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A JOINT MACRO- AND MICROSCOPIC AUTHENTICATION KEY FOR PHARMACOGNOSTIC ANALYSIS OF CROCI SATIVI STIGMA AND ITS COMMON ADULTERANTS ON THE BULGARIAN MARKET

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Abstract. One of the most expensive spices and herbal substances – the saffron threads, is produced from the stigmas of *Crocus sativus* L. The low number of stigmas per flower, labour-intensive harvesting and high demand drive its high cost but also make it a significant target for adulteration. The current study aims to develop a key for rapid macroscopic and microscopic screening of Croci sativi stigma samples, which can distinguish between the herbal substance and its most common adulterants. A literature review was conducted to determine known adulterants of saffron and relevant identification techniques. Vaucher materials were acquired from Bulgarian markets and analysed. An authentication key was developed and then tested by students enrolled in the Pharmacognosy courses in the master’s program in Pharmacy at Sofia University “St. Kliment Ohridski”. Of 15 samples, 12 were adulterated. Using the provided key, 12 out of 13 students managed to correctly identify provided samples.

Keywords: saffron adulteration; Croci sativi stigma authentication; pharmacognosy

Introduction

Saffron (*Crocus sativus* L., Iridaceae) is considered an important medicinal plant, yielding a valuable and expensive herbal substance and spice both in a historical and contemporary context. The morphological part in question is the brightly red-coloured, aromatic stigmas of the plant of which only three are produced per

flower. The saffron threads (Croci sativi stigma) are prized as a natural remedy and foodstuff and are cultivated in Greece, Asia Minor, Persia, India, Pakistan, China, Spain and even England and Bulgaria (Evans 2009, Peter 2012, Mohtashami et al. 2021). In contemporary practice, quality standards for the herbal substance have been adopted as monographs into different national and international pharmacopoeias, such as Ph. Eur.[1] (only for homoeopathic preparations), ChP, USP etc. and as standards by the ISO[2,3]. Regardless, due to its high price determined by the growing demand, laborious cultivation and harvesting, and low yield after primary processing, Croci stigma is subject to extensive and creative adulteration attempts. These include, though are not limited to, self-adulteration (the stamens or styles of saffron), using exhausted and dyed plant materials (carmine, Sudan red G), adding honey, glycerol or other substances to increase the mass of the commercially available material, replacing some or all of the herbal substance with dried morphological parts of other plants or dried meats, replacing some or all of the plant material with dyed natural or synthetic fibres, plastics etc. (Anon 2021). In response, multiple analytical methods ranging from hyphenated techniques to genomic and metabolomic analysis have been developed in an attempt to better authenticate saffron threads and achieve sufficient quality control (Raina et al. 2024; Malavi et al. 2024; Long et al. 2023; Younis et al. 2023). Unfortunately, albeit advanced, these techniques all come with a myriad of disadvantages, namely that they either require expensive apparatuses and/or reagents, extensively trained and highly-skilled personnel or have low throughput (Kumari et al. 2021). Concurrently, macroscopic and microscopic examination, coupled with qualitative colour reactions and/or TLC/HPTLC, continue to be the approach of choice for determining herbal substance identity[4]. Additionally, microscopic analysis requires relatively inexpensive equipment for observation in bright field. It must be remarked however, that the chloral-hydrate solution (a commonly used clearing and mounting agent) for observation in bright field is considered hazardous and is subject to additional regulation in different parts of the world. The primary goal of the present study is to construct an authentication key based on the contemporarily applied methods of analysis for Croci sativi stigma and its adulterants of herbal origin identified in samples of the substance obtained from the Bulgarian marketplace. A secondary goal of the study was to assess the performance of the created authentication key by personnel trained in pharmacognostic analysis but with no prior experience in the identification of Croci sativi stigma and its adulterants.

Materials and methods

Pharmacognostic textbooks and articles published in the Web of Science and ScienceDirect databases were analysed in an effort to determine common saffron adulterants of herbal origin. Keywords used for the search were saffron, adulteration, analysis, *Crocus sativus* and Croci stigma. The taxonomic and pharmacog-

nostic nomenclature used in these works was standardised using The World Flora Online database[5]. Quality standards outlined in herbal substance monographs in the Ph. Eur. 11.0 were used to determine unique Croci sativi stigma and adulterant diagnostic characteristics[6,7,8]. A market study was carried out during the month of May 2022. Traditional and modern herbal stores, supermarkets and pharmacies, as well as open market stalls in Sofia, Plovdiv and Pazardzhik, were used to source samples of Croci sativi stigma. A total of 15 samples were acquired and analysed. Voucher materials were archived and are kept in the voucher collection of the laboratory of Pharmacognosy, Department of Organic chemistry and Pharmacognosy, Faculty of Chemistry and Pharmacy, Sofia University "St. Kliment Ohridski". The herbal substances were cultivated domestically (Bulgaria – 2), imported (China – 1, Crete – 1, Iran – 4, Spain – 1, Türkiye – 5) or were of undeclared origin (1), based on the information provided on the packaging, ranged from 0.50 g to 1.00 g in weight, and were priced from BGN 0.11 to BGN 38.80 per gram. Macroscopic analysis was carried out with unaided vision and a Zeiss Stemi 305 Stereo Zoom Microscope. Microscopic analysis was carried out with a Zeiss Primostar 1 Compact microscope in bright field with 4 g/mL chloral hydrate (Honeywell, 99.5%) aqueous solution as the mounting and clearing agent. Mortars and pestles were used to reduce samples to powder and sample preparation followed the Ph. Eur. methodology[9].Concentrated sulfuric acid (Merk, 99.8%) was used for the qualitative colour reaction. After developing the authentication key, 13 students split into two groups were presented with a hypothetical scenario in which they were assigned the roles of experts who would have to determine the identity of provided samples based on the key. Inclusion criterion for participation was determined to be a score of at least 60% (19.8 of 33 points) on the Pharmacognosy 1 practical exam. All student volunteers met the inclusion criterion. For each group, one of the samples was of Croci sativi stigma and the others were common adulterants (Arnicae flos, Calendulae flos, Carthami flos, Maydis stigma and Rhus coriariae fructus) from the Pharmacognosy laboratory herbal substance collection. Samples were assigned at random and sample numbers were changed between groups. Students were also handed out sheets on which they had to note their age, gender, sample number, Pharmacognosy 1 practical exam score, start and end time of the analysis, macroscopic, microscopic, and qualitative reaction determination, and conclusion on sample identity. They were also asked to note whether they have studied the herbal substance during the Pharmacognosy 1 practicums and if they considered to possess extensive knowledge of the herbal substance from their personal experience.

Results

The published literature gives numerous examples of morphological parts of botanical origin used to adulterate saffron threads, namely: self-adulteration with anthers

and/or perigonium of *Crocus sativus* L., the dried flowers of safflower (Carthami flos, *Carthamus tinctorius* L.), the dried flowers of Calendula (Calendulae flos, *Calendula officinalis* L.), the stigma of other *Crocus* sp. (*Crocus vernus* L. and *Crocus speciosus* M.Bieb.), the petals of the common poppy (Papaveris rhoeados flos, *Papaver rhoeas* L.), the flowers of pomegranate (Punicae flos, *Punica granatum* L.), the flowers of arnica (Arnicae flos, *Arnica montana* L.), the flowers of the Spanish oyster (*Scolymus hispanicus* L.), stamens of different species of carnation (*Dianthus* sp.), ground red pepper fruit (Capsici fructus, *Capsicum annuum* L.), roots of leek (Allii porri radix, *Allium ampeloprasum* L.), red sandalwood powder (*Pterocarpus santalinus* L.f.), ground turmeric rhizome (Curcumae longae rhizoma, *Curcuma longa* L.), corn silk (Maydis stigma, *Zea mays* L.) and fruits of sumac (Rhus fructus, *Rhus coriaria* L.). Only some of these were discovered in the samples acquired from Bulgarian markets and not all contained Croci sativi stigma (Table 1). The table denotes the voucher material number, the herbal substance country of origin, as declared on the packaging, whether saffron threads and adulterant(s) were identified in the sample and the reaction the samples gave when treated with concentrated sulfuric acid.

Table 1. Croci sativi stigma Bulgarian market samples adulteration analysis

Voucher material code:	Declared origin on sample packaging:	Croci sativi stigma:	Adulterant(s):	Reaction with conc. H_2SO_4:
VMCSS1	Crete	Yes	None	Blue
VMCSS2	Spain	Yes	None	Blue
VMCSS3	Iran	Yes	None	Blue
VMCSS4	Undeclared	No	Yes (elongated, red plastic fragments and Carthami flos)	Reddish- brown
VMCSS5	Türkiye	No	Yes (Carthami flos)	Blue
VMCSS6	Türkiye	No	Yes (Carthami flos)	Blue
VMCSS7	China	No	Yes (Carthami flos)	Blue
VMCSS8	Türkiye	No	Yes (Carthami flos)	Blue
VMCSS9	Türkiye	No	Yes (Carthami flos)	Blue
VMCSS10	Türkiye	No	Yes (Carthami flos)	Blue
VMCSS11	Bulgaria	Yes	Yes, self-adulteration (anthers)	Blue
VMCSS12	Bulgaria	Yes	Yes (Maydis stigma)	Blue
VMCSS13	Iran	Yes	Yes (Calendulae flos)	Blue
VMCSS14	Iran	Yes	Yes, self-adulteration (perigonium)	Blue

VMCSS15	Iran	Yes	Yes (Arnicae flos, visible foreign fibres, sand and plastic pieces)	Blue

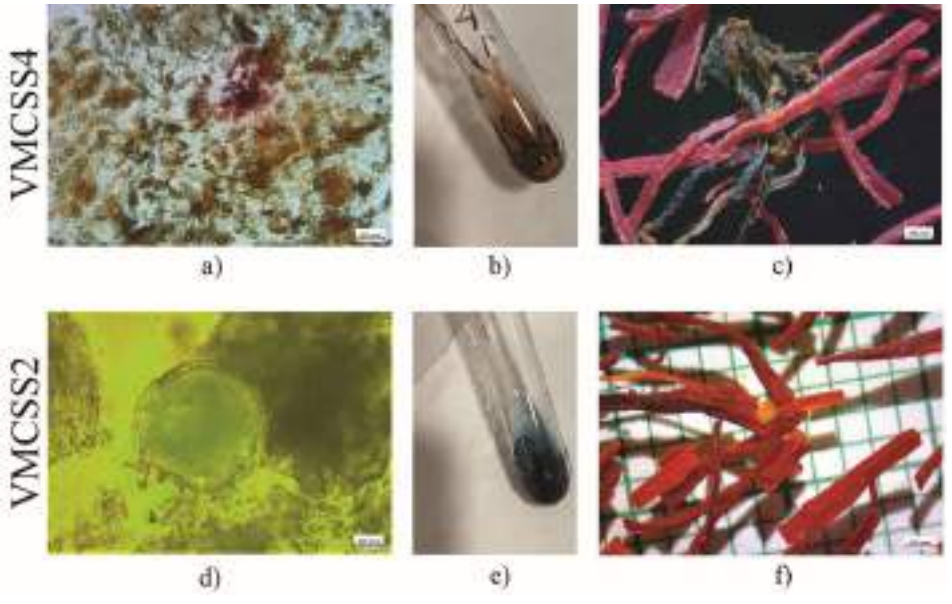

Figure 1. Photographic materials of analysed samples from Bulgarian markets. VMCSS2: d) Pollen from *Crocus sativus* L. attached to papillae, 400x magnification; e) colour reaction with conc. H_2SO_4; f) Stigma of *Crocus sativus* L., 4x magnification. VMCSS4: a) amorphous, non-botanical fragments, chloral hydrate with no yellow tint, 40x magnification. b) colour reaction with conc. H_2SO_4; c) red plastic fragments and discoloured safflower flower, 4x magnification.

Of the analysed samples only 3 (20%) contained no visible adulterants upon macroscopic and microscopic identification. Only VMCSS4, comprised primarily of plastic and a few dried and discoloured safflower flowers, failed the sulfuric acid test. By far the most common adulterant present, even replacing the declared contents entirely, was Carthami flos. Photographic materials of the analysis results are provided (Figure 1). Based on the observations made on the market samples and available quality standards for Croci sativi stigma and its adulterants, several macroscopic and microscopic diagnostic characteristics were highlighted as crucial for the rapid authentication of saffron threads. Macroscopically organoleptic characteristics of saffron (specific red colour and smell) are important and are typically not encountered in its adulterants. Size and shape also play a key role

(Croci sativi stigma are typically funnel-shaped and range in length from 20 to 50 mm). While saffron threads treated with concentrated sulfuric acid give an intense blue colour, the isolated morphological fragments used as its adulterants do not. Microscopically, the orange-yellow colour of the powdered saffron threads and the yellow tint imparted on the mounting agent are very characteristic of the substance (though of the known adulterants in literature, turmeric roots and safflower flower can also possess similar organoleptic characteristics). This is why it is important to visually confirm the presence of large (100 μm in diameter), spherical pollen grains with a smooth exine of *Crocus sativus* L. Smaller pollen with a spherical to elliptical shape and a spiky exine can be encountered in adulterants of the Asteraceae family. The presence of trichomes, bristles, fibres, sclereids, starch granules and cork cells are other characteristics that can be indicative of the presence of botanical adulterants of various morphological parts of different origins. The developed authentication key takes into account the outlined features and is provided in the form of two decision trees (Figure 2 and Figure 3).

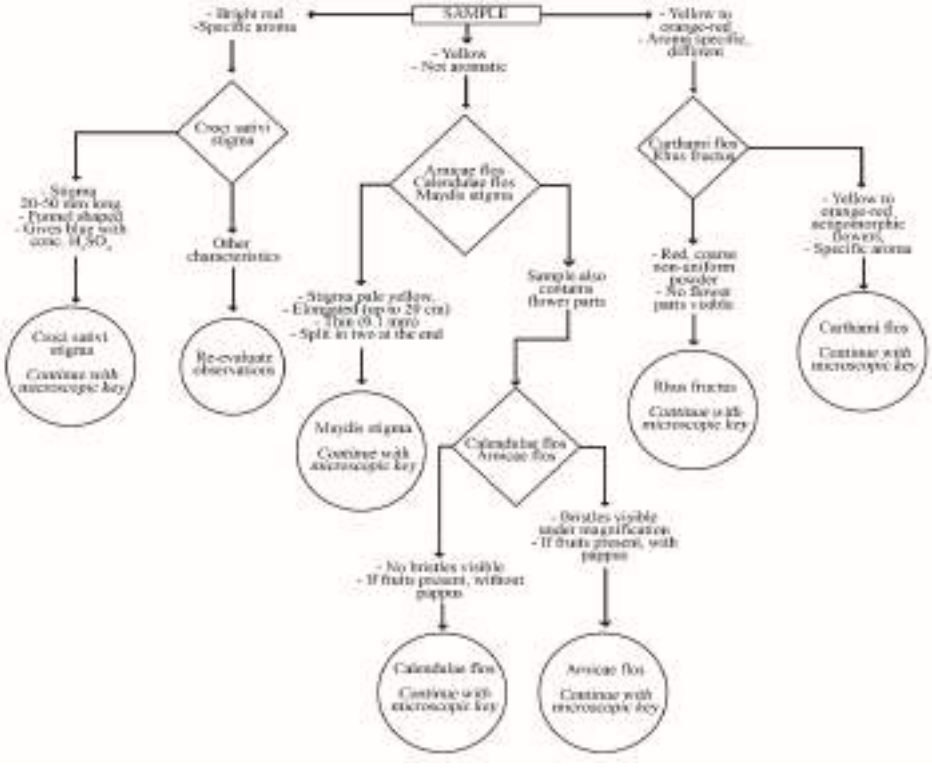

Figure 2. Decision tree – macroscopic authentication key for Croci sativi stigma samples

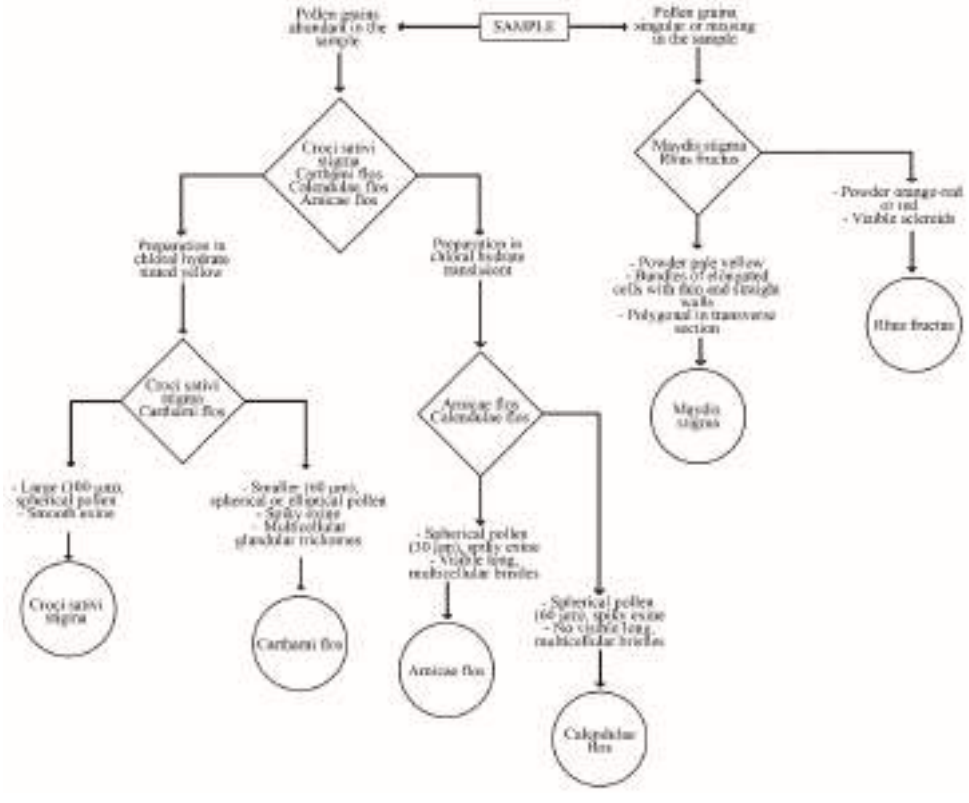

Figure 3. Decision tree – microscopic authentication key for Croci sativi stigma samples

Student volunteers were both male and female and were of 21 – 23 years of age. Their practical exam scores ranged from 100% (33 points) to 63% (20.8 points). Average analysis time was approximately 46 minutes. The fastest analysis time was 20 minutes for a sample of Maydis stigma, for which the student declared to have studied extensively during Pharmacognosy 1 practicums. The longest analysis time was 68 minutes for a sample of Calendulae flos. Using the provided decision trees, 12 of 13 students managed to correctly identify their samples. Student decision tree-based analysis results are provided (Table 2).

Table 2. Student decision tree-based analysis results. To ensure anonymity each student was provided a code name denoting the group they participated in (G1 or G2) and participant number (R1 to R7):

Student:	Practical exam result:	Analysis duration [min]:	Macroscopic identity	Microscopic identity:	Colour reaction with conc. H_2SO_4:	Correct ID:
G1R1	27.5	68	Calendulae flos	Calendulae flos	Brown-red	Yes
G1R2	20.8	50	Carthami flos	Carthami flos	Yellow-orange	No (Rhus fructus)
G1R3	30.0	50	Arnicae flos	Arnicae flos	Dark green	Yes
G1R4	38.0	37	Carthami flos	Carthami flos	Brown-black	Yes
G1R5	23.0	35	Croci stigma	Croci stigma	Blue	Yes
G1R6	21.5	20	Maydis stigma	Maydis stigma	Red	Yes
G1R7	23.0	30	Maydis stigma	Maydis stigma	Red	Yes
G2R1	32.7	59	Arnicae flos	Arnicae flos	Greenish-brown	Yes
G2R2	32.0	60	Rhus fructus	Rhus fructus	Brown-red with bubbles	Yes
G2R3	29.7	50	Maydis stigma	Maydis stigma	Redish-brown	Yes
G2R4	33.0	40	Carthami flos	Carthami flos	Red-brown	Yes
G2R5	28.0	50	Calendulae flos	Calendulae flos	Dark brown	Yes
G2R6	33.0	43	Croci stigma	Croci stigma	Blue	Yes

Conclusion

Common adulterants of botanical origins of Croci sativi stigma were determined based on published literature and market analysis in three Bulgarian cities. Unique macroscopic and microscopic diagnostic characteristics for these herbal substances were determined and an authentication key was developed based on the market analysis results. Students with demonstrated skills in Pharmacognostic analysis, but little prior knowledge of these morphological parts, were able to successfully determine randomly provided sample identity based on the decision tree authentication keys. While the longest analysis time was 68 minutes, it must be remarked that sample analysis times also include the reduction of samples to a fine powder and microscopic sample preparation. Further analysis with a larger pool of volunteers and multi-component samples can be carried out to further determine the applicability of the developed authentication key.

NOTES

1. Ph. Eur. 11.0, 1624 (07/2014)
2. International standard ISO 3632-2: Saffron (*Crocus sativus* L.) test methods
3. International standard ISO 3632-1: Saffron (*Crocus sativus* L.) specification
4. Guide for the elaboration of monographs on HERBAL DRUGS AND HERBAL DRUG PREPARATIONS, EDQM, 2023
5. WFO (2024): World Flora Online. Published on the Internet; http://www.worldfloraonline.org. Accessed on: 29 Feb 2024
6. Ph. Eur. 11.0, 1391 (07/2022), corrected 11.2
7. Ph. Eur. 11.0, 1297 (04/2020)
8. Ph. Eur. 11.0, 2386 (07/2014)
9. Ph. Eur. 11.0, 20823 (04/2010)

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