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Real-Time PCR Assays for the Quantitation of rDNA from Apricot and Other Plant Species in Marzipan

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Supporting Information

ABSTRACT: Marzipan or marzipan raw paste is a typical German sweet which is consumed directly or is used as an ingredient in the bakery industry/confectionery (e.g., in stollen) and as filling for chocolate candies. Almonds (blanched and pealed) and sugar are the only ingredients for marzipan production according to German food guidelines. Especially for the confectionery industry, the use of persipan, which contains apricot or peach kernels instead of almonds, is preferred due to its stronger aroma. In most of the companies, both raw pastes are produced, in most cases on the same production line, running the risk of an unintended cross contamination. Additionally, due to high almond market values, dilutions of marzipan with cheaper seeds may occur. Especially in the case of apricot and almond, the close relationship of both species is a challenge for the analysis. DNA based methods for the qualitative detection of apricot, peach, pea, bean, lupine, soy, cashew, pistachio, and chickpea in marzipan have recently been published. In this study, different quantitation strategies on the basis of real-time PCR have been evaluated and a relative quantitation method with a reference amplification product was shown to give the best results. As the real-time PCR is based on the high copy rDNA-cluster, even contaminations <1% can be reliably quantitated.

KEYWORDS: *marzipan, persipan, real-time PCR, absolute quantitation, relative quantitation, Prunus*

INTRODUCTION

Almond paste, which is made only from ground blanched pealed almonds and sugar (≤35%), is defined as marzipan raw paste according to the German Food Guidelines.[1] Persipan raw paste is a related product in which the almonds are replaced by ground blanched pealed apricot or peach kernels. Whereas marzipan raw paste is mostly processed to marzipan by further addition of sugar and is directly offered to the consumer in local markets, persipan raw paste is mainly used as ingredient in the bakery and confectionary industry. Most of the raw paste producers are small- and medium-sized companies producing both marzipan and persipan raw paste, in most cases on the same production lines. According to an agreement of the German raw paste producers, the unintended dilution of marzipan raw paste with apricot kernels has to be kept below 0.5%. An efficient cleaning of the production lines between marzipan and persipan raw paste production is therefore mandatory. Existing analytical methods based on the protein pattern or the α/γ-tocopherol ratio of almonds and apricot kernels do not allow a reliable quantitation at this low concentration range.[2−5] Published DNA-based methods for the detection and quantitation of apricots and almonds are based on sequence differences in a nonspecific lipid transfer protein gene,[6,7] in the lipid transfer protein gene 1,[8] or give no further details on primer design.[5] In the present work, the nuclear rDNA (rDNA) region was used for primer design. The rDNA is a high copy region[9] allowing detection and quantitation even in the lower concentration range.[10] Additionally, the rDNA exhibits heterologous and homologous sequence regions, which enable the design of the specific primer pairs as well of the reference primer pair on adjacent sequences. Similar efficiencies of both polymerase chain reactions (PCR) reactions seem to be likely. Besides the development of apricot and peach specific real-time PCR systems, specific primer pairs for plant species (pea, bean, lupine, soy, cashew, pistachio, and chickpea), which can be considered as possible adulterants for marzipan,[11] are also introduced.

MATERIALS AND METHODS

Sample Collection. Marzipan and persipan raw pastes and other plant seed materials are described in Bruening et al.[11] and are listed in Table 1. The seeds were lyophilized and ground to fine powder (sample powder).

Spiking of Marzipan Raw Pastes. For the spiking of marzipan raw pastes, an Unguator was used as described earlier.[11] Marzipan raw pastes were spiked with different amounts (0.01−64.0%, m:m) of plant sample powder (dry weight) or persipan (total weight). Additionally, a "blind study" was performed using five marzipan samples spiked with different amounts of persipan which were prepared and supplied by one of the project partners (Lübecker Marzipan-Fabrik v. Minden & Bruhns GmbH & Co. KG, Stockelsdorf/Lübeck, Germany).

Total Genomic DNA Isolation. DNA was isolated as described by Bruening et al. performing a 2-propanol precipitation step followed by a purification step using silica spin columns (Epoch Biolabs, Sugar Land, USA).[11] This method was shown to be excellent for the efficient isolation of high-quality DNA from raw pastes or kernels. DNA purity was determined photometrically (absorbance ratio 260 nm/280 nm), DNA concentration was determined fluorimetrically (excitation, 395

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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	5′	sequence 3′	target	product size [bp]
apricot	*Prunus armeniaca*	PA	FW	GGGCCGTCTCGGCGT	*AF318756* rDNA	64
			RW	TTGTACGCCCGGAAGGGTAT		
peach	*Prunus persica*	PP	FW	AGGGAAAGATGTTTGGTAGC	*EU155160* R2R3MYB	110
			RW	CACCACCACATCTTCTAGG		
pea	*Pisum sativum*	PS	FW	AACGACAAAATGCGTTCTCTT	*GQ260108.1* rDNA	89
			RW	CAGATGTGCTCAGAGCAAA		
fava bean	*Vicia faba*	VF	FW	TTCCACACCTCGGCTGA	*FJ212318.1* rDNA	119
			RW	GCGCTCAGAGCAAAATTTAAA		
soy	*Glycine max*	GM	FW	GGGAGGGGATGACCAC	*FJ980442* rDNA	113
			RW	CCGGGAGTCGCACTTAA		
lupine	*Lupinus* sp.	LS	FW	AGCCTCACAAGCAGTGC	*AF007481.1* rDNA	105
			RW	CAGGGTGTGGCGCCT		
cashew	*Anacardium occidentale*	AO	FW	GCGGCGCGTCAACGAA	*AB071690.1* rDNA	113
			RW	AATGAAAGGAGGCAACGCT		
pistachio	*Pistacia vera*	PV	FW	TGCCCGTGTGCCTCCA	*EF193089.1* rDNA	183
			RW	ATAATGAAAGAAGGCTACCCA		
chick pea	*Cicer* sp.	CS	FW	TTGAACACCTCGGCCCAA	*AB198904.1* rDNA	120
			RW	GTGCGCACATAACAAAGTTTT		
all species		reference	5.8SFW	GACTCTCGGCAACGGATATC	rDNA	115[11]
			5.8SRW	CGCAACTTGCGTTCAAAGACTCGA		
additional species used for specificity tests						
almond	*Prunus dulcis* *Prunus dulcis* var. Amara					
cherry	*Prunus sargentii* *Prunus avium* *Prunus salicina*					
plum	*Prunus domestica*					
hazel nut	*Corylus* spec.					

nm; emission, 425 nm) using SYBR Green I and a plasmid (pBluescript II SK(−), Fermentas GmbH, St. Leon-Rot, Germany, c = 10 ng/μL) dilution series for calibration. Absorbance and fluorescence were measured in a plate reader (SpectraMax M2, Molecular Devices, Sunnyvale, USA) in 96 or 384 well plates, respectively. DNA concentration was adjusted to 15 ng/μL, samples were stored at −20 °C until use.

Real-Time PCR. The primer sequences used in this study are listed in Table 1. Real-time PCR experiments were performed in an iQ5 multicolor real-time PCR thermocycler (Bio-Rad Laboratories GmbH, Munich, Germany) using the following temperature program: initial denaturation for 180 s at 95 °C; 40 cycles with denaturation for 20 s at 95 °C, annealing for 20 s at 59 °C (peach: 55 °C), elongation for 20 s at 72 °C, and fluorescence detection for 15 s at 80 °C; final elongation for 600 s at 72 °C. Subsequently, for melting curve analysis a temperature gradient (55−95 °C, 1 °C/s) was added. Reaction mixtures contained 1× DreamTaq buffer (Fermentas GmbH, St. Leon-Rot, Germany), 0.5 U DreamTaq polymerase (Fermentas GmbH, St. Leon-Rot, Germany), dNTP-Mix (0.2 mM each, Bioline GmbH, Luckenwalde, Germany), 625 nM of each primer (Life Technologies GmbH, Darmstadt, Germany), SYBR-Green I (1:32,000, Life Technologies GmbH, Darmstadt, Germany), and 75 ng of DNA template in a total volume of 20 μL. For quantitation, different strategies were used: (a) absolute quantitation using an external calibration curve (a-1) with a DNA dilution series or (a-2) with DNA from spiked marzipan samples, (b) absolute quantitation (cf. a-1 respectively a-2) using a normalization step (ΔC_T-method), (c) relative quantitation using a normalization step and an external reference sample (DNA-dilution or spiked marzipan sample) ($\Delta\Delta C_T$-method).

Agarose Gel Electrophoresis. For the analysis of PCR products, 3% agarose gels were run in TAE buffer (40 mM Tris/acetate, 2 mM EDTA, pH 8.2) at 150 V (Powerpac 1000, Biorad Laboratories Inc., Hercules, USA). The DNA was stained using a 0.01% ethidium bromide solution and visualized under UV light (Biostep, Felix 1040, Biostep GmbH, Jahnsdorf, Germany).

RESULTS AND DISCUSSION

Primer Design. Primers were designed manually on basis of the rDNA sequences of all plant species under study, except for peach. As can be seen in Figure S1 of the Supporting Information, specific primer pairs hybridize in the variable ITS1 (internal transcribed spacer 1) region, whereas the highly conserved 5.8 S region was used for the development of the reference primer pair. As the peach sequence showed no significant differences to the almond sequence, the specific peach primer pair "PP" was based on the sequence coding for a polygalacturonase inhibitor protein (PGIP). Caused by the production process, the DNA of raw pastes could be highly fragmented.[11] Hence, all primer pairs were designed to lead to PCR products ≤120 bp (except for pistachio). Primer pairs are listed in Table 1. Generally, the high genomic copy number of rDNA sequences allows a reliable quantitation even in the lower concentration range ≤1%.

PCR Optimization. PCR conditions were optimized in order to get a standardized PCR protocol for all plant species to be detected. Briefly, different concentrations of primers (50−600 mM), $MgCl_2$ (2−5 mM), SYBR Green I (1:10,000−1:64,000 dilution), and polymerase (0.1−2 U/reaction) were tested in real-time PCR experiments and were evaluated by the observed C_T values and dimer production (melting curve analysis). Furthermore, the annealing temperature could be unified for all PCR setups, except for peach. The final reaction

parameters are given in the Material and Methods section. As primer dimer formation cannot be avoided and has a negative influence on the quantitative interpretation of SYBR Green I real-time experiments, an additional detection point was included in the PCR program (80 °C/15 s, cf. Materials and Methods). It could be observed that the melting temperature of the primer dimers was between 75 and 80 °C, whereas the melting temperatures of the specific PCR products were >85 °C (cf. Figure 1). Consequently, at 80 °C, only the specific PCR products should be detectable with the SYBR Green system.

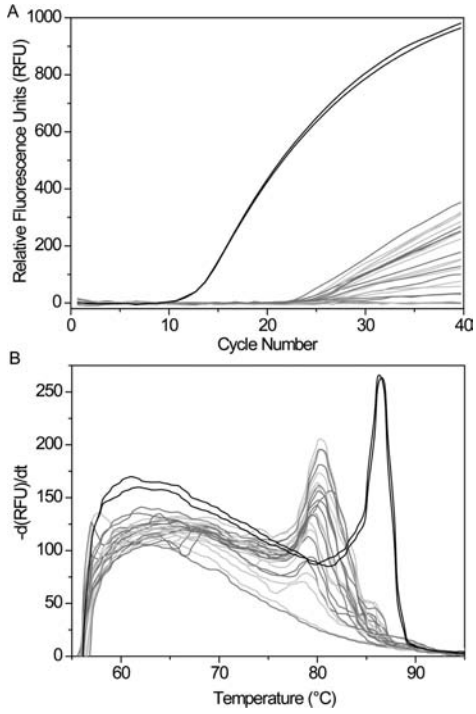

Figure 1. Real-time PCR experiments using the specific primer pair PA for apricot and DNA from all species listed in Table 1. (A) reaction kinetics and (B) melting curve analysis. Black, apricot DNA as template; gray, DNA from all other species. RFU, relative fluorescence unit.

Primer Specificity. The specificity of each of the manually designed primer pairs was tested using DNA from all species under study. In Figure 1 the results from the real-time experiments with the specific apricot primer pair "PA" and DNA from all species listed in Table 1 are exemplarily shown. The specific apricot amplificate formation can be clearly identified by an early C_T and a characteristic melting point. For all primer pairs, no cross-reactivity was observed. All extracted DNA was first checked by the reference primer pair for amplifiability.

Calibration Parameters. To keep an eye on possible intra- and interspecific variations of the rDNA copy number, different apricot (2 Turkish, 5 Chinese, 1 Syrian and 1 Iranian) and almond (4× Nonpareil (USA), 3× Butte (USA), 4× Mission (USA), 2× Carmel (USA), 1× California (USA), 1× Padre (USA), 1 Moroccan, 1 Turkish, 1 Spanish, and 1 Syrian (bitter almond)) samples were tested. All apricot cultivars showed identical C_T values for the target and the reference reaction when using 75 ng of DNA for PCR experiments. The same result was observed for the reference reaction of all almond cultivars. Consequently, for the samples under study, variations of the rDNA copy number can be ruled out. In subsequent experiments, linear working ranges and efficiencies were estimated using dilution series of DNA from all plant species and either the corresponding specific primer pair or the reference primer pair, respectively. The results for the calibrations with the specific primer pairs are given in Table 2. In all cases, a linear working range over at least 3 orders of magnitude could be achieved with efficiencies between 90.3% (peach) and 120.7% (cashew). For the reference primer pair, the same linear working ranges could be observed with efficiencies between 87.9% and 152.4% (data not shown). In a next step, the same experimental setup was performed using DNA from spiked marzipan raw pastes (0.01−64% spiking material). The linear working ranges as well as efficiencies are given in Table 3. In summary, it could be shown that even the matrix calibration experiments lead to satisfying reaction parameters, clearing the way for a reliable quantitation of plant contaminants in raw pastes.

Table 2. Linear Working Ranges and Efficiencies of Real-Time PCR Calibration Experiments with DNA Dilution Series

species	primer pair	linear working range [ng DNA/reaction]	efficiency [%]
apricot	PA	0.075−75	95.1
peach	PP	0.2−200	90.3
pea	PS	0.02−50	113.3
fava bean	VF	0.02−50	109.3
soy	GM	0.02−50	97.1
lupine	LS	0.02−50	94.6
cashew	AO	0.02−50	120.7
pistachio	PV	0.1−100	102.2
chick pea	CS	0.02−50	95.9

Table 3. Linear Working Ranges and Efficiencies of Real-Time PCR calibration Experiments with Matrix Samples

species	primer pair	linear working range [g/100 g marzipan raw paste]	efficiency [%]
apricot	PA	0.03−25.0	115.4
peach[a]	PP	0.05−34.0	110.7
pea	PS	0.03−18.6	135.9
fava bean	VF	0.01−42.5	95.3
lupine	LS	0.01−18.1	92.3
cashew	AO	0.13−27.7	107.8
pistachio	PV	0.13−64.0	86.5
chick pea	CS	0.01−8.3	107.7

[a]No reference primer pair available.

Quantitation of Persipan Amounts in Marzipan Raw Paste. For a reliable quantitation, different interpretation strategies were performed and compared. First, a matrix calibration (persipan in marzipan) was performed without and with normalization using the reference primer pair (Figure S2 of the Supporting Information). DNA from each of the seven calibration standards was isolated at five different days and was used for real-time PCR experiments in triplicates at the same day (5 × 3 = 15 C_T values for each calibration standard). For the normalization, the difference (ΔC_T) between the C_T mean values of the specific reactions (Figure S2A of the Supporting Information) and the reference reactions (Figure

S2B of the Supporting Information) were calculated and plotted against log(concentration persipan [%]) (Figure S2C of the Supporting Information).

$$\Delta C_T = C_T(\text{specific reaction}) - C_T(\text{reference reaction}) \quad (1)$$

As can be seen, the normalization step leads to significantly improved calibration parameters (efficiency, 108% → 103%; R^2, 0.979 → 0.997). This normalized absolute quantitation permits a compensation of inaccuracies of the fluorimetric determination of the DNA concentration.

To compare different quantitation strategies, four samples with different persipan amounts in marzipan were prepared by our project partners and were provided for a "blind study". For the quantitation of the apricot amount in these "unknown samples", a dilution series of apricot DNA in almond DNA (0.08%, 0.4%, 2%, 10%), DNA from a matrix reference sample spiked with known persipan amount (0.5% persipan in marzipan), and DNA from a pure marzipan sample was used (negative control). Real-time experiments were performed with the apricot specific primer pair PA and the reference primer pair in all cases. The "unknown samples" were subjected to real-time PCR experiments in duplicates, also using both primer pairs. The apricot amount was estimated (1) by absolute quantitation using the DNA dilution series, (2) by normalized absolute quantitation using the DNA dilution series, (3) by relative quantitation using the 0.4% apricot DNA in almond DNA sample as reference, and (4) by relative quantitation using the 0.5% persipan in marzipan sample as reference. For the relative quantitation, the ΔC_T of the sample was compared with the ΔC_T of the reference sample ($\Delta\Delta C_T$ method):

$$\text{apricot amount (\%)} = 2^{-(\Delta C_T(\text{sample}) - \Delta C_T(\text{reference sample}))} \quad (2)$$

The results were handed over to our project partners, who compared them to the real values. The results are given in Figure 2. The absolute quantitation led to the worst results confirming the importance of a normalization step, which clears inaccuracies in the determination of the DNA concentration. The other three methods show comparable retrieval rates. Even the lowest apricot amount of 0.2% could be reliably quantitated. The use of a reference sample instead of a calibration curve would be the most-favored procedure for a simple application and fast quantitation in routine analysis.

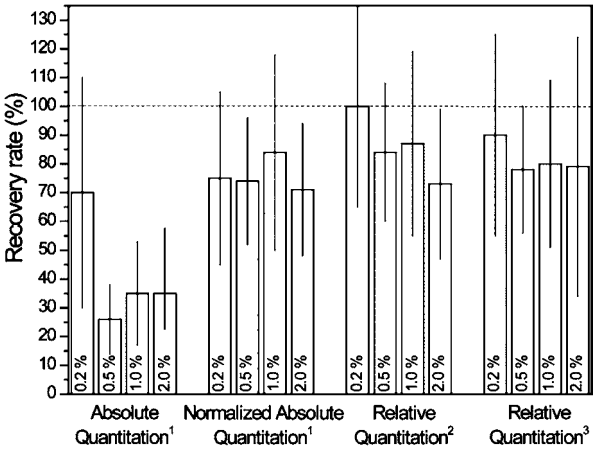

Figure 2. Quantitation of apricot amounts in "unknown" samples by different strategies. [1] Using an apricot DNA dilution series: 0.08%, 0.4%, 2%, 10% apricot DNA in almond DNA. [2] Using an apricot DNA dilution reference: 0.4% apricot DNA in almond DNA. [3] Using a matrix reference: 0.5% persipan in marzipan) and comparison to the real value (0.2%, 0.5%, 1.0%, and 2.0%).

A recommended complete procedure is given as follows: (i) The DNA isolation should be performed according to Bruening et al..[11] (ii) DNA-concentration should be quantitated fluorimetrically. (iii) 75 ng (or at least an identical amount for all samples) of DNA should be used as template for a PCR in a total volume of 20 μL. (iv) The concentration of the contaminant could roughly be estimated by a DNA dilution series. For the approximate assessment, even an adequate calibration curve stored in the database is sufficient. (v) For a proper quantitation, the use of a standard (DNA mixture or matrix standard) within the same concentration range as estimated in the sample is recommended as shown above. The quantitation should be carried out using the described $\Delta\Delta C_T$ method with the C_T values of experiments with the specific primer pair as well as the reference primer pair. As the reference primer pair as well as the specific primer pairs hybridize on the rDNA (cf. Figure S1 of the Supporting Information, except for peach), it is assumed that reaction efficiencies are the same for PCRs with the specific primer pair and the reference primer pair. Additionally, with the use of matrix reference samples or dilution series of apricot DNA in almond DNA, matrix effects affecting the reaction efficiencies were considered at the best. However, in cases where different reaction efficiencies are expected, different methods are described to perform an efficiency correction.[12−16] It would also be possible, to calculate the apricot content relatively to the almond content by the implementation of an almond specific primer pair.

For a consistent quantitation, the use of a (certified) reference material with a defined amount of plant contaminant in marzipan raw paste is recommended.

For many applications, real-time PCR detection systems with a species specific fluorescence, e.g., Taqman probes are used. However, for the design of a specific Taqman probe, sequence differences between the primer hybridization sites are necessary. As can be seen in Figure S1 of the Supporting Information, due to the close relationship of all *Prunus* species under study, the design of an apricot specific probe would not be possible. Thus, the authors would recommend the use of fluorescently labeled primers if a species specific fluorescence is desired. Future work will focus on the upgrade of the developed methods using the Plexor system (labeling of species specific primers).

■ ASSOCIATED CONTENT

Supporting Information

Alignment of the rDNA (ITS1 and 5.8 S regions, indicated by arrows) sequences from fava bean (accession number = AN FJ212318.1), pea (AN GQ260108.1), chickpea (AN AB198904.1), lupine (AN AF007481.1), soy (AN FJ980442.1), cashew nut (AN AB071690.1), pistachio (AN AY677201.1), apricot (AN AF318756.1), peach (AN DQ006276.1), and almond (AN HE806329.1). Specific primer binding sites are marked by arrows and hybridization sites of the universal primers are highlighted by gray boxes. Calibration curves resulting from real-time PCR experiments with five marzipan samples spiked with different amount of persipan. (A) C_T values using the apricot specific primer pair PA. (B) C_T values using the reference primer pair. (C) Normalized calibration curve using $\Delta C_T = C_T$ (primer pair PA) − C_T

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(reference pair). Each concentration was measured in 5-fold replicates, standard deviations are shown as whiskers. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

AN, accession number; ITS1, internal transcribed spacer 1; PCR, polymerase chain reaction; PGIP, polygalacturonase inhibitor protein

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