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Extraction, purification, antioxidant properties and stability conditions of phytomelanin pigment on the sunflower seeds

Önder Özdemir [1], Yüksel Keleş [*2]

[1]Mersin University, Science Enstitute, Biotechnology Department, Mersin, Turkey
[2]Mersin University, Education Faculty, Biology Education Department, Mersin, Turkey

Abstract: Phytomelanin pigment, a rare secondary matabolite in plants, has potential for use in the cosmetic and food sectors. This pigment has antioxidant, antimicrobial and ultraviolet (UV) light absorbing properties, so it can be used as natural food coloring and in the field of cosmetics. In this study, extraction, purification, antioxidant properties and stability conditions of phytomelanin pigment found in sunflower (*Helianthus annuus* L.) seed coat were investigated. NaOH, KOH and NH_4OH solutions were tested at different concentrations for extraction. It has been determined that the most suitable solvent for extraction is 0.3 M NaOH. The purification process involves precipitation with HCl followed by washing with ethyl alcohol, ethyl acetate and acetone. The findings show that the proportion of phytomelanin in sunflower seeds is 1.95% and that the antioxidant capacity is 9.8% ascorbic acid equivalent. The purity degree of the purified phytomelanin pigment and that of the synthetic phytomelanin pigment were compared by thin layer chromatography (TLC). Chromatography findings have shown that the purification performance is quite high. It was determined that the pigment was slowly deteriorated in temperature and light, and was not affected by air. Consequently, sunflower seed coat can be a convenient and economical source of producing pure phytomelanin for industrial use.

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1. INTRODUCTION

The use of colorants in the food and cosmetics production has begun in ancient times. There are many examples of the use of natural colorants in recorded history [1]. Today, there is an increasing interest in food colorants derived from natural sources as an alternative to synthetic dyes due to legal regulations and consumer demand [2]. Pigments have an important place in food additives and the demand for pigments has increased in recent years. Natural pigments are regarded as safe compared to synthetic pigments in terms of nutritional value and therapeutic effects. At present, most natural pigments are obtained from plant material by appropriate extraction methods [3].

Melanin is a black, insoluble pigment that is not degraded by concentrated acids but can be whitened with oxidizing agents. This pigment can be found in plants, animals and microorganisms. The form found in plants is called phytomelanin or phytomelan [4].

CONTACT: Yüksel Keleş ykeles@mersin.edu.tr Mersin University, Education Faculty, Biology Education Department, Mersin, Turkey

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Phytomelanin is especially found in the seed coats of Asteraceae species. There are only a few published literature on the structure, chemical composition and formation of the composite outer zone, as known for over 50 years. Pigment; polymeric, highly insoluble, resistant to alkali and acid, and has a black color at high concentration. Although the natural function is not fully known, it has been suggested that black coated seeds can survive for years under the soil, and may be resistant to weather conditions, soil acidity and microbial attack [5, 6].

Melanins are high-molecular weight polymers formed by the oxidation of phenols. These nitrogenous compounds which are derived from tyrosine, are called eumelanin [7]. Complex polymeric structured melanin compounds are classified as: eumelanin, pheomelanin and allomelanin. Allomelanines are black pigments produced by plants and fungi. It is found in black spots of leaves, in flowers, in fruits and seeds (eg *Osmanthus fragrans*), in fungal spores (eg *Tuber melanosporum*). Fungal melanins are produced by *Aspergillus nidulans* and *A. niger*, *Alternaria alternata*, *Cryptococcus neoformans*, and *Wangiella dermatitis* [8]. Phytomelanins are isolated from leaves of *Tea sinensis* and their radical scavenger properties are shown [9]. Phytomelanin is found in fruit pericarp of species belonging to *Eupatorium* and *Helianthus* genus from Asteraceae family. In these tissues, it forms a mechanically hard, durable layer of brown-black color [6]. Phytomelanin acts as a protective layer that protects the pericarp, providing external protection against attacks by insects, insects and macro-micro organisms. In addition to the destructive effects of insect pests, it is also resistant to bacterial deterioration [10].

Phytomelanin is used especially in cosmetics sector and has usage potential in many different sectors. Various sources indicate that both antioxidant, antimicrobial and ultraviolet light absorptive properties are present, as well as their potential for use as natural food colorants [8, 9].

In this study, optimization of extraction and purification methods of phytomelanin pigment in sunflower seeds were carried out. In addition, antioxidant properties of phytomelanin pigment were determined and evaluated for stability in various conditions such as air, light and temperature.

2. MATERIAL AND METHODS

2.1. Material

In these experiments, black colored seeds produced by Cypriot variety of sunflower (*Helianthus annuus* L.) were used. Seeds were obtained from seed markets in Mersin/Turkey.

2.2. Optimization of extraction conditions

Melanin in animal and fungus species is highly stable in acidic solutions, but soluble in dimethyl sulfoxide (DMSO) at low levels. Phytomelanin can be successfully extracted from various plant tissues by standing in alkaline solutions for 24 hours [11]. Three different alkaline chemicals (NH_4OH, KOH and NaOH for analysis from MERCK) were used to determine the optimum solvent and concentration for extraction. The prepared 0.5 M stock solutions were then diluted to 0.3 M, 0.1 M, and 0.05 M, respectively.

1.0 g of sunflower seeds were placed in 100 ml of solution in the absence of oxygen and at room temperature for 24 hours. The extract was filtered through filter paper (Whatman No 1) and then centrifuged at 3500 xg for 5 minutes. The extract was diluted 1/3 with distilled water (Comecta destilation 3.1) and the absorbance at 280 nm was measured [11, 12]. The amount of melanin in the samples was calculated using standard curve prepared with pure melanin pigment (Sigma Aldrich M8631).

The most suitable solute / solvent ratio was determined by making experiments with seed quantities ranging from 1.0 to 5.0 g. The most successful extractions were with 4.0 g seeds in 100 mL of solvent. The subsequent extractions were carried out at these ratios. In order to determine the effect of temperature and time on extraction, 4.0 g of seed was incubated for 24 hours at 4, 24, 35 and 50 ˚C in 100 ml of 0.3 M NaOH solution. At specific time intervals, 50 µl samples were taken and final volume was adjusted to 3.0 ml with distilled water and absorbance at 280 nm was measured by spectrophotometer.

2.3. Purification process

The phytomelanin extract was filtered and adjusted to pH between 1.0 and 3.0 with a 2.0 M HCl solution. After being allowed to stand at room temperature for 2 hours, it was centrifuged at 4000 xg for 5 minutes. The supernatant was removed and the precipitated material was taken up with a spatula. It was boiled in 6.0 M HCl for 2 hours to remove carbohydrates and proteins, cooled and then centrifuged at 4000 xg for 5 minutes. The supernatant was removed and then suspended with distilled water, vortexed and centrifuged at 4000 xg for 5 min. The supernatant was removed and the precipitated melanin pigment collected using a spatula. The obtained melanin pigment was shaken for 30 minutes by adding ethyl alcohol and centrifuged at 5000 xg for 15 min. The supernatant was removed and washed with distilled water. The same process was repeated with ethyl acetate and acetone [13]. The purified phytomelanin was dried in a glass petri dish at 100 °C and stripped from the glass surface.

2.4. Antioxidant capacity

The prufied fitomelanin pigment was dissolved in methanol and quantified. 20, 40, 60, 80, 100 µg ml^{-1} ascorbic acid solutions were prepared to form a standard curve. 0.3 ml of melanin and ascorbic acid solutions were mixed with 2.7 ml of the reagent solution (containing 0.6 M H_2SO_4, 28.0 mM Na_2HPO_4 and 4.0 mM ammonium molybdate in water). The reaction mixture was kept at 90 °C for 90 min and then cooled. Finally, absorbance measurements were performed at 695 nm spectrophotometer (Chebios Optimum One UV-VIS) [14].

2.5. Stability tests

10 mg of pure melanin was dissolved in 250 ml of 0.1 M NaOH to determine the stability of the melanin pigment in high temperature conditions. The melanin solution was exposed to a temperature of 100 ° C for 2 hours. The volume was adjusted to 250 ml before sampling for measurement. The samples were taken at the beginning and 30 min intervals. 0.1 ml of the sample was diluted with 2.9 ml of 0.1 M NaOH and the absorbance was determined at 280 nm on spectrophotometer.

Stability tests of melanin pigment in presence of light and oxygen were performed at room temperature. 0.1 ml of the melanin pigment solution was taken and diluted with 2.9 ml of 0.1 M NaOH solution and the absorbance at 280 nm was measured by spectrophotometer. The solution transferred into the four different tubes was kept in the light, dark, air + light and air + dark conditions. The tubes were fully filled and closed to create an airless environment. Measurements of absorbance were performed at varying intervals for 35 days.

2.6. Thin layer chromatography (TLC)

Thin layer chromatography was performed to compare the pigment of the purified phytomelan with the pigment purity of the synthetic melanin [15]. Purified and dried phytomelanin pigment and synthetic melanin pigment dissolved in 0.1 M NaOH solution. solvent system of n-butanol: acetic acid: pure water (70:20:10) was used [16]. It was carried out on silica gel layers (MERCK TLC Silica Gel 60 F254) [17]. Purity levels were compared with the spots on the chromatogram.

2.7. Determination of purity level

A synthetic melanin pigment from Sigma-Aldrich was used to determine the amount of pigment of the purified phytomelan. 500 mg L^{-1} melanin stock solution was prepared for analysis. This solution was diluted to give serial solutions such as 0, 10, 20, 40, 80 and 100 mg L^{-1}. The absorbance at 280 nm was measured on spectrophotometer. Quantitative analysis was performed on the standard curve generated by the measured absorbance values.

2.8. Statistics

All analysis were repeated at least three times. The significance levels of the difference between groups were determined with ANOVA test. Results of statistics analyses were shown with P values and significance levels in Table and Figure legends. Data were indicates as arithmetic means and standard deviations (±SD) in Tables and Figures.

3. RESULTS

Different concentrations of three alkaline chemicals were tested in order to determine the most suitable solvent for pigment extraction of phytomelanin pigment and the findings are shown in Table 1. The measurement results showed that the most suitable solvent was 0.5 M NaOH solution. However, in order to avoid problems resulting from high concentrations of NaOH, 0.3 M NaOH solution was used in subsequent processing.

Table 1. Mean absorbance values of phytomelanin pigment extracted at different concentrations of three alkaline solvents at 280 nm wavelength on a UV-VIS Spectrophotometer. Values are given as averages and standard deviations of three repetitions. (Statistics: Solvent P = 0.031 (*), concentration P = 0.00 (**), interaction P = 0.00 (**)).

Concentrations (M)	NaOH	KOH	NH_4OH
0	0.32±0.04	0.32±0.04	0.32±0.04
0.05	1.05±0.15	0.58±0.06	0.74±0.05
0.1	1.07±0.17	0.97±0.08	0.92±0.12
0.3	1.50±0.25	1.30±0.16	1.28±0.18
0.5	2.05±0.22	1.34±0.12	1.58±0.21

1.0-5.0 g of sunflower seeds were incubated in 0.3 M 100 mL NaOH solution, 24 h, at room temperature to determine the optimum amount of phytomelanin pigment that could be dissolved in solvent. A slight difference between 4.0 and 5.0 g was observed with an increase from 1.0 g to 4.0 g (Figure 1). The ideal material / solvent ratio was determined as 4.0 g seed / 100 ml.

Experimental results to determine the appropriate temperature and duration for extraction are given in Figure 2. Measurement was carried out at four different temperatures (4, 24, 35 and 50˚C) during one hour of the experiment. Findings show that the most successful extraction takes place in 7 hours at a temperature of 50 ˚C. The increase in phytomelanin after 6 hours is considerably reduced.

Total antioxidant capacity analysis was performed by phosphomolybdenum complex formation method in order to determine the antioxidant properties of phytomelanin pigment. The antioxidant capacity was determined to be the ascorbic acid equivalent. The antioxidant capacity of the purified 1000 mg L^{-1} phytomelanin pigment was determined to be 98 mg L^{-1} ascorbic acid equivalent, i.e., 9.8%.

In order to determine the stability of the purified phytomelanin pigment at high temperature, the phytomelanin solution was boiled for 2 hours at 100 °C. Absorbance measurements were made every 30 minutes from the start and the amount of phytomelanin was

calculated. The concentration of phytomelanin pigment was not significantly changed for 90 minutes at 100 ˚C but decreased after 120 minutes (Figure 3).

The levels of stability of the purified phytomelanin pigment in daylight and in the air have been determined. The amount of phytomelanin in the solutions was measured with a spectrophotometer at certain time intervals and the results are given in Table 2. At the end of the 35th day, loss of phytomelanin was determined as 61.6% in light and 25.3% in darkness. The loss of phytomelanin in air exposed samples was found to be less than those in air-free controls. These findings show that the destructive effect of light is stronger than the effect of air.

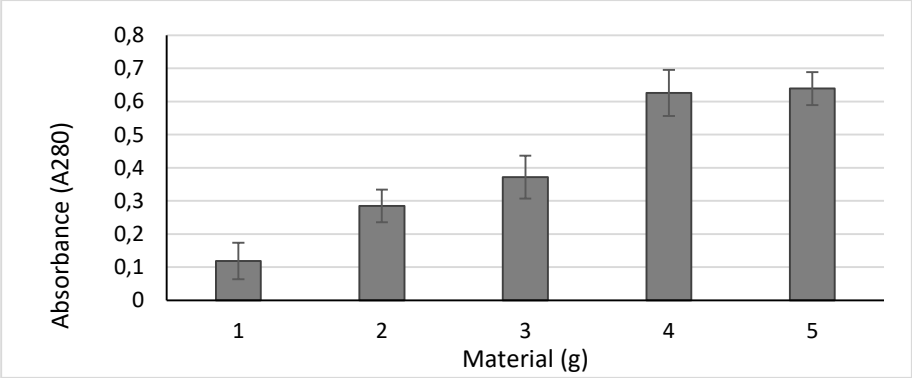

Figure 1. Absorbance values measured at 280 nm wavelength on extracts of phytomelanin from sunflower seeds in different amounts (1.0-5.0 g). Data represent the mean and standard deviation of the triplicate measurements.

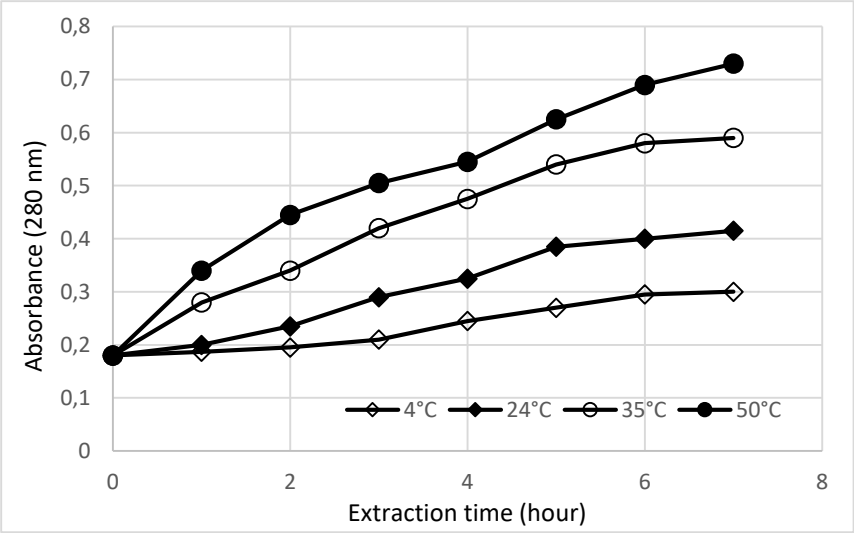

Figure 2. Temperature and time-dependent change in extraction performance of phytomelanin pigment. Extraction was carried out with 0.3 M NaOH solution. (Statistics: Temperature P = 0.00 (**), time P = 0.00 (**), interaction P = 0.00 (**)).

Thin layer chromatography was applied to compare the purity level of phytomelanin pigment purified from sunflower seeds with synthetic melanin pigment. Since the melanin pigment is insoluble in the acidic mobile phase, the applied chromatographic process distinguishes the impurities. The comparison results obtained are given in Figure 4. It has been determined that the procedure applied in this study provides 90% purification.

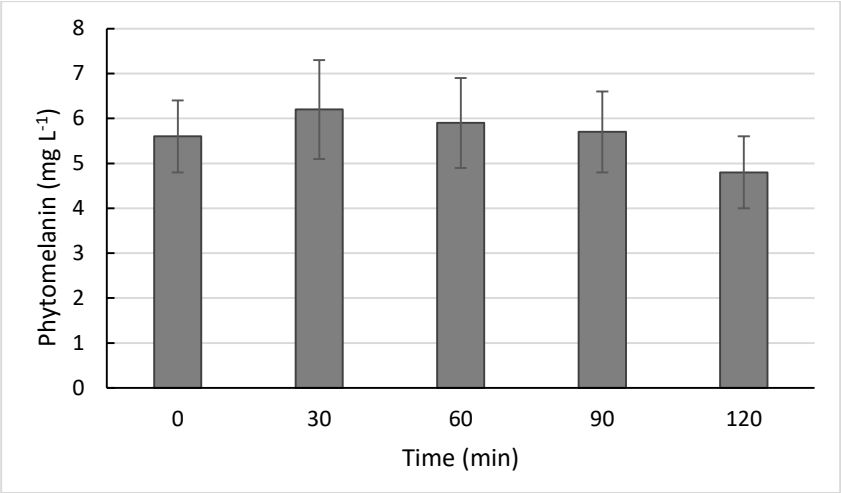

Figure 3. Time-dependent changes of phytomelanin pigment concentration at 100 ˚C. Data represent the mean and standard deviation of the triplicate measurements.

Table 2. Time-dependent changes in amount of phytomelanin pigment (mg Kg^{-1}) exposed to daylight and oxygen. (Statistics: light P = 0.034 (*), air P = 0.563, time P = 0.29, interaction P = 0.151).

Time (day)	Light	Light+Air	Dark	Dark+Air
0	11.1±0.0	11.1±0.0	11.1±0.0	11.1±0.0
1	11.1±0.2	11.2±0.2	11.1±0.1	11.1±0.1
2	11.2±0.2	10.9±0.2	10.9±0.2	11.5±0.3
3	11.3±0.3	11.1±0.2	10.8±0.2	11.1±0.1
7	6.6±0.5	10.3±0.3	9.2±0.3	11.4±0.3
14	6.5±0.5	9.5±0.4	9.2±0.3	11.3±0.3
21	6.1±0.5	9.1±0.5	8.5±0.4	11.1±0.3
35	4.3±0.8	8.3±0.6	8.3±0.5	10.9±0.4

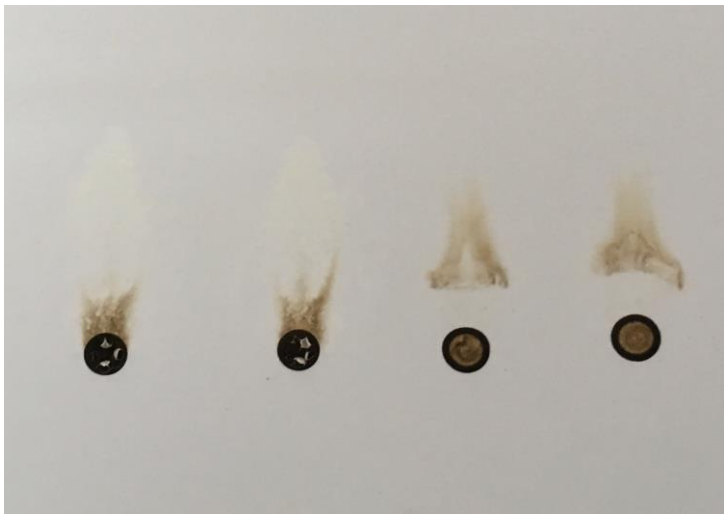

Figure 4. Time-dependent changes of phytomelanin pigment concentration at 100 ˚C. Data represent the mean and standard deviation of the triplicate measurements.

4. DISCUSSION

In plants, phytomelanin can be solubilized at a low level with dimethyl sulfoxide (DMSO), although it is highly stabilized in pigmented acidic solutions. Phytomelanin dissolved in water

and organic solvents is completely soluble in 0.5 M NaOH or KOH. The dark brown material which can be extracted with alkali solutions is precipitated when hydrochloric acid is adjusted to pH 2 or when the medium $FeCl_3$ is added. For this reason, precipitation is an important step in the purification process of phytomelanin. [11]. It has been observed that sedimentation at high temperatures takes place in a shorter time. Coloring is achieved with oxidation agents such as $KMnO_4$, H_2O_2, and color can be reproduced with $AgNO_3$ and ammonia solution [13].

Because of the difficulty of extraction and purification processes, the economic value of pure phytomelanin pigment is high. For this reason, it is important to obtain abundant and good quality pigment in a short time by optimizing pigment purification processes. The success rate has been increased by making some changes in the extraction and purification processes in the literature. Thin layer chromatography was used to determine the degree of purity of the obtained pigment [16]. Chromatograms taken with the acid-solvent mixture on the silica gel layer indicate that the pigment is above 90% of the purity level. This purity grade is sufficient for industrial use. For analytical use further purification by thin layer or column chromatography is necessary.

The pigment of phytomelanin is very resistant to deterioration due to its inert nature. However, when exposed to heat and light, it was observed to be damaged in a certain rate. The most common problems in the use of pigments in the food sector are stability and toxicity. In order to obtain the desired color in the formulations, the formulation experts must know the interactions with the other compounds and other factors that determine the color qualities. Although the pigment of phytomelanin is a natural product, toxicity tests are required [5].

Antioxidants may delay or prevent the staling of foodstuffs and the deterioration of their taste. Antioxidants inhibit degradation in two ways: primary antioxidants are effective by scavenging free radicals (such as phenolic compounds), while secondary antioxidants are effective by binding metal ions, converting hydroperoxides to non-radical products, UV absorbing, or singlet oxygen deactivation [18]. The antioxidant activity of phytomelanins may well be due to a combination of chelating and scavenging charecteristics [9, 19].

Park et al. [20] show that phytomelanins accumulate mainly in the outer epidermis and palisade layers of the wild-type *Ipomoea purpurea* seed coats, The outermost epidermal layer, in which phytomelanins accumulated extensively, appeared to overlap with the proanthocyanidin accumulating layers. Phytomelanin deposition in the outer epidermis and palisade layers, probably because the precursors of phytomelanins can migrate from synthesized cells into the space where phytomelanins are polymerized [21]. The biosynthesis and/or accumulation of seed phytomelanins are controlled by *bHLH2* gene, even though both the chemical properties and biosynthetic pathway of phytomelanins remain unknown [20].

5. CONCLUSION

Natural melanin found in plants and animals affects various biological activities with its antioxidant properties. Due to the antioxidant properties of melanin, it is of interest in the protection of health and the production of useful foods. Consequently, sunflower seed coat can be a convenient and economical source of producing pure phytomelanin for industrial use. Phytomelanin posses a potential to the development of new products in cosmetics and food industries.

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ORCID

Yüksel Keleş https://orcid.org/0000-0001-8651-8385

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