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Marzipan: Polymerase Chain Reaction-Driven Methods for Authenticity Control

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Supporting Information

ABSTRACT: According to German food guidelines, almonds are the only oilseed ingredient allowed for the production of marzipan. Persipan is a marzipan surrogate in which the almonds are replaced by apricot or peach kernels. Cross-contamination of marzipan products with persipan may occur if both products are produced using the same production line. Adulterations or dilutions, respectively, of marzipan with other plant-derived products, for example, lupine or pea, have also been found. Almond and apricot plants are closely related. Consequently, classical analytical methods for the identification/differentiation often fail or are not sensitive enough to quantify apricot concentrations below 1%. Polymerase chain reaction (PCR)-based methods have been shown to enable the differentiation of closely related plant species in the past. These methods are characterized by high specificity and low detection limits. Isolation methods were developed and evaluated especially with respect to the matrix marzipan in terms of yield, purity, integrity, and amplificability of the isolated DNA. For the reliable detection of apricot, peach, pea, bean, lupine, soy, cashew, pistachio, and chickpea, qualitative standard and duplex PCR methods were developed and established. The applicability of these methods was tested by cross-reaction studies and analysis of spiked raw pastes. Contaminations at the level of 0.1% could be detected.

KEYWORDS: marzipan, persipan, PCR, DNA isolation, *Prunus*

INTRODUCTION

According to German food guidelines, raw pastes that are declared as "marzipan" may only contain almonds (*Prunus dulcis*) as an oilseed ingredient. If other seeds are used, declaration of the respective ingredient is required. Possible alternatives to almonds are kernels of apricot (*Prunus armeniaca*) and peach (*Prunus persica*). In Germany, those products have to be declared as "persipan".[1]

Two reasons can be defined for the presence of plant ingredients other than almond in marzipan: (i) An unintentional contamination can be caused by raw products of minor quality (purity) or by insufficient cleaning procedures of the production line after a product change (e.g., from persipan to marzipan). In similar ways, other contaminants, for example, with allergenic potential, can end up in marzipan. (ii) An intentional addition of cheaper plant materials at all steps of the production process is also supposable. In this context, it should be noted that a marzipan-like product with lupine is legally distributed on the European market.

Producers of raw pastes have to guarantee the quality and the declaration of their products. For comprehensive control of raw materials (almonds), semifinished goods (raw pastes), and products (marzipan), reliable analytical methods are necessary. Different classical techniques are on the market, but they either lack in specificity (*P. armeniaca* vs *P. persica* vs *P. dulcis*) or their limit of detection is too high for the detection of contaminations occurring in lower concentration levels. Typically, the analysis of the tocopherol pattern is still used for the detection and semiquantitative determination of apricot in marzipan.[2,3] This method has a limit of detection of about 5% and is not applicable on marzipan products containing cocoa butter. In the last years, DNA-based methods have been developed and published.[4,5] The advantages of polymerase chain reaction (PCR) driven methods are low limits of detection and high specificity of the reaction.

The goal of our studies was the development of DNA-based methods for the detection of low concentrated (<1%) contaminations in marzipan or marzipan-derived products. Established DNA isolation methods were used as a starting point for the development of methods especially adapted on the marzipan matrix. The presented highly specific PCR methods enable the detection of apricot, peach, pea, bean, lupine, soy, cashew, pistachio, and chickpea (Table 1). All methods were optimized for the matrix of marzipan raw pastes.

MATERIALS AND METHODS

Sample Collection. Nine marzipan raw pastes, two persipan raw pastes, and two almond and two apricot kernels were provided by our project partners (Zentis GmbH & Co. KG, Lübecker Marzipan-Fabrik v. Minden & Bruhns GmbH & Co. KG, Georg Lemke GmbH & Co. KG) or purchased from the local market. In addition, as possible

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Table 1. Overview of the Species under Study, Primer Sequences, Accession Numbers, Target Sequences, and PCR Product Sizes

species		primer pair		sequence 5′−3′	target	product size (bp)
apricot	*P. armeniaca*	1	FW	AAAGCTCAAGAGCCTGAAGTTC	*AF196922.1* PGIP	180
			RW	ACGGTCTAGATGAAGAGCATCTAA		
		2	FW	TTATCTGCGTCAAGCTCACA	(4)	130
			RW	GATCATTGAAATTTTGGTCTAGC		
peach	*P. persica*	3	FW	AGGGAAAGATGTTTGGTAGC	*EU155160* R2R3MYB	110
			RW	CACCACCACATCTTCTAGG		
pea	*P. sativum*	4	FW	ACTTTGCTCTGAGCACATCTG	*GQ260108.1* rDNA	280
			RW	CCACCCTGCACAATACCTG		
fava bean	*V. faba*	5	FW	CGGGATGTGTTTTGACACATGA	*FJ212318.1* rDNA	215
			RW	CAGGGAAATTGGCAAGGAGG		
soy	*G. max*	6	FW	AGTCGTCACGACACAACA	*AJ011337.1* rDNA	230
			RW	CGCACAACATTGTTACATG		
lupine	*Lupinus spec.*	7	FW	CGTTGCGACACGCTTATCCT	*AF007481.1* rDNA	265
			RW	CCAACCGTGAGACATTGCTC		
		8	FW	CCTCACAAGCAGTGCGA	(22)	130
			RW	TTGTTATTAGGCCAGGAGGA		
cashew	*A. occidentale*	9	FW	GGAAGCGTTGCCTCCTTTCA	*AB071690.1* rDNA	285
			RW	GAGGACTCGCGTTTGGGC		
pistachio	*P. vera*	10	FW	CTTAACGAGAGAGCTCGCTC	*AY677201.1* rDNA	270
			RW	GATCGCAAGATTTTGGGCGG		
		11	FW	TGCCCGTGTGCCTCCA		180
			RW	ATAATGAAAGAAGGCTACCCA		
chick pea	*Cicer* sp.	12	FW	TTGAACACCTCGGCCCAA	*AB198904.1* rDNA	120
			RW	GTGCGCACATAACAAAGTTTT		
all species		uni-1	5.8SFW	GACTCTCGGCAACGGATATC	rDNA	115
			5.8SRW	CGCAACTTGCGTTCAAAGACTCGA		
		uni-2	18SFW	GTCGCGAGAAGTCCACTGAA		350
			5.8SRW	AGAGCCGAGATATCCGTTGC		
		uni-3	18SFW	AGAACGACCCGAGAACTAGTTTC		600
			26SRW	TTAAATTCAGCGGGTAACCCCG		
Additional Species Used for Specificity Tests						
almond	*P. dulcis, P. dulcis* var. *amara*	cherry		*Prunus sargentii, Prunus avium, Prunus salicina*	plum	*Prunus domestica*
					hazel nut	*Corylus* sp.

contaminants/adulterants, seeds of the following plants (purchased from the local market) were included in the studies (cf. Table 1): peach (*P. persica*), soy (*Glycine max*), pea (*Pisum sativum*), chick pea (*Cicer arietinum*), fava bean (*Vicia faba*), lupine (*Lupinus* spec.), cashew (*Anacardium occidentale*), and pistachio (*Pistacia vera*). Leaf material was used from *P. persica*, *P. sargentii*, *P. avium*, and *P. domestica*. All samples with exception of the raw pastes were lyophilized and ground to a fine powder (sample powder).

Spiking of Marzipan Raw Pastes. Marzipan raw paste was mixed with equal amounts of persipan raw paste or with prepared sample powders, respectively. After homogenization using an Unguator (apparatus normally used for the homogeneous compounding of pharmaceutical formulations), a part of the spiked raw paste was diluted in series (1:3) with unspiked marzipan raw paste.

DNA Isolation. State of the art DNA isolation methods based on different DNA extraction strategies [cell lysis with SDS or cetyl-trimethyl-ammonium-bromide (CTAB), DNA precipitation with CTAB or alcohol, or the use of a silica suspension] were used as starting points for the development of methods especially adapted on marzipan matrix.[6−13] In addition, two commercial DNA isolation kits (Stool Kit; Plant Kit, Qiagen GmbH, Hilden, Germany) were applied. On the basis of these results, four raw paste matrix-adapted methods (Cp, Cp + S, Ip + S, and S) were developed. In all cases, in a first step, 200 mg of the sample was incubated with 1 mL of buffer 1 (0.1 M Tris/HCl, 55 mM CTAB, 1.4 M NaCl, and 20 mM EDTA, pH 8.0) at 65 °C for 30 min in 2 mL reaction tubes. A 300 μL amount of chloroform was added, and after it was centrifuged at 10000*g* for 5 min, the supernatant was transferred to a new 2 mL reaction tube. The subsequent treatment of this solution 1 differed for each method.

Precipitation with CTAB (Method Cp). DNA was precipitated in solution 1 by the addition of 1 mL of buffer 2 (13.7 mM CTAB, 40 mM NaCl), incubation for 60 min at room temperature, and centrifugation at 10000*g* for 10 min. After the supernatant was discarded, the DNA pellet was dissolved in 350 μL of 1.2 M NaCl, mixed with 350 μL of chloroform by vortexing, and centrifuged (10000*g* for 2 min). The supernatant was transferred to a new 1.5 mL reaction tube, and the DNA was precipitated with 200 μL of isopropyl alcohol at 4 °C for 30 min. A centrifugation step at 10000*g* for 10 min was followed by washing the DNA pellet with

Table 2. Primer Pairs for the Detection of Persipan (Peach and/or Apricot)

		sequence			
target species		5′	3′	target sequence	product size (bp)
apricot/peach	13	FW	TGAGTGTGTGTAATGATGAGTT	EU153578.1	100
		RW	GGGAGTTTCACTAAAACACC		
	14	FW	ACCCAAAATCAACCCCAAAGTCAA	EU153578.1	100
		RW	AACACACCCAAAACTCATCATTAC		
	15	FW	AGAGGCTTAATCTGTTG	AF134732.1	135
		RW	ATACCCATTCTTCTTCTCA		
	16	FW	CCACATACTTAGTTGCATTG	AF206634.1	130
		RW	AGCCCACTGTTAGGAC		

500 μL of 70% ethanol and centrifugation at 10000*g* for 10 min. The DNA pellet was vacuum-dried for 30 min and finally dissolved in 50 μL of water.

Precipitation with CTAB and Subsequent Silica Adsorption (Method Cp + S). DNA was precipitated in solution 1 by the addition of 1 mL of buffer 2 as described above. The supernatant was discarded, and the DNA pellet was dissolved in 600 μL of binding buffer 3 (20 mM Tris/HCl, 5.5 M guanidin hydrochloride, pH 6.6). The solution was transferred on top of an EconoSpin All-in-1 mini Spin Column (Epoch Biolabs, Sugar Land, TX) and centrifuged at 10000*g* for 1 min. The flow-through was discarded. Two washing steps using 500 μL of buffer 4 (20 mM Tris/HCl, 1 mM EDTA, and 50 mM NaCl in 50% ethanol, pH 7.4) and 500 μL of 70% ethanol were performed by centrifugation at 10000*g* for 1 min. For a complete removal of the ethanol, the column was centrifuged at 10000*g* for 2 min. Finally, the spin column was transferred to a new 1.5 mL reaction tube, and the DNA was eluted with 50 μL of water.

Precipitation with Isopropyl Alcohol and Subsequent Silica Adsorption (Method Ip + S). A 700 μL amount of chloroform was added to solution 1 and mixed by vortexing. After centrifugation at 10000*g* for 2 min, the supernatant was transferred to a new 2 mL reaction tube. Again, 700 μL of chloroform was added, and the extraction step was repeated. A 700 μL amount of ice-cold isopropyl alcohol was added to the aqueous phase, and the solution was incubated at 4 °C for 30 min. After centrifugation at 10000*g* for 10 min, the supernatant was discarded, and the DNA pellet was dissolved in 600 μL of buffer 3. The solution was placed on an EconoSpin All-in-1 mini Spin Column and centrifuged at 10000*g* for 1 min. Washing steps were performed as described in Method Cp + S. Finally, the DNA was eluted with 200 μL of water.

Silica Adsorption (Method S). A 650 μL amount of solution 1 was directly placed on an EconoSpin All-in-1 mini Spin Column and centrifuged at 10000*g* for 1 min. Washing steps were performed as described in Method Cp + S. Finally, the DNA was eluted with 50 μL of water.

Quality Assessment of Isolated DNA. For the determination of the DNA purity, the ratio of absorbance at 260 and 280 nm (ideally 1.7−1.9) was estimated (SpectraMax M2, Molecular Devices, Sunnyvale, CA).

Fluorimetric Determination of the DNA Concentration. For the determination of the DNA concentration, a SYBR Green I assay was used. A 90 μL amount of a SYBR Green I solution (1:6,250) was pipetted into each well of a 96-well fluorescence plate. Five microliters of the DNA isolates was added. For calibration, 2, 4, 6, 8, or 10 μL of a plasmid stock solution (pBluescript II SK(-), Fermentas GmbH, St. Leon-Rot, Germany, c = 10 ng/μL) was pipetted into the wells. Finally, the volume was completed with water to a final volume of 100 μL, and the fluorescence intensity was determined at 425 nm (excitation: 395 nm). If necessary, DNA isolates were diluted into the linear working range.

PCR. PCR was performed using a Biometra thermocycler (T3000, Biometra, Göttingen, Germany). After an initial denaturation at 95 °C for 3 min, 35 cycles at 95 °C for 20 s, 59 °C for 20 s, and 72 °C for 20 s were performed. For a terminal elongation, the reaction batches were incubated for 3 min at 72 °C.

Standard PCR. Reaction mixtures contained *Taq*-Polymerase [1 U *Taq* (in house production) or 0.5 U Euro-*Taq* (Euroclone, Pero, Italy)], 1× reaction buffer [10 mM Tris/HCl, 0.01% Triton X-100, 50 mM KCl, pH 8.8 or Euro-*Taq*-buffer: 16 mM, $(NH_4)_2SO_4$, 67 mM Tris/HCl, and 0.01% Tween-20, pH 8.8], 3 mM $MgCl_2$, 0.2 mM concentration of each deoxynucleoside triphosphate (Bioline GmbH, Luckenwalde, Germany), 1 μM concentration of each primer (VBC biotech GmbH, Wien, Austria), and 5 μL of the DNA solution (set to 15 ng/μL) in a total volume of 25 μL. The primer sequences are listed in Tables 1 and 2.

Duplex PCR. For the simultaneous detection of two potential contaminations/adulterations, two primer pairs were used in one reaction mixture (see Figure 6) in concentrations of 625 nM each. All other reagents were used in the same concentrations as described above in a total reaction volume of 25 μL.

Agarose Gel Electrophoresis. The analysis of DNA isolates was performed on 0.75% agarose gels at 75 V (Powerpac 1000, Biorad Laboratories Inc., Hercules, CA) using TAE-buffer (40 mM Tris/acetate, 2 mM EDTA, pH 8.2). For the analysis of the PCR products, 3% agarose gels were used (150 V). For the detection of DNA, the gels were incubated for 25 min in ethidium bromide solution (0.01%). After the gels were rinsed, the results were documented under UV light (Biostep, Felix 1040, Biostep GmbH, Jahnsdorf, Germany).

RESULTS AND DISCUSSION

Primer Design. Ribosomal DNA sequences (rDNA) were found in the National Center for Biotechnology Information (NCBI) Database for all organisms under study. On the basis of alignments (clustalw algorithm), six universal primers (uniprimers) were generated with hybridization sites on conserved regions of the rDNA genes coding for 18S, 5.8S, and 28S rRNA (Table 1). These primer combinations could be used for quality assessment of isolated DNA. The internal transcribed spacer (ITS) regions were used for the design of specific primer pairs for fava bean, pea, soy, lupine, cashew nuts, pistachio, and chickpea. The primer sequences are given in Table 1; binding sites can be seen in the alignment in Figure S1 in the Supporting Information. The alignment of rDNA regions of the *Prunus* species under study showed a high sequence homology in line with their close degree of relationship (Figure S2 in the Supporting Information). The design of specific primer pairs based on these sequences was not possible. For the specific detection of apricot kernels, the sequence coding for a polygalacturonase inhibitor protein

(PGIP) was selected (Table 1). Primers for the detection of peach kernels are based on the sequence of the R2R3MYB transcription factor gene (MYB) (Table 1).

DNA Isolation. In several publications, the influence of isolation protocols or manufacturing processes on the DNA respectively on PCR results is described.[14−16] Marzipan raw pastes commonly contain a minimum sugar content of about 50% and a minimum almond fat content of about 30%. Both ingredients have to be removed during DNA isolation. Additionally, the influence of the raw paste production (milling) on the DNA molecules should be investigated. In contrast to that, published methods for the detection of contaminations in marzipan used a classical CTAB isolation protocol,[4,5] which was not optimized for the matrix under study disregarding the fact that the isolation of high-quality DNA is the prerequisite for a reliable PCR-based analysis. According to the minimum performance requirements for analytical methods of GMO testing defined by the European Network of GMO Laboratories (ENGL), the quality of DNA depends on the average length, structural integrity, and chemical purity of the extracted DNA.[17] Thus, different DNA isolation methods were adapted to raw paste matrix and were evaluated in terms of (i) DNA amount, (ii) DNA purity, (iii) fragmentation, and (iv) DNA amplificability. Apart from these analytical requirements, the parameters time, costs, and workload were also taken into account. Preliminary results with published methods showed that (i) cell lysis by CTAB is most effective, (ii) comparable purities were observed with all precipitation methods and all silica adsorption methods, (iii) precipitation leads to higher amounts, and (iv) use of silica spin columns ensure an easy handling and save time. After this first evaluation step, a modified CTAB method (method Cp) based on the German official collection of test methods in accordance with Article 64 of the German Food Act was used as a reference method. Three DNA isolation protocols were generated by combination of different steps from the cited methods with the goal to reach an optimal performance in marzipan raw pastes or kernels (methods Cp + S, Ip + S, and S). Method Cp + S combined two basic principles for DNA isolation: First, the DNA is precipitated with CTAB followed by an adsorption of the DNA to silica spin membranes. In method Ip + S, isopropyl alcohol was used as a precipitation agent instead of CTAB. The use of alcohol instead of an CTAB buffer is frequently described in literature,[18,19] for example, for the DNA extraction from Cashew nuts.[12] The repeated chloroform extraction was performed to remove fat and proteins.[20] Method S contained no precipitation step. Binding of DNA to the silica membrane proceeded directly after the cell lysis for a simple and fast protocol.

DNA of 11 raw pastes, two apricot kernels, and two almond kernels was extracted five times with all four methods evaluating the most practicable isolation method for DNA that is suitable for subsequent PCR experiments. Generally, no differences between raw pastes of different producers were observed. Thus, the data were combined for final interpretation.

On the basis of the specific intercalation of SYBR Green I to dsDNA, the fluorimetric method was used to compare the yields of extracted DNA. In accordance with the ENGL requirements the DNA concentration should be higher than the working concentration described in the PCR protocol of the detection method.[17] Hence, a DNA concentration of 15 ng/μL was considered as the minimum amount for a successful extraction. In Figure 1A, the average DNA yields of each method are shown as box and whiskers plots. Comparing the indicated medians, it is obvious that the application of method Ip + S yielded the highest amount of DNA. It seemed that the precipitation with isopropyl alcohol was more effective than with CTAB. Even the lowest obtained DNA concentration of 50 ng/μL (minimum value raw pastes) was about three times higher than the required concentration of 15 ng/μL. Because of the subsequent dilution step, less coisolated compounds (e.g., inhibitors) will end up in the PCR. This is a benefit of this method because effects by possible inhibitors on the PCR will be reduced.[21] The box plots of methods Cp and Cp + S indicate that in many cases the amount was too low for a dilution step. With method S, unsatisfactory amounts of DNA were extracted.

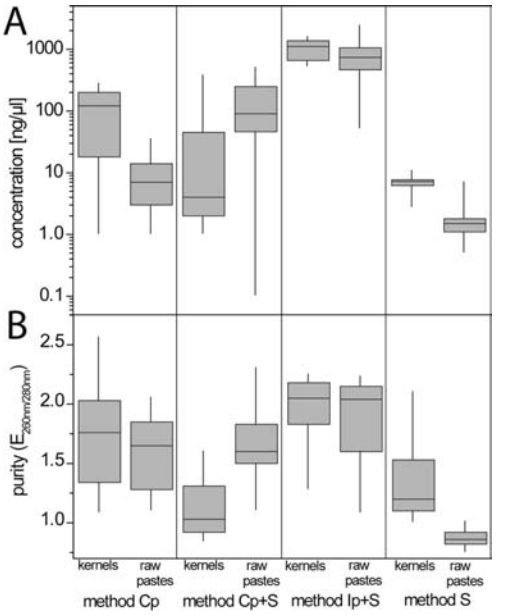

Figure 1. (A) DNA yield (box and whisker plots) of the four isolation methods in dependence of the DNA source (kernels or raw pastes) and (B) DNA purity (ratio 260 nm/280 nm, box and whisker plots) of the four isolation methods in dependence of the DNA source (kernels or raw pastes).

The purity of the extracted DNA was determined photometrically (OD ratio 260 nm/280 nm). Comparing the box and whisker plots of the experiments (Figure 1B), two groups could be identified. Because of the low DNA yields (cf. A) of method S (kernels and raw pastes) and method Cp + S (kernels), no absorption maxima were detected [theoretical OD_{260} (5 ng/μL) = 0.01 for a path length of 1 mm]. This resulted in an $OD_{260/280}$ quotient of about 1.0. DNA extracted with one of the other methods showed higher purities reflected by interquartile ranges including the optimum of $OD_{260/280}$ = 1.9. There was no significant difference observed using CTAB or isopropyl alcohol precipitation. The whiskers in Figure 1B show in all cases a high range between the min and the max, indicating the occurrence of random method failures. Method Ip + S showed a lower divergence in the whisker plots than method Cp + S. Thus, with method Ip + S, reproducible purities and amplification results could be expected.

In Figure 2, electropherograms of DNA extracted with the three methods Cp, Cp + S, and Ip + S are shown. As described above, amounts of DNA isolated with method S were too low for the detection on agarose gels. Genomic DNA extracted from raw

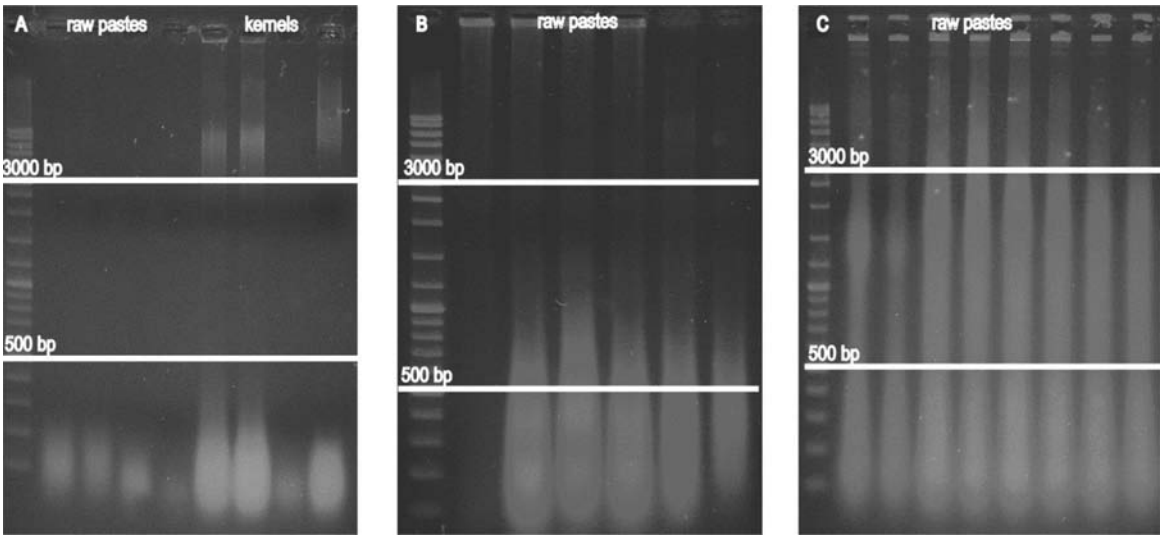

Figure 2. Electropherograms of genomic DNA isolated from raw pastes or kernels. (A) Method Cp, (B) method Cp + S, and (C) method Ip + S.

pastes with method Cp was more fragmented than DNA isolated from kernels with method Cp (Figure 2A). Also, with method Cp + S, the size of the extracted DNA from raw pastes was predominantly smaller than 500 bp (Figure 2B). On the contrary, the size of DNA extracted with method Ip + S was up to 3000 bp (Figure 2C). The only difference to method Cp + S was the use of isopropyl alcohol for DNA precipitation instead of CTAB. The observations suggest that besides the expected DNA fragmentation during raw paste production, different DNA extraction methods possibly lead to an enrichment of DNA of different size. This influence of chemical and physical parameters of the extraction method on the DNA size was already described by other research groups.[22,23]

PCR experiments were performed in triplicate using uniprimer pairs 1−3 (Table 1). In Figure 3, the yield of positive PCR results based on DNA templates from the different extraction methods and three different uniprimer pairs is shown. PCR experiments with primer combinations uni-1 and uni-2 led to reproducible results irrespective if the DNA was extracted from kernels as well as from raw pastes. It can be concluded that PCR was not affected by matrix components or added reagents. Focusing on PCR results using DNA of kernels and raw pastes isolated with either method Cp, Cp + S, or S, it could be observed that the amplification of large DNA fragments (uniprimer pair 3) predominantly failed in the case of raw pastes, presumably caused by fragmentation of the DNA during raw paste production but also during isolation.

The isolated DNA using methods Cp, Cp + S, and Ip + S showed an excellent quality in terms of amplificability and purity (260/280 nm ratio). Nearly all amplifications with uni-1 and uni-2 showed the expected products. Hence, reliable PCR results can be expected in routine analysis.

PCR Optimization. Optimal PCR conditions were defined by a visual assessment of the PCR products on the electropherograms. PCR optimization was performed for a fast and consistent protocol and included modification of the annealing temperature (finally 59 °C), $MgCl_2$ concentration (finally 3 mM), primer concentration (finally 1 μM for simplex and 0.625 μM for duplex reactions), initial and final elongation times (finally 3 min), and the time of all cycle steps (finally 20 s each).

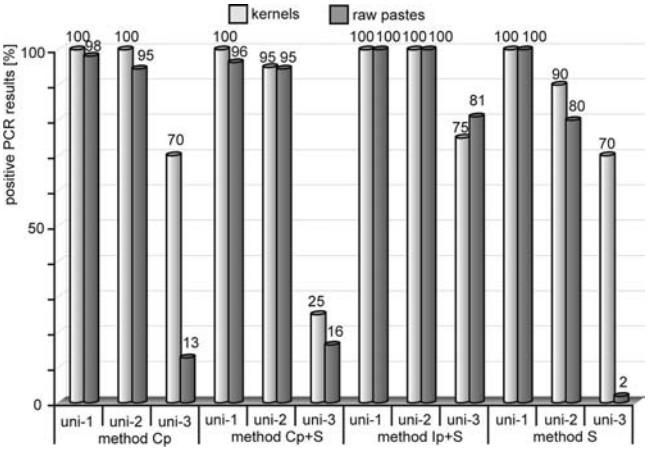

Figure 3. Overview of positive PCR results (%) in dependence of the extraction method and primer pairs.

Primer Specificity. The manually designed primer pairs were first checked with the primer blast tool within NCBI database for specificity.[24] Additionally, the primers were used in PCR with extracted DNA from the species denoted in Table 1. Isolated DNA from every single species was tested with each primer pair from Tables 1 and 2 in PCR experiments. To avoid false negative results, the absence of inhibitors was checked with primer combination uni-1. Only DNA extracts with positive PCR results were used in specificity tests. Despite a specific primer blast search, some of the primers showed cross-reactions with other species. As expected, especially the differentiation of the closely related *Prunus* species was difficult.[25] The primer pairs given in Table 2 could serve for the detection of persipan contaminations if differentiation of peach and apricot was not necessary. The final primer pairs listed in Table 1 gave specific PCR results in simplex as well as in duplex PCR experiments. No cross-reactions with the DNA from other species under study were observed, and the PCR products showed the expected sizes given in Table 1. The results from the specificity tests (simplex and duplex PCR) with the primer pairs for apricot (primer pair 1) and peach

Table 3. Almond Kernels Considered for Cross-Reactivity

	country of exportation	additional information
P. dulcis var. *amara*	Morocco	
	Syria	
P. dulcis	Spain	Maracas
		Larguetas
		Valencias
	United States	California
		Padre
		Nonpareil
		Camel
		Mission selected
		Butte selected
		Nonpareil
	Turkey	

(primer pair 3) are exemplarily shown in Figure S3 in the Supporting Information.

Because of reported unspecific PCR results in cross-reactivity tests of different seeds from *Prunoideae*,[25] it was important to analyze different cultivars with the apricot and peach primer pairs for interspecies specificity and intraspecies uniformity. DNA from 12 almond (Table 3), six apricot, and two peach cultivars and multiple marzipan or persipan raw pastes were used as templates. In Figure S4 in the Supporting Information, PCR results with primer pair 1 (apricot) and isolated DNA from six apricot cultivars of different origins are shown. Primer pair 1 proved to be applicable on all apricot cultivars tested. Analogous results were achieved with the second specific primer pair for apricot (2) and the specific primer pair for peach (3).

DNA Dilution Series and Spiked Raw Pastes. The developed PCR methods should be able to detect less than 1% of any contamination/adulteration in the raw paste matrix according to the zero-tolerance of other plant ingredients than almonds in German marzipan. To get a first indication on the sensitivities of the developed methods, dilution series (0.005−50 ng DNA per reaction) of the extracted DNA from each species were subjected to PCR experiments. Results for apricot, soy, lupine, and fava bean are given in Figure 4. In all cases, 0.05 ng of DNA (per reaction) could be clearly detected. Furthermore, the strong influence of coextracted matrix components on the PCR results has already been described.[26,27] Therefore, the analysis of real matrices was important to check the sensitivity of the developed methods. Hence, raw pastes were spiked with the possible contaminations/adulterations (see the Materials and Methods). On the basis of economic considerations, it is very unlikely that marzipan raw pastes contain more than one contaminant/adulterant. Therefore, spiking of marzipan samples with only one analyte seems to be sufficient in terms of realistic applications of the methods. An example of a duplex PCR for the detection of lupine is given in Figure 5. Regardless of the method (simplex or duplex PCR), the lowest spiked concentrations (0.1%) could be detected. Additionally, five marzipan raw pastes that were intentionally contaminated with different amounts of persipan raw paste (0, 0.2, 0.5, 1.0, and 2.0%) were provided by one of our project partners and analyzed as "unknown samples". The four samples containing apricot kernels could be clearly identified by the apricot specific PCR method.

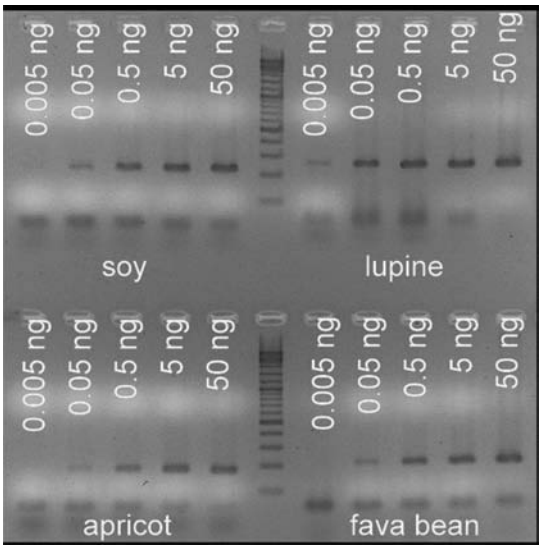

Figure 4. PCR results from DNA dilution series (ng DNA/reaction). DNA was isolated from seeds from soy, lupine, apricot, and fava bean and amplified with the specific primer pairs 1 (apricot), 5 (fava bean), 6 (soy), and 7 (lupine), respectively.

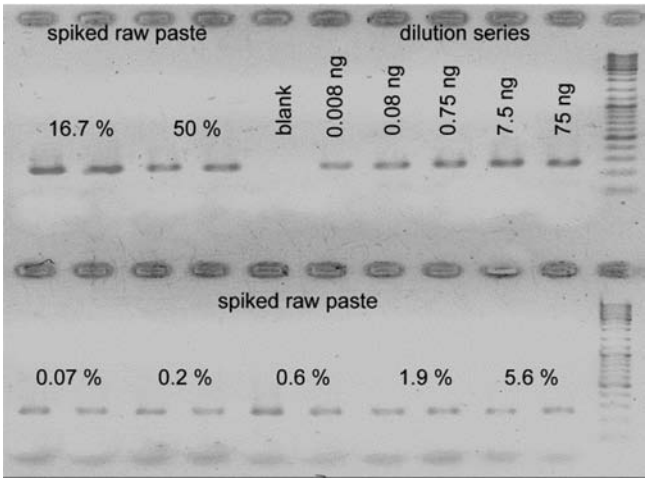

Figure 5. Results of duplex PCR experiments for the detection of soy and lupine. Lupine DNA (0.008−75 ng) or DNA isolated from marzipan raw paste spiked with different amounts of lupine powder (0.07−50%) was used as a template.

In conclusion, qualitative PCR methods for the detection of commercially or technologically relevant contaminations or adulterations in marzipan raw materials or raw pastes were developed. Method Ip + S was shown to be excellent for the isolation of high-quality DNA from raw pastes or kernels with little effort. In Figure 6, a flowchart is given for the analysis of marzipan raw pastes. The numbering of the primer pairs refers to Table 1. If no information about the possible contaminant is available, a screening with four duplex PCR reactions in parallel is suggested (Figure 6a), saving time and costs. All methods can be performed with the same thermocycler program. The primer pairs and reaction parameters were adapted to duplex reaction conditions, and different product sizes enable clear species identification. If only one single species has to be detected, a standard simplex PCR is recommended (Figure 6b). The presented

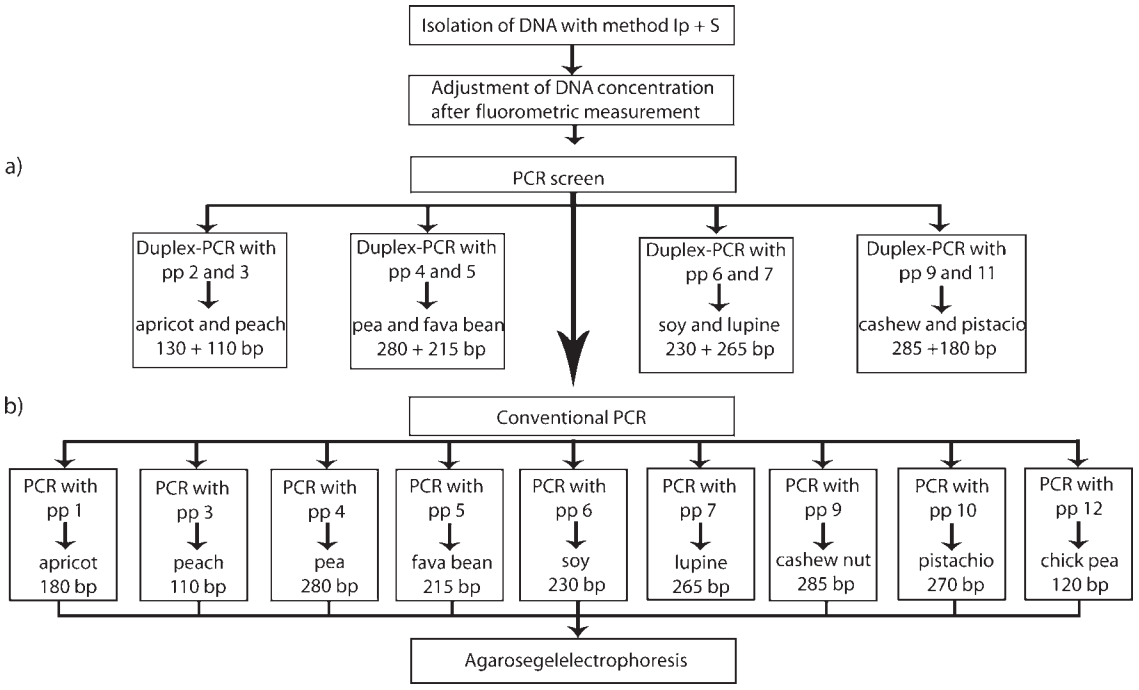

Figure 6. Flowchart for the detection of contaminations or adulterations in marzipan raw pastes. Sequences of the primer pairs (pp) are given in Table 1.

methods could serve as perfect tools for the screening of contaminations/adulterations [down to the low percentage range (<1%)] in routine quality analysis of marzipan raw pastes. Future work will focus on the development of real-time PCR methods for quantitative analysis.

■ ASSOCIATED CONTENT

Supporting Information. Figures of overview of the rDNA organization and the positions of the designed primer pairs and alignment of the rDNA sequences, alignment of rDNA regions from different *Prunus* species, specificity tests for the primer pairs 1 (apricot) and 3 (peach), and intraspecies uniformity of primer pair 1 (apricot). This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

CTAB, cetyl-trimethyl-ammonium-bromide; ENGL, European Network of GMO Laboratories; ITS, internal transcribed spacer; method Cp, precipitation with CTAB; method Cp + S, precipitation with CTAB and subsequent silica adsorption; method Ip + S, precipitation with isopropyl alcohol and subsequent silica adsorption; method S, silica adsorption; MYB, R2R3MYB transcription factor gene; NCBI, National Center for Biotechnology Information; PGIP, polygalacturonase inhibitor protein; rDNA, rDNA; SDS, sodium dodecylsulfate; uniprimers, universal primers

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