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Multiple tests on saffron find new adulterant materials and reveal that Ist grade saffron is rare in the market

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ABSTRACT

Among spices, Saffron is among the most extensively interrogated for purity and authenticity. Numerous methods have been recommended for authentication of Saffron samples and for detection of adulterants for codex compliance. However, none of these methods can fulfill both of these important quality criteria. This study describes a three step approach to achieving this goal by including the established ISO3632 method and two additional methods based on microscopic examination and DNA barcoding. We provide results showing the utility of these methods both independently and in combination for quality evaluation of 36 commercial saffron samples. Our results show that use of the ISO3632 approach alone can reveal the color and aroma but not the genetic origin of the material or distinguish between synthetic components versus natural ingredients. Also, the microscopic observation method can give a preliminary indication of saffron authenticity, but used alone it is unable to quantify purity. Finally, a relatively new method based on the use of DNA barcodes can authenticate the biological origin of the saffron, but here results may be misleading if auto-adulterating materials are present. Overall, our study reveals that through the combined use of all three methods, saffron authentication can substantially improved.

1. Introduction

A wide variety of herbal materials used for food flavoring, coloring or preservation are popularly referred as “spices”. These materials may contain ample nutraceutical properties, including providing a rich source of antioxidants, minerals and vitamins. For these reasons, among others, spices have been extensively used in food, cosmetic and pharma industries as well as for several religious rituals (Srinivasan, 2014). The spice material may be obtained from various dried parts of specific plant/herbs including the root, stem, bark, leaves, flower, fruit and seed.

As such a condiment, saffron has been used in our day to day life since antiquity. Historically, among 85 known spices in the world, saffron is relatively rare and expensive, but it is used extensively in the food industry as well as in pharmaceuticals (Thakur & Sharma, 2014). Additionally, when processed using traditional methods, the value of saffron is significantly enhanced (Melnyk, Wang, & Marcone, 2010). Saffron has been described from ancient times to possess strong medicinal benefits including cardiotonic, carminative, aphrodisiac, diuretic, diaphoretic, stimulant, nervine tonic and sedative properties (Chopra, Nayar, & Chopra, 1956). In this context, saffron is an excellent example of a potentially valuable product in terms of health benefits, flavoring and traditional uses (Thakur & Sharma, 2014).

Saffron is produced using the dried stigmas of the cultivated plant *Crocus sativus* L. Major saffron producing countries include Iran, Spain, Greece, Italy, Turkey, and India. Of these countries, almost 90% of all saffron is produced in Iran. However, saffron is also among the spices most commonly questioned for its quality and purity. Considering its limited production and cultivation in areas from high altitude ranges and cold climatic conditions and the laborious nature of the extraction process, saffron is a potential candidate for commercial motivated fraud (Johnson, 2014). Cases of adulteration of saffron and other spices have been described where a variety of inferior quality plant materials which are similar in appearance, less expensive and more readily available

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were noticed. Such practices are by no means only contemporary phenomena, in fact they are likely to be as old as the evolution of culinary art itself (Hong et al., 2017).

Adulterations of saffron can be classified into three specific practices. These are: 1) Substitution of similar materials; 2) augmentation of saffron mass and 3) use of coloring agents. Each of these will be described in more details as follows:

1.1. Substitution of similar materials

1. Substitutions of other plant /herbal materials such as beet fibres, pomegranate fibers, red-dyed silk fibers, safflower petals, marigold petals, calendula petals, turmeric powder are among the most frequently encountered (Soffritti et al., 2016).
2. Auto adulterations refer to cases where other parts of the *C. sativus* plant itself including flower styles, stamens, and petals are used as substituting materials (Soffritti et al., 2016).

1.2. Augmentation of saffron mass

1. To increase the saffron mass, in this type of adulteration saffron fibers are soaked in honey, vegetable oils, or glycerin (Remington, Horatio, & Wood, 1918).

1.3. Use of dyes

1. Natural coloring extracts (dyes) can be used for fraudulent coloring of adulterants. Fraudulent materials most commonly used are gardenia fruit extracts and dyes from the buddleja flower (Soffritti et al., 2016). These adulterants have pigments which are similar to the saffron pigment known as crocin (Soffritti et al., 2016).
2. Use of artificial dyes including water-soluble colourants such as erythrosine, ponceau 4R, and tartrazine are also common (Petrakis & Polissiou, 2017). Also, fat-soluble compounds, such as banned Sudan dyes, are also used for improving the appearance of old and inferior quality saffron (Petrakis & Polissiou, 2017).

The use of these adulteration practices can make authentication of legitimate saffron products a challenging task. However, recently, interest and motivation for establishing appropriate methods for evaluation of the quality of saffron available in the market has increased.

As described, some of the most important parameters to be considered for establishment of saffron quality are the coloring strength and the aroma. The metabolite crocin produces color and the saffranal produces aroma. Both metabolites are carotenoid derivatives (Soffritti et al., 2016).

Saffron color and aroma are commonly measured using different laboratory methods including UV–Vis spectrometry, HPLC, and gas chromatography-mass spectrometry (GC–MS) (Sereshti, Heidari, & Samadi, 2014). However, these methods are expensive and require trained experts. Also, the recent addition of artificial colourants to adulterated materials has raised further concerns for use of quantification of color and aroma as a measure of quality control. As a general rule, according to the ISO 3632 standards (ISO, 2011), artificial colourants should be not be present in saffron. However, the ISO 3632-2 test (ISO, 2010) uses chromatographic (i.e. TLC, HPLC) methods to test methods for detection of artificial water-soluble acid colourants, but not for fat-soluble compounds. This suggest that certain artificial colourants such as Sudan dyes that may be used for improving appearance of adulterated inferior quality materials, may still escape detection.

Moreover, in addition to the detection of adulterated materials and the active chemical components of interest (FSSAI, 2013; SSAFE, 2016), codex compliance claims require knowledge of the origin of any adulterating materials (Codex Alimentarius, 2004; FSSAI, 2013; SSAFE, 2016). None of the methods as of yet available can meet both of these requirements. Here, our study tested the utility of combining a microscopic method for primary detection of adulterated materials with DNA barcode methodology to determine the biological origin of putative saffron samples. Also, an effort was made to evaluate the ability of each method, namely the ISO 3632-2 (International Organization for Standardization) recommended spectrophotometric methods, the microscopic method and the DNA barcode methods for detecting the biological origin of adulterants for compliance of Codex Alimentarius applicable to material from different countries both independently and in combination.

2. Materials and methods

This study tested 36 saffron samples collected directly from retailers and through online shopping. All store locations are shown in Fig. S1. Original purchase bills and receipts were also retained. Collected samples were processed in triplicate to ensure the capture of all types of the adulterant materials, if any. All samples were tested for their authenticity using three methods of analysis *viz*. microscopy, DNA barcoding using the rbcL gene and spectrometric methods as prescribed by ISO 3632-1, 2011 and as detailed below. The rbcL gene was selected for the barcode tracking of biological origin of samples since it is extant to all terrestrial plants which have photosynthetic ability. In this method, variant forms of this chloroplast gene can be compared within and between species in order to determine the genetic identity of material (Freeman, 2008).

2.1. Microscopy

A few saffron fibers were randomly selected from each sample. These were kept on a clean glass slide, hydrated with double distilled water and cover slips were mounted avoiding air bubbles using gentle pressure. Slides were observed under the microscope (objectives 4×, ocular 10×; Evos, Thermo Fisher Scientific, USA) with a final magnification of 40×. Images were captured and compared.

2.2. DNA barcoding

2.2.1. DNA extraction, PCR and sequencing

DNA was extracted from all samples in triplicate using 10 mg samples using the Wizard genomic DNA purification kit (Promega, Madison, WI, USA) and following manufacturer's instructions. Extracted DNA was quantified and diluted to a final concentration of 50 ng/μL. DNAs were also checked using1% agarose gels.

A portion of the rbcL gene was amplified in a 25 μL reaction volume containing 2 μL of 250 μM dNTP, 0.5 μL 25 mM $MgCl_2$, 0.5 μL 10 pM primers rbcLa-F – ATGTCACCACAAACAGAGACTAAAGC and rbcLa-R – GTAAAATCAAGTCCACCRCG (Kress, Erickson, Jones, Swenson, & Perez, 2009) each, 1U *Taq* Polymerase (Kapa Biosystems, USA) using a Veriti thermocycler (Applied Biosystems, Foster City, USA). All PCR products were visualized on 1.2% agarose gel (Fig. 2). Selected samples were used for bidirectional sequencing using a Sanger sequencing platform (ABI 3730xl genetic analyzer, Applied Biosystems, Foster City, USA).

2.2.2. Sequence analysis

Sequences of rbcL PCR products were edited visually following base calling and aligned by Codon-code aligner v 3.0 (CodonCode Corporation, MA, USA). All edited and assembled sequences were checked using BLAST on the NCBI and BOLD identification systems. Using MEGA 7.0, variant versions of rbcL gene sequences were as input to produce a dendrogram for saffron and its allied adulterants to observe genetic relatedness.

2.3. Spectrophotometric analysis of saffron following ISO 3632-1:2011

With slight modifications, the ISO method of Hadizadeh et al.