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Identification by HPLC-DAD and SEM-EDX Analyses of Lake Pigments from *Helichrysum arenarium*

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In this study, natural organic lake pigments were producted in various colors and with different mordants from *Helichrysum arenarium* dye plant. $Fe(SO_4)_2 \cdot 7H_2O$, $SnCl_2 \cdot 2H_2O$, $KAl(SO_4)_2 \cdot 12H_2O$ and $Ca(NO_3)_2 \cdot 4H_2O$ metal compounds were used. The highest yield was obtained from the lake pigments with alum and used together with alum and calcium mordants. A reversed-phase high-performance liquid chromatography with diode-array detection was utilized for the identification of the lake pigments.The lake pigments were hydrolyzed with a 37 % hydrogen chloride/methanol/water (2:1:1; v/v/v) mixture. *Naringenin*, *apigenin* and their derivatives dyestuffs were detected in the analysis result done by HPLC-DAD. Coloring compounds of obtained lake pigments were detected. The microstructure and chemical homogeneity of the natural lake pigments were analyzed by scanning electron microscopy equipped with energy dispersion spectroscopy (SEM-EDX). The elements were identified for each pigment. Color measurements of the lake pigments were taken by CIEL*a*b* spectrophotometer/colorimeter.

Keywords: *Helichrysum arenarium*, HPLC-DAD, SEM-EDX, Color measurement, Lake pigment.

INTRODUCTION

Helichrysum arenarium is a herb that blooms yellow in color, can grow up to 50- 60 cm. It has golden yellow flowers all year round, are in globe form and are 15 to 20 cm in size. It is usually grown in every region of the country, especially the Black Sea Region, Southern Anatolia, Aegaen and Marmara. It grows by itself in open areas of forests, and on the slopes of foot hills. The leaves of this herb's life is two years, are also wooly and have a bitter taste. This plant contains flavonoids, resin, cumarin and bitter substances with a large amount of essential oils and an unidentified antibacterial compound (named arenarin). The herb also has good anti-inflammatory, antiviral and anti-allergic properties. Although there are many natural antimicrobial agents against common human pathogens, very few studies have been reported regarding the antimicrobial properties on textile materials especially with respect to the human pathogenic strains.

Natural dyes have the advantage that their production implies the use of renewable resources, causes minimum environmental pollution and has a low risk factor in detection (DAD)[1-3] and mass spectrometric detection (MS)[4,5]. For a long time, natural dyes have been used for purposes such as the coloring of wool, cotton and silk natural fibers as well as fur and leather[6-10]. For the identification of dyestuffs in the pigments or the dye plants, several analytical techniques have been used, high performance liquid chromatography, thin layer chromatography[11-22], UV-visible spectrophotometry[23,24], FT-IR spectroscopy[25,26], Raman spectroscopy[27], capillary electrophoresis with electrospray mass spectrometric detection[28] and gas chromatography/mass spectrometry[29]. A reversed-phase high performance liquid chromatography using a diode-array detection (DAD) is ideally suited for the identification of dyestuffs[30,31].

Scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM/EDX) is the best known a powerful analytical techniques in the examination of these materials. It is most widely-used of the surface analytical techniques. High resolution images with a good depth of field can also analyse individual crystals or other features. A high-resolution SEM image can show detail down to 25 Angstroms, or better.

The CIEL*a*b* (1976)-system was introduced to describe color as a result of these three factors. This system is a three-dimensional space, with coordinate axes L*, a* and b*. L* denotes the brightness of the color (L*=0: black, L*=100: white), a* represents the green-red axis (a* negative: green, a* positive: red) and b* represents the blue-yellow axis (b* negative: blue, b* positive: yellow). Each color can be represented

as a set of values for L*, a* and b*, and conse-quently as a point in this color space[32].

EXPERIMENTAL

Helichrysum arenarium dye plant was obtained from the Turkish Cultural Foundation, Cultural Heritage Preservation and Natural Dyes Laboratory Natural Dyes, Istanbul, Turkey. The following standard dyestuffs were used as references: *apigenin* and *naringenin* from Carl Roth (Karlsrube, Germany). Potassium-aluminum sulphate (alum), ferrous sulfate, stannous chloride, calcium nitrate, hydrochloric acid (37% fuming HCl), acetonitrile (HPLC grade), trifluoroacetic acid (HPLC gradient grade) and methanol (HPLC gradient grade) were obtained from Merck (Darmstadt, Germany).

Extraction: 4 g of dried and powdered dye plant were weighed and transferred into a beaker. Then 300 mL distilled water was added. The mixture of dye plant was heated to 100 °C with a magnetic mixer. The mixture was held at low temperature for 1 h. Then the mixture was filtered to eliminate impurities and obtained the dye plant extract at 25-30 °C.

Pigment formation: 15 % $KAl(SO_4)_2{\cdot}12H_2O$ (alum) solution and 3 L *Helichrysum arenarium* plant extracts were heated respectively to 90 and 60 °C. 250 mL from alum solution at 90 °C was added to *Helichrysum arenarium* extract at 60 °C. Afterward, 0.1 M K_2CO_3 solution was added to neutralize the medium. The mixture was cooled to room temperature to precipitate the obtained pigment. After subsidence, the mixture was filtrated and dried. Precipitate was washed with distilled water. The same process was repeated respectively for 250 mL 15 % $KAl(SO_4)_2{\cdot}12H_2O$ (alum) and 7.5 % $Ca(NO_3)_2{\cdot}4H_2O$ solutions to each part of 3 L *Helichrysum arenarium* plant extract. Afterwards process was repeated respectively for 100 mL 2 % $Fe(SO_4)_2{\cdot}7H_2O$, 100 mL 2 % $SnCl_2{\cdot}2H_2O$ solutions to each part of 1.5 L *Helichrysum arenarium* extract.

Color measurements of lake pigments: All color measurements were performed using Minolta 3600D spectrophotometer (D65 illuminant, specular included, 10° observer angle). The spectrophotometer was equipped with software, which is able to automatically calculate CIEL*a*b* and color measurement (L*, a*, b*) values from the reflectance values at the appropriate wavelength for each dyeing (Fig. 1).

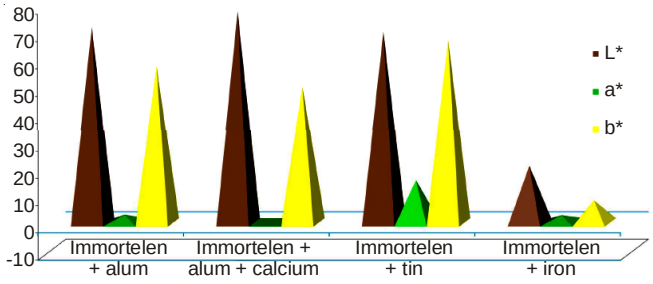

Fig. 1. L*, a* and b* graph of obtained pigments with different metals

Preparation of lake pigments for HPLC analysis: The dyestuff extraction from the natural pigments was carried out using the previously described method[33-35]. Weights were measurements of aluminium (7.1 mg), calcium with aluminium together (7.2 mg), tin (7.4 mg) and iron natural pigments. They were hydrolyzed in 400 mL of a mixture of 37 % HCl/MeOH/H_2O (2/1/; v/v/v) until evaporating in conical glass tubes for precisely 8 min in a water bath at 100 °C to extract the organic dyes. After rapid cooling under running cold water, the solution was evaporated just to dryness in a water-bath at 55-65 °C under a gentle stream of nitrogen. The dry residues were dissolved in 400 mL of a mixture of MeOH/H_2O (2/1; v/v). Then 10 mL of the supernatant were injected into the HPLC apparatus.

HPLC equipment: Chromatographic experiments were carried out using an Agilent 1200 series system (Agilent Technologies, Hewlette Packard, Germany) including a G1329A ALS autosampler, a G1315A diodearray detector. Chromatograms were obtained by scanning the samples from 191 to 799 nm with a resolution of 2 nm and the chromatographic peaks were monitored at 255 nm. A G1322A vacuum degasser and a G1316A thermostatted column compartment were used. The data was analyzed using Agilent Chemstation. A Nova-Pak C18 analytical column (3.9 × 150 mm, 4 mm, 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 by using the previously described method[36,37]. Chromatographic separations of the hydrolyzed samples were performed by using a gradient elution program that utilizes two solvents: solvent A: H_2O -0.1 % trifluoroacetic acid and solvent B: CH_3CN - 0.1 % trifluoroacetic acid. The flow rate was 0.5 mL/min and the applied elution program is the same as the previously performed program.

SEM-EDX equipment (scanner electron microscopy-energy dispersive X-ray): In this study, the obtained lake pigments from *Helichrysum arenarium*dye plant were investigated using a TESCAN VEGA3 Easy Prob SBU (FESEM) equipped with energy dispersive spectroscopy (EDX with detector Bruker 410-M, software: Esprit 1.9).

RESULTS AND DISCUSSION

HPLC analysis: In the present study, natural lake pigments were obtained as the complexes formed by adding aluminum(III); aluminum(III) and calcium(II) together; iron(II) and tin(II) solutions to *Helichrysum arenarium* extract. The identification of dyestuffs present in the natural pigments was analyzed qualitatively by reversed phase high performance liquid chromatography (RP-HPLC). The HPLC chromatograms of acid hydrolyzed pigments are shown in Fig. 2. Identified dyestuffs are shown in Fig. 3. *Apigenin* dyestuff was detected in the obtained lake pigments with aluminium and calcium together, aluminium and iron together. Both *naringenin* and *apigenin* dyestuffs were detected in the obtained lake pigments with tin.

SEM-EDX analysis (scanner electron microscopy-energy dispersive X-ray): The lake pigments were analyzed as loose powders on carbon stubs. Back-scattered electron mode was employed to examine the nature, homogeneity and microstructure of the samples. EDX was applied to achieve the elemental composition of pigments with accelerating voltage at 30 kV and the working distance at between 14-16 mm without coating.In this study, obtained four natural lake pigments collected were characterized (Fig. 4) and the results were shown in Table-1.

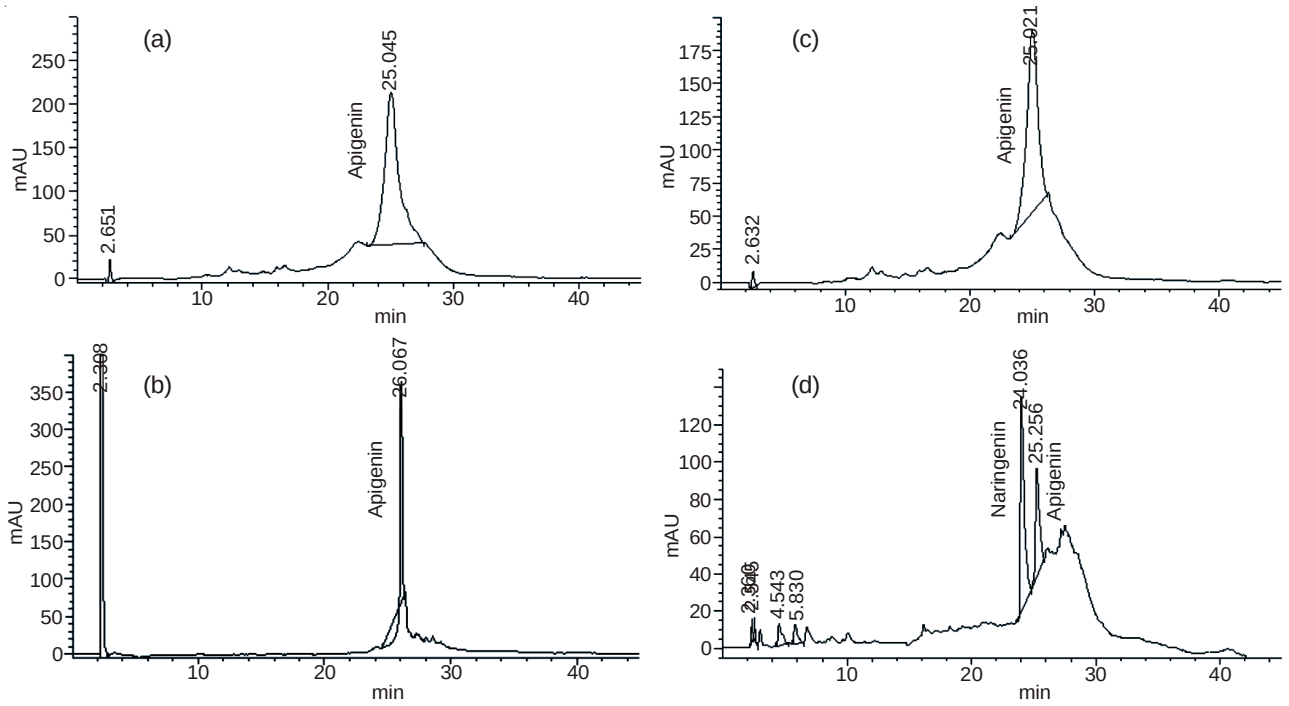

Fig. 2. HPLC chromatograms of the obtained natural lake pigments. (a) with aluminum, (b) with together aluminum and calcium, (c) with iron and (d) with tin

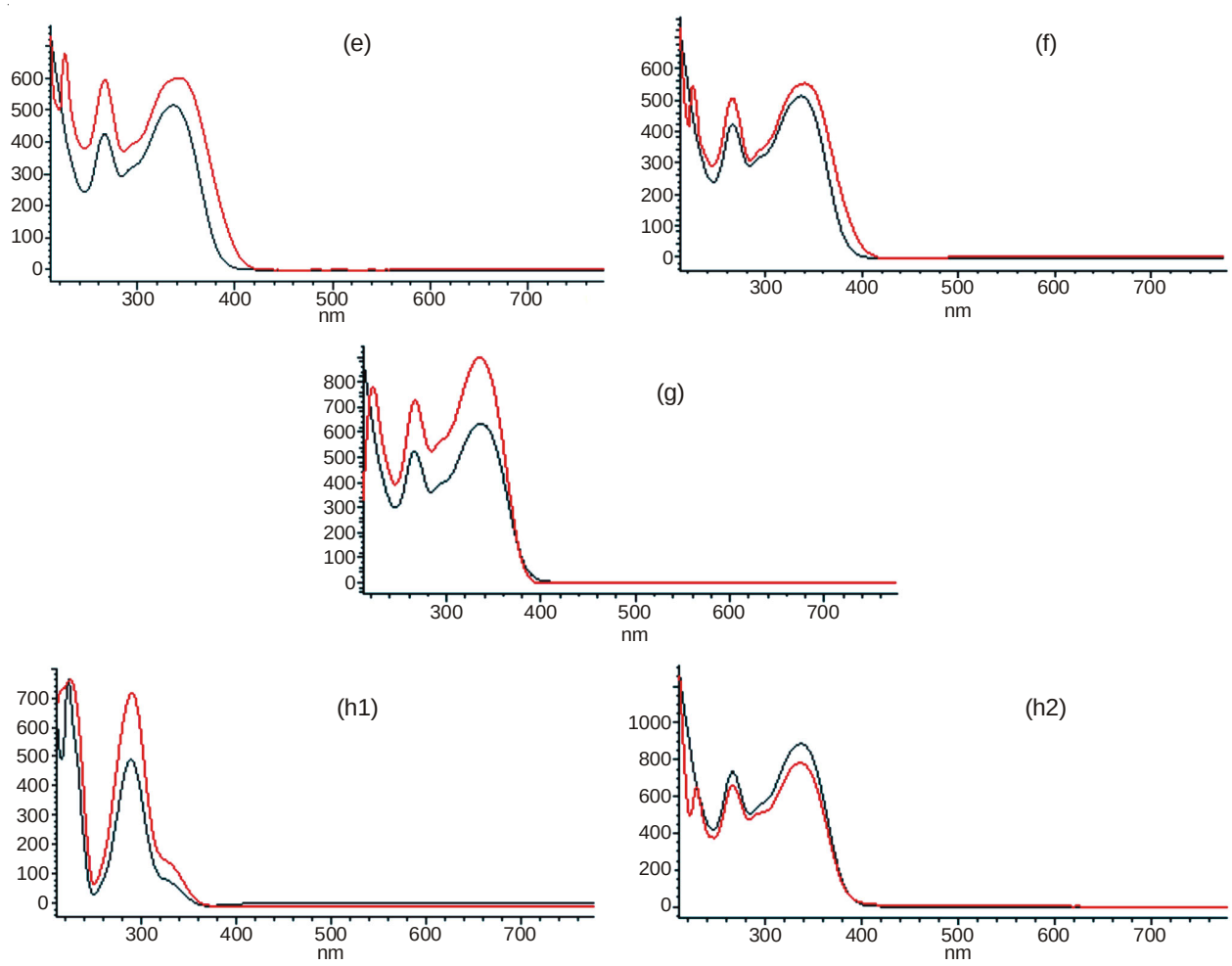

Fig. 3. Identified spectrum in the obtained natural lake pigments, (e) spectra of apigenin standard and spectra in the lake pigment with aluminum, (f) spectra of apigenin standard and spectra in the lake pigment with aluminum and calcium, (g) spectra of apigenin standard and spectra in the lake pigment with iron, (h1) spectra of naringenin standard and spectra in the lake pigment with tin, (h2) spectra of apigenin standard and spectra in the lake pigment with tin

Color measurement of obtained lake pigments: L*, a* and b* values for the four lake pigments were measured with Konica Minolta CM-2300d Software Spectra Magic NX (6500 K, 45°). L*, a* and b* values were shown in the Table-2.

Conclusion

The *Helichrysum arenarium* dye plant with aluminum, aluminum + calcium, tin and iron metals were obtained containing natural organic lake pigments. The results of the pigments obtained show that aluminum + calcium pigments were observed at high yields, whereas iron was observed at low yields. The analysis done on these four pigments were done by using HPLC-DAD and SEM-EDX. With the assistance of HPLC, it was identified that the lake pigments genereted using aluminum, aluminum + calcium, and iron detected only *apigenin* pigment, whereas the lake pigment genereted using tin detected *apigenin* and *naringenin* pigments.

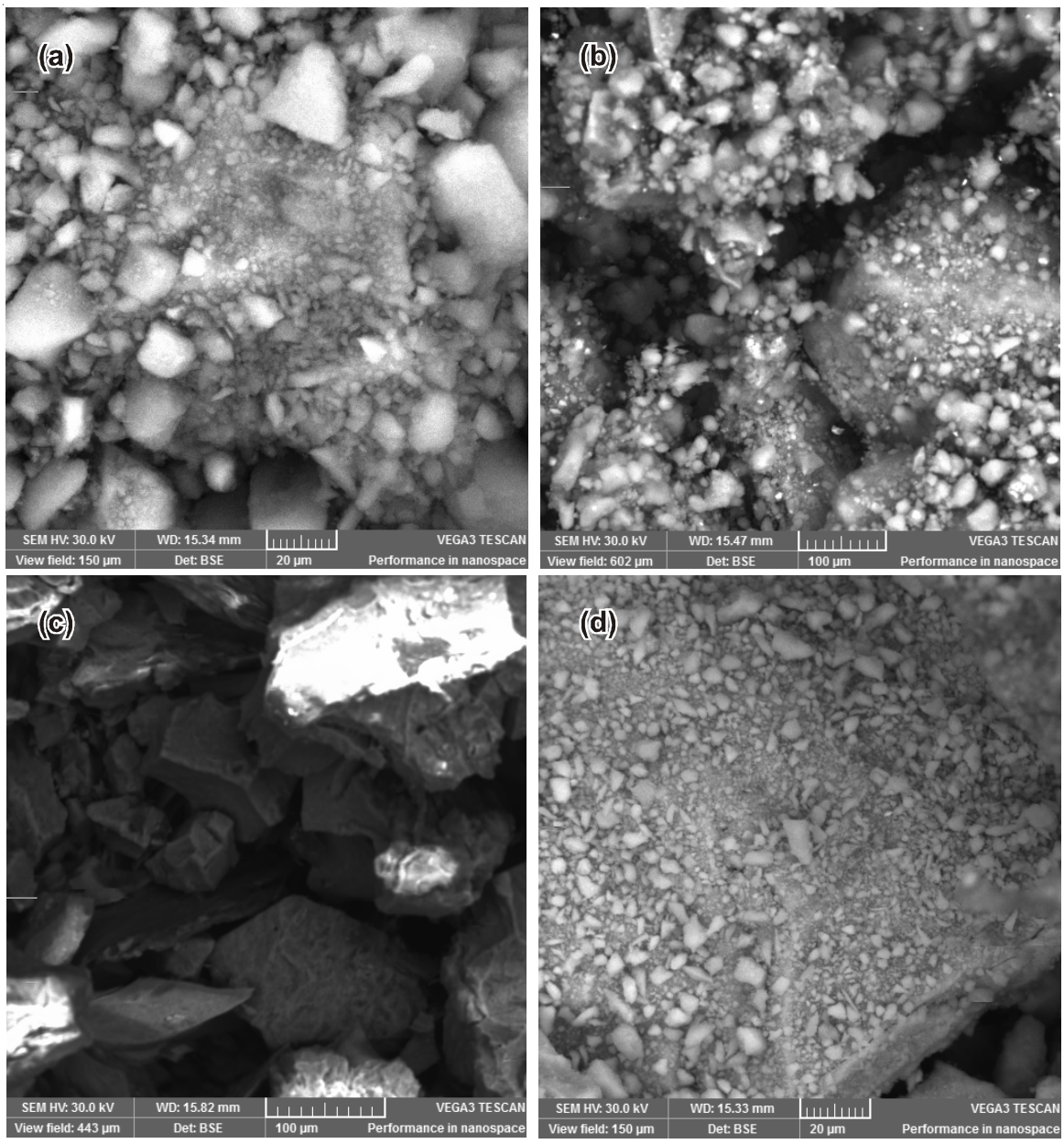

Fig. 4. SEM-EDX of (a) aluminum pigment (b) aluminum and calcium pigment (c) iron pigment and (d) tin pigment

TABLE-1
ELEMENTAL COMPOSITIONS OF THE FOUR NATURAL LAKE PIGMENTS

Name of pigment	Identified elements and their Wt %					
	C	O	Al	Ca	Fe	Sn
Aluminum pigment	30.19	51.95	17.86	-	-	-
Aluminum and calcium pigment	29.00	52.32	14.73	3.94	-	-
Iron pigment	49.65	42.44	-	-	7.91	-
Tin pigment	57.73	23.99	-	-	-	18.27

TABLE-2
L*, a* AND b* VALUES FOR THE OBTAINED FOUR LAKE PIGMENTS

Name of pigment	L*	a*	b*
Aluminum pigment	70.58	2.82	56.46
Aluminum and calcium pigment	76.93	-0.80	49.50
Iron pigment	21.30	2.67	8.27
Tin pigment	69.64	16.71	64.72

Elementel analysis using SEM-EDX shows the metals in every pigment was determined. However, carbon and oxygen that is located in the pigment forms a complex with *Helichrysum arenarium* was determined. The results are shown in Table-2.

CIEL*a*b* color measurements made with a colorimeter defined that aluminum, aluminum + calcium, and tin pigments gave shades of yellow, but the iron pigment gave the color black. The brightest color is the aluminum + calcium lake pigment; but the tin lake pigment has been identified to have the most yellowness value.

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