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ORIGINAL PAPER

Duplex digital droplet PCR for the determination of apricot kernels in marzipan

René Köppel[1] · Regula Ledermann[1] · Franziska van Velsen[1] · Arthika Ganeshan[1] · Patrick Guertler[2]

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Abstract
Marzipan is a mixture of almonds, sugar, and water. Almonds can be replaced by apricot kernels, which results in a similar product called persipan. Depending on the prices of almonds, profit can be maximized using apricot kernels instead of almonds. If not specified on the product, this kind of substitution is illegal and is prosecuted by official food control authorities. Likewise, also commercial buyers would like to know which product they bought. Real-time PCR systems for the determination of apricot DNA are already available, however, real-time PCR requires the use of external standards, which have a direct impact on the accuracy of the measurement. Currently, such standards are not available. In contrast to real-time PCR, digital PCR does not require external standards and exhibits a smaller measurement uncertainty as shown in recent publications. Therefore, we developed a duplex droplet digital PCR system to measure the proportion of apricot in marzipan without the use of external standards. We present validation data and results of an international proficiency test, underlining the applicability of this system.

Keywords Duplex · Apricot · Marzipan · Persipan · Digital PCR

Introduction

Marzipan is made from almonds (*Prunus dulcis*), whereas persipan is made from apricots (*Prunus armeniaca*). Both plants belong to the family *rosacea* and genus *prunus* and are very close relatives to cherries, plums, mirabelles, and peaches. The kernels look very similar. Bitter almonds (Prunus amara) are a genetic variation of the sweet almond trees (*Prunus dulci*s) and contain a higher proportion of cyanides. Their proportion should be kept low as high consume of cyanide leads to intoxication. The close relationship between almonds and apricots makes it difficult to distinguish these species from each other. Cross-breeding between the different *Prunus* cultivars even increases these difficulties. In case of processed products like marzipan/persipan, it is impossible to distinguish these cultivars from each other by eye, chemical or physical methods. But this is possible by PCR and such PCR-systems have already been published [1–6]. They exploit minor sequence variations between the two cultivars. In principle, kinds of marzipan can also be produced from other prunus kernels or nuts. However, the aroma may differ from marzipan and is therefore no source of fraud of marzipan.

For quantification, real-time PCR (qPCR) is suitable, but exhibits often an intralaboratory measurement uncertainty of 30%. This makes it impossible to prosecute minor fraud. Furthermore, it is highly dependent on the availability of reference material with defined almond/apricot concentrations, which has to be used as an external calibrator for an absolute quantification. Digital PCR has gained importance in recent years. Several applications in the field of food analysis showed that results generated by digital PCR may have a smaller measurement uncertainty compared to qPCR [7–9]. Therefore, we decided to develop a digital PCR for this purpose. We used primers and probes from proven specific qPCR systems [2, 10] and adapted them for droplet digital PCR on the QX200 System from BioRad. The QX200 allows the measurement of two fluorescence signals at once, one for FAM labeled probes and one for e.g., HEX labeled probes. We developed a duplex dPCR system for specific

✉ René Köppel
rene.koeppel@kl.zh.ch

1 Food Control Authority of the Canton Zürich, Zürich, Switzerland

2 Molekularbiologie (LH), Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit, Veterinärstr. 2, 85764 Oberschleißheim, Germany

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apricot DNA and one for prunus species like almonds in general. Here we present this duplex dPCR system and its performance during validation in a proficiency test and when analyzing routine samples.

Materials and methods

Marzipan samples

Almond and apricot kernels were bought from local food suppliers. A wide scale of low price and high price marzipan/persipan and products containing a mixture of marzipan and persipan were bought from retailers and confectioneries. Pure marzipan and persipan products were used to produce defined mixtures to generate matrix adapted reference material.

Production of marzipan and persipan samples for the proficiency test

To produce defined material for the proficiency test we produced also marzipan/persipan mixtures. Marzipan/persipan was produced according to a common recipe using different proportions of almond and apricot kernels. The almonds and apricots were purchased as unground single kernels unpeeled. Before milling they were cooked for 10 min and peeled. Milling (Grindomix Nr. 2174, e.g., 7 × time 0.02/speed 7.0 × 1000 rpm) was done until a fine powder was gained using liquid nitrogen. To 100 g of milled almond/apricot mixture 80 g of sugar was added and milled again. Then 2 g of water was added. This mixture was kneaded until a dough arise. From this dough (marzipan) the DNA was isolated.

DNA extraction

DNA extraction was performed using the column-based Wizard Plus Miniprep DNA purification system (Promega, Madison, USA). 200 mg of ground sample material was extracted, and the DNA was eluted into 50 µl elution buffer according to the supplier's manual. The concentration was determined spectrophotometrically and adjusted to 20 ng/µl by dilution using PCR-grade water.

Primers

Primers and hydrolysis-probes have already been published [2, 10]. All primers and hydrolysis probes were synthesized by Microsynth AG, Balgach, Switzerland. The sequences and sources of primers and probes as well as the labelling are listed in Table 1. To achieve an optimal fluorescence signal for both systems we titrated the primer and probe concentrations as the standard concentrations lead to heavy rain.

Droplet digital PCR procedure

5 µl of DNA (10 ng/PCR) was added to 17 µl of dPCR reaction mix, consisting of 11 µl of 2 × QX200 dPCR Supermix for probes (Bio-Rad Laboratories, USA) and 6 µl of primers. Primer sequences and optimized concentrations are listed in Table 1. 20 µl of this mixture was transferred to the DG8 droplet generator cartridge (Bio-Rad Laboratories, USA) and droplets were generated with the QX200 Droplet Generator (Bio-Rad Laboratories, USA) according to the manufacturer's protocol. Droplets were transferred to PCR tubes and PCR was performed on a Mastercycler Gradient (Eppendorf, Germany) according to the following cycling protocol: enzyme activation step at 95 °C for 5 min, followed by 50 cycles at 95 °C for 30 s and 60 °C for 60 °C s, followed by a signal stabilization step at 4 °C for 5 min and 90 °C for 5 min, final hold at 4 °C. Droplet counting was performed on the QX200 Droplet Reader (Bio-Rad Laboratories, USA). For droplet analysis, the QuantaSoft Software (Bio-Rad Laboratories, USA) was used. Figure 1 shows a typical readout of the droplets.

Table 1 Duplex dPCR system for the simultaneous determination of almond and apricot DNA

Primer	Final concentration	Sequence (5′–3′)	Amplicon size	Accession no./literature
Apricot specific primers and probe				
AprLF1	0.9 µM	TTA TCT GCG TCA AGC TCA CA	121 bp	[2] AP019302.1
AprLR1	0.9 µM	GAT CAT TGA AAT TTT GGT CTA GC		
AprLS1 Fam	0.125 µM	FAM-TGT TGA CAA TTA AAT GCG GAA TAT T-BHQ1		
Almond specific primers and probe				
Madl F	0.3 µM	CCT AGC GGA GGA TCC ATC ATC	129 bp	[10] AM493970
Madl R	0.3 µM	GTA GGT CTC AAT GAG CTT GAA GAG		
Madl Joe	0.25 µM	JOE-AGA GCA CCA GCC ACT ACC ACA CCA-BHQ1		

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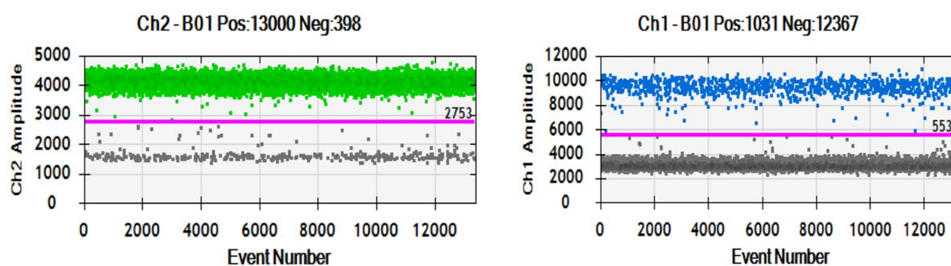

Fig. 1 Example of a typical probe dPCR picture, generated with the QuantaSoft Software analysis tool (Bio-Rad Laboratories, USA) with positive and negative populations in each channel (left Fam-channel for apricot, right hex-channel for almonds)

Specificity

To test the specificity of the presented dPCR method, DNA of a wide range of plants was analyzed. To save costs and material, four to ten samples of the related/non-related organisms where pooled, per reaction. If an unexpected positive signal would occur, the single samples of the correspondent pool would be subjected to re-analysis to assess the exact species cross reacting.

DNA of the following organisms was tested for cross-reactivity

Animal DNA (10–25 ng total DNA per reaction): sheep (*Ovis aries*), pig (*Sus scrofa*), horse (*Equus caballus*), goat (*Capra aegagrus*), buffalo (*Bubalis bubalus*), cow (*Bos taurus*), chicken (*Gallus gallus*), men (*Homo sapiens*), cod (*Gadus morhua*).

Plant DNA (10–25 ng total DNA per reaction): onion *(Allium cepa)*, carrot (*Daucus carota sativus*), parsley (*Petroselinum crispum*), nutmeg (*Myristica fragrans*), cinnamon (*Cinamomum verum*), lentil (*Lens culinaris*), peas (*Pisum sativum*), mung beans (*Vigna radiata*), chickpeas (*Cicer arietinum*), lupine (Lupinus), peanut (*Arachis hypogaea*), beans (*Phaseolus vulgaris*), borlotti beans (Vicia faba), garlic (*Allium sativum*), celery (*Apium graveolens*), pepper white (*Piper nigrum*), aniseed (*Illicium anisatum*), soy (*Glycine max*), wheat (*Triticum aestivum*), rice (*Oryza spp*), maize (*Zea mays*), potato (*Solanum tuberosum*), cinnamon (cinnamon spp), cloves *(Syzygium aromaticum)*, curcuma (*Curcuma longa*), parsley *(Petroselinum crispum),* aniseed anise (*Illicium anisatum*), coconut *(Cocos nucifera)*, chive *(Allium schoenoprasum), lavender (Lavandula angustifolia),* saffron *(Crocus sativa),* curry leaves *(Bergera koenigii)*, thyme *(Thymus vulgaris),* strawberry *(Fragaria ananassa),* rasberry *(Rubus spp).*

Genus prunus*:* plum *(Prunus prunus),* mirabelle *(Prunus domestica subsp. Syriaca)* peach *(Prunus persica),* bitter almond *(Prunus amara),* cherry *(Prunus avium).*

Calculation of apricot-DNA content and elaboration of a conversion factor (*F*)

DPCR is an endpoint PCR. The QX200 dPCR system allows the measurement of two different fluorescent signals. Amplification of apricot specific DNA is measured in the FAM channel, whereas amplification of prunus DNA is measured in the HEX/VIC channel. The individual copy number per analyte (apricot and prunus) can be determined using the ration of positive and negative partitions. The proportion of the copy numbers gained by droplet dPCR can be different from the true values weight per weight.

Therefore, a conversion factor (*F*) has to be determined.

Relative proportion of the apricot DNA in percent can be calculated by a simple formula:

$$\text{ApricotDNAratio\%} = \frac{F \times 100\% \times \text{apricot} - \text{DNA}}{\text{prunus} - \text{DNA}}$$

apricot-DNA as copies/µl, prunus-DNA as copies/µl.*F* conversion factor to convert DNA proportions to weight to weight proportion.

Calculation of the factor: apricot DNA ratio as known % of apricot in the whole persipan as % w/w, apricot-DNA as copies/µl, prunus-DNA as copies/µl

$$F = \frac{\text{ApricotDNAratio\%} \times prunus - \text{DNA}}{100\% \times \text{apricot} - \text{DNA}}$$

Example with real dataset

Apricot DNA ratio as known % of apricot in the whole persipan as % w/w: 10%, apricot-DNA: 129copies/µl, prunus-DNA: 632 copies/µl

$$F = 0.489 = \frac{10\% \times 632}{100\% \times 129}$$

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Table 2 To determine the intralaboratory performance, marzipan/persipan was produced by mixing of pure persipan with pure marzipan (purchased) in the different proportions w/w

	1% Persipan in marzipan	3% Persipan in marzipan	10% Persipan in marzipan	30% Persipan in marzipan	50% Persipan in marzipan Matrix S3	20% Persipan in marzipan Matrix S4
Mean value % (w/w)	1.1	3.6	9.9	24	41	25
Precision ± %	12	11	5	3	5	4
Accuracy ± %	14	19	1	20	9	26
Measured uncertainty ± %	18	22	5	20	10	26

The DNA was isolated and taken to assess the LOD, precision and accuracy. The values represent mean values from ten single experiments at Persipan contents of 1–50%. To convert the droplet concentrations into w/w contents a conversion factor of 0.5 was applied for apricot DNA for all samples. Matrix S3 and S4 were made from different pure marzipan and persipan material (purchased)

Determination of the factor

These calculations were done several time, like described in Table 2. The average factor was used for all these samples and was assigned as final factor.

Data for intralaboratory sensitivity, precision, and measurement

We purchased unadulterated marzipan and persipan (verified by qPCR) and mixed them to produce combinations with known proportions. After DNA isolation of these combinations we determined a conversion factor. Such was done by comparing the average difference between true value weight by weight and proportion of the copies/µl measured by dPCR. The conversion factor leading to minimal difference between these values was assigned as conversion factor.

Proficiency test

Do assess the influence of different equipment and the robustness of the dPCR system, a proficiency test was performed. Eight samples had to be analyzed in triplicates (3 separate runs). The samples contained in house produced (see above) marzipan with different additions of persipan (see Table 3) and were analyzed by eight laboratories. Four laboratories applied the QX200 of Bio-Rad, one laboratory the QX100, two laboratories used the Rain-Dancer platform and one laboratory used the Naica-system by Stilla Technology.

Table 3 Marzipan/persipan was produced according to a common recipe using different proportions of almond and apricot kernels

Samples of the proficiency test (persipan content) (w/w)	Mean measured persipan content	RSD %	Deviation %	MU %
Apricots 0%	0.1			
Apricots 1%	1.2	21.9	14.4	26.2
Apricots 3.2%	3.7	22.4	12.9	25.8
Apricots 10%	8.7	7.8	14.9	16.8
Apricots 50%	53.5	5.7	6.6	8.7
Apricots 90%	88.0	5.4	2.2	5.9
Apricots 96%	99.4	6.8	3.4	7.6
Apricots 100%	104.6	8.8	4.4	9.8

This marzipan/persipan samples (see Table 3) were sent to seven Laboratories. They isolated the DNA applying their individual method and determined the content of the DNA. Per Assay 10 ng of DNA was used. These results were collected and taken to assess the interlaboratory LOD, precision (RSD), accuracy (deviation) and measurement uncertainty (MU). The MU is not extended and counts for a single measurement

Results and discussion

Specificity

None of the pooled non-target samples showed an unspecific amplification using our duplex dPCR method. The cross-reactivity test therefore fulfilled the requirements. In addition, ten none template controls (NTC) were analyzed in each single run during the method validation. None of them resulted in an amplification signal.

Genus Prunus

DNA of cherry exhibited a 1% cross-reactivity in the apricot-system. DNA of plum was not detected by both systems. Hence, analyzing samples of marzipan/persipan is not biased by these findings.

Intralaboratory sensitivity, precision, and measurement uncertainty

The data for intralaboratory sensitivity, precision, and measurement uncertainty are compiled in Table 2. All samples were detectable and quantifiable within the assigned dilution down to a proportion of 1% of persipan content w/w. As a

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tolerance of 1% is accepted for such samples. A maximum measurement uncertainty or 26% was observed for a single determination (not extended).

The exact LOD was not determined (e.g., CCβ-method). We estimate the LOD to be below 1% persipan content. At 1% proportion of persipan the measurement uncertainty still is acceptable (18%) indicating that this point is still in the quantification range. A conversion factor of 0.5 was applied to convert proportion of copies/µl to w/w. The factor was the same for all used materials and combinations. The material consisted of purchased marzipan and persipan, where the exact content of e.g., water and sugar is unknown. Therefore, we speculate that the factor may be matrix specific.

Proficiency test

Marzipan/persipan samples were prepared and sent to eight laboratories to assess the interlaboratory test sensitivity, precision and measurement uncertainty (see confirmations). A total of seven data sets were collected and the results are summarized in Table 3. It is noteworthy that the data were generated by three different digital PCR platforms: QX100/200, RainDancer and Naica. One laboratory used their adapted in-house method and therefore could not be included in the results. The raw data (not shown) differed greatly between the laboratories. The values for copies/ul ranged from more than 15,000 to 150 cp/µl for apricot or almond DNA. This was partly to be expected, since the DNA was isolated according to the laboratory's individual protocol. After the calculation, the final result was well comparable between the laboratories. The measurement uncertainty was 26% for 1% persipan in marzipan. In contrast to the findings in the intralaboratory validation, no conversion factor was required. This may be an indication that there is a difference between mixing material of the same production and mixtures of marzipan/persipan from different productions (as in the intralaboratory validation).

Conclusion

The proposed duplex digital droplet PCR was able to detect and determine the proportion of apricot DNA in marzipan. The LOD is estimated to be 1% persipan content. A maximum intralaboratory measurement uncertainty of 26% was observed for a single determination over different matrixes.

Using self-produced mixtures containing persipan and marzipan, which were produced identically, no conversion factor was required to adjust the accuracy. The measured values can be directly converted into w/w values. In this case, the maximum interlaboratory measurement uncertainty at 1% persipan in marzipan was also 26%. This interlaboratory measurement uncertainty includes the DNA isolation and three different platforms (QX100/200, Rain Dancer and Naica), which is remarkable. The absolute numbers of positive droplets varied considerably between the laboratories (not shown). After calculation of the proportions, this difference disappeared. Reasons for the differences may be different DNA isolation methods, different DNA determination methods and the use of different platforms. To elucidate the true reason, leading to the differing raw data, further experiments are required. However, the final proportion is not biased, and the system therefore showed to be fit for purpose.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Compliance with ethics requirements This article does not contain any studies with human or mammalian subjects.

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