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Identification of Dyestuffs in the Natural Pigments Produced with Al^{3+}, Fe^{2+} and Sn^{2+} Mordant Metals from Cochineal (*Dactylopius coccus* Costa) and Walloon oak (*Quercus ithaburensis* Decaisne) by HPLC-DAD

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In this study, the natural pigments from the cochineal (*Dactylopius coccus* Costa) dye insect and the walloon oak (*Quercus ithaburensis* Decaisne) dye plant were produced by using $KAl(SO_4)_2 \cdot 12H_2O$ (alum), $FeSO_4 \cdot 7H_2O$ and $SnCl_2 \cdot 2H_2O$ mordants. A reversed-phase high performance liquid chromatography (HPLC) with diode array detection (DAD) method was utilized for the identification of dyestuffs in the natural pigments. The dyestuff extractions from the pigments were carried out with HCl/MeOH/H_2O (2:1:1; v/v/v) solution.

Key Words: Natural pigment, HPLC, Dyestuff analysis, Walloon oak, Cochineal.

INTRODUCTION

Walloon oak (*Quercus ithaburensis* Decaisne) is a tree growing to 15-20 m in open forests in Turkey and Greece[1]. Their acorn caps contain 25-35 % tannin[2,3]. The complicated chemistry of tannins has been explained by Schweppe[4]. The hydrolyzable tannin *i.e.*, ellagic acid is found in the bark of oak[5]. Cochineal (*Dactylopius coccus* Costa) feeds on the cactus species *Nopalea coccinellifera* in Central and South America. Carminic acid which main dyestuff component may exhibit approximately 20 % by weight of the total insect body mass[6]. Carminic acid was identified in cochineal insect by several authors[7-17]. Metal-tannin or metal-anthraquinone complexes are generated by the reaction of metals like Al(III); [$KAl(SO_4)_2 \cdot 12H_2O$], Sn(II); [$SnCl_2 \cdot 2H_2O$] and Fe(II); [$Fe(SO_4) \cdot 7H_2O$] with the dyestuffs[18] obtained from the plant or insect dye sources[19-24]. For the identification of dyestuffs in the pigments or the dye plants, several analytical techniques have been used *e.g.*, high performance liquid chromatography[25-28], thin layer chromatography[19,29],

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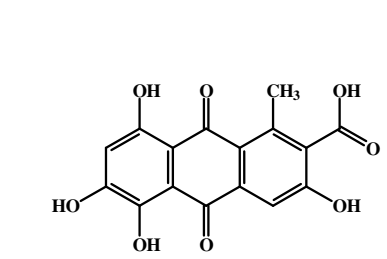
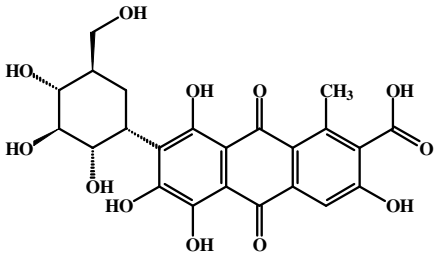
UV-visible spectrophotometry[30,31], FT-IR spectroscopy[32,33], Raman spectroscopy[34], capillary electrophoresis with electrospray mass spectrometric detection[35] and gas chromatography/mass spectrometry[36]. A reversed-phase high performance liquid chromatography using a diode-array detection (DAD) is ideally suited for the identification of dyestuffs[37,38].

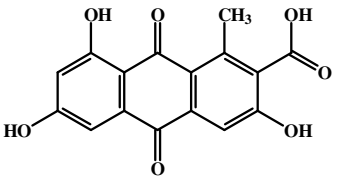
The aim of present study is to obtain natural pigments which complexes formed with the mordant solutions [$KAl(SO_4)_2 \cdot 12H_2O$ (alum), $FeSO_4 \cdot 7H_2O$ and $SnCl_2 \cdot 2H_2O$] of the dyestuffs in cochineal (*Dactylopius coccus* Costa) dye insect and walloon oak (*Quercus ithaburensis* Decaisne) dye plant. However, the natural pigments are to analyze qualitatively with a reversed-phase high performance liquid chromatography (HPLC) with diode-array detection (DAD).



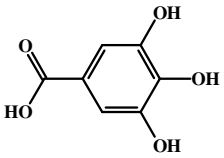
Kermesic acid



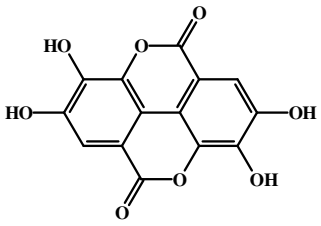
Carminic acid



Flavokermesic acid



Gallic acid



Ellagic acid

EXPERIMENTAL

Dye insect and dye plant: Cochineal (*Dactylopius coccus* Costa) dye insects and the acorn caps of walloon oak (*Quercus ithaburensis* Decaisne) dye plant were obtained from Laboratory for Natural Dyes, Faculty of Fine Arts, Marmara University.

Stannius chloride, ferrous sulphate, potassium carbonate, hydrochloric acid and methanol, gallic acid and ellagic acid were purchased from Merck (Darmstadt, Germany); alum (potassium aluminum sulphate) was provided by Pancreac (Madrid, Barcelona). Flavokermesic acid and kermesic acid were obtained from University of Jordan.

Agilent 1200 series system, Heraeus D-6450 Hanau Oven, WiseStir MSH-20A Daihan Scientific Co. Stirrer, Shimadzu AEX-200G, Gesellschaft für Labortechnik (GFL) were used in the study.

Extraction of dyestuffs from walloon oak (*Quercus ithaburensis* Decaisne) and cochineal (*Dactylopius coccus* Costa): 30 g of the highly granulated walloon oak (*Quercus ithaburensis* Decaisne) acorn caps were transferred into a 2000 mL beaker. 2 g of the granulated cochineal (*Dactylopius coccus* Costa) insects were transferred into another 2000 mL beaker. 1000 mL distilled water was added and the mixtures were heated to 100 °C for 1 h by using a magnetic stirrer. Finally, the mixtures were filtered to obtain the walloon oak and the cochineal extract (non-acid hydrolyzed).

Preparation of aluminium-cochineal-walloon oak natural pigment: 15 % $K_2SO_4 \cdot Al_2(SO_4)_3 \cdot 12H_2O$ solution, walloon oak extract and cochineal extract were heated separately to 90 °C (for solution) and 60 °C (for extracts), respectively. 30 mL of walloon oak and 30 mL of cochineal extract at 60 °C were added to 30 mL alum solution at 90 °C. K_2CO_3 (1 M) solution was added to adjust the pH of the mixture between 6 and 7[21,39]. The mixture was cooled to room temperature to allow the precipitation of the aluminium-walloon oak-cochineal natural pigment. After settling down, the mixture was filtered and the precipitate was washed with distilled water and dried on a filter paper at 100 °C for 0.5 h. The dried aluminium-cochineal-walloon oak natural pigment precipitate was then powdered. These experiment was repeated to precipitate the natural pigments by using 3 % $FeSO_4 \cdot 7H_2O$ and 3 % $SnCl_2 \cdot 2H_2O$ solutions. 30 mL of iron solution mixed with 30 mL of walloon oak and 30 mL of cochineal extract were used to prepare iron-walloon oak-cochineal natural pigment, 30 mL tin solution mixed with 30 mL of walloon oak extract and 30 mL of cochineal extract were used to prepare tin-walloon oak-cochineal natural pigment.

Dyestuff extraction procedure for HPLC analysis: The dyestuff extraction from the dye insect, the dye plant and the natural pigments was done by using the previously described method[7,8,40].

The samples were prepared as follows: For the dyestuff extraction from cochineal (*Dactylopius coccus* Costa) insect and walloon oak (*Quercus ithaburensis* Decaisne) plant, two procedure (1º and 2º) were performed (each one approximately 2.5 mg). 1º: First procedure for the dyestuff extraction from cochineal dye insect and walloon oak dye plant was achieved in 400 µL of the mixture of MeOH:H_2O (2:1; v/v) in a conical glass tube without heating. 2º: As second procedure, cochineal insect and walloon oak plant were hydrolyzed by using 37 % HCl:MeOH:H_2O (2:1:1; v/v/v; 400 µL) until evaporating in a porcelain croze on a water-bath at 100 ºC to extract organic dyes. After rapid cooling under running cold water, the solution was evaporated just to dryness in a water-bath at 55-65 ºC under a gentle stream of nitrogen. The dry residues were dissolved in 400 µL of the mixture of MeOH:H_2O (2:1; v/v). 3º: For the acid hydrolysis of aluminium, tin and iron-walloon oak-cochineal pigments (each one approximately 2.5 mg) was utilized according to procedure presented in 2º step. Then 10 µL and/or 100 µL of the supernatant were injected into the HPLC apparatus.

HPLC Instrumentation: Chromatographic experiments were carried out using an Agilent 1200 series system (Agilent Technologies, Hewlett-Packard, Germany) including a G1329A ALS autosampler, a G1315A diode-array detector. Chromatograms were obtained by scanning the sample from 191-799 nm with a resolution of 2 nm and the chromatographic peaks were monitored at 255 and 268 nm. A G1322A vacuum degasser and a G1316A thermostatted column compartment were used. The data were analyzed using an Agilent Chemstation. A Nova-Pak C_{18} analytical column (3.9 mm × 150 mm, 4 µm, Part No. WAT 086344, waters) protected by a guard column filled with the same material was used. Analytical and guard columns were maintained at 30 °C. The HPLC gradient elution was performed using the method of Halpine *et al.*[9] and Karapanagiotis *et al.*[41]. Chromatographic separations were accomplished using a gradient elution program that utilizes two solvents: solvent A: H_2O-0.1 % TFA (trifluoroacetic acid) and solvent B:CH_3CN (acetonitrile)-0.1 % TFA. The flow rate was 0.5 mL/min and the applied elution program is described in Table-1.

TABLE-1
PARAMETERS OF GRADIENT ELUTION PROGRAM

Time (min)	H_2O + 0.1 % TFA	CH_3CN + 0.1 % TFA
0.0	95	5
1	95	5
20	70	30
25	40	60
28	40	60
33	5	95
35	5	95
45	95	5

RESULTS AND DISCUSSION

In the present study, the complexes formed with adding aluminium(III), iron(II) and tin(II) solutions to walloon oak (*Quercus ithaburensis* Decaisne) extract mixed with cochineal (*Dactylopius coccus* Costa) extract were obtained as natural pigments. The identification of dyestuffs present in the natural pigments was analyzed qualitatively by reversed phase high performance liquid chromatography (RP-HPLC). According to RP-HPLC analysis, gallic acid was identified in the non-acid hydrolyzed, acid hydrolyzed walllon oak extracts, aluminium-cochineal-walloon oak and tin-cochineal-walloon oak natural pigments. Carminic acid was determined in the non-acid hydrolyzed, acid hydrolyzed cochineal insect extracts, iron-cochineal-walloon oak and tin-cochineal-walloon oak natural pigments. Otherwise, ellagic acid was identified in the non-acid hydrolyzed, acid hydrolyzed walloon oak extracts, aluminium-cochineal-walloon oak and iron-cochineal-walloon oak natural pigments. Additionally, flavokermesic acid was determined in the acid hydrolyzed cochineal insect extract. The HPLC chromatograms of acid hydrolyzed

and non-acid hydrolyzed samples were shown in Figs. 1-7. The spectra relating to the these samples was given in Figs. 8-11. The retention times and the related spectra were in good agreement with data given in the literature for gallic acid, ellagic acid, carminic acid and flavokermesic acid[8-10,17,42]. It is known that cochineal dye insects are rich in carminic acid, while kermesic acid and flavokermesic acid are rarely found in this dye source[6,12,13]. Otherwise, tannin compounds *i.e.*, gallic acid and ellagic acid are determined in oak acorn caps[5,43-45].

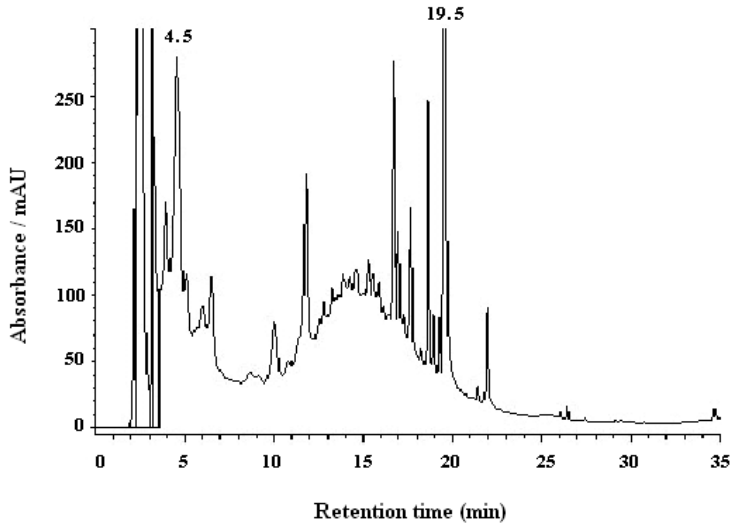


Fig. 1. HPLC chromatogram of walloon oak plant dissolved in (2:1; MeOH:H_2O; 400 µL) at 255 nm. Gallic acid (4.5 min) and ellagic acid (19.5 min) are identified

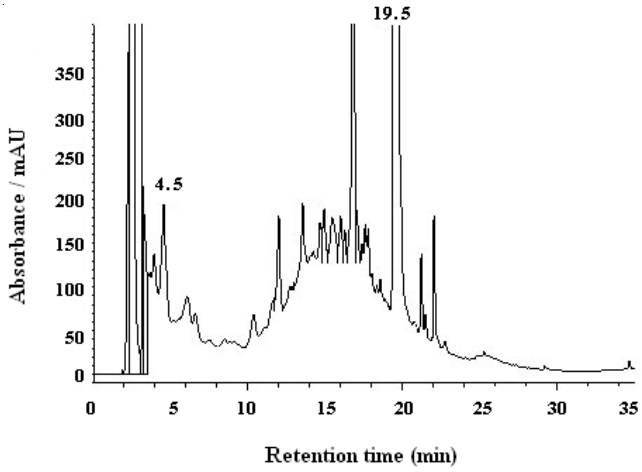


Fig. 2. HPLC chromatogram of acid hydrolyzed walloon oak plant at 255 nm. Gallic acid (4.5 min) and ellagic acid (19.5 min) are identified

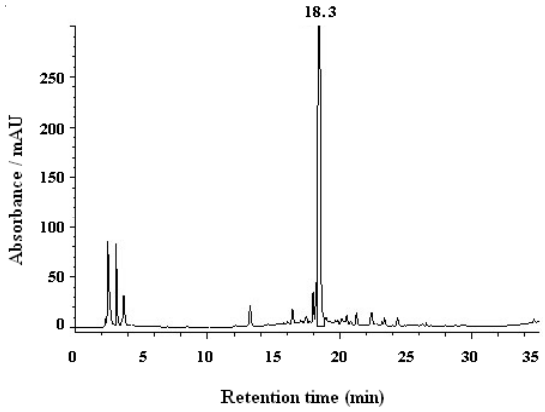


Fig. 3. HPLC chromatogram of cochineal insect dissolved in (2:1; MeOH:H_2O; 400 µL) at 255 nm. Carminic acid (18.3 min) is identified

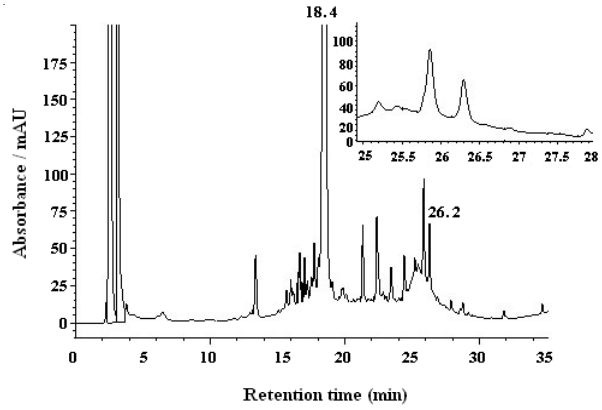


Fig. 4. HPLC chromatogram of acid hydrolyzed cochineal at 255 nm. Carminic acid (18.4 min) and flavokermesic acid (26.2 min) are identified

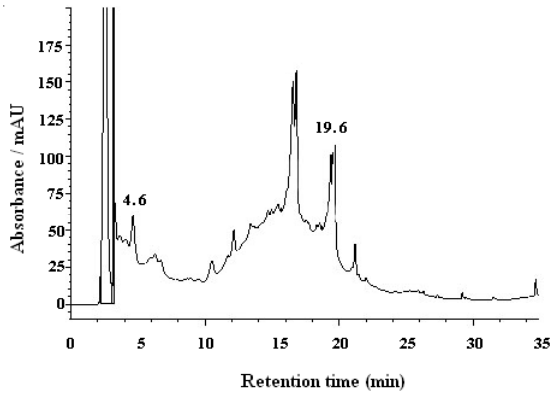


Fig. 5. HPLC chromatogram of acid hydrolyzed aluminium-cochineal-walloon oak natural pigment at 255 nm. Gallic acid (4.6 min) and ellagic acid (19.6 min) are identified

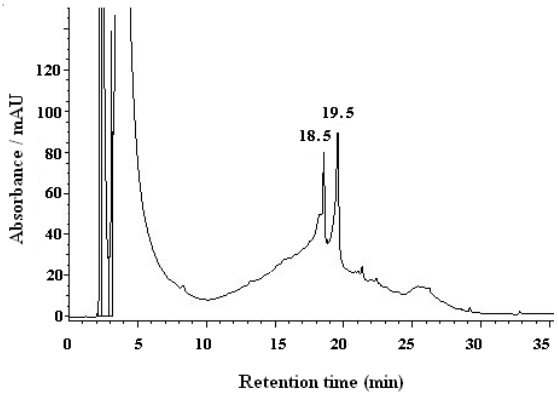


Fig. 6. HPLC chromatogram of acid hydrolyzed iron-cochineal-walloon oak natural pigment at 268 nm. Carminic acid (18.5 min) and ellagic acid (19.5 min) are identified

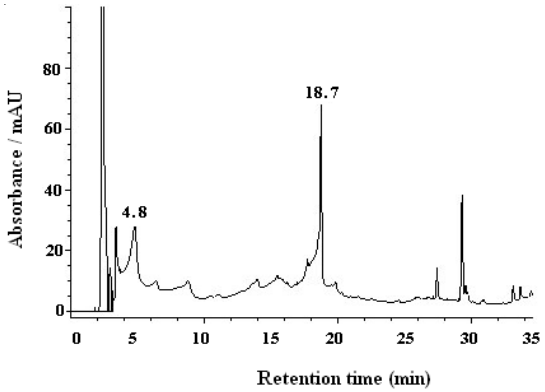


Fig. 7. HPLC chromatogram of acid hydrolyzed tin-cochineal-walloon oak natural pigment at 255 nm. Gallic acid (4.8 min) and carminic acid (18.7 min) are identified

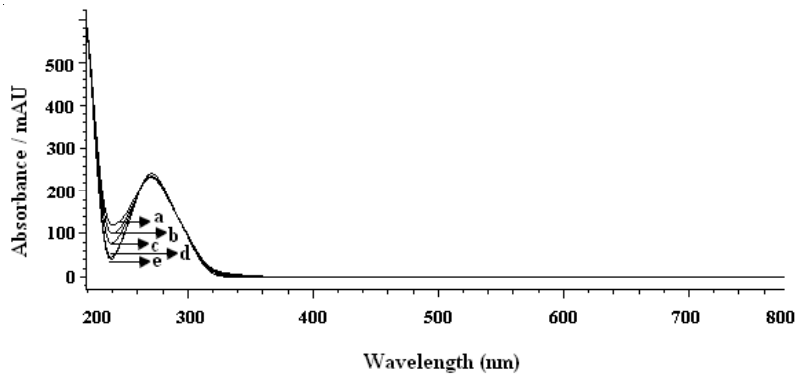


Fig. 8. Photodiode array spectra of peaks of 4.5 min retention time in Fig. 1 (a), 4.6 min retention time in Fig. 5 (b), 4.5 min retention time in Fig. 2 (c), 4.8 min retention time of gallic acid standard (d) and 4.8 min retention time in Fig. 7 (e)

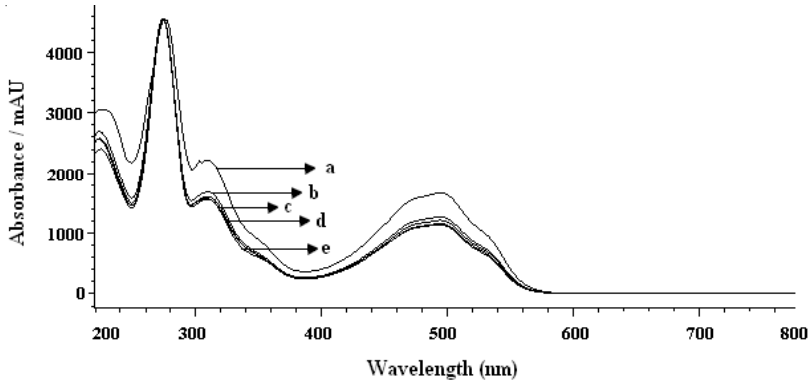


Fig. 9. Photodiode array spectra of peaks of 18.4 min retention time in Fig. 4 (a), 18.3 min retention time in Fig. 3 (b), 18.5 min retention time of carminic acid standard (c), 18.7 min retention time of Fig. 7 (d) and 18.5 min retention time in Fig. 6 (e)

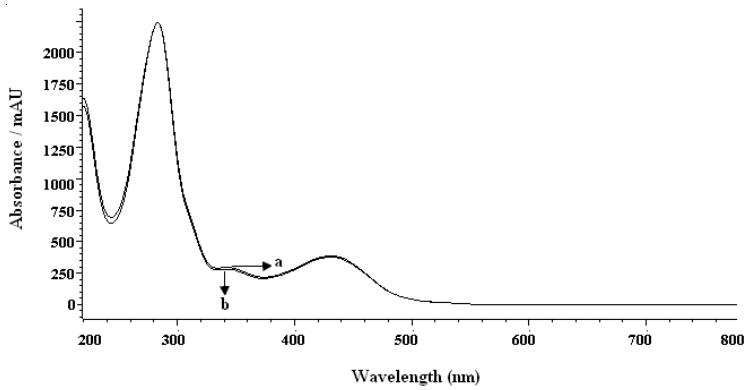


Fig. 10. Photodiode array spectra of peaks of 26.3 min retention time of flavokermesic acid standard (a) and 26.2 min retention time in Fig. 4 (b)

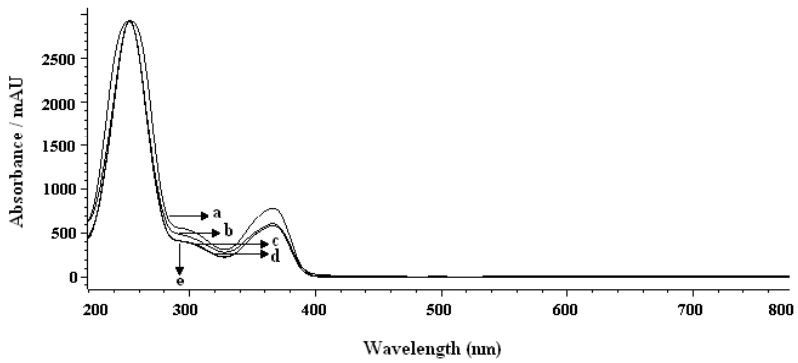


Fig. 11. Photodiode array spectra of peaks 19.5 min retention time in Fig. 2 (a), 19.6 min retention time in Fig. 5 (b), 19.5 min retention time of ellagic acid standard (c), 19.5 min retention time in Fig. 6 (d) and 19.5 min retention time in Fig. 1 (e)

Conclusion

In this study, the reaction of the dyestuffs present in walloon oak plant and cochineal insect with aluminium(III), iron(II) and tin(II) has been used to prepare natural pigments. The dyestuffs present in the natural pigments were analyzed qualitatively by a reversed-phase high performance liquid chromatography (HPLC) with diode array detection (DAD) in comparison with the standard dyestuffs. The identified dyestuffs from the plant, insect and pigments are given in Table-2.

TABLE-2
IDENTIFIED DYESTUFFS IN THE PLANT, INSECT AND PIGMENTS

Identified dyestuff	Non-hydrolyzed walloon oak extract	Acid hydrolyzed walloon oak extract	Non-hydrolyzed cochineal extract	Acid hydrolyzed cochineal extract	Aluminium cochineal walloon oak pigment	Iron cochineal walloon oak pigment	Tin cochineal walloon oak pigment
Gallic acid	+	+	–	–	+	–	+
Carminic acid	–	–	+	+	–	+	+
Ellagic acid	+	+	–	–	+	+	–
Kermesic acid	–	–	–	–	–	–	–
Flavokermesic acid	–	–	–	+	–	–	–

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