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RAPD-Based Method for the Quality Control of Mediterranean Oregano and Its Contribution to Pharmacognostic Techniques

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A pharmacognostic survey of 84 commercial samples of Mediterranean oregano, obtained from wholesale traders between 2001 and 2007, pinpointed the presence of extraneous plant material in 90.5% of the samples. In 59% of them extraneous material of plant origin was above 20%. Two major groups of botanical foreign matter were identified: oregano-like flavored plants (*Satureja montana* L., *Origanum majorana* L.) and plants lacking a clearly detectable essential oil profile (*Rubus* sp., *Cistus incanus* L., *Rhus coriaria* L.), added as bulk extraneous material. A random amplified polymorphic DNA (RAPD) method was developed to make the detection of the second group of adulterants easier and speed pharmacognostic analysis of large batches of samples. Thirteen primers discriminating between *Origanum* spp. and *Rubus caesius*, *R.coriaria*, and *C. incanus* were individuated, allowing their detection in oregano samples with a limit of detection of 1%. The utilization of RAPD as a reliable test to probe the authenticity of Mediterranean oregano or previously screen the presence of specific contaminants is proposed as a complementary approach to pharmacognostic and phytochemical screening.

KEYWORDS: DNA molecular markers; RAPD; *Origanum* sp.; spice adulteration; *Cistus* sp.; *Rubus* sp.; *Rhus* sp.

INTRODUCTION

The worldwide spice market was worth U.S. $2.97 billion, and a corresponding 1.55 billion metric tons was globally exported in 2004, outlining a steady upward trend (*1*). However, authenticity and quality control may represent a critical issue, as sophistications are frequently discovered in both ground and dried spices, giving rise to issues related to the spice industry's reputation and credibility (*2*−*6*). In the specific case of oregano the scenario is complicated by the large heterogeneity of the *Origanum* genus and by the denomination of different botanical genera under a single generic name, namely *Origanum* (Lamiaceae) in the Mediterranean area and *Lippia* (Verbenaceae) in Mexico, a fact that has led to market distinction between Mediterranean oregano and Mexican oregano (*7*). Different standards are in use to define the oregano quality, and in order to regulate and standardize the phytopharmaceutical market of oregano, the European Pharmacopoeia (PhEur) narrowed the number of species that can be marketed as true oregano to two: *Origanum vulgare* L. subsp. *hirtum* (Link) Ietsw. and *Origanum onites* L. Within the food market, specifications internationally approved by the American Spice Trade Association (ASTA) and European Spice Association (ESA) define quality minima for spices such as oregano and are limited to the amount and phytochemical profile of the essential oil. Further authentication analyses required are not discriminative (weight by weight, acid-insoluble ash) or extremely time-consuming when the samples to be screened are large, as in case of traditional pharmacognostic analysis. Besides the adulteration with species belonging to the same *Origanum* genera and/or with similar essential oil profiles [*Origanum majorana* L., *Origanum syriacum* L., *O. vulgare* L. subsp. *virens* (Hoffmanns & Link) Ietsw., *Satureja montana* L., *Thymus capitatus* L.], Mediterranean oregano has recently also been adulterated with plants having similar silvery gray color and size of leaves, as in the case of *Rhus coriaria* L. and *Cistus* spp. (*3*, *8*). These plants are added as bulk, cheap material concealed to illicitly increase volume and, subsequently, producers' or traders' income. Whereas essential oil bearing plants can be detected by routine GC-MS and other chromatographic or spectroscopic techniques (*9*−*13*), the detection of nonaromatic contaminants relies almost completely on manual

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pharmacognostic assays; screening methods allowing fast detection of potentially adulterated samples are not available. When multiple samples have to be evaluated, pharmacognostic probing has significant constraints in terms of time, efficacy, and cost, due to the large manpower needed. Recently, the cheaper availability of biomolecular assays made the employment of DNA-related techniques affordable in a large number of food-related applications (*14*−*17*). The recourse to DNA molecular markers in the specific control of herbal drugs and spices is gaining popularity, evidencing a good deal of interest in the application of molecular techniques for the authentication and detection of sophistication in commercial plant material (*18*−*25*). RAPD-PCR (*26*, *27*) can be considered a useful starting point with regard to its low operating cost and good reliability of RAPD markers to distinguish between different botanical species. Moreover, RAPD-PCR is a fast assay in which no sophisticated technology and no previous sequence informations are needed; it also allows good performance levels when the DNA to be probed is in low quantities, as is very common with dried commercial spice samples.

This paper reports a survey of the wholesale market of dried Mediterranean oregano imported in Europe from different Mediterranean countries. Eighty-four samples collected between 2001 and 2007 were probed by means of microscopic pharmacognostic methods. Subsequently, a method based on RAPD-PCR was set up to evaluate its effectiveness as a complementary, fast, and reliable assay to probe the presence of specific contaminants and speed pharmacognostic analysis of large batches of samples.

MATERIALS AND METHODS

Plant Material. A total of 84 dried Mediterranean oregano samples were purchased in uniform 250 g batches between 2001 and 2007 from international wholesale traders exporting to the European Union. Once acquired, and in order to simulate real commercial conditions, samples were stored in dry and dark conditions at 5 °C until macroscopic, microscopic, and molecular analyses were performed. Fresh oregano samples necessary to set up the RAPD-PCR method were obtained from seeds of different *Origanum* species and cultivars [*O. onites* L., *O. vulgare* L., *O. vulgare* L. subsp. *hirtum* (Link) Ietsw., *O. vulgare* L. var. *aureum*, *O. heracleoticum* L., *O. vulgare* L. subsp. *virens* (Hoffmanns & Link) Ietsw., *O. majorana* L.] and cultured on silty soil in a greenhouse (Botanical Garden of the University of Parma, Italy). Seeds were kindly provided by Giardino delle Erbe Officinali di Casola Valsenio (Ravenna, Italy), NPGS (Ames, IA), and IPK (Gatersleben, Germany). Plant material for contaminant species was collected in the field (*Rubus caesius* L.) or supplied by the Botanical Gardens of Ferrara (*Cistus incanus* L.) and Palermo (*Rhus coriaria* L.). Fresh plant material was collected and 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.

Pharmacognostic Identification. From each Mediterranean oregano sample three equal parts were obtained as described in WHO guidelines (*28*). Twenty milligrams from each part was carefully weighed and observed by means of a microscope and a stereomicroscope (Stemi SV 11, Zeiss, Germany). Quali-quantitative determinations were made by direct recognition and manual isolation of morphological traits of each species by discrimination of flower, bract, leaf, and stem portions. Recognition of Mediterranean oregano was made according to PhEur specifications (*29*), whereas *C. incanus*, *R. coriaria*, and *Rubus* sp. were identified by detection of specific pharmacognostic markers and comparison with specific pure drug samples. Fragments from each botanical species were separately pooled and weighed.

DNA Extraction. Genomic plant DNA was isolated from fresh and dried material through a protocol modified after that of Murray and Thompson (*30*). Milder extraction conditions were preferred, and for each sample 100 mg of powdered material was incubated overnight at room temperature, under horizontal shaking, in 6 mL of buffer containing 50 mM Tris-HCl (pH 8), 10 mM EDTA, (pH 8), 0.7 M NaCl, 2% w/v CTAB, 2% w/v PVP, 0.3% w/v activated charcoal, and 2% v/v β-mercaptoethanol added before using. The high β-mercaptoethanol concentration (*31*) and the combination of activated charcoal and PVP (*32*) were developed to prevent the oxidation of polyphenols and the solubilization of polysaccharides in the DNA extracts. Samples were then repeatedly extracted with an equal volume of chloroform/isoamyl alcohol (24:1, v/v), until the aqueous phase was clear. The DNA was then precipitated with 2-propanol and dissolved in 300 μL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) supplemented with 5 μg of RNase, DNase free, and incubated for 45 min at 37 °C. After RNase digestion, CTAB and NaCl were added at final concentrations of 1% w/v and 0.7 M, respectively (*33*), and the samples were incubated for 15 min at 65 °C to inactivate RNase and remove carbohydrates. A further extraction with an equal volume of chloroform/isoamyl alcohol 24:1 was performed. The DNA was then ethanol precipitated and dissolved in sterile distilled water. DNA quality and concentrations were evaluated by both spectrophotometric analysis and visual comparison with DNA standards in ethidium bromide-stained agarose/TAE gels and adjusted to approximately 10 ng/μL. Agarose gels were analyzed and quantified with a Kodak DC40 camera (Kodak) using the Kodak digital science 1D Image analysis software (Eastman Kodak Co., Rochester NY).

DNA Purification. DNA from dried samples was partially degraded, and gel elution was required to collect the unimpaired molecular fraction. Gel elution was carried out with a NucleoSpin Extract II Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions. DNA was eluted in 150 μL of sterile distilled water, pH 8.5, and ethanol precipitated.

RAPD-PCR. RAPD reaction was performed in a 25 μL volume containing 75 mM Tris-HCl (pH 9.0), 2 mM $MgCl_2$, 50 mM KCl, 20 mM $(NH_4)_2SO_4$, 1 unit of Taq DNA polymerase (Biotools B&M Laboratories, S.A., Madrid, Spain), and 25 pmol of arbitrary decamer primer. In this work, 20 RAPD primers were used (see **Table 2**). After preliminary experiments with different amounts of DNA, the reaction was standardized using 10 ng of DNA from a single species. Amplification was performed as follows: 94 °C for 5 min, 40 cycles of 92 °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 Reasearch Inc.). An aliquot (10 μL) of the amplification product was resolved by electrophoresis on 2% agarose gel and detected by ethidium bromide staining. RAPD analysis with discriminating primers was conducted on DNA of *Origanum* mixed with 1, 2, and 5% DNA extracted from contaminants, and then it was extended to genomic DNA extracted from mixtures of *Origanum* containing a similar percentage of the contaminant species (prepared by both fresh and dried material) and to DNA extracted from commercial samples. The PCR reactions from mixtures was conducted using 20 ng of DNA template.

RESULTS AND DISCUSSION

The pharmacognostic survey of 84 Mediterranean oregano commercial samples conducted in accordance with PhEur specifications allowed the detection of various amounts of botanical species different from *O. vulgare* and *O. onites* and the quantification of plant material other than flowers and leaves (**Table 1**). Less desirable *O. onites* and *O. vulgare* parts such as stems were present above 2% in almost all samples, and their amount varied from 1.5 to 12% of total weight (average abundance = 5.3%). Only 9.5% of the examined samples were strictly compliant with PhEur in terms of botanical authenticity, whereas 27.4% were in the 95−100% range of purity and 59% were below 80% purity. The samples evaluated contained an overall average of 66.8% of authentic Mediterranean oregano, and a considerable 72.6% of them repeatedly contained an amount of contaminants far above the 2% allowed and liable to rejection by European control authorities. The majority of the samples contained more than one contaminant, and the most common contaminants detected were *O. majorana* in 41

Table 1. Authenticity of Mediterranean Oregano Samples ($n = 84$) and Distribution of Contaminants Detected by Microscopic Pharmacognostic Assay

purity[a] (w/w %)	plant identified	no. of samples	min—max (w/w %)
>98[b]	Mediterranean oregano	8 (9.52%)[c]	
>95	Mediterranean oregano	23 (27.38%)	
contaminants			
group I[d] (88.1%)[c]	*Satureja montana*	65 (77.38%)	1.2−76.9
	Origanum majorana	41 (48.81%)	1.3−95.2
	other Lamiaceae	10 (11.90%)	1.7−13.1
group II[e] (16.7%)	*Cistus incanus*	4 (4.76%)	8.9−52.1
	Rhus coriaria	7 (8.33%)	1.0−83.0
	Rubus sp.	4 (4.76%)	1.0−57.8

[a] Most samples contained more than one contaminant; values were referred to every single species detected. [b] In accordance with PhEur specifications. [c] In parentheses: sample percentage. [d] Oregano-like flavored plants (Lamiaceae.) [e] Plants lacking a clearly detectable essential oil profile.

samples, with amounts ranging from 1.3 to 95.2% of the total weight (average = 10.5%); *S. montana*, in 65 samples, ranging from 1.2 to 76.9% (average = 17.5%); and nonidentified Lamiaceae in 10 samples, ranging from 1.7 to 13.1% (average = 8.1%). *C. incanus* was detected in 4 samples, ranging from 8.9 to 52.1% (average = 30.9%); *Rhus coriaria* in 7 samples, ranging from 1.0 to 83.0% (average = 18.7%); and *Rubus* sp. in 4 samples, ranging from 1.0 to 57.8% (average = 30.45%). The presence of *Rubus* sp. leaves as contaminants in Mediterranean oregano had not been reported before (*4*, *9*). Unknown plant material above 2% was detected in 21% of the samples (average = 9.4%). Nonvegetal foreign matter was absent in all of the samples evaluated.

Two major groups of adulterants were identified: (I) essential oil bearing plants with oregano-like flavor, mostly belonging to the Lamiaceae family (*S. montana*, *O. majorana*) (88.1% of the total) and (II) plants lacking a clearly detectable essential oil profile, added as bulk extraneous material (*Rubus* sp., *C. incanus*, *R. coriaria*) (16.7% of the total). Those plants seem to be added only when the essential oil content of pure oregano batches is adequately high to fall within regulatory standards (i.e., 25 mL/kg) even after weight increase of the drug with foreign plant matter. The above-mentioned plants do not seem to constitute a significant risk for the consumers. However, Mediteranean oregano batches in which they are concealed could be subject to regulatory actions that may weigh on manufacturers, should a regulatory agency determine that sanctions are warranted. Their presence being a potential issue for the spice industry, and given the need for reliable methods that could support and speed pharmacognostic analysis, we have developed an RAPD-based protocol, enforceable in the screening of large numbers of samples, for the fast detection of *Rubus* sp., *C. incanus*, and *R. coriaria* in stocks of Mediteranean oregano.

PCR amplification of DNA from species rich in essential oil and secondary polyphenolic metabolites, such as *Origanum*, is strongly impaired when oxidized polyphenols bind the nucleic acids, making DNA unavailable for subsequent enzymatic reaction. Moreover, in the comparison of DNA from different species, a unitary protocol of extraction should be chosen and adjusted to the requirements of the most difficult species to extract. These constraints determined the need for the development of a DNA extraction protocol suitable for each species involved in the analysis and applicable to both fresh and dried material. DNA samples of suitable quality extracted from *Origanum* species and *R. coriaria*, *Rubus* sp., and *C. incanus*

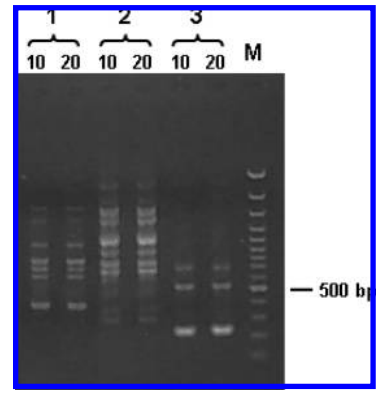

Figure 1. RAPD amplification patterns generated by the primer OPB02 with 10 and 20 ng of DNA template. Lanes: 1, *Origanum vulgare*; 2, *Cistus incanus*; 3, *Rubus caesius*; M, 100 bp DNA ladder.

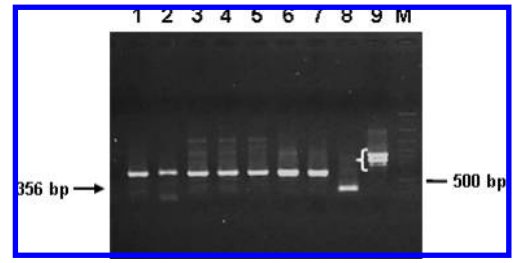

Figure 2. RAPD amplification patterns obtained with the primer OPA07. Lanes: 1, *O. vulgare*; 2, *O. vulgare* var. *aureum*; 3, *O. vulgare* subsp. *hirtum*; 4, *O. vulgare* subsp. *virens*; 5, *O. heracleoticum*; 6, *O. onites*; 7, *O. majorana*; 8, *Cistus incanus*; 9, *Rubus caesius*; M, 100 bp DNA ladder. Black arrow indicates the position of the specific band of 356 bp obtained from *Cistus incanus* (lane 8); white bracket indicates the three specific amplicons of 1142, 980, and 842 bp obtained from *Rubus caesius* (lane 9).

were obtained both by the contemporary use of an elevated concentration of β-mercaptoethanol, PVP, and activated charcoal, which prevented polyphenol oxidation, and by repeated CTAB/NaCl treatment, for carbohydrate removal. These buffer implementations produced samples that allowed subsequent PCR amplification and were particularly useful for the DNA extraction from *Cistus*, which was otherwise both hardly quantifiable and not amplifiable at all. Preliminary reactions were conducted with both 10 and 20 ng of DNA template, which gave rise to a good reproducibility of the amplicon patterns (**Figure 1**)., Subsequent reactions were thus standardized using 10 ng of DNA template. RAPD amplification products were scored irrespective of the intensity. To individuate bands specifically produced by the contaminant plants belonging to group II and absent in the genus *Origanum*, RAPD profiles obtained from *Rubus caesius*, *C. incanus*, and *R. coriaria* were compared with the profiles obtained from seven *Origanum* taxa, namely *O. onites* L., *O. vulgare* L. subsp. *hirtum* (Link) Ietsw. and its putative synonymous *O. heracleoticum* L. and *O. vulgare* L., *O. vulgare* L. subsp. *virens* (Hoffmanns & Link) Ietsw., *O. vulgare* L. var. *aureum*, and *O. majorana* L. (considered as possible contaminants). Twenty random primers were used to perform RAPD analysis, and 13 of them produced one or more amplicons, which were at the same time absent in all of the considered *Origanum* taxa and specific for at least one contaminant plant (**Table 2**). To achieve an easier identification of contaminants, primers giving a low heteromorphic profile for *Origanum* were preferred. Moreover, because bands of high molecular size are quite difficult to amplify from dried and potentially not completely integer material, primers giving rise

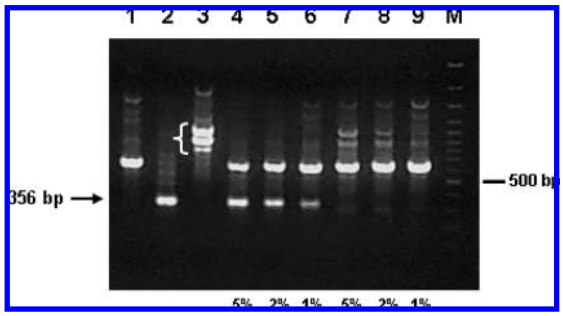

Figure 3. RAPD analysis of individual species and mixture of genomic DNA of *O. vulgare* subsp. *hirtum* with genomic DNA of *Cistus incanus* and *Rubus caesius*. PCR reactions were performed with the primer OPA07. Genomic DNA of each contaminant species was mixed in three different percentages (shown at the bottom of the figure) with *Origanum* DNA. Lanes: 1, *O. vulgare* subsp. *hirtum*; 2, *Cistus incanus*; 3, *Rubus caesius*; 4−6, mixtures of *Origanum* and *Cistus incanus*; 7−9, mixtures of *Origanum* and *Rubus caesius*; M, 100 bp DNA ladder. Black arrow indicates the position of the single specific band (356 bp) amplified from *Cistus incanus* (lanes 2, 4, 5, 6); white bracket indicates the position of the three specific amplicons (1142, 980, and 842 bp) generated from *Rubus caesius* (lanes 3, 7, 8, 9).

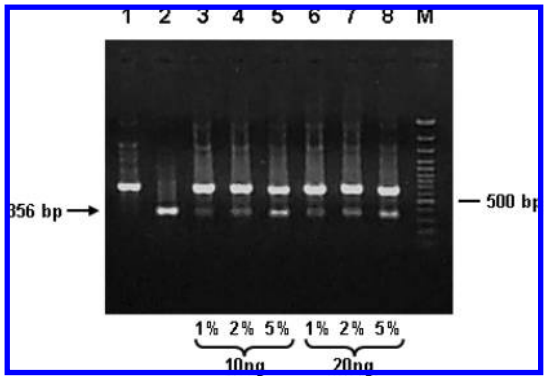

Figure 4. RAPD analysis of individual fresh leaves and mixtures of *O. vulgare* subsp. *hirtum* and the contaminant species *Cistus incanus*. Fresh leaves of the contaminant species were mixed in three different percentages (shown at the bottom of the figure) with fresh leaves of *Origanum*, before genomic DNA was extracted. PCR reactions were performed with the primer OPA07 and were conducted with both 10 ng (lanes 3−5) and 20 ng (lanes 6−8) of DNA template. Lanes: 1, *O. vulgare* subsp. *hirtum*; 2, *Cistus incanus*; 3−8, mixtures of *Origanum* and the contaminant species; M, 100 bp DNA ladder. Black arrow indicates the position of the single specific band (356 bp) amplified from *Cistus incanus* (lanes 2−8).

to most obvious discriminating bands of relative low dimension were chosen. As an example, in **Figure 2** are shown the RAPD profiles obtained with OPA07, which gave a monomorphic band of high intensity for each one of the *Origanum* species (lanes 1−7), a single intense band of 356 bp for *C. incanus* (lane 8), and three amplicons of elevated molecular weight (1142, 980, 842 bp) for *R. caesius* (lane 9). For the reasons stated above, the primer OPA07 was thus chosen to distinguish *C. incanus* but not *R. caesius*, for which the primer OPA20 was preferred, with particular regard to the shorter amplicon (317 bp) of the three specific bands produced by this primer in *Rubus*. The primer OPA11, which gave rise to a 286 bp specific band, was utilized for the detection of *R. coriaria*.

The reliability and the limits of detection of the chosen RAPD markers were tested on DNA extracted independently from fresh

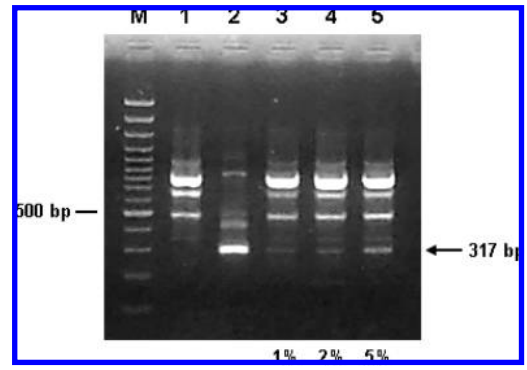

Figure 5. RAPD analysis of individual fresh leaves and mixtures of *O. vulgare* subsp. *hirtum* and the contaminant species *Rubus caesius*. Fresh leaves of the contaminant species were mixed in three different percentages (shown at the bottom of the figure) with fresh leaves of *Origanum*, before extraction of genomic DNA. PCR reactions were performed with the primer OPA20. Lanes: 1, *O. vulgare* subsp. *hirtum*; 2, *Rubus caesius*; 3−5, mixtures of *Origanum* and the contaminant species; M, 100 bp DNA ladder. Black arrow indicates the position of the specific band (317 bp) amplified from *Rubus caesius* (lanes 2−5).

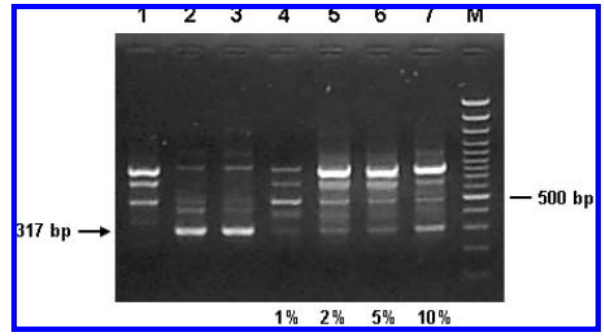

Figure 6. RAPD analysis of individual dried leaves and mixtures of *O. vulgare* subsp. *hirtum* and the contaminant species *Rubus caesius*. Dried leaves of the contaminant species were mixed in four different percentages (shown at the bottom of the figure) with dried leaves of *Origanum*, before extraction of genomic DNA. PCR reactions were performed with the primer OPA20. DNA from fresh leaves of *Rubus caesius* were used as further control (lane 2). Lanes: 1, *O. vulgare* subsp. *hirtum*; 2, *Rubus caesius* fresh leaves; 3, *Rubus caesius* dried leaves; 4−7, mixtures of *Origanum* and the contaminant species; M, 100 bp DNA ladder. Black arrow indicates the position of the shorter specific band (317 bp) amplified from *Rubus caesius* (lanes 2−7).

leaves of *O. vulgare* subsp. *hirtum* and from each contaminant species and subsequently mixed to obtain percentages of 1, 2, and 5% of a single contaminant DNA. As shown in **Figure 3** contaminations are clearly identifiable by the presence of the specific amplicons even when the contaminant DNA is present at the lowest percentage (lane 6, 1% *C. incanus*; lane 9, 1% *R. caesius*). The DNA extraction was subsequently performed on mixtures prepared from fresh material. Fresh leaves of the contaminant species were mixed in three different percentages (1, 2, and 5%) with fresh leaves of *Origanum*, before the extraction of genomic DNA. In **Figure 4** the results are shown of PCR reactions performed with either 10 ng (lanes 3−5) or 20 ng (lanes 6−9) of DNA extracted from mixtures of fresh *Origanum* and *Cistus* leaves. Because a better detection of the lowest percentage of the contaminant species was achieved by using 20 ng of DNA template, subsequent analyses were all carried out with 20 ng of DNA template. **Figure 5** shows the results obtained from PCR reaction with 20 ng of DNA extracted

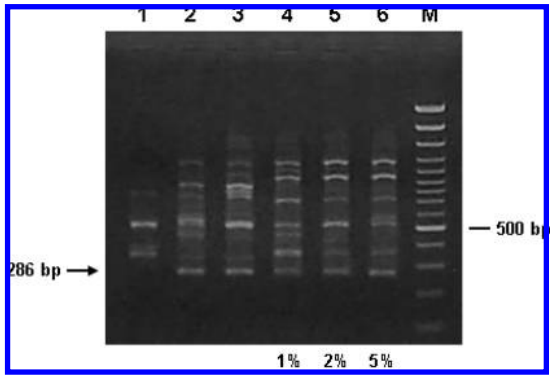

Figure 7. RAPD analysis of individual dried leaves and mixtures of *O. vulgare* subsp. *hirtum* and the contaminant species *Rhus coriaria*. Dried leaves of the contaminant species were mixed in three different percentages (shown at the bottom of the figure) with dried leaves of *Origanum*, before extraction of genomic DNA. PCR reactions were performed with the primer OPA11. DNA from fresh leaves of *Rhus coriaria* was used as further control (lane 2). Lanes: 1, *O. vulgare* subsp. *hirtum*; 2, *Rhus coriaria* fresh leaves; 3, *Rhus coriaria* dried leaves; 4−6, mixtures of *Origanum* and the contaminant species; M, 100 bp DNA ladder. Black arrow indicates the position of the shorter specific band (286 bp) amplified from *Rhus coriaria* (lanes 2−6).

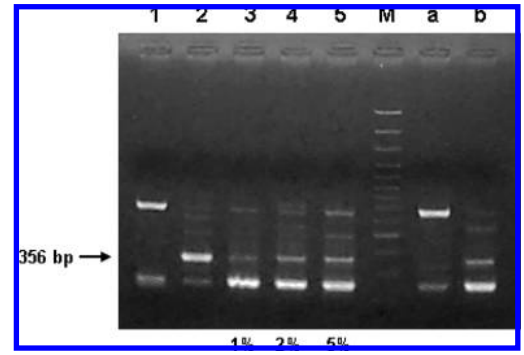

Figure 8. RAPD analysis of individual dried leaves and mixtures of *O. vulgare* subsp. *hirtum* and the contaminant species *Cistus incanus*. Dried leaves of the contaminant species were mixed in three different percentages (shown at the bottom of the figure) with dried leaves of *Origanum*, before extraction of genomic DNA. PCR reactions were performed with the primer OPA07. DNA from two *Origanum* commercial samples was also analyzed (lanes a, b). Lanes: 1, *O. vulgare* subsp. *hirtum*; 2, *Cistus incanus*; 3−5, mixtures of *Origanum* and the contaminant species; M, 100 bp DNA ladder; a, *Origanum* commercial sample without *Cistus incanus*; b, *Origanum* commercial sample with high percentage (<10%) of the contaminant species. Black arrow indicates the position of the specific band (356 bp) amplified from *Cistus incanus* (lanes 2−5, b).

from mixtures prepared by fresh material containing 1, 2, and 5% of *R. caesius*.

To transfer the above-described protocol to the analysis of commercial samples, the analysis was extended to DNA extracted both from mixtures prepared with dry material (see Materials and Methods) and from commercial samples in which different percentages of plant contaminant were evidenced through pharmacognostic examination. Before the analysis of DNA extracted from dry material, an enrichment of the integer DNA fraction available for PCR reaction was obtained through gel purification. Despite causing a loss of material, this step induced an enrichment in the integer DNA fraction available for PCR reaction due to the elimination of both the tail of degraded DNA and the polyphenol-linked DNA, which remained blocked into the gel wells (data not shown). After checking that identical banding patterns were obtained from fresh and dried material and from gel-eluted DNA, we analyzed the DNA extracted from artificial mixtures and from some commercial samples.

In **Figures 6−8** the results are shown of these amplifications which, even in complex patterns, allowed the detection of up to 1% of *Rubus* (**Figure 6**, lane 4), *Rhus* (**Figure 7**, lane 4), and *Cistus* (**Figure 8**, lane 3), respectively, as evidenced by the specific amplicons (indicated by arrowhead) visible in the RAPD profile of the single contaminant species and its mixtures but absent in the profile of *Origanum* alone. In **Figure 8** (lanes a, b) is shown an example of the analysis conducted on two *Origanum* commercial samples. Microscopical analysis indicated for sample a the absence of contaminants and for sample b the presence of 16% of *Cistus* plus 8% of *Satureja*. The PCR reaction conducted with OPA07 to check *Cistus* contamination confirmed the pharmacognostic analysis evidencing the absence

Table 2. Primers and Contaminant-Specific Bands, Characterized by Molecular Mass (bp)[a]

primer	sequence	*Cistus incanus*	*Rubus* sp.	*Rhus coriaria*
OPA01	5′-d[CAGGCCCTTC]-3′	984	471	—[b]
OPA03	5′-d[AGTCAGCCAC]-3′	—	—	—
OPA04	5′-d[AATCGGGCTG]-3′	444	444	—
OPA05	5′-d[AGGGGTCTTC]-3′	—	—	682; 507; 388
OPA07	5′-d[GAAACGGGTG]-3′	356	1142; 980; 842	1566; 1141; 851; 442
OPA09	5′-d[GGGTAACGCC]-3′	1309	2365; 1848; 1615	868
OPA10	5′-d[GTGATCGCAG]-3′	1270	—	428; 280
OPA11	5′-d[CAATCGCCGT]-3′	456	1110	286
OPA12	5′-d[TCGGCGATAG]-3′	—	—	—
OPA16	5′-d[AGCCAGCGAA]-3′	344	—	801
OPA20	5′-d[GTTGCGATCC]-3′	1293; 1021	1021; 806; 317	1298; 1072; 956; 559
OPB02	5′-d[TGATCCCTGG]-3′	893	513; 231	—
OPB08	5′-d[GTCCACACGG]-3′	549; 445	—	—
OPB10	5′-d[CTGCTGGGAC]-3′	—	—	—
OPB12	5′-d[CCTTGACGCA]-3′	—	—	—
OPB13	5′-d[TTCCCCCGCT]-3′	—	—	594; 484; 385
OPB16	5′-d[TTTGCCCGGA]-3′	—	—	—
OPB19	5′-d[ACCCCCGAAG]-3′	—	—	—
OBB20	5′-d[GGACCCTTAC]-3′	—	—	—
OPP10	5′-d[TCCCGCCTAC]-3′	—	2096; 1799; 1213; 604	—

[a] The amplicons listed in the table were found in the RAPD profiles generated by the contaminant plants *Rubus caesius*, *Rhus coriaria*, or *Cistus incanus* and absent in the RAPD profiles obtained from the *Origanum* taxa used as controls. [b] Contaminant-specific bands not detected.

of *Cistus*-specific bands in sample a and identifying a strong contamination in sample b.

In conclusion, the scenario depicted by the market survey highlighted a high level of heterogeneity and a frequent contamination of Mediterranean oregano shipped from foreign producers to the European Union, thus suggesting that specific attention must be given to the authentication of Mediterranean oregano by European wholesale buyers. The development of a reliable method suitable for the detection of contaminants could allow a faster and accurate quality control of a large number of Mediterranean oregano samples. The method proposed requires the contemporary amplification of DNA extracted from the contaminant species and from *Origanum* samples; it is reliable and presents a limit of detection equal to 1% for a single contaminant. It can be used for primary routine screening of large number of batches, allowing the immediate rejection of suspect samples and thus narrowing the number of samples to be subjected to pharmacognostic analyses.

ABBREVIATIONS USED

CTAB, hexadecyltrimethylammonium bromide; PVP, polyvinylpyrrolidone, EDTA, ethylenediaminetetraacetic acid.

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LITERATURE CITED

(1) *World Markets in the Spice Trade 2000−2004*; International Trade Centre: Geneva, Switzerland, 2006.
(2) American Spice Trade Association, Spice Adulteration—White Paper, available online at http://www.astaspice.org/i4a/pages/index.cfm?pageid=3395 (accessed August 2008).
(3) Kintzios, S. E. Oregano. In *Handbook of Herbs and Spices*, 1st ed.; Peter, K. V., Ed.; Woodhead Publishing: Cambridge, U.K., 2004; Vol. 2, pp 215−229.
(4) Singhal, R. S.; Kulkarni, P. R. Herbs and spices In *Food Authenticity and Traceability*, 1st ed.; Lees, M., Ed.; Woodhead Publishing: Cambridge, U.K., 2003; pp 386−414.
(5) Tremlova, B. Evidence of spice black pepper adulteration. *Czech. J. Food Sci.* **2001**, *19*, 235–239.
(6) Winterhalter, P.; Straubinger, M. Saffron—renewed interest in an ancient spice. *Food Rev. Int.* **2000**, *16*, 39–59.
(7) Kintzios, S. E. Profile of the multifaceted prince of the herbs. In *Oregano: The Genera Origanum and Lippia*, 1st ed.; Kintzios, S. E., Ed.; CRC Press: London, U.K., 2002; pp 3−10.
(8) Tainter, D. R.; Grenis, A. T. The spices. In *Spices and Seasonings: A Food Technology Handbook*, 2nd ed.; Tainter, D. R., Grenis, A. T., Eds.; Wiley-VCH: New York, 2001; pp 116−119.
(9) Baranska, M.; Schulz, H.; Krüger, H.; Quilitzsch, R. Chemotaxonomy of aromatic plants of the genus *Origanum* via vibrational spectroscopy. *Anal. Bioanal. Chem.* **2005**, *381*, 1241–1247.
(10) Nhu-Trang, T. T.; Casabianca, H.; Grenier-Loustalot, M. F. Deuterium/hydrogen ratio analysis of thymol, carvacrol, γ-terpinene and *p*-cymene in thyme, savory and oregano essential oils by gas chromatography−pyrolysis−isotope ratio mass spectrometry. *J. Chromatogr., A* **2006**, *1132*, 219–227.
(11) Huie, C. W. A review of modern sample-preparation techniques for the extraction and analysis of medicinal plants. *Anal. Bioanal. Chem.* **2002**, *373*, 23–30.
(12) Schultz, H.; Quilitzsch, R.; Kruger, H. Rapid evaluation and quantitative analysis of thyme, origano and chamomile essential oils by ATR-IR and NIR spectroscopy. *J. Mol. Struct.* **2003**, *661−662*, 299–306.
(13) Moller, J. K. S.; Catharino, R. R.; Eberlin, M. N. Electrospray ionization mass spectrometry fingerprinting of essential oils: spices from the Labiatae family. *Food Chem.* **2007**, *100*, 1283–1288.
(14) Lockley, A. K.; Bardsley, R. G. DNA-based methods for food authentication. *Trends Food Sci. Tech.* **2000**, *11*, 67–77.
(15) Weder, J. K. P. Identification of plant food raw material by RAPD-PCR: legumes. *J. Agric. Food Chem.* **2002**, *50*, 4456–4463.
(16) Lenstra J. A., DNA Methods for identifying plant and animal species in food. In *Food Authenticity and Traceability*, 1st ed.; Lees, M., Ed.; Woodhead Publishing: Cambridge, U.K., 2003; pp 386−414.
(17) Popping, B. The application of biotechnological methods in authenticity testing. *J. Biotechnol.* **2002**, *98*, 107–112.
(18) Zhang, Y. B.; Wang, J.; Wang, Z. T.; But, P. P. H; Shaw, P. C. DNA microarray for identification of the herb of *Dendrobium* species from Chinese medival formulation. *Planta Med.* **2003**, *69*, 1172–1174.
(19) Sasikumar, B.; Syamkumar, S.; Remya, R.; Zachariah, T. J. PCR based detection of adulteration in the market samples of turmeric powder. *Food Biotechnol.* **2005**, *18*, 299–306.
(20) Kalpana, J.; Preeti, C.; Dnyaneshwar, W.; Bhushan, P. Molecular markers in herbal drug technology. *Curr. Sci.* **2004**, *87*, 159–165.
(21) Hudson, J.; Altamirano, M. The application of DNA micro-arrays (gene arrays) to the study of herbal medicines. *J. Ethnopharmacol.* **2006**, *108*, 2–15.
(22) Techen, N.; Crockett, S. L.; Khan, I. A.; Scheffler, B. E. Authentication of medicinal plants using molecular biology techniques to compliment conventional methods. *Curr. Med. Chem.* **2004**, *11*, 1391–1401.
(23) Cheng, K. T.; Tsay, H. S.; Chen, C. F.; Chou, T. W. Determination of the components in a Chinese prescription, Yu-Ping-Feng San by RAPD analysis. *Planta Med.* **1998**, *64*, 563–565.
(24) Wolf, H. T.; Zündorf, I.; Winckler, T.; Bauer, R.; Dingermann, T. Characterization of *Echinacea* species and detection of possible adulterations by RAPD analysis. *Planta Med.* **1999**, *65*, 773–774.
(25) Nieri, P.; Adinolfi, B.; Morelli, I.; Breschi, M. C.; Simoni, G.; Martinotti, E. Genetic characterization of the three medicinal *Echinacea* species using RAPD analysis. *Planta Med.* **2003**, *69*, 685–686.
(26) Williams, J. G.; Kubelik, A. R.; Livak, K. J.; Rafalski, J. A.; Tingey, S. V. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **1990**, *18*, 6531–6535.
(27) Welsh, J.; McClelland, M. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.* **1990**, *18*, 7213–7218.
(28) *Quality Control Methods for Medicinal Plant Materials*; WHO Library Cataloguing in Publication Data: Geneve, Switzerland, 1998.
(29) Oregano Monograph 01/2005:1880. In *European Pharmacopoeia*, 5th ed.; Council of Europe: Strasbourg, France, 2005; pp 2155−2156.
(30) Murray, M. G.; Thompson, W. F. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* **1980**, *8*, 4321–4325.
(31) Li, Y. X.; Su, Z. X.; Chen, F. Rapid extraction of genomic DNA from leaves and bracts of dove tree (*Davidia involucrata*). *Plant Mol. Biol. Rep.* **2002**, *20*, 185–185.
(32) Križman, M.; Jakše, J.; Baričevič, D.; Javornik, B.; Prošek, M. Robust CTAB-activated charcoal protocol for protocol for plant DNA extraction. *Acta Agric. Slovenica* **2006**, *87*, 427–433.
(33) Pateraki, I.; Kanellis, A. K. Isolation of high-quality nucleic acids from *Cistus creticus* ssp. *creticus* and other medicinal plants. *Anal. Biochem.* **2004**, *328*, 90–92.

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