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Quality Control of Saffron (*Crocus sativus* L.): Development of SCAR Markers for the Detection of Plant Adulterants Used as Bulking Agents

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ABSTRACT: A method based on sequence-characterized amplified regions (SCARs) was developed from random amplified polymorphic DNA markers (RAPDs) specific for *Arnica montana* L., *Bixa orellana* L., *Calendula officinalis* L., *Carthamus tinctorius* L., *Crocus vernus* L. (Hill), *Curcuma longa* L., and *Hemerocallis* sp. to detect these common bulking agents in commercial saffron (*Crocus sativus*). The method enabled the unequivocal detection of low amounts (up to 1%) of each adulterant, allowing the preemptive rejection of suspect samples. Its enforcement limits the number of samples to be subjected to further evaluation with pharmacognostic or phytochemical analyses, especially when multiple batches have to be evaluated in a short time. The dimension of the amplicons is suitable for the analysis of degraded DNA obtained from dried, stored, processed, and finely ground commercial material. Proper SCAR markers may represent a fast, sensitive, reliable, and low-cost screening method for the authentication of dried commercial saffron material.

KEYWORDS: *saffron, quality control, molecular markers, bulking agents, adulteration*

INTRODUCTION

Saffron, the spice obtained from the dried stigmas of *Crocus sativus* L. (family Iridaceae) flowers, is renowned as one of the most valuable seasonings; its market price ranks among the highest in the food and flavoring sector and is also the object of ongoing scientific research related to potential medicinal properties. Saffron is also sparingly used also as a dye or in perfumery.[1−3] The worldwide market of saffron is worth nearly $1 billion, and approximately 300 tons were globally produced in 2007, despite a downward trend mostly related to labor costs and socio-agricultural constraints rather than to actual demand.[4−7]

As a consequence of its high market value, perceived value, and demanding production, saffron has been regularly coupled with an unrivaled degree of adulteration, performed in both ground and whole stigmas with the most diverse materials and strategies. Artificial and natural dyes, mineral compounds, other spices, and plant stuff with similar color and morphology have been deliberately added as bulking agents in *C. sativus* dried stigmas, to increase the volume and weight of commercial lots.[4,5,8] The most frequently encountered materials are *Carthamus tinctorius*, *Calendula officinalis*, and *Arnica montana* flowers, *Bixa orellana* ground seeds, *Hemerocallis* sp. tepals, *Curcuma longa* powdered rhizomes, and *Crocus vernus* stigmas.[5,7,9] A wide array of strategies have been enforced to prevent malicious practices, and at present the issue can be tackled with two different approaches, the first related to purity and authentication of saffron and the second mostly related to its grading. The latter is usually certified in the international trade market by reference to the ISO 3632-2 standard and to its technical specification ISO 3632/TS. These protocols define four different grades for saffron according to its coloring strength, calculated by means of a combination of UV measurements of picrocrocin, safranal, and crocin.[10] ISO 3632-2 provides limits and methods for the definition of saffron taste, fragrance, color, floral waste, foreign matter, moisture, mineral, and exogenous dyes but, as highlighted by recent literature, it offers weak reliability in the detection of some typologies of vegetal foreign matter. In particular, the addition in calibrated amounts of other plant materials rich in carotenoids may easily go undetected, and it has been recently demonstrated that a contamination of dried saffron with amounts up to 20% of *Calendula* or *Carthamus* flowers or ground turmeric rhizome may not be revealed by the ISO/TS 3632-2 method.[11] Instead, saffron authentication relies mostly on traditional pharmacognostic analysis performed with microscopic observations of morphological traits, which are extremely time-consuming when the number of samples to be screened is large. These procedures imply the recognition of traits such as starch granules, epidermal stigma residues, pollen granules, or upper end stigma residues with epitheliums, the proper identification of which requires the availability of trained and experienced personnel and involves a certain degree of subjective interpretation.[9] This scenario paved the way for a wide range of chromatographic and spectroscopic approaches, which have been suggested as potential candidates for a more reliable evaluation of saffron quality and, on a few occasions, for its authentication.[12−20] The interest in these determinations is also strenghtened by the questionable reliability and accuracy of the ISO method in the quantitation of both safranal and crocins, the substances responsible for the aroma, color, and bitterness of saffron.[21] However, only a few of the above-mentioned methods resolve suitably the issue of bulking agents

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of plant origin, and most of them offer a more careful and reliable strategy for the grading of saffron according to its phytochemical content or for the detection of synthetic and natural dyes, frequently used as adulterants as well. Proper screening techniques allowing fast detection of potentially adulterated samples at a reasonable operational price are actually not available or their sensitivity is not adequate. For example, an HPLC/PDA/ESI-MS method has been recently suggested for the detection of bulking agents in saffron, but its sensitivity in commercial samples has reached 5% for *C. tinctorius* and *C. officinalis* and 2% for *C. longa*, whereas the highest amount of foreign matter allowed in third-grade saffron by ISO protocols is 1%.[11]

Recently, the cheaper availability of biomolecular assays made the recourse to DNA-related techniques affordable in a wide array of food-related applications, de facto allowing the achievement of operating costs similar to those of UV detection.[22] As a consequence, DNA markers have become a popular means for the identification and authentication of a steadily increasing range of food products, spices, and medicinal or aromatic plants.[22,23] Random amplified polymorphic DNA markers (RAPDs) can be considered a useful starting point with regard to operating cost and reliability in distinguishing between different botanical species. They also allow good performance levels in a short time when the DNA to be probed is in low quantity or partially degraded, as is very common with dried commercial spices.[24,25] Despite these considerable advantages, this technique is difficult to reproduce due to its high sensitivity to reaction conditions.[26] To get around this drawback, any polymorphism established by means of the RAPD assay can be made more robust by converting it into a more specific and reliable marker, known as sequence-characterized amplified regions (SCARs).[27] Therefore, SCARs offer various advantages for routine quality control in particular when traditional techniques pose constraints in terms of time, subjective interpretation of the results, or lack of properly trained personnel.[28−30] Despite its potential, the application of genetic analyses to quality control of saffron has been somewhat limited. Previous work regarded the development of a method for the sole detection of *C. tinctorius* and *Hemerocallis* sp. as bulking agents.[31]

Thus, the objective of the present study was to develop robust SCAR markers for seven vegetable bulking agents commonly spotted in the saffron trade, to confirm their presence as adulterants. The final goal is to obtain a diagnostic tool for the preemptive rejection of suspect samples, thus reducing the number of samples to be evaluated by means of pharmacognostic or phytochemical analyses and providing useful data for further molecular diagnostic tools. The method was optimized with specific respect to a reliable application on plant material of commercial grade (i.e., dried, stored), to complement existing pharmacognostic and chemical methods.

MATERIALS AND METHODS

Plant Material. Bulbs of *C. sativus* were kindly supplied by the Botanical Gardens of Cagliari and cultured in pots to provide fresh material (leaves) for DNA extraction. Six dried commercial samples of *C. sativus* marketed as whole stigmas and from different geographic proveniences (two from Morocco, two from Spain, one from Afghanistan, and one from Italy) were also purchased and used as control after validation by microscopic analysis. Plant material for contaminant species was collected in the field (*C. officinalis*, *A. montana*) or kindly supplied by the Botanical Gardens of Parma (*C. vernus*, *Hemerocallis* sp.). Seeds of *C. tinctorius*, kindly provided by Giardino delle Erbe Officinali di Casola Valsenio (Ravenna, Italy), were sown, and plants were cultured on silty soil in a greenhouse (Botanical Garden of the University of Parma, Italy). *B. orellana* seeds and dried *C. longa* rhizomes were collected by agronomists of Fundacion Chankuap (Macas, Ecuador) in January 2008 on the outskirts of Macas, Ecuador, and positively identified by the National Herbary of Pontificia Universidad Catolica del Ecuador (J. Jaramillo). Fresh plant material was collected, immediately freeze-dried in liquid nitrogen, and stored at −80 °C until molecular analysis. Dried material of each species was prepared from fresh material by drying at 40 °C for 1 week.

Extraction of PCR-Compatible Genomic DNA. Genomic plant DNA was isolated from fresh and dried material as previously described except for the initial incubation in CTAB buffer that was performed at 65 °C for 30 min instead of overnight at room temperature.[29] DNA concentration and purity ($A_{260/280}$ and $A_{260/230}$) were evaluated by spectrophotometric analysis. The suitability of DNA for RAPD analysis was also checked by ethidium bromide-stained agarose/TAE gels, which allowed both the evaluation of DNA integrity and further confirmation of DNA quantitation by visual comparison with DNA standards. Agarose gels were analyzed and quantitated with a Kodak DC40 camera (Kodak) using Kodak digital science 1D Image analysis software (Eastman Kodak Co., Rochester, NY, USA). DNA samples were adjusted to approximately 20 ng/μL prior to using them in PCR reactions.

RAPD Analysis and Marker Selection. Seven potential contaminants of commercial saffron were compared with seven fresh or dried samples of *C. sativus* from different geographic origins to develop RAPD markers.[24] PCR was conducted on approximately 20−40 ng of DNA template and performed in a 25 μL volume containing 67 mM Tris-HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 2 mM $MgCl_2$, 1 U SubTherm Taq DNA Polymerase (Fisher Molecular Biology, Trevose, PA, USA), and 25 pmol of each primer. To avoid inhibition due to coprecipitation of secondary metabolites with DNA, the PCR amplification was improved through the addition of 0.4% BSA and a nonionic detergent, Tween 20 0.5%.[32,33] Reaction was performed as follows: 94 °C for 5 min, 40 cycles of 94 °C for 40 s, 36 °C for 40 s, 72 °C for 2 min, followed by 1 cycle of 72 °C for 10 min (PTC-100, MJ Research Inc.). A total of 19 random primers (Operon Technologies OPA01, OPA03, OPA04, OPA05, OPA07, OPA09, OPA10, OPA11, OPA12, OPA16, OPA20, OPB02, OPB08, OPB10, OPB12, OPB13, OPB16, OPB20, and OPP10) were utilized for RAPD analysis. RAPD patterns were compared to select amplicons present in the contaminants and absent in *C. sativus* RAPD profiles. Most of the suitable marker bands were obtained with the primers OPA10, OPA11, OPB08, and OPB10 and are listed in Table 2.

Cloning and Sequencing of Putative RAPD Markers. The selected marker bands were excised from 2% agarose gels, purified using a JET-Sorb Gel Extraction Kit (Genomed, Löhne, Germany), and cloned in pGEM-T Easy Vector (Promega Corp., Madison, WI, USA). The transformed bacterial colonies were screened through colony PCR, and clones carrying correctly sized inserts were sequenced with M13 forward and M13 reverse primers.

Sequence Analysis. Sequencing of the positive clones was carried out following the protocol "CEQ 2000 dye terminator cycle sequencing" (Beckman Coulter, Fullerton, CA, USA), by means of the automatic sequencer CEQ 2000 (Beckman Coulter). Database searches of sequence homology were performed using the programs BlastN, BlastX, and PSI-BLAST set to standard parameters.[34,35] The sequences were deposited into GenBank (Table 2).

SCAR Primer Design and Optimization of PCR Conditions. The sequence data were used for the SCAR primer design and homology searches by the BLAST program. SCAR primers 20−22 nucleotides long were designed for stringent conditions of annealing temperature (about 60 °C) and, with the exception of $ScCv_{304}$ reverse primer, did not overlap the sequence of the original RAPD primer (Table 2). The annealing temperature was first calculated as 4-fold the number of GC (guanidine/cytosine) plus 2-fold the number of AT (adenine/thymine). Each primer pair was tested up to 2 °C over and under its annealing temperature to obtain the optimum. PCR reactions

Table 1. Number and Molecular Weight Range of Bands Generated as Products of PCR Amplification with Different Primers and DNA from *Crocus sativus* and Contaminant Species *Arnica montana*, *Bixa orellana*, *Calendula officinalis*, *Carthamus tinctorius*, *Crocus vernus*, *Curcuma longa*, and *Hemerocallis* sp.

		no. of bands									
primer	sequence (5′→3′)	*C. sativus* (fresh leaves)	*C. sativus* (dried stigmas)	*A. montana*	*B. orellana*	*C. officinalis*	*C. tinctorius*	*C. vernus*	*C. longa*	*Hemerocallis* sp.	size range (bp)
OPA03	AGTCAGCCAC	19	19	20	19	20	24	19	25	14	270−2740
OPA04	AATCGGGCTG	12	12	16	9	13	13	18	15	15	280−2390
OPA05	AGGGGTCTTC	8	8	11	2	9	4	12	9	11	290−>3000
OPA07	GAAACGGGTG	19	19	12	16	4	8	16	12	16	280−2970
OPA09	GGGTAACGCC	20	14	9	13	13	12	15	12	20	190−1930
OPA10	GTGATCGCAG	12	12	11	15	17	15	14	12	9	260−>3000
OPA11	CAATCGCCGT	13	10	2	16	6	3	17	13	16	250−2070
OPA12	TCGGCGATAG	11	6	4	7	1	13	10	6	17	240−2900
OPA20	GTTGCGATCC	10	9	6	11	4	11	13	5	10	260−1910
OPB08	GTCCACACGG	13	12	21	18	12	11	12	6	11	230−2950
OPB10	CTGCTGGGAC	8	8	19	12	11	16	14	15	9	190−1730
OPB12	CCTTGACGCA	14	14	4	8	5	4	10	11	11	230−2800
OPP10	TCCCGCCTAC	4	3	1	8	1	3	1	2	1	320−1530

were conducted on approximately 20 ng of DNA template, were performed in a 25 μL volume containing 67 mM Tris-HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 2 mM $MgCl_2$, 1 U SubTherm Taq DNA Polymerase (Fisher Molecular Biology, Trevose, PA, USA), 12.5 pmol specific primers, and were enhanced through the addition of 0.4% BSA and 0.5% Tween 20.[33] Unless not otherwise specified, amplification was performed as follows: 94 °C for 5 min, 35 cycles of 94 °C for 40 s, 55 °C for 40 s, 72 °C for 40 s, followed by 1 cycle of 72 °C for 10 min (PTC-100, MJ Research Inc.). An aliquot (10 μL) of the amplification product was resolved by electrophoresis on 1.5% agarose gel and detected by ethidium bromide staining.

Validation of the SCAR Markers. DNA samples of the seven contaminant species were analyzed with the specific primer pairs defined for each species and with fresh and dried *C. sativus* samples to exclude possible cross-reactions leading to misinterpretation of the results. Once the primer specificity was confirmed, SCAR analysis was conducted on genomic DNA extracted from mixtures of *C. sativus* dried stigmas containing 1, 2, and 5% of the contaminant species added as dried material (flowers for *A. montana*, *C. officinalis*, and *C. tinctorius*; tepals for *Hemerocallis* sp.; seeds for *B. orellana*; stigmas for *C. vernus*; and rhizomes for *C. longa*). The integrity and availability of DNA from dried commercial samples for SCAR analysis were checked by performing an amplification with a couple of primers, constructed on a RAPD amplicon (OPA11Cs_{791}) obtainable from the amplification with the primer OPA11 on *C. sativus* DNA. The band, nearly 790 bp long, was cloned and sequenced. The sequence was deposited into GenBank (accession number JQ952665). The SCAR primers ScCs_{263} forward 5′-AATAGCCTTGCATGAAGATCC-3′ and ScCs_{263} reverse 5′-CGATGGTTTTGCTTGAGTGG-3′, gave rise to an amplicon 263 bp long suitable as positive control for PCR reactions.

RESULTS AND DISCUSSION

The detection of commercial frauds in foods and spices is a challenging task, due to the fact that physical, chemical, and organoleptic properties are not always easily identifiable without investing substantial amounts of experimental time and/or recourse to costly technologies.[34] This happens in particular when the plant material is finely ground or when the spice is added to a seasoning as an ingredient mixed with others. Besides the obvious trade issues, deceitful adulteration of saffron may also represent a matter of concern when plant materials are evaluated for research purposes, because it may lead to misleading results from chemical, biological, and therapeutic standpoints. Undeclared constituents, in particular when commercial samples are evaluated, may in fact jeopardize the scientific evidence. As our goal was the development of a reliable method based on molecular genetic markers, we proceeded by the identification of RAPD markers discriminating *C. sativus* from each of its most common bulking agents and then strengthening the replicability by their transformation into SCAR markers. These markers were finally evaluated in artificial mixes of plant material at different concentrations to evaluate the sensitivity and accuracy of the method.

Development of SCAR Markers. We have previously analyzed 180 RAPD profiles obtained by amplification with 20 primers on DNA extracted from 2 samples of *C. sativus* (1 consisting of fresh leaves from corms and 1 from dried commercial sample in stigmas previously checked by microscopic examination) and 7 plant species indicated in the literature as among the most frequent adulterants found in commercial saffron samples, namely, *A. montana*, *B. orellana*, *C. officinalis*, *C. tinctorius*, *C. vernus*, *C. longa*, and *Hemerocallis* sp. Of the 20 primers tested, 13 produced clear and scorable amplification products for *C. sativus* (Table 1) and were thus taken into account to select discriminating bands in the RAPD profiles of each contaminant. The polymorphic bands to be cloned were selected on the basis of their reproducibility, the amount of DNA obtained in a single RAPD reaction, and the absence of other bands in the proximity to avoid contamination. Seven RAPD fragments specific for a contaminant species and absent in the RAPD profiles of the two *C. sativus* samples (OPA11Am_{748}, OPB10Bo_{483}, OPB10Co_{590}, OPB08Ct_{780}, OPA10Cv_{397}, OPB10Cl_{412}, and OPB10Hsp_{392} specific for *A. montana*, *B. orellana*, *C. officinalis*, *C. tinctorius*, *C. vernus*, *C. longa*, and *Hemerocallis* sp., respectively, and obtained from amplification with the primers OPA10, OPA11, OPB08, and OPB10, which provided the most suitable bands) were selected (Figure 1). RAPD amplicons were cloned and sequenced, and their sequences were deposited into GenBank (Table 2). A further RAPD amplicon (OPA11Cs_{791}) present in *C. sativus* and absent in all contaminant profiles was also selected to be used as positive control in the analysis of commercial samples. The BLAST of the nucleotide sequences did not have homology with any coding sequences of structural genes. These discriminating RAPD bands were subsequently converted into SCAR markers. To increase the amplification specificity, the SCAR primers were designed internally to the

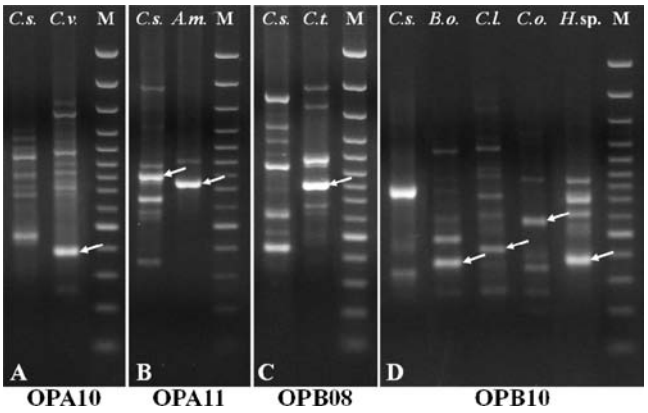

Figure 1. RAPD analyses of genomic DNA from *C. sativus* and the contaminant species *A. montana*, *B. orellana*, *C. officinalis*, *C. tinctorius*, *C. vernus*, *C. longa*, and *Hemerocallis* sp.: (A) amplification profiles obtained with the primer OPA10; (B) amplification profiles obtained with the primer OPA11; (C) amplification profiles obtained with the primer OPB08; (D) amplification profiles obtained with the primer OPB10. *C.s.*, DNA from dried stigmas of *C. sativus*; M, 100 bp DNA ladder; *C.v.*, DNA from fresh leaves of *C. vernus*; *A.m.*, DNA from dried flowers of *A. montana*; *C.t.*, DNA from dried flowers of *C. tinctorius*; *B.o.*, DNA from dried seeds of *B. orellana*; *C.l.*, DNA from dried rhizomes of *C. longa*; *C.o.*, DNA from dried flowers of *C. officinalis*; *H.*sp., DNA from dried tepals of *Hemerocallis* sp. White arrows indicate the positions of species-specific RAPD amplicons selected to be converted to SCAR markers.

sequence of the original amplicons and did not contain the sequences of the original RAPD primers, with the exception of ScCv_{304} reverse primer, thus giving rise to amplification products shorter than the respective RAPD markers. The relatively short dimension of the amplification targets offers a further advantage, these SCAR markers being more suitable for the analysis also when DNA is partially degraded. This event is extremely likely to occur in dried plant material stored at room temperature often for a rather long time, a fact that represents routine practice in the spice market. The primer sequences, their annealing temperature, and the dimensions of the RAPD and SCAR amplicons are listed in Table 2.

Validation of Selected Markers. SCAR marker specificity was validated by testing each primer pair in amplification on DNA from fresh and dried *C. sativus*, from the contaminant species for which each specific primer pair was defined, and from the other six contaminant species. None of the tested primer pairs generated an amplification product when applied to *C. sativus* or gave cross-reaction when tested on the DNA of the other contaminants (data not shown). The SCAR marker ScCs_{263} selected as positive control for subsequent analyses on commercial samples was further validated on DNA extracted from *C. vernus* and *C. sativus* (leaves) and from six saffron commercial dried stigma samples from different geographical origins, previously checked by microscopy observation. The ScCs_{263} marker identified *C. sativus* in each sample in which it was present and gave rise to a clearly distinguishable amplicon, because it was nearly 100 bp longer in *C. vernus* (Figure 2A, lane *C.v.*). This SCAR marker represents a useful tool for routine analyses of commercial saffron powdered samples, allowing the evaluation of the accuracy of the whole procedure of DNA extraction and downstream assays. It may also act as a positive control by preventing the misinterpretation of false negatives due to bad-quality DNA. The weaker amplification obtained with the sample in lane 1 (Figure 2A) was probably due to the longer aging of this sample, which presumably caused greater DNA degradation. The marker ScCs_{263} could also offer the advantage to identify both *C. sativus* and *C. vernus* with the same reaction. However, when the presence of *C. vernus* is low enough, the amplification of the longer amplicon can be easily overwhelmed by the amplification of the 263 bp band of *C. sativus*, masking a potential adulteration. Thus, because the main form of adulteration in samples commercialized as whole stigmas comes from the addiction of dried stigmas from other *Crocus* species, the same samples were tested with the ScCv_{304} marker designed on a RAPD amplicon specific for *C. vernus*. This marker amplified with DNA of *C. vernus* (Figure 2B, lane *C.v.*) but not with the saffron stigma samples, confirming that they were not contaminated by the addition of *C. vernus* stigmas. Once the purity of the stigma

Table 2. Species-Specific SCAR Markers and Primer Specifications

species	orginal RAPD amplicon	GenBank accession no.	SCAR marker	SCAR primer sequence (5′→3′)		annealing temperature (°C) calculated	annealing temperature (°C) working	amplicon length (bp)
Crocus sativus	OPA11Cs_{791}	JQ952665	ScCs_{263}	forward	AATAGCCTTGCATGAAGATCC	60	60	263
				reverse	CGATGGTTTTGCTTGAGTGG	60		
Arnica montana	OPA11Am_{748}	JX239751	ScAm_{190}	forward	GATTAGCAGCAGCCATCTCG	62	60	190
				reverse	GATGAAGAAACGGGCACTCC	62		
Bixa orellana	OPB10Bo_{483}	JQ952666	ScBo_{267}	forward	ACTTTTCAAAGCCGACACGC	60	60	267
				reverse	ATCTGGACAATAGCTTTAACGC	62		
Calendula officinalis	OPB10Co_{590}	JX239749	ScCo_{390}	forward	TCGACGTTGATTCTTGGACC	60	60	390
				reverse	GACGATACAGATCGAAGAGG	60		
Carthamus tinctorius	OPB08Ct_{780}	JQ952667	ScCt_{131}	forward	ACAACCATTGGAGATTCCGG	60	60	131
				reverse	AGTGAGCACTCTTAGTTAACC	60		
Crocus vernus	OPA10Cv_{397}	JQ952668	ScCv_{304}	forward	AAATTCATCAAACCCGTGCC	58	60	304
				reverse	CAGCTGAAGAAGAGTTACCC	60		
Curcuma longa	OPB10Cl_{412}	JQ952669	ScCl_{289}	forward	AACTTGAAGTGGGAGCTAGC	60	58	289
				reverse	CATCTGGTAAAGTCTCCTCC	60		
Hemerocallis sp.	OPB10Hsp_{392}	JX239750	ScHsp_{354}	forward	GACAAGGGCTAAAATCACTTG	60	60	354
				reverse	AGACTTTGTGCAGGTTCACC	60		

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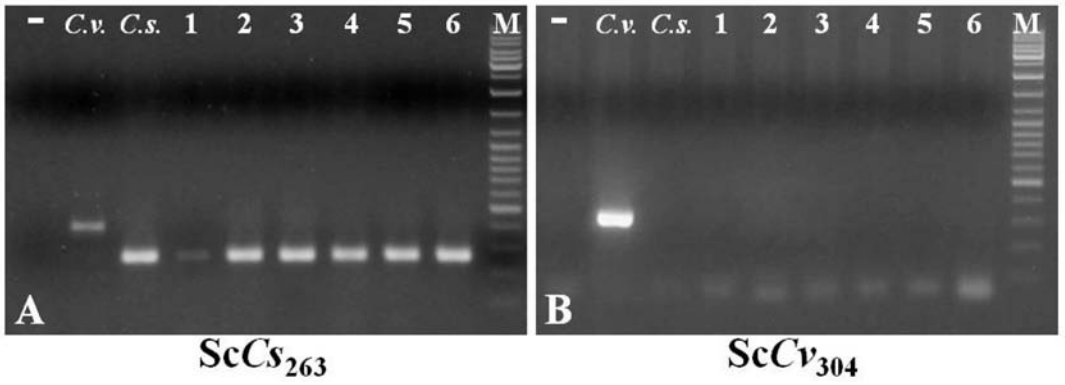

Figure 2. ScCs_{263} and ScCv_{304} SCAR marker validation on saffron commercial dried stigma samples from different geographical origins: (A) PCR performed with ScCs_{263} primer pair specific for *Crocus sativus* to validate the identity of samples and to certify PCR availability of DNA; (B) reactions performed with ScCv_{304} primer pair specific for *Crocus vernus*. Each commercial sample was authenticated by pharmacognostic examination. −, negative control, amplification with no template DNA; *C.s.*, DNA from fresh leaves of *C. sativus*; *C.v.*, DNA from fresh leaves of *C. vernus*; 1−6, DNA from saffron commercial dried stigma samples from different geographical proveniences; M, 100 bp DNA ladder.

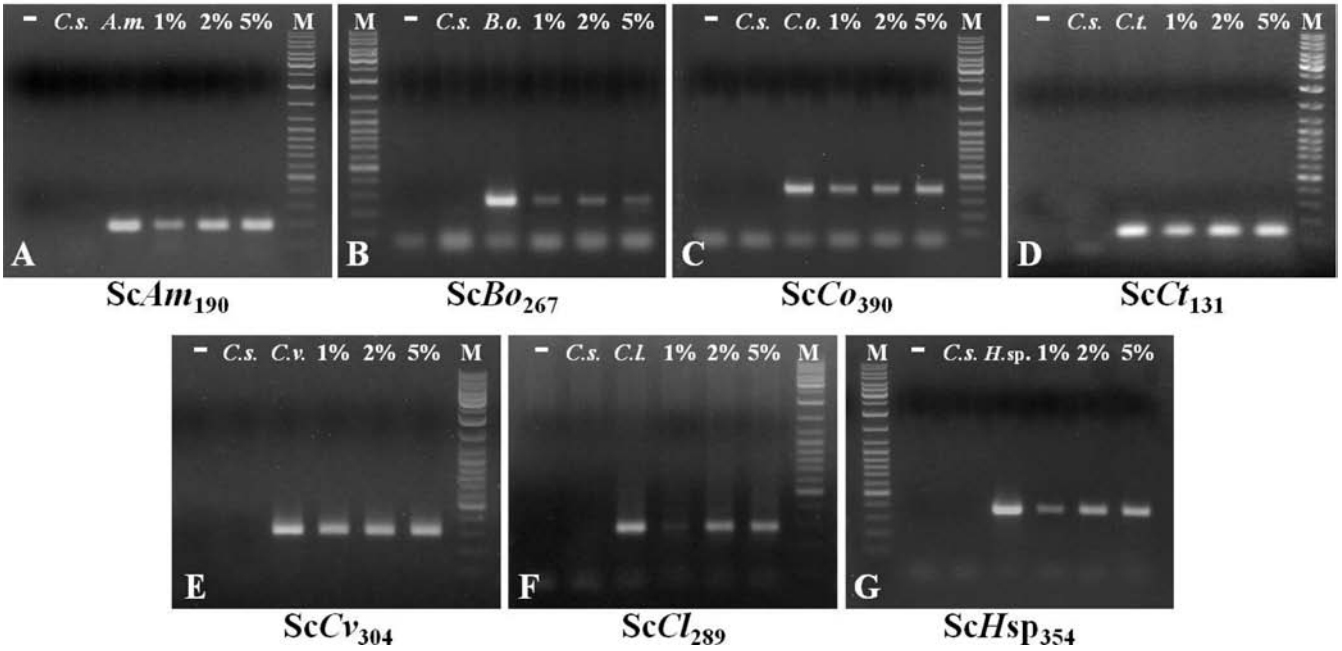

Figure 3. SCAR marker sensitivity assay: (A) PCR performed with ScAm_{190} primer pair specific for *Arnica montana* (1, 2, and 5%, DNA from mixtures of *C. sativus* and *A. montana*); (B) PCR performed with ScBo_{267} primer pair specific for *Bixa orellana* (1, 2, and 5%, DNA from mixtures of *C. sativus* and *B. orellana*); (C) PCR performed with ScCo_{390} primer pair specific for *Calendula officinalis* (1, 2, and 5%, DNA from mixtures of *C. sativus* and *C. officinalis*); (D) PCR performed with ScCt_{131} primer pair specific for *Carthamus tinctorius* (1, 2, and 5%, DNA from mixtures of *C. sativus* and *C. tinctorius*); (E) PCR performed with ScCv_{304} primer pair specific for *Crocus vernus* (1, 2, and 5%, DNA from mixtures of *C. sativus* and *C. vernus*);; (F) PCR performed with ScCl_{289} primer pair specific for *Curcuma longa*; (G) PCR performed with ScHsp_{354} primer pair specific for *Hemerocallis* sp. (1, 2, and 5%, DNA from mixtures of *C. sativus* and *Hemerocallis* sp.). −, negative control, amplification with no template DNA; *C.s.*, DNA from dried stigmas of *C. sativus*, as further negative control; *A.m.*, DNA from dried flowers of *A. montana*, as positive control; *B.o.*, DNA from dried seeds of *B. orellana*; *C.o.*, DNA from dried flowers of *C. officinalis*; *C.t.*, DNA from dried flowers of *C. tinctorius*; *C.v.*, DNA from dried stigmas of *C. vernus*; *C.l.*, DNA from dried rhizomes of *C. longa* (1, 2, and 5%, DNA from mixtures of *C. sativus* and *C. longa*; *H.*sp., DNA from dried tepals of *Hemerocallis* sp.; M, 100 bp DNA ladder.

samples was established both by microscopy and PCR analyses, sample 3 in Figure 2 (originating from Morocco) was utilized in the preparation of the artificial mixtures for the sensitivity assay. *C. sativus* is a triploid sterile plant that is vegetatively propagated by means of bulbs (or corms). Its intraspecific genetic variability has been proved to be limited worldwide, and thus it represents an ideal candidate for quality control based on molecular markers, as the differences do not represent a constraint in terms of method development and analysis of the results.[36,37] As further proof, the RAPD and SCAR primers developed in our work were tested for uniform response in authentic saffron samples from some of the most frequent commercial sources, namely, Spain, Iran, Greece, Italy, Morocco, and Afghanistan, providing uniform responses.

Sensitivity Assay. After marker specificity had been verified, the method sensitivity was validated to define its limit of detection for each adulterant. To create conditions similar to those of commercial samples, artificial counterfeit

mixtures were prepared by mixing saffron stigmas with the parts of each contaminant species usually utilized as bulking agents, namely, flowers for *A. montana*, *C. officinalis*, and *C. tinctorius*, tepals for *Hemerocallis* sp., stigmas for *C. vernus*, seeds for *B. orellana*, and rhizomes for *C. longa*. Figure 3 reports the results obtained with DNA extracted from artificial mixtures containing *C. sativus* (from Morocco) and 1, 2, or 5% of *A. montana* (panel A), *B. orellana* (panel B), *C. officinalis* (panel C), *C. tinctorius* (panel D), *C. vernus* (panel E), *C. longa* (panel F), and *Hemerocallis* sp. (panel G) subjected to amplification with the primer pairs for Sc*Am*$_{190}$, Sc*Bo*$_{267}$, Sc*Co*$_{390}$, Sc*Ct*$_{131}$, Sc*Cv*$_{304}$, Sc*Cl*$_{289}$, and Sc*H*sp$_{354}$, respectively. The selected SCAR markers allowed an easy detection up to 1% of each contaminant, as shown by the amplification of an individual specific band absent in the lanes of *C. sativus* (Figure 3, lanes *C.s.*) and present in the reactions both with the individual contaminant species (panels A–F, lanes *A.m.*, *B.o.*, *C.o.*, *C.t.*, *C.v.*, *C.l.*, and *H.*sp.) and with its mixtures with *C. sativus* (panels A–F, lanes 1%, 2%, and 5%).

A method based on SCAR markers was developed from RAPD specific for seven common bulking agents used as saffron adulterants (*A. montana*, *B. orellana*, *C. officinalis*, *C. tinctorius*, *C. vernus*, *C. longa*, and *Hemerocallis* sp.) to authenticate their presence and set up a fast, sensitive, reliable, and low-cost screening of dried commercial saffron samples. The method enabled the unequivocal detection of low amounts (up to 1%) of both artificial and commercial batches, compliant with ISO requirements that the official protocols cannot detect without having recourse to burdensome and expensive analyses. The method meets the needs of the present market and may be used to screen large saffron batches before their grading, preemptively excluding those counterfeited with bulking agents that may otherwise go undetected. The availability of genetic sequences could also ease the development of technological devices such as gene chip or specific kits to be enforced in routine control, as their response is not affected by phenotype, environment, and process or storage variability.

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Notes

The authors declare no competing financial interest.

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