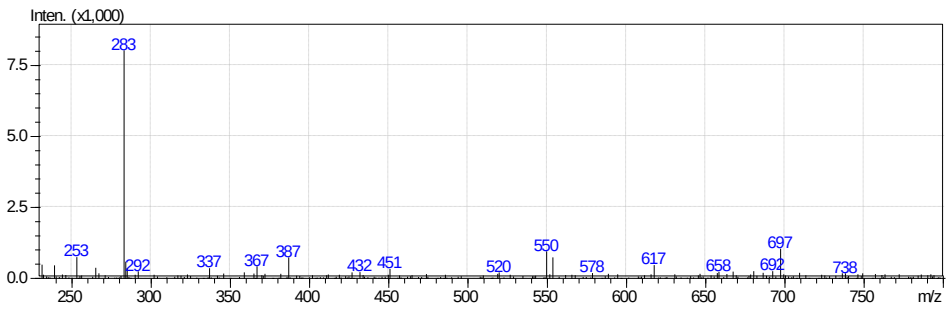


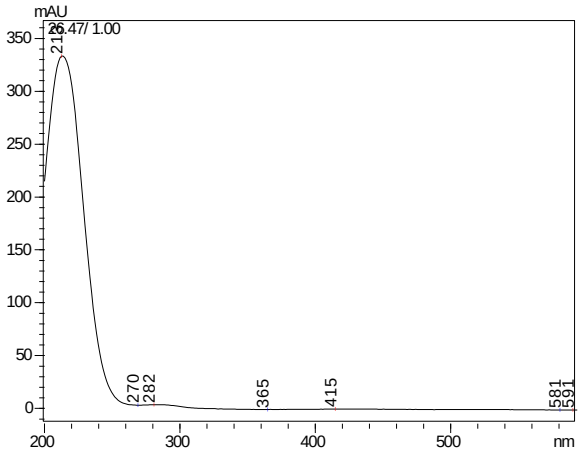

(3) Rubiadin primeveroside (traces): Retention time of 23.90 min; $[M - H]^-$ at m/z 547.

The separation result is not distinguishing.

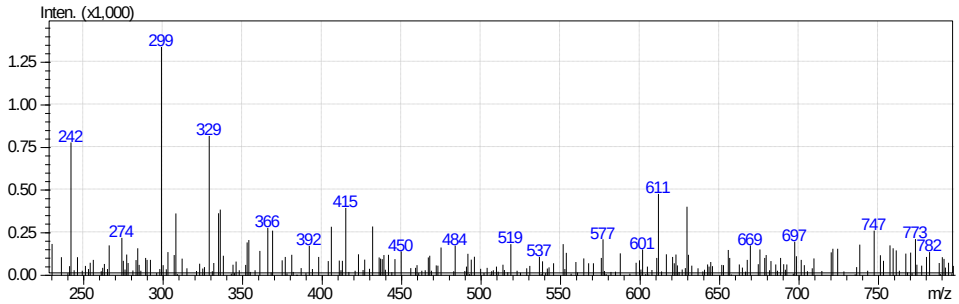


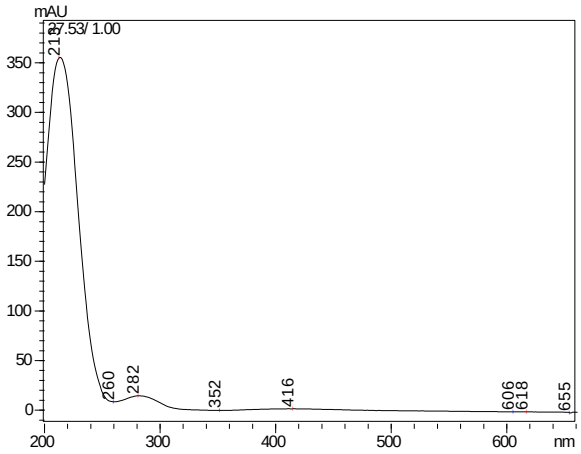

(4) Munjistin: Retention time of 24.47 min; $[M - H]^-$ at m/z 283.



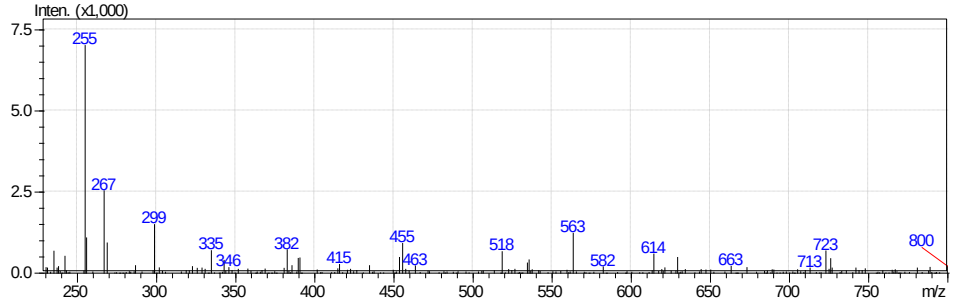


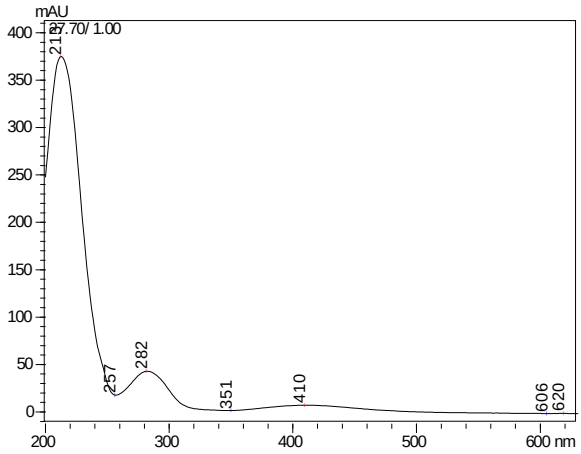

(5) Pseudopurpurin: Retention time of 27.53 min; $[M - H]^-$ at m/z 299.



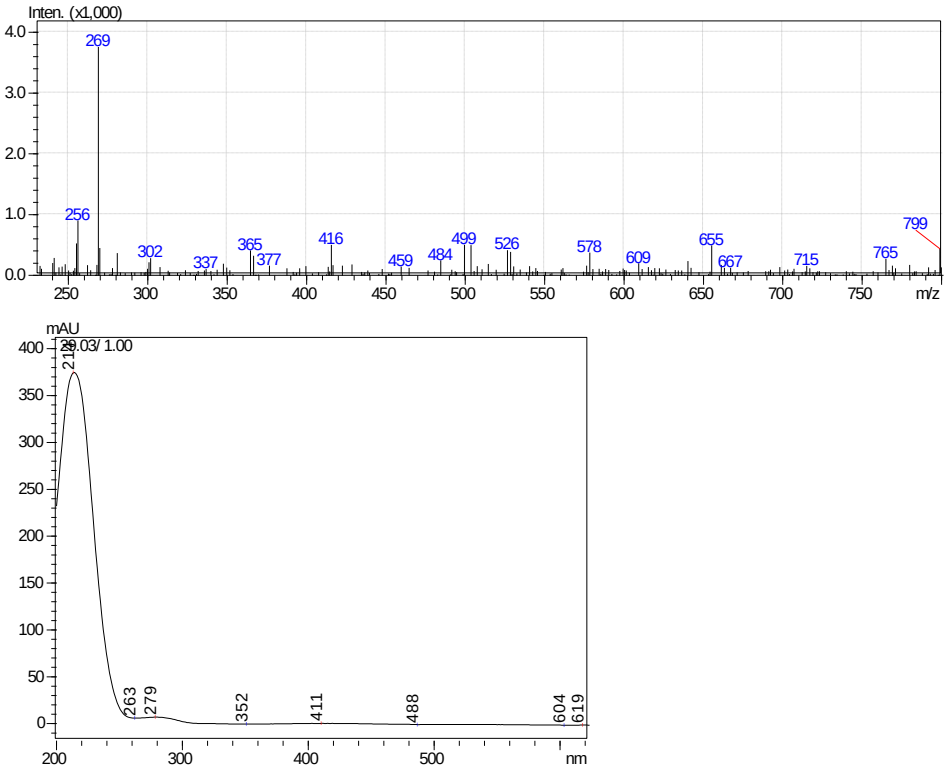




(6) Anthragallol: Retention time of 27.70 min; $[M - H]^-$ at m/z 255.

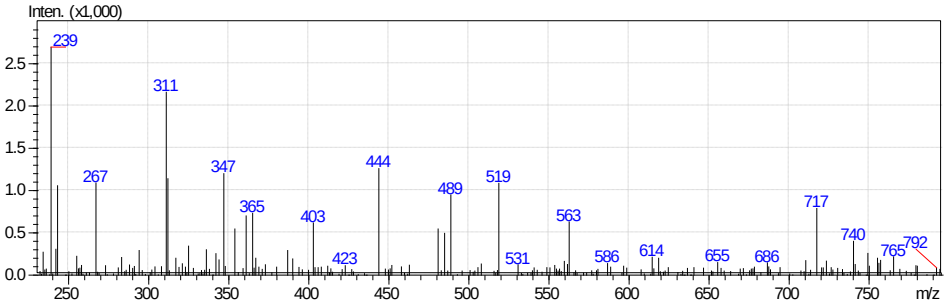


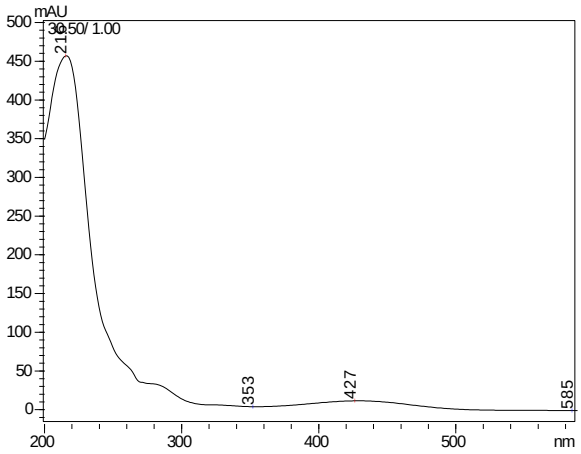



(7) Lucidin: Retention time of 29.03 min; $[M - H]^-$ at m/z 269.

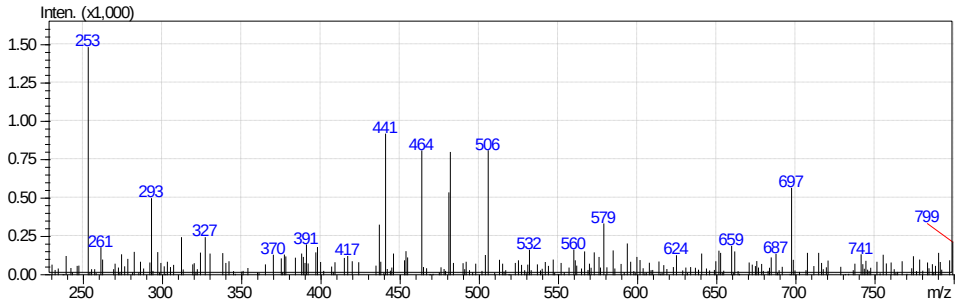


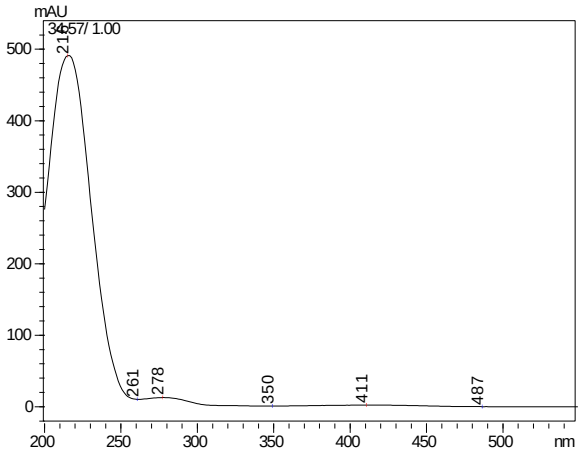

(8) Alizarin: Retention time of 30.50 min; $[M - H]^-$ at m/z 239.






(9) Rubiadin: Retention time of 34.57 min; $[M-H]^-$ at m/z 253.




### *American Cochineal*

In the investigation of American Cochineal extract of the silk sample, totally 4 compounds were identified, including: (1) ca at 491 m/z, (2) dcIV at 491 m/z, (3) dc7 at 611 m/z and (4) fa at 313 m/z. Details are shown in Figure 8 and Table 8.

**Fig. 8:** LC-PDA chromatogram (λ= 490nm) of compounds in the silk sample dyed with American Cochineal.

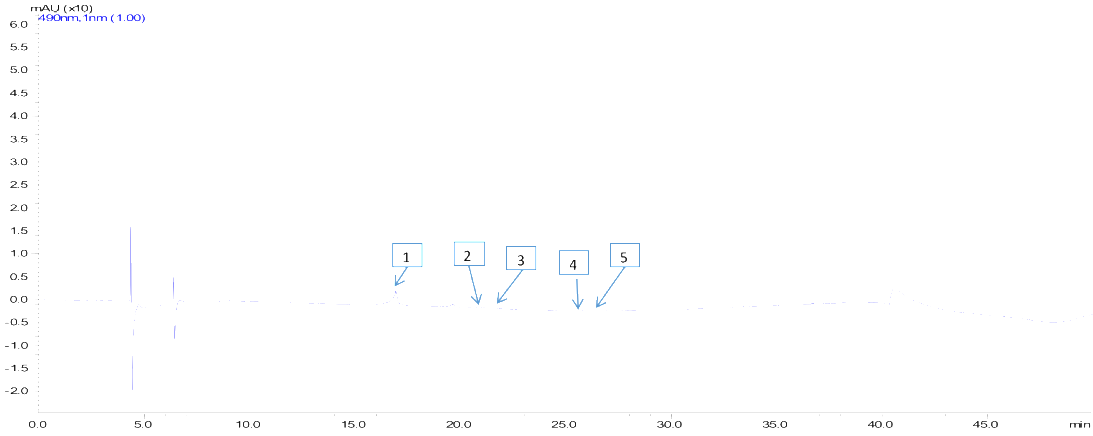


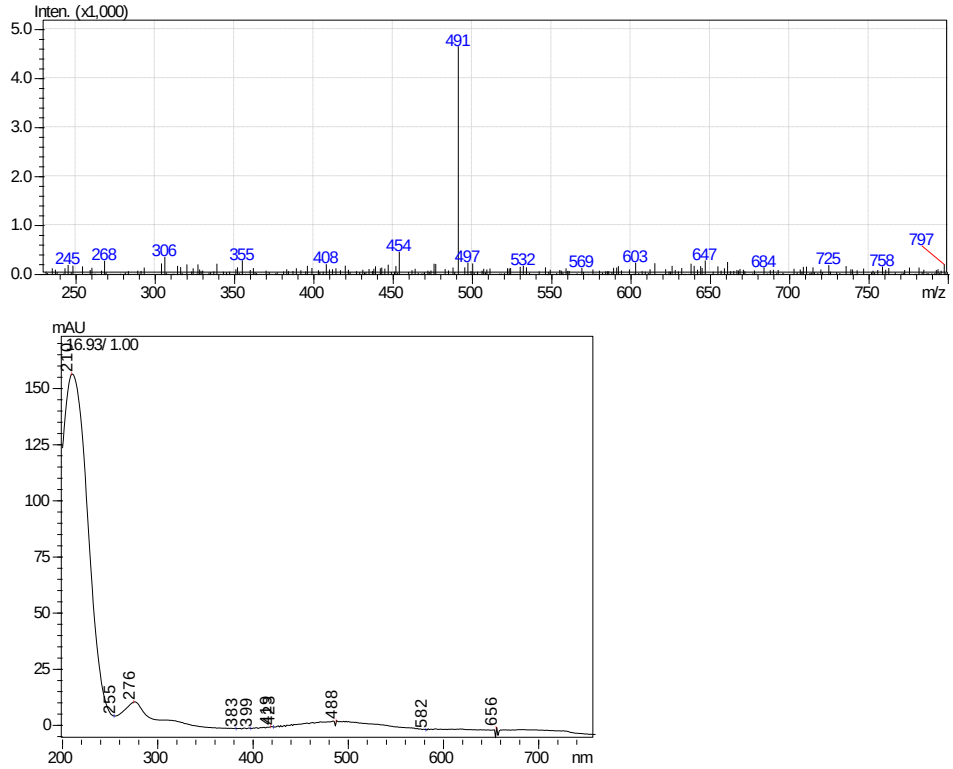

**Table 8:** Spectro-chromatographic data of compounds in the silk sample dyed with American Cochineal.

| Peak No. | Compound Name | $t_R$ (min) | UV λmax (nm) | $[M-H]^-$ (m/z) |
|---|---|---|---|---|
| **1** | ca: Carminic acid (*C*-glucopyranoside of kermesic acid) | 16.93 | 210/276/488/420/656 | 491 |
| **2** | dcIV: *C*-glucofuranoside of kermesic acid (isomer of carminic acid) | 21.00 | 211/488/276/656/420 | 491 |
| **3** | dc7: *C*-glucoside, carboxyphenyl ester of carminic acid ((carminyloxy)benzoic acid) | 21.23 | 211/488/276/656/531 | 611 |

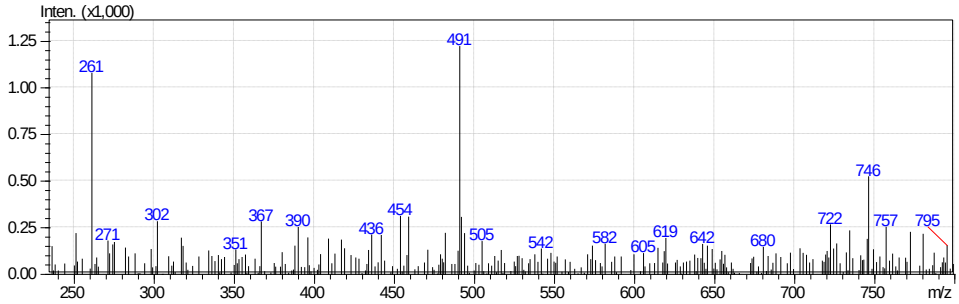
| | | | | |
|---|---|---|---|---|
| **4** | fa: Flavokermesic acid | 25.77 | 213/433/276/656/617 | 313 |

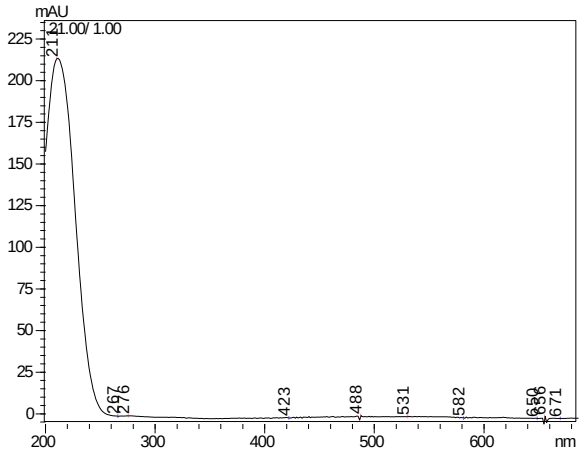
The qualitive analysis of American Cochineal extract from the silk sample by selective Program B was based on the retention time for each eluted peak, ESI(-) mass spectral data and UV-vis absorption below:

(1) Carminic acid: Retention time of 16.93 min; $[M-H]^-$ at m/z 491.

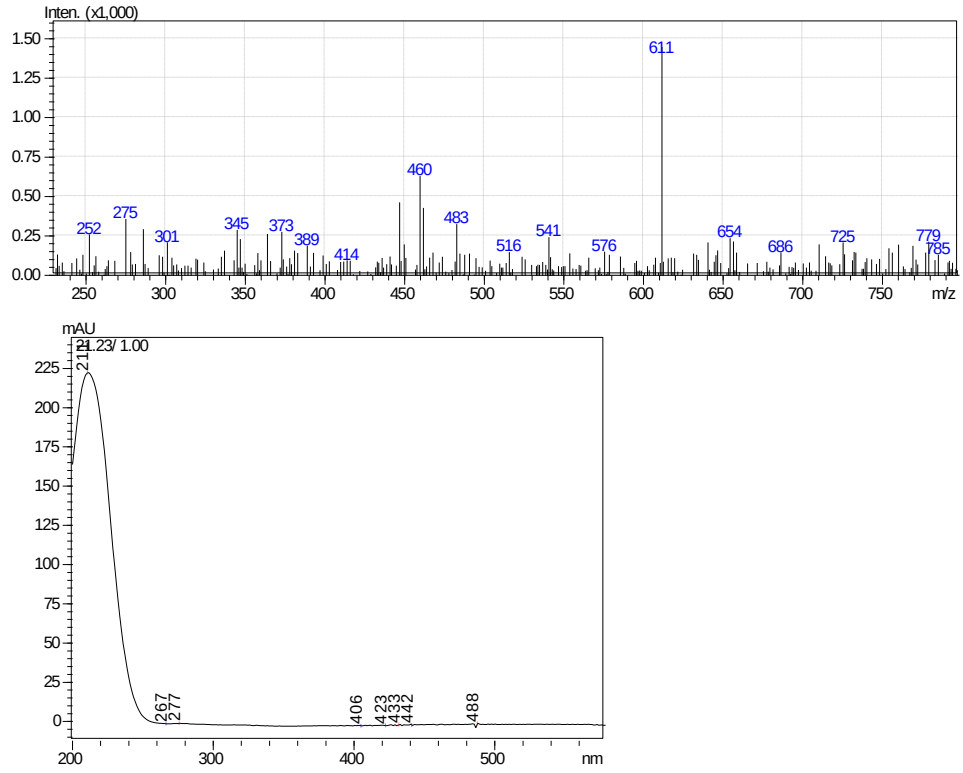



(2) dcIV: Retention time of 21.00 min; $[M-H]^-$ at m/z 491.

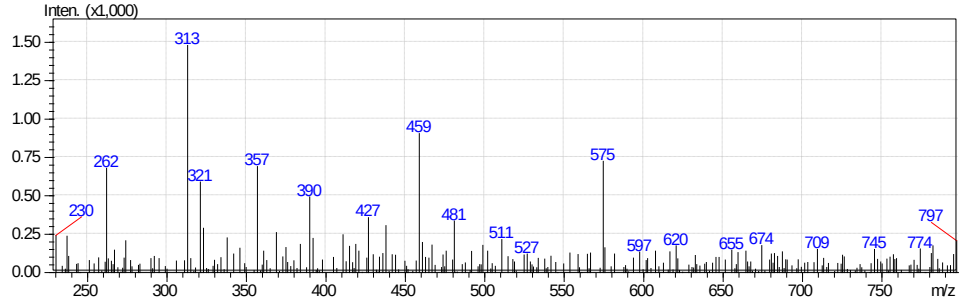


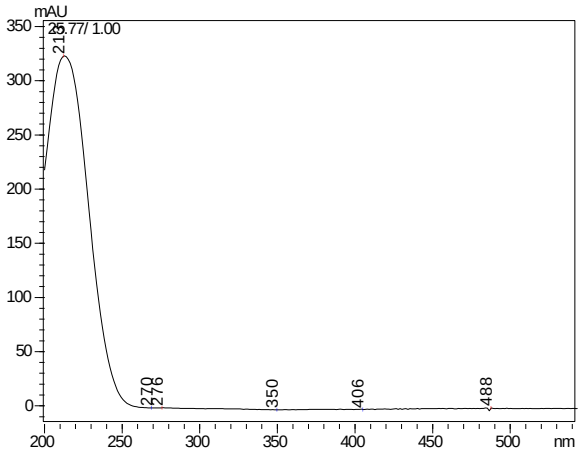



(3) dc7: Retention time of 21.23 min; $[M - H]^-$ at m/z 611.




(4) fa: Retention time of 25.77 min; $[M - H]^-$ at m/z 313.






***Rhamnus***

The selective Program B has done a very good investigation on the identification of Rhamnus compounds, especially for the species of *R. petiolaris*. There are totally 13 compounds identified, including: (1) Kaempferol-3-*O*-acetyl-rhamnoside at 781 m/z, (2) Quercetin-3-*O*-rhamninoside at 755 m/z, (3) Kaempferol-3-*O*-rhamninoside at 739 m/z, (4) Quercetin-3-*O*-robinoside at 609 m/z, (5) Quercetin-3-*O*-rhamnoside at 447 m/z, (6) Isorhamnetin-3-*O*-rhamninoside at 769 m/z, (7) Rhamnocitrin-3-*O*-rhamninoside at 753 m/z, (8) Rhamnazin-3-*O*-rhamninoside at 783 m/z, (9) Rhamnetin-*O*-robinoside at 623 m/z, (10) Rhamnetin-3-*O*-rhamnoside at 461 m/z, (11) Rhamnazine at 329 m/z, (12) Kaempferol at 285 m/z and (13) Emodin at 269m/z. The largest peak was (6) Isorhamnetin-3-*O*-rhamninoside at 769 m/z, which eluted at 21.70 min with absorption maxima at 211nm. In addition to Peak no. 6, Peak no. 2, 3, 7, 8 are assigned as flavonol-3-*O*-rhamninosides including: Quercetin-3-*O*-rhamninoside, Kaempferol-3-*O*-rhamninoside, Rhamnocitrin-3-*O*-rhamninoside and Rhamnazin-3-*O*-rhamninoside respectively, based on similar fragmentation patterns and features of the UV spectra similar to those of Isorhamnetin-3-

*O*-rhamninoside. Moreover, Peak no. 4, with the quasi-molecular ion at m/z 611, can be identified as Quercetin-3-*O*-robinoside, which has been discovered in R. *disperma* and reported on in the previous study. Peak no. 2 refers to Kaempferol-3-O-rhamninoside. According to the reference literature, Peak no. 2 and 7 can be used as markers for distinguishing between *R. petiolaris* and *R. utilis*. Furthermore, a minor peak in the beginning corresponding to Kaempferol-3-O-acetyl-rhamnoside, eluting at 16.20 min with absorption maxima at 210 nm is a significant marker, rather than Kaempferol-3-O-rhamninoside. Comparison of the profiles in Fig. 2 shows that quercetin-3-O-robinoside (Peak no. 4) and quercetin-3-O-rhamnoside (Peak no. 5), were present in the rage of 19.93-20.83 min regarded as the extract of *R. petiolaris*. (Liu et al., 2018) Details are shown in Figure 9 and Table 9.

**Fig. 9:** LC-PDA chromatogram (λ= 350nm) of compounds in the silk sample dyed with Rhamnus.

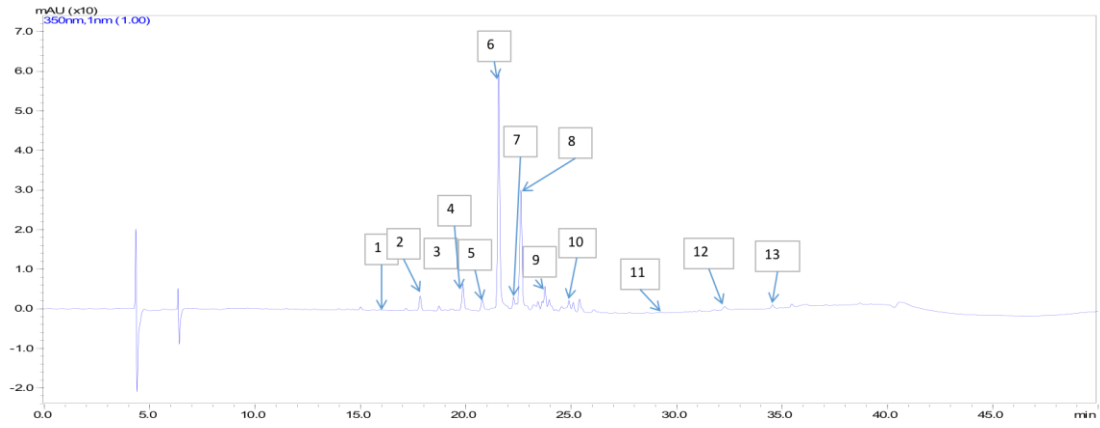
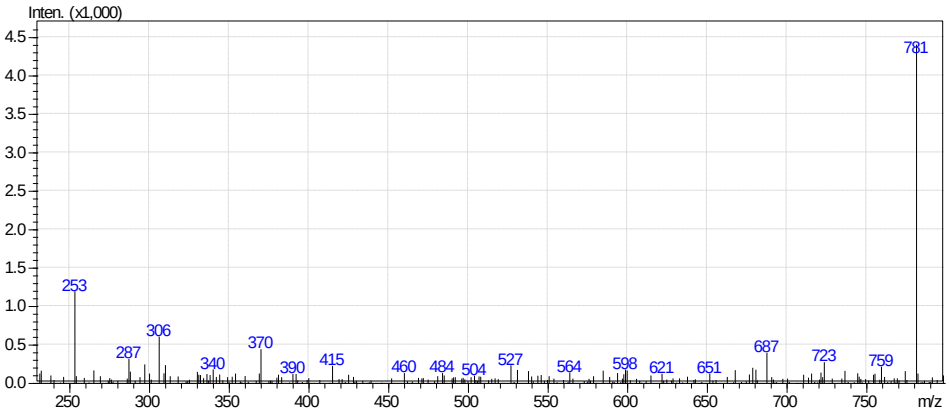


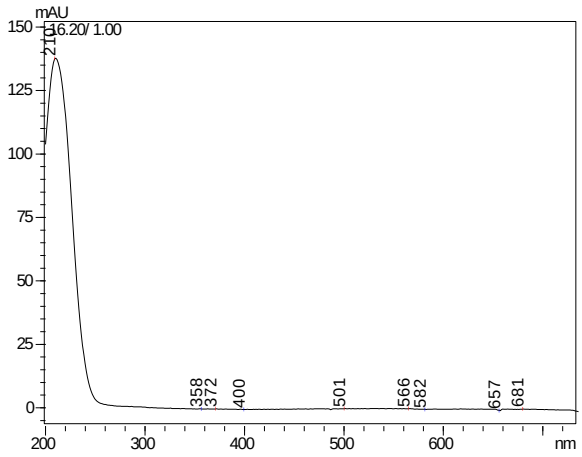

**Table 9:** Spectro-chromatographic data of compounds in the silk sample dyed with Rhamnus.

| Peak No. | Compound Name | $t_R$ (min) | UV λmax (nm) | $[M-H]^-$ (m/z) |
|---|---|---|---|---|
| **1** | Kaempferol-3-*O*-acetyl-rhamnoside | 16.20 | 210/358/618/681 | 781 |
| **2** | Quercetin-3-*O*-rhamninoside | 17.93 | 210/354/300/544/475 | 755 |
| **3** | Kaempferol-3-*O*-rhamninoside | 19.37 | 211/353/501/618/727 | 739 |
| **4** | Quercetin-3-*O*-robinoside | 19.93 | 210/347/300/472 | 609 |
| **5** | Quercetin-3-*O*-rhamnoside | 20.83 | 211/350/300/544/472 | 447 |
| **6** | Isorhamnetin-3-*O*-rhamninoside | 21.70 | 208/256/355/471/660 | 769 |
| **7** | Rhamnocitrin-3-*O*-rhamninoside | 22.37 | 211/350 | 753 |
| **8** | Rhamnazin-3-*O*-rhamninoside | 22.73 | 210/355/660/681/727 | 783 |
| **9** | Rhamnetin-*O*-robinoside | 23.83 | 211/347/544/618/681 | 623 |
| **10** | Rhamnetin-3-*O*-rhamnoside | 24.97 | 212/358/544/501/681 | 461 |
| **11** | Rhamnazine | 29.37 | 214/353/501/681/727 | 329 |
| **12** | Kaempferol | 32.83 | 215/326/377/485/566 | 285 |
| **13** | Emodin | 34.63 | 216/286/437/643/727 | 269 |

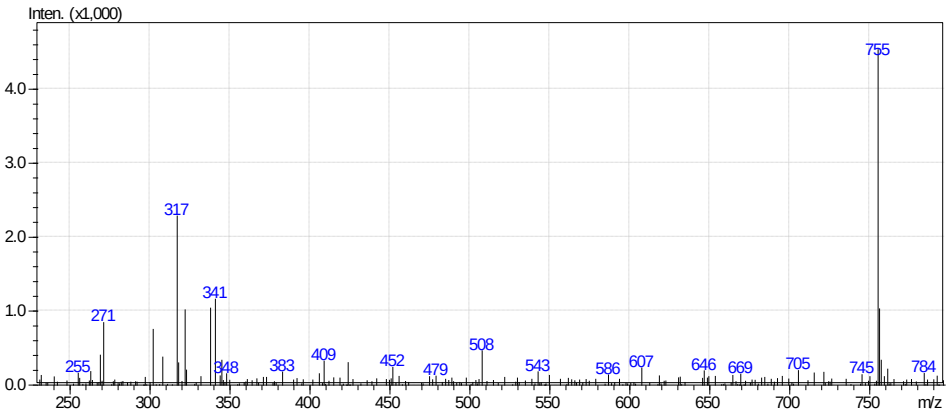
However, as for the details of each compound observed in the chromatographic program (Selective Program B), the qualitive analysis based on the retention time for each eluted peak, ESI(-) mass spectral data and UV-vis absorption is also briefly clarified below:

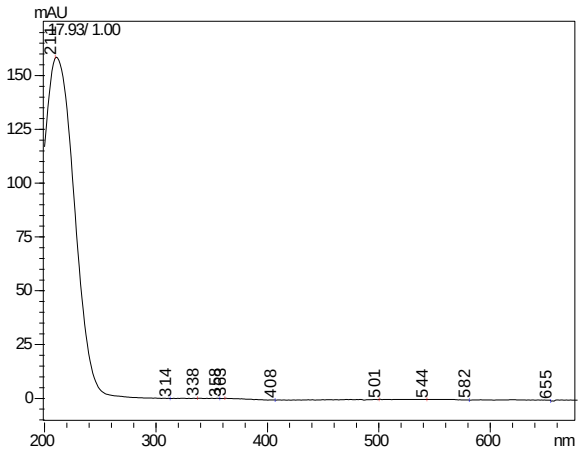
(1) Kaempferol-3-*O*-acetyl-rhamnoside: Retention time of 16.20 min; $[M-H]^-$ at m/z 781.



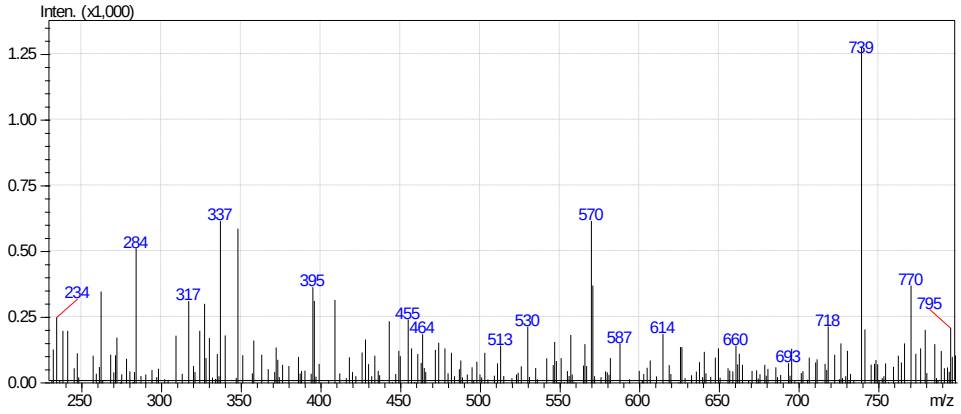



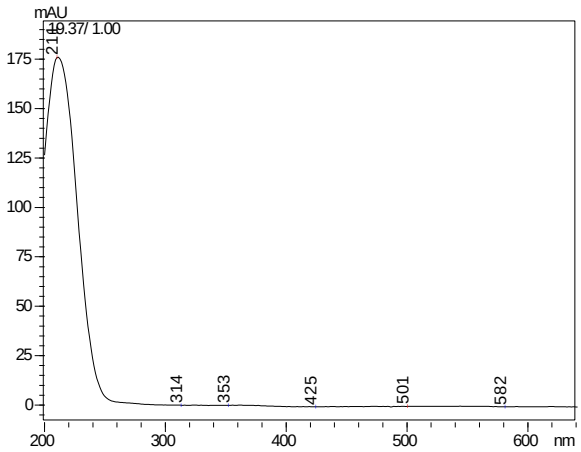

(2) Quercetin-3-*O*-rhamninosid: Retention time of 17.93 min; $[M-H]^-$ at m/z 755.



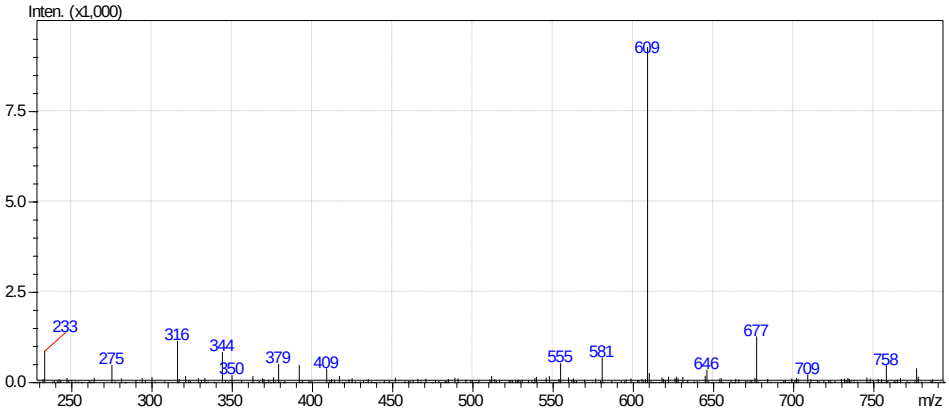


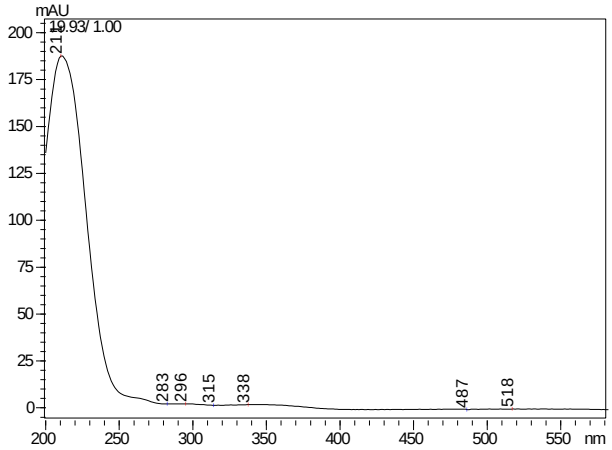

(3) Kaempferol-3-*O*-rhamninoside: Retention time of 19.37 min; $[M - H]^-$ at m/z 739.



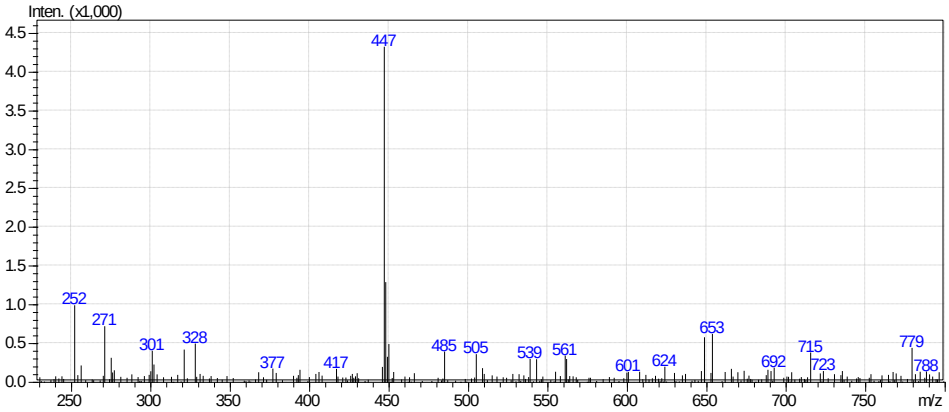



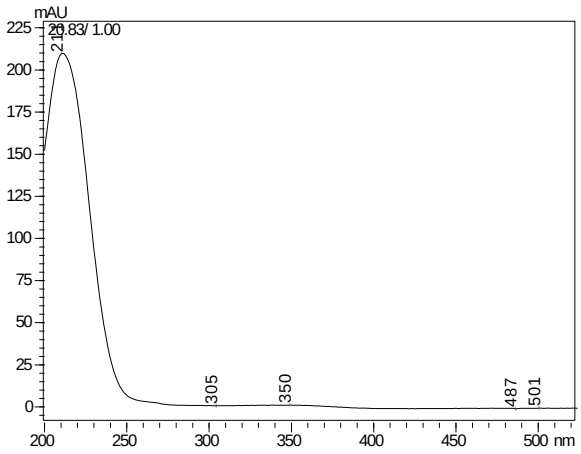

(4) Quercetin-3-*O*-robinoside: Retention time of 19.93 min; $[M - H]^-$ at m/z 609.



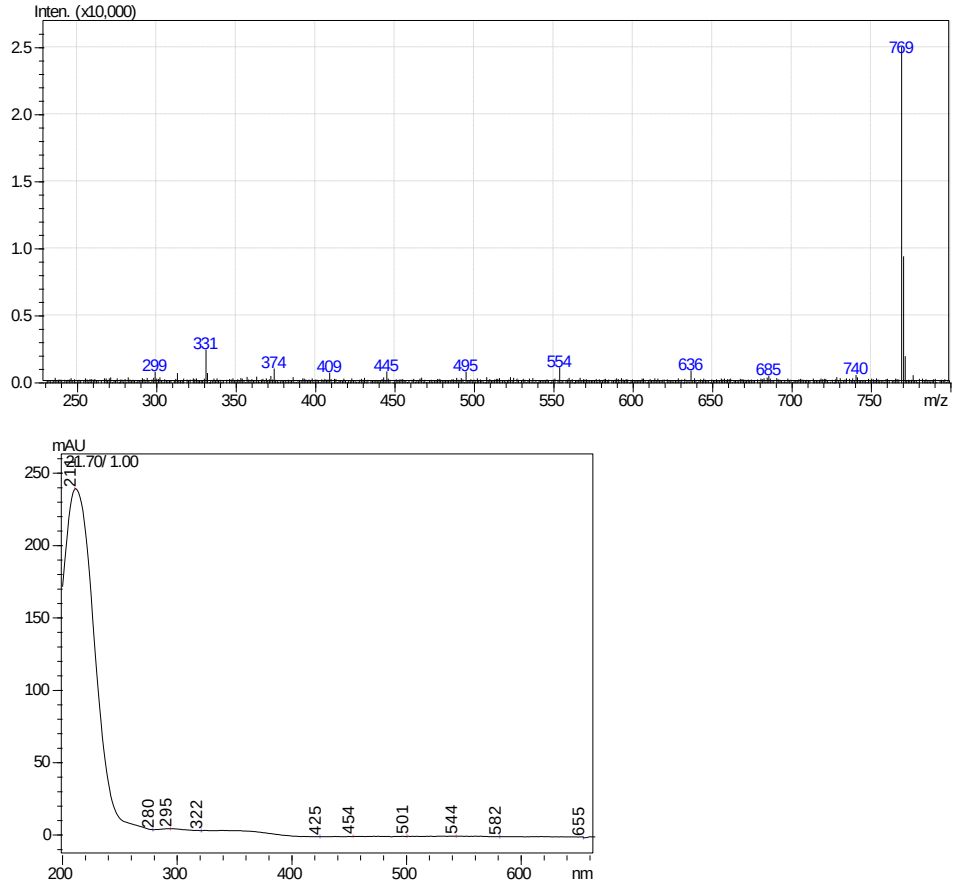




(5) Quercetin-3-*O*-rhamnoside: Retention time of 20.83 min; $[M-H]^-$ at m/z 447.

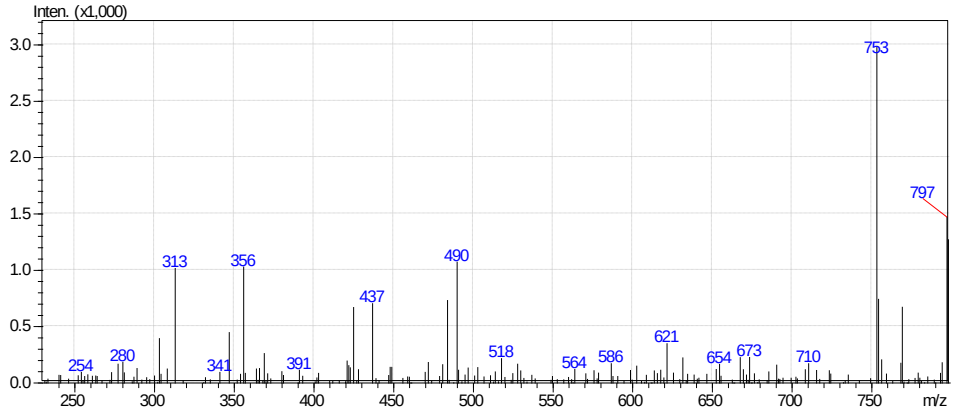


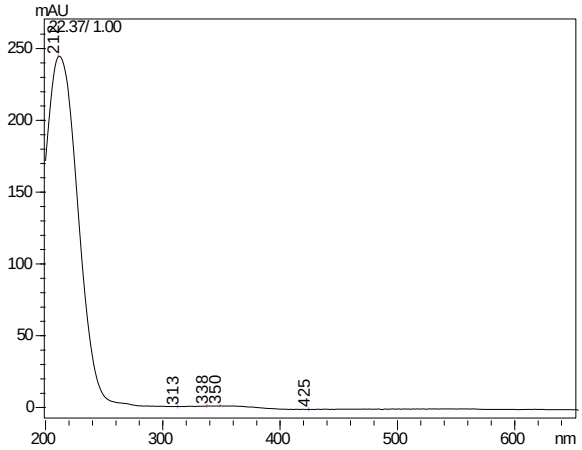



(6) Isorhamnetin-3-*O*-rhamninoside: Retention time of 21.70 min; $[M-H]^-$ at m/z 769.

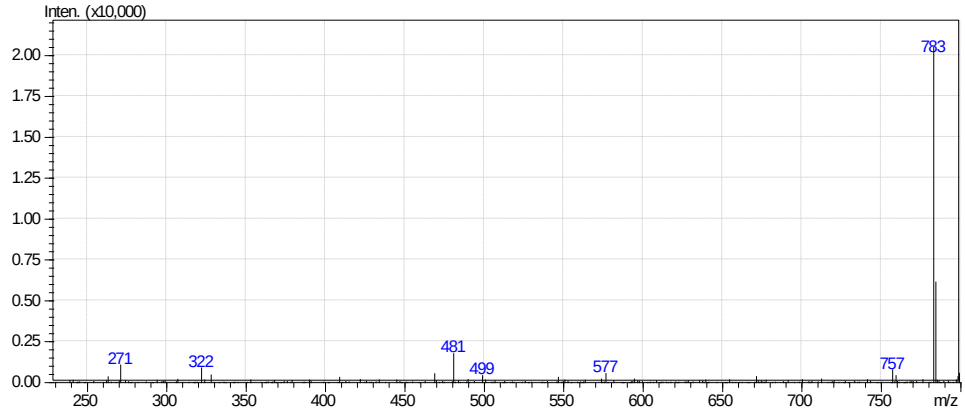


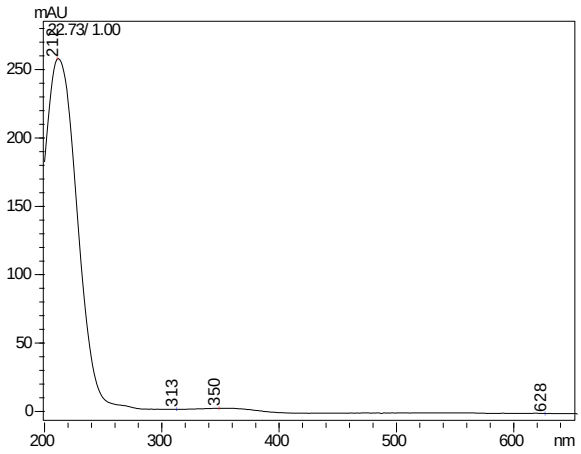

(7) Rhamnocitrin-3-*O*-rhamninoside: Retention time of 22.37 min; $[M-H]^-$ at m/z 753.



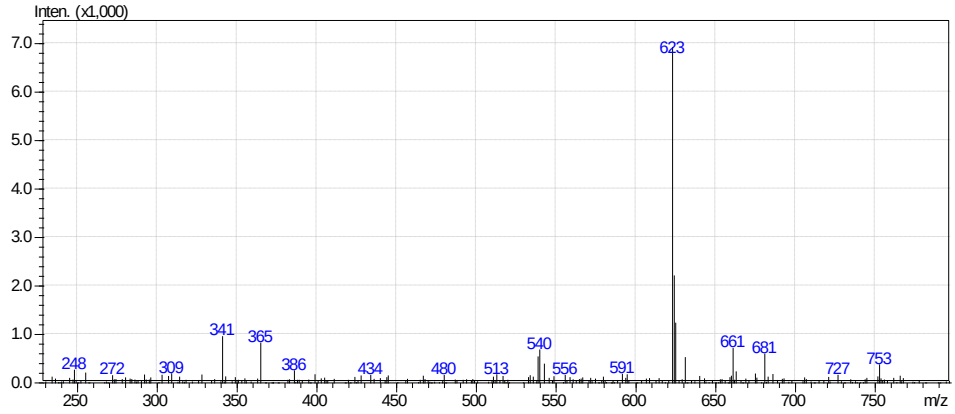



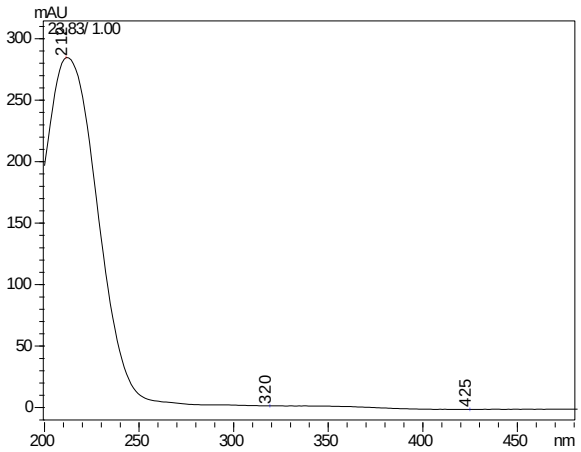

(8) Rhamnazin-3-*O*-rhamninoside: Retention time of 22.73 min; $[M - H]^-$ at m/z 783.



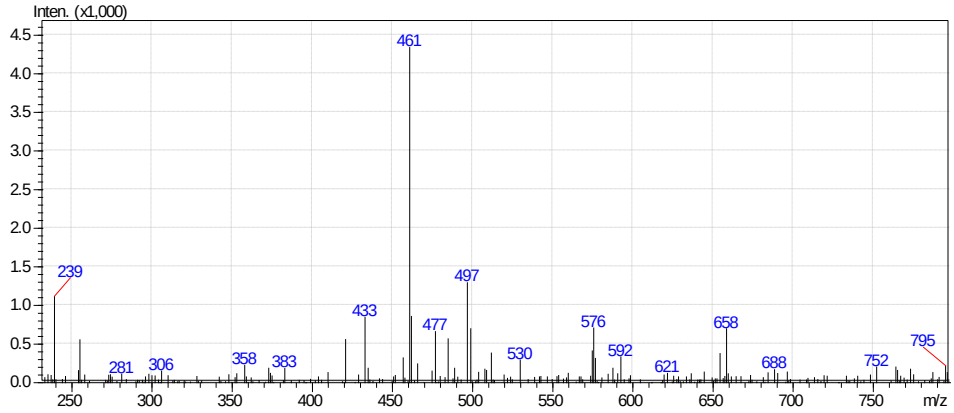


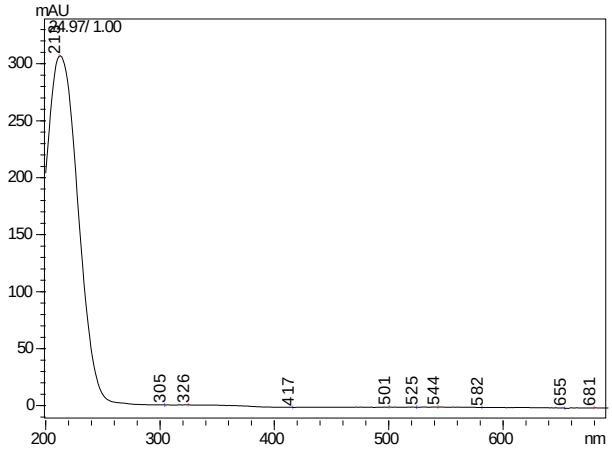

(9) Rhamnetin-*O*-robinoside: Retention time of 23.83 min; $[M-H]^-$ at m/z 623.



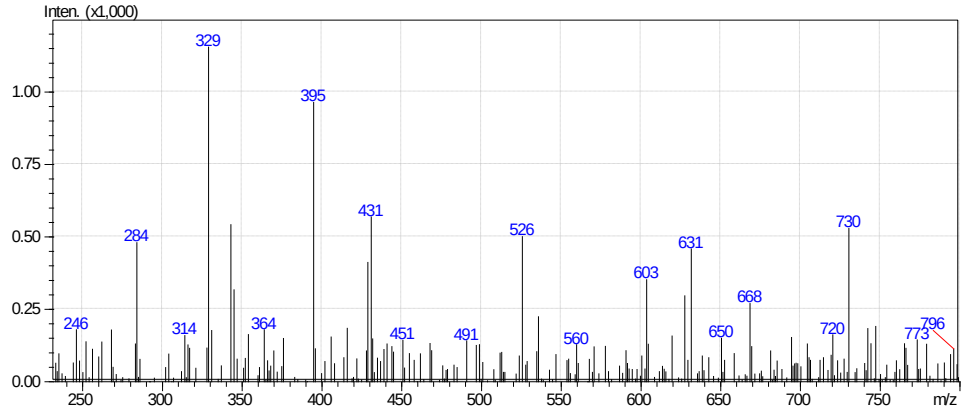



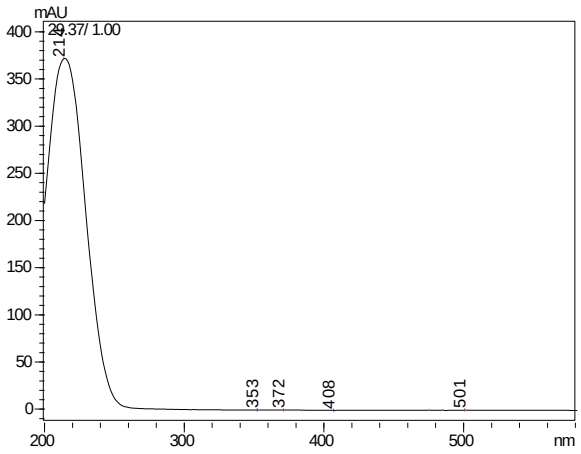

(10) Rhamnetin-3-*O*-rhamnoside: Retention time of 24.97 min; $[M-H]^-$ at m/z 461.



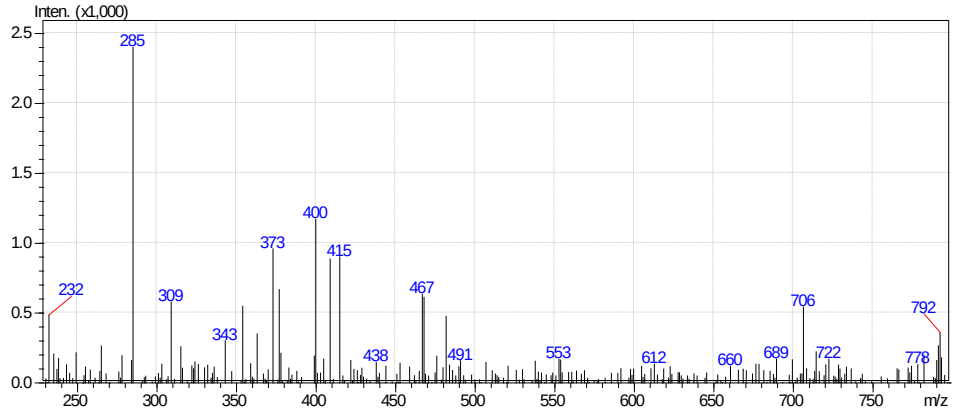



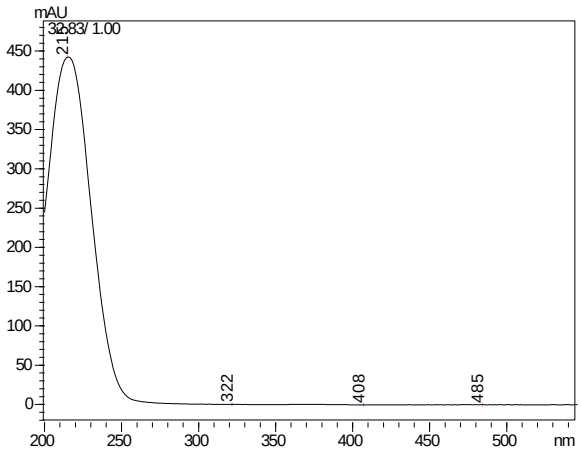

(11) Rhamnazine: Retention time of 29.37 min; $[M - H]^-$ at m/z 329.



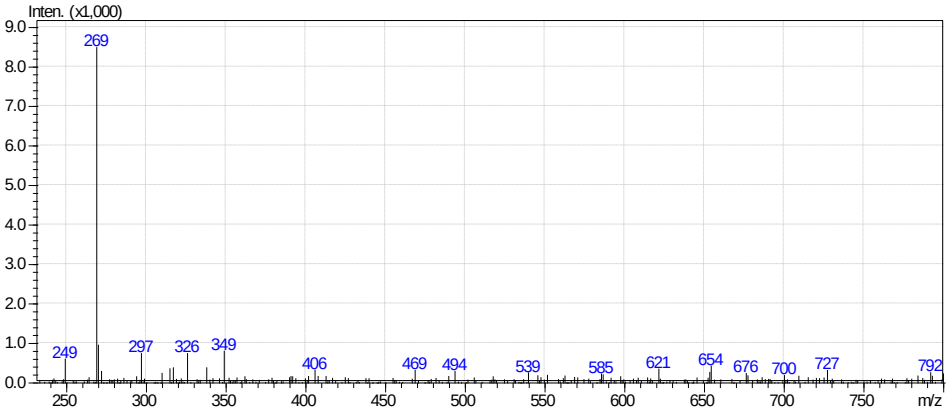


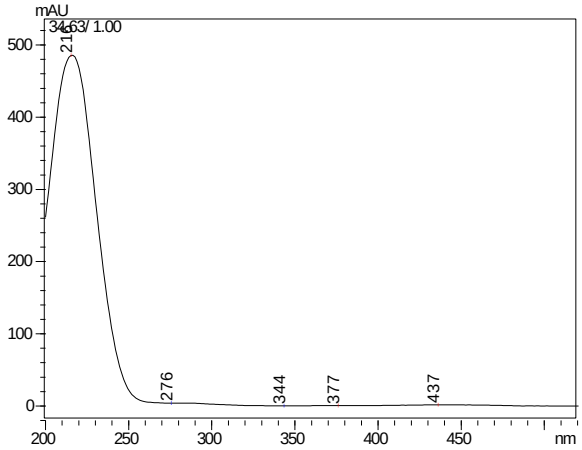

(12) Kaempferol: Retention time of 32.83 min; $[M - H]^-$ at m/z 285.




(13) Emodin: Retention time of 34.63 min; $[M - H]^-$ at m/z 269.




Therefore, according to the chromatographic data and the separation results shown above, selective Program B has generally applied successfully on the identification on the silk samples dyed with Madder, American Cochineal and Rhamnus, and the qualitative analysis of Rhamnus compounds has shown the powerfulness of this chromatographic program. Thus, Program B not only had the good separation in both red and yellow standard samples, but also proved suitable to effectively conduct an qualitative analysis of the most common red and yellow dyes from silk samples.

## 6. Conclusion

This thesis work has presented a comprehensive analysis of the most common organic natural dyes used in Mediterranean region since historical times, which can help to strengthen the interdisciplinary collaborations between archaeology and analytical chemistry. Since dyes and textiles have a great artistic and historic significance in human civilization, of which studies is also the key to reconstructing the story of dyes and elucidating the circumstances that dyes have been produced. In addition, investigation of the origin, nature and chemical behavior of these colorants for producing historical textiles may shed new light on their color and appearance. Thus, a standard database of dyes for qualitative analysis is required. In this study, 5 pure dye materials in terms of Madder, American Cochineal, Brazilwood, Weld and Rhamnus were extracted by ACN/water solvent system to largely preserve their chemical information as reference samples. Furthermore, improved mild extraction with formic acid and methanol enabled the observation of intact organic dyes in silk samples dyed with Madder, American Cochineal and Rhamnus. The pre-treatment has been done successfully, which has effectively facilitated the release of dyes and preserved glycoside linkages of flavonoids. Importantly, Liquid chromatography couple with photodiode array detector and mass spectrometer was employed for the identification of compounds from 5 standard dye extracts in 3 different chromatographic programs: Program A, B and C respectively under same conditions, and Program B has been selected to be applied in the real samples from silk textiles due to its general effectiveness of separation in all standard samples including yellows and reds. Thus,

the identification of compounds was actually performed by comparison of retention times, UV and mass spectra in the negative ionization mode (ESI(-)) to those obtained for the compounds found in Madder, American Cochineal and Rhamnus (*R. petiolaris)* extracts to those of 5 standard reds and yellows under the same chromatographic conditions. The selected Program B, again, has done an overall good qualitative analysis on these most common red and yellow dyes from silk samples. Therefore, the mild extraction using relevant soft solution (ACN/water, formic/methanol) for natural organic dyes, combined with LC-PDA-MS technique and chromatographic program B, has contributed a comprehensive database of the most common red and yellow dyes and verified the effectiveness of the program for qualitative analysis of natural organic dyes.

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