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Determination of Natural Colors by Thin Layer Chromatography

[1]Wegdan M. Lotfi, [2]Rasmia A. Hassan, [2]Wafaa. A. Tawfik and [2]Amira A. Habib

[1]American University in Cairo, Egypt.
[2]Phytochemistry Dept., National Research Centre, Cairo, Egypt.

Abstract: Application of TLC scanning analysis is a rapid and simple method for identifying natural products. It permits a simplified approach to this kind of compounds as UV spectra can be measured directly on the developed plates without previous elution. Natural mixtures can include minor constituents, which may be laborious to isolate. *Bixa orellana* L. and *Opuntia tuna* Mill. are two important sources for natural colors. Bixin, betanin, isobetanin and indicaxanthin were qualitatively and quantitatively determined using TLC densitometric analysis. The obtained results suggest that TLC densitometry is suitable for the identification and quantification of carotenoids and betalines as an advanced analytical technique, comparable with HPLC.

Key words: TLC densitometry; Carotenoids; Betalines; Bixin-*Bixa orellana*; Betanin-*Opuntia tuna*

INTRODUCTION

Carotenoids are a family of natural fat/oil soluble pigments found principally in chloroplasts where they function as accessory pigments to chlorophyll. The majority of carotenoids have a 40 – carbon polyene chain serving as the backbone molecule. Carotenes [alpha – beta – delta – and gamma] are hydrocarbon carotenoids whereas oxygenated derivatives of these hydrocarbons are known as xanthophylls, e.g. lutein, the major yellow pigment in marigold petals. Carotenoids play an essential role in human health due to their provitamin A activity and their function as biological antioxidants, protecting tissues and cells from the destructive effects of free radicals and quenches the reactive oxygen species ROS[1,2].

Bixin is a well-known apocarotenoid and is extracted from annatto seed coats. Annatto [*Bixa orellana*] is a native central and tropical South American tree. Annatto is one of the foremost economically important natural colors mainly used to color dairy products[3,4]. *Opuntia tuna* Mill. is from a family of cacti, cactaceae[5], that consists of more than 360 species. Common names are Bunny Ears, Barbary Fig and Prickly Pear. *Opuntia tuna* is also named elephantear prickly pear. *Opuntia cactus* and Swiss chard are sources of betanin as is beetroot. Betanin is a betalain pigment, along with probetanin, isobetanin and neobetanin. Betanin is the natural, commercial food colorant. It is used in frozen food products only because they have a short shelf life as it undergoes degradation once subjected to heat, light and oxygen.

Several analytical techniques have been established for the identification and quantification of natural products; however, they are known to include tedious, time-consuming procedures, high operational cost and need high technical skills together with sophisticated equipment[6]. Quantitative planar chromatography with scanning densitometry has gained acknowledgement as the most appropriate instrumental, analytical method for herbal medicinal extracts[7,8,9] due to simplicity of operation, reproducibility of results, quantification at any interval of time with variable parameters, and cost effective as a number of samples can be analyzed simultaneously on a single plate with a small amount of solvent[10].

The purpose of this study is to perform a simple method for determination of bixin in forms of Annatto different extracts as well as betalines isolated from *Opuntia tuna*.

MATERIALS AND METHODS

Plant Material: Seeds of *Bixa orellana* L. were collected from the Botanical Garden in Aswan. *Opuntia tuna* L. Mill was collected from Kafr-Elsheikh, Egypt and from that cultivated at the National Research Centre Botanical Garden, Egypt. The yellow petals of the flowers were separated, air dried and powdered. The plants were kindly identified by Prof. Dr. Sayed F. Khalifa, Botany Department, Faculty of Science, Ain Shams University, Egypt.

Corresponding Author: Rasmia A. Hassan, Phytochemistry Dept., National Research Centre, Cairo, Egypt.
E-mail: dr.rasmia@hotmail.com

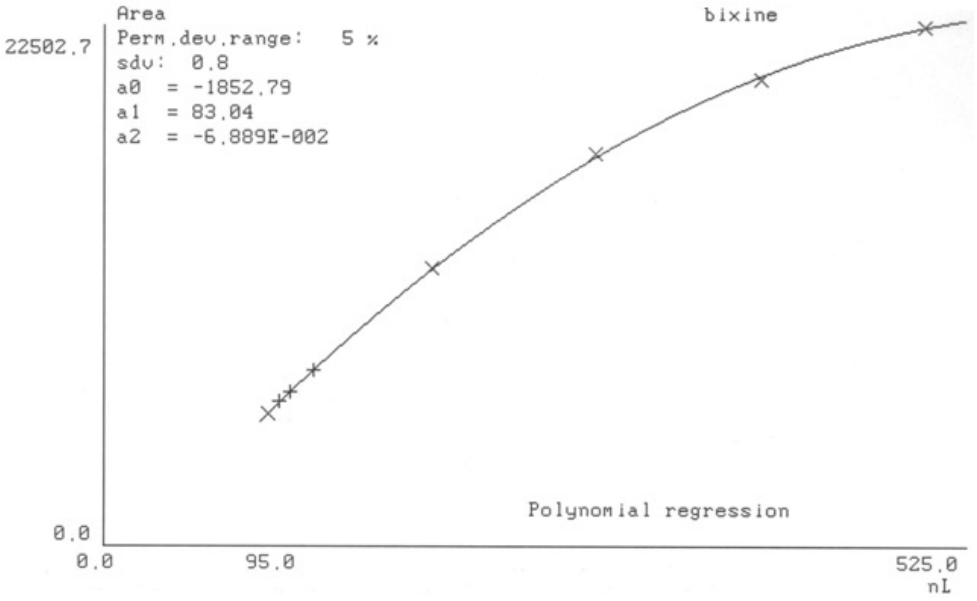


Fig. 1: Standard Curve of Bixin.

Chemicals: Bixin was obtained from Sigma, Germany. Silicagel 60 F254 plates and solvents were obtained from Merck, Darmstadt, Germany.

Extraction and Scanning Conditions:
Standard Solution: 1% stock solution of standard bixin was prepared. Different application volumes of standard bixin were applied on the TLC stationary layer starting with 100 nL to 500 nL to illustrate the standard curve (Fig. 1).

Bixa Samples: Five grams aliquots of seeds powder of *Bixa orellana* were extracted with EtOH, CH_2Cl_2 and EtOH-CH_2Cl_2 (1:1). The extracts were evaporated *in vacuo* at 40°C. 1% solution of each extract was prepared.

10 µL bixin samples were applied bandwise onto the plates using the automated TLC sampler III (Camag, Switzerland). The optimal chromatographic conditions adopted for the study were achieved by adjusting the instrumental parameters as such;

- Distance from the base edge = 20 mm
- Band length = 8 mm
- Track distance = 20 mm
- № of applications = 8
- After development in $CHCl_3$ – MeOH (95:5), the plates were measured at 460 nm with a TLC Scanner III (Camag, Switzerland).
- Fig. 2 represents densitometric TLC chromatograms of Ethanol sample (a), Chloroform sample (b) and Chloroform : Ethanol, 1:1 (c).

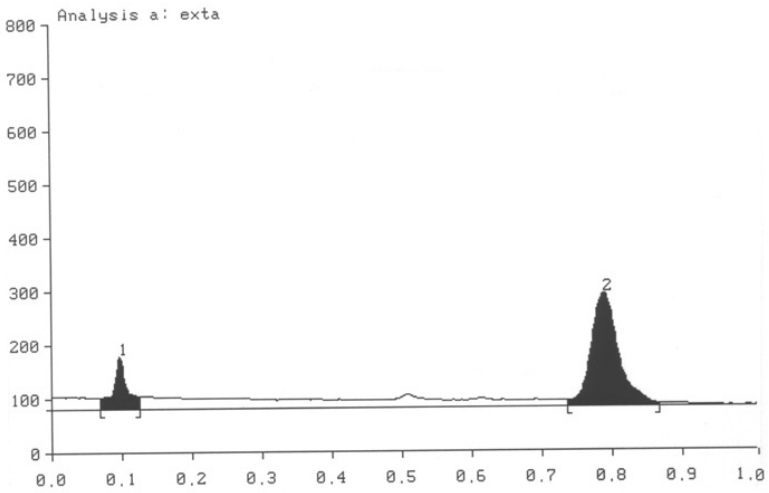
Betalaine Samples: Five grams of *Opuntia tuna* Mill. fruits were extracted with 80% ethyl alcohol in a blender. The homogenate was heated at 40° for 5 min and filtered then adjusted to pH 4.5 and centrifuged to remove the brown precipitate. The clear dark red violet pigment was concentrated *in vacuo* at 40°C and then fractionated with ethanol. and stored at 4°C overnight, then filtered. The filtrate was evaporated at 40°C *under vacuum* to give a yellowish orange pigment (Pigment I). Further extraction of the remaining precipitate was carried out with acidified ethanol then neutralized and concentrated under vacuum at 40°C to give a red-violet pigment (Pigment II).

The two isolated betalaine pigments were subjected to UV and densitometer spectral analyses under the following conditions:

- Distance from lower edge = 15 cm
- Band length = 8 mm
- Track distance = 20 mm
- № of applications = 4.
- 100 µL were applied for each sample.

After development in CH_3COOH-MeOH (40:60), the plates were measured at 538 nm. Fig 3 represents densitometric TLC chromatograms of pigment I (a) and pigment II (b).

Ultra-Violet Spectroscopy: UV spectra of the betalaine pigments were measured in absolute spectroscopic methanol in the region of 250-600 nm. The UV absorption spectrum of the yellowish-orange


Analysis a: exta
800
700
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500
400
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200
100
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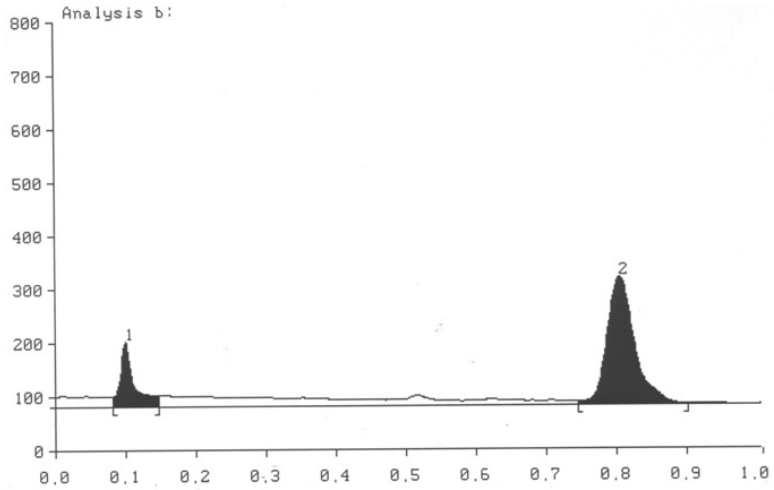
0.8
0.9
1.0

(2a)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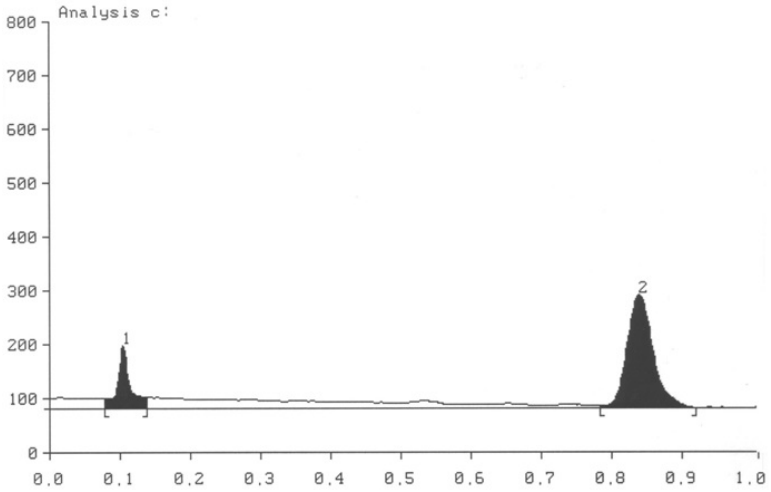
Analysis c:
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0.7
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0.9
1.0

(2c)

Fig. 2:

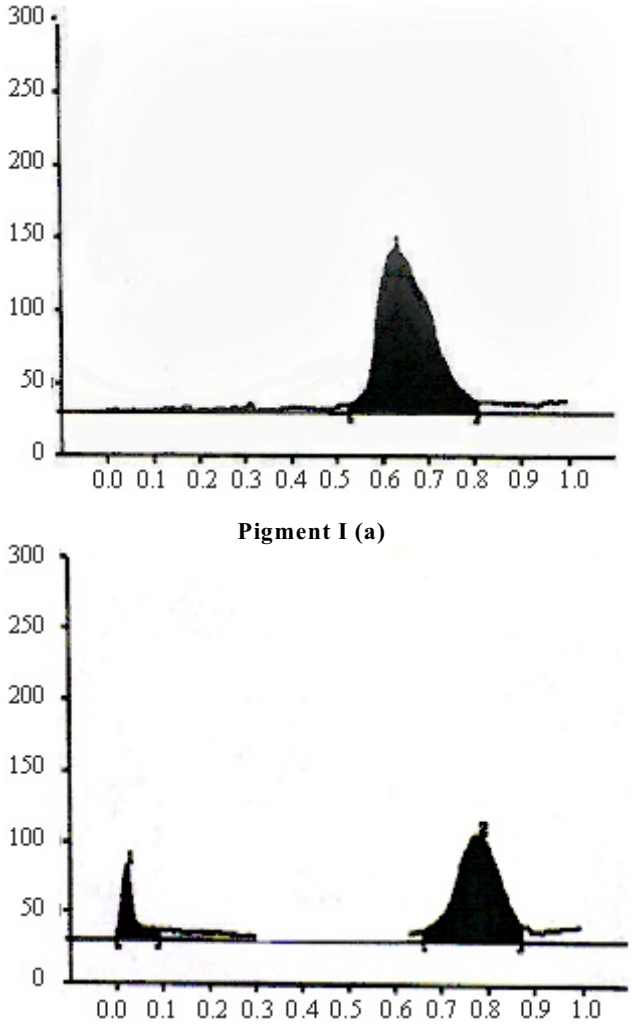

Fig. 3:

(Pigment I) exhibited an absorption band at 475 nm, while the red-violet pigment (Pigment II) showed an absorption band at 534 nm. These data were in accordance with that reported before[11]. (Table 1).

RESULTS AND DISCUSSION

The objective of this work was to establish the eligibility of this analytical tool for the identification and quantitative determination of bixin, indicaxanthin, betanin and isobetanin upon extraction of these specific constituents.

The standard bixin has been subjected to TLC-densitometric evaluation under the suitable conditions proved that this technique is suitable for the detection of these molecules under the selected conditions. Different application volumes of standard bixin were applied on the TLC stationary layer to illustrate the standard curve (Fig 1). The samples under investigation were analyzed under the same conditions as the standard (Fig. 2). Concentrations of bixin in the different prepared samples were calculated automatically using CATS 4 Software.

The results indicated that the highest percent of bixin was found in sample extracted with 100 % EtOH (1.37 %) followed by the sample extracted by EtOH/$CHCl_3$ (1:1) (1.23 %) and finally the sample extracted with 100 % $CHCl_3$ (1.16 %). These results are in agreement with that obtained previously by HPLC[12].

The betaline samples; Indicaxanthin (Pigment I), Betanin and Isobetanin (Pigment II) were analyzed under the previously mentioned conditions. Pigment I showed maximum absorption at 475 nm which was in accordance with that reported for indicaxanthin. Also, pigment II showed maximum absorption at 534 nm which was in accordance with that reported for betanin and isobetanin[11].

Table 1: UV-Spectrum of Betalaine Pigments

UV-Data of Isolated Compounds	UV-Data of The reported Compounds	Isolated Compounds
475	475	Indicaxanthin (Pigment I)
534	538	Betanin and Isobetanin (Pigment II)

It was found that the red violet betanin is usually accompanied by minor amounts of its diastereoisomer isobetanin. This fact was confirmed by TLC-Densitometeric analysis.

Comparison of the TLC-densitometric results with those reported and with those obtained by spectrophotometric measurements showed no significant differences, confirming the reliability of the TLC-densitometric data.

Conclusion: TLC densitometry proved to be a precise and simple analytical method for the chromatographic densitometric determinations. Simultaneous determinations were achievable by this method and one can conclude that TLC densitometry is a powerful and useful tool and can be considered as an alternative to HPLC. The most important features of this analytical method is its sensitivity and rapidity which ultimately renders it appropriate for routine analysis of plant extracts.

In conclusion, for the routine analysis of betanine and bixin the TLC method using precoated silica plates coupled with densitometric determination can be used as an appropriate screening method. The proposed method, TLC densitometry, can be widely practiced as a standard technique for quick and precise qualitative and quantitative determinations of plant extracts.

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