



Analytical Methods

Detection of mandarin in orange juice by single-nucleotide polymorphism qPCR assay

Miriam Aldeguez^{a,c}, María López-Andreo^b, José A. Gabaldón^{a,c}, Antonio Puyet^{b,*}^a Centro Tecnológico Nacional de la Conserva, Molina de Segura, Murcia, Spain^b Departamento de Bioquímica y Biología Molecular IV, Facultad de Veterinaria, Universidad Complutense de Madrid, 28040 Madrid, Spain^c Departamento de Ciencia y Tecnología de Alimentos, Universidad Católica San Antonio de Murcia (UCAM), Murcia, Spain

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ABSTRACT

A dual-probe real time PCR (qPCR) DNA-based analysis was devised for the identification of mandarin in orange juice. A single nucleotide polymorphism at the *trnL-trnF* intergenic region of the chloroplast chromosome was confirmed in nine orange (*Citrus sinensis*) and thirteen commercial varieties of mandarin, including *Citrus reticulata* and *Citrus unshiu* species and a mandarin × tangelo hybrid. Two short minor-groove binding fluorescent probes targeting the polymorphic sequence were used in the dual-probe qPCR, which allowed the detection of both species in single-tube reactions. The similarity of PCR efficiencies allowed a simple estimation of the ratio mandarin/orange in the juice samples, which correlated to the measured difference of threshold cycle values for both probes. The limit of detection of the assay was 5% of mandarin in orange juice, both when the juice was freshly prepared (not from concentrate) or reconstituted from concentrate, which would allow the detection of fraudulently added mandarin juice. The possible use of the dual-probe system for quantitative measurements was also tested on fruit juice mixtures. qPCR data obtained from samples containing equal amounts of mandarin and orange juice revealed that the mandarin target copy number was approximately 2.6-fold higher than in orange juice. The use of a matrix-adapted control as calibrator to compensate the resulting C_T bias allowed accurate quantitative measurements to be obtained.

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1. Introduction

Sensitive and reliable methods are increasingly needed for authentication of food products, which may help to prevent either accidental or fraudulent substitutions during food manufacturing or commercialization. Inadequate cleaning of food process equipment and containers may result in accidental substitutions. Fraudulent substitution commonly arises as a result of the deliberate extension of products with cheaper additives. Common adulteration in fruit juices may be caused by water addition or excessive dilution of concentrates, sugar or pulp wash addition, the use of colorants or other additives to give the impression of another juice, which sells for a higher price, or mixing with lower priced juices, or the sale of pasteurized or juice from concentrate as fresh squeezed juice (US Food and Drug Administration, 2011). Misrepresentation or substitution of the fruit species in juices may be detected by traditional chemical analysis such as high-performance liquid chro-

matography (HPLC) or gas chromatography (del Castillo, Caja, & Herraiz, 2003; Hammond, 2001). The use of Fourier transform infrared (FTIR) spectroscopy or piezoelectric sensors have also been proposed as analytical tools (Kuchmenko, Lisitskaya, & Bobrova, 2008; Vardin, Tay, Ozen, & Mauer, 2008). DNA-based polymerase chain reaction (PCR) methods are currently used for food species authentication in a wide variety of meat, fish or milk products (Asensio, 2007; Ballin, Vogensen, & Karlsson, 2009; Calvo, Osta, & Zaragoza, 2002; Intrieri, Muleo, & Buiatti, 2007). However few reports have shown the applicability of these approaches to fruit juice (Han et al., 2012; Mooney, Chappell, & Knight, 2006; Scott & Knight, 2009), and no real-time PCR-based method for the differential detection of closely related species as orange (*Citrus sinensis*) and mandarin (*Citrus reticulata*) has been reported.

Mandarin may be added to improve the quality of orange juice. In addition, seasonal surplus of mandarin, or fruits unsuitable for retail, may be channeled to the manufacturing of concentrates. Due to the low demand of pure mandarin juice, these concentrates may be used in the formulation of other fruit juices. Although these additions are not unlawful, they must be declared to prevent fraud.

The differentiation of *C. sinensis* and *C. reticulata*/*Citrus unshiu* by DNA-based methods is not straightforward, as the species are

Abbreviations: MGB, minor groove binding; FC, juice from concentrate; NFC, juice not from concentrate; Tm, melting temperature.

* Corresponding author. Tel.: +34 913943827; fax: +34 913943824.

E-mail address: apuyet@vet.ucm.es (A. Puyet).

closely related and few usable polymorphisms in either species are available for differential screening. Any candidate species-specific sequence should be confirmed in a wide variety of orange and mandarin cultivars and hybrids that are currently commercialized. An assay for mandarin detection in orange juices, based on the formation of heteroduplex DNA after amplification of a chloroplast *trnT-trnL* intergenic spacer containing an eight base-pair indel, has been previously reported (Mooney et al., 2006). The method makes use of polyacrylamide gel electrophoresis analysis for the detection of heteroduplex, which is time-consuming and laborious. Although the assay has subsequently been adapted to a Laboratory-on-a-Chip capillary electrophoresis system (Scott & Knight, 2009) and, more recently, to Denaturing High Performance Liquid Chromatography (DHPLC) (Han et al., 2012), it still relies on a skilled procedure.

qPCR-based methods in food analysis are well developed for the detection and quantitation of genetically modified organisms (Marmioli et al., 2008). A wide variety of qPCR methods for the detection of species in foods and food products have also been reported (Ballin et al., 2009; Laube et al., 2010; Puyet & Bautista, 2009). Some of these approaches are likewise potentially suitable for the quantitation of target materials in foods containing different species (Eugster, Ruf, Rentsch, & Koppel, 2009; Laube, Zagon, & Broll, 2007; López & Pardo, 2005). Most of these approaches make use of qPCR detectors based on conserved primers and species-specific fluorescently labeled hydrolysis probes targeting highly divergent sequences. qPCR technologies may, however, also be adequate for detection of closely-related species in foods. Single nucleotide polymorphisms (SNP) genotyping is routinely performed by qPCR (Gibson, 2006) and can be adapted as a tool for species detection in food products. SNP detection by qPCR, however, requires more stringent reaction conditions than those used in the detection of highly divergent targets to avoid cross-amplifications leading to the appearance of false positives. Such conditions may be difficult to establish in food products due to the heterogeneity of materials and processing methods used in their manufacture.

In this report, a single-nucleotide polymorphism at the chloroplast chromosome *trnL-trnF* intergenic region has been used to differentiate *C. sinensis* from both *C. reticulata* (mandarin) and *C. unshui* (Satsuma mandarin) DNA. The specificity of the polymorphism was assessed in a wide selection of common orange and mandarin cultivars commercialized in Spain, and a real-time PCR (qPCR) assay was devised for the detection of mandarin in orange juices. qPCR technology allows the development of relatively simple analytical methods, both qualitative and quantitative. Using a dual-probe approach, a single-tube reaction containing species-specific probes and shared primer oligonucleotides allowed the detection of mandarin both in juices not from concentrate (NFC) and juices prepared from orange and mandarin concentrates (FC). In a recent report (López-Andreo, Aldeguer, Guillén, Gabaldón, & Puyet, 2012), we have shown feasibility of using single matrix-adapted standards to obtain reliable quantitative data by qPCR. Here, we have tested this approach for the acquisition of quantitative data in orange/mandarin mixed juices.

2. Materials and methods

2.1. Orange, mandarin and juice samples

Leaves from orange and mandarin cultivars, shown in Table 1, were kindly provided by the Murcian Institute for Agricultural and Food Research and Development (IMIDA).

Fresh orange and mandarin juices were prepared from Valencia Late and Fortune cultivars, respectively. Juicing was carried out in

Table 1

Orange and mandarin cultivars used in this work.

Species	Cultivar	Id.#
<i>Citrus sinensis</i>	Newhall	1
	Navelate	2
	W. Navel	3
	Vernalina	4
	Santomera	5
	Sanguinelli	6
	Valencia Late	7
	Lanelate	8
	Salustiana	9
<i>C. reticulata</i>	Orogrande	12
	Marisol	13
	Arrufetina	14
	Clemenules	15
	Oronules	16
	Hernandina	17
	Fortune ^a	19
	Fedele	20
	Tardino	21
	Seedless common mandarine	22
<i>C. unshui</i>	Okitsu	10
	Clauselina	11
Hybrid <i>C. reticulata</i> x Tangelo (<i>C. reticulata</i> x <i>C. máxima</i>)	Nova	18

^a Hybrid of two *C. reticulata* varieties, "Fina" and "Dancy".

our lab by squeezing raw samples or prepared from concentrates provided by JUVER SLU, Murcia, Spain. When juice was from concentrates (65% w/w), samples were reconstituted with water up to 12% w/w. Mixed juices containing different proportions of mandarin were prepared by mixing the corresponding volumes of fresh, not from concentrate juices (NFC series) or reconstituted concentrates (FC series). Commercial fruit juices were purchased in local markets.

2.2. DNA extraction

To obtain DNA from leaf tissue, approximately 5 mm² of leaf were excised with a cork borer and grinded. Total DNA was isolated using UltraClean® Plant DNA Isolation Kit from MoBio following manufacturer's instructions. DNA was resuspended in 50 µL of distilled water and the concentration was determined after staining with Picogreen dsDNA quantification reagent (Molecular Probes) by comparing the fluorescence with that from DNA standards of known concentration using a Perkin-Elmer LS-50B fluorescence spectrophotometer.

2.3. DNA sequencing

Sequencing of the 50990–51720 bp section of chloroplast DNA, according to the reference Genbank entry NC_008334, was carried out using the DNA obtained from leaves from all cultivars. The primer pairs LF2: 5'-GCTTCTATCGAATATCGAACTC/LR2: 5'-CTTGAA CCCTCAGATTTC, and LF3: 5'-GAAATCGTGAGGGTTCAAGTC/LR3: 5'-GATTTGAACTGGTGACACGAG were used both for amplification and sequencing of the resulting DNA fragments at the Genomics Service of the Universidad Complutense, using an Applied Biosystems 3730 DNA sequencer. DNA similarity searches were carried out using BLAST at the NCBI.

2.4. Detection and quantification PCR assays

DNA detection was performed by amplification using the real-time sequence-detection system 7000 from Applied Biosystems.

Primers used were: CTR02F: 5'CTCTTATCACAAGCCGTATGGTCTATACGATATATGTAGA/CTR02R: 5'TATGAGCAATAATATGGATTGGGAATCATTAACG, which hybridize both orange and mandarin, and the specific MGB probes were: Sin3FAM: (FAM)-5'CTTTGAGCAAGGA-(TAMRA) for orange and Ret2VIC: (VIC)-5'CACCTTTGATCAAGGAATC-(TAMRA) for mandarin.

Reactions were carried out in duplicate using 96-well plates. The 25 μ L reaction mixtures contained 12.5 μ L of TaqMan[®] Universal PCR Master Mix, No AmpErase[®] UNG, 300 nmol/L of each primer oligonucleotide and 200 nmol/L TaqMan[®] MGB probes (Sin3FAM for orange and Ret2VIC for mandarin) and 2 μ L of purified DNA. qPCR conditions were: enzyme activation, 10 min at 95 °C followed by 40 cycles of denaturation at 95 °C for 15 s, and re-annealing polymerization step at 59 °C for 1 min.

The method to quantify the proportion of mandarin in orange/mandarin binary mixtures was adapted from a previously published procedure (López-Andreo et al., 2012). Briefly, serial dilutions containing 0.32–200 pg of DNA obtained from leaves were amplified with the dual-probe qPCR to obtain the corresponding standard curves by plotting C_T vs. log DNA, and the slope m and intercept b were calculated for both species. qPCR amplifications of DNA from juice binary mixtures were carried out in duplicate and the mean C_T , obtained with the Sin3FAM probe (C_{Tsin}) were interpolated in the corresponding standard curve to obtain the orange DNA content in the sample (N_{sin}). The juice NFC mixture containing 50% mandarin was used as matrix reference to calibrate the differences in DNA content for equal amounts of orange and mandarin. This difference, D , was defined as the number of cycles that needed to be subtracted from the experimental C_T obtained with the Ret2VIC probe (C_{Tret}) and interpolated on the corresponding standard curves to obtain the same amount of orange DNA detected in the 50% sample. The adjusted amount of mandarin DNA in any sample can be inferred from:

$$N_{ret} = 10^{(C_{Tret} - b_{ret} - D)/m_{ret}} \quad (1)$$

where b_{ret} and m_{ret} are the C_T axis intercept and slope of the mandarin standard curve, respectively.

Finally, the proportion of mandarin DNA in the sample was calculated as

$$\% \text{mandarin} = 100 \times N_{ret} / (N_{sin} + N_{ret}) \quad (2)$$

The experimental error was calculated as the percent of the difference between real and measured mean mandarin content.

3. Results and discussion

3.1. Selection of species-informative nucleotide polymorphisms

The chloroplast chromosome *trnL-trnF* intergenic region has been used for taxonomic and population studies in the *Rutaceae* family (Bayer et al., 2009; Groppo, Pirani, Salatino, Blanco, & Kalunki, 2008; Li, Cheng, Yi, & Deng, 2006). To identify nucleotide polymorphisms that could potentially be used for the detection of mandarin in orange, a segment of 746 bp encompassing orange plastid DNA (reference Genbank sequence NC_008334, nucleotides 50974 to 51720) was sequenced in all nine orange and 13 mandarin cultivars used in this work. All orange cultivars showed complete identity in this region with the NC_008334 reference sequence. The corresponding sequences in the mandarin cultivars matched those previously reported in Genbank sequences EU369560, EU369550 (Nanfengmiju cultivar) and EU369537 (Hua-piju cultivar) (Li, Cheng, Tao, & Deng, 2007). All mandarin cultivars sequenced displayed a 5 bp (AGAAAA) insertion repeat, which was not present in orange cultivars. In addition, two SNPs were identified within the region: A $\rightarrow$ G at position 51028, and G $\rightarrow$ T at posi-

tion 51542, which is found in most CR reported sequences (Bayer et al., 2009; de Araujo, de Queiroz, & Machado, 2003; Li et al., 2006). Although it would be technically possible to design a qPCR fluorescent probe overlapping the 5 bp AGAAAA repeat, the high adenosine content would likely result in some unspecific binding, which would affect the sensitivity and specificity of the assay. Remarkably, the G/T 51542 polymorphism was also found in the two Satsuma mandarin varieties, and in the Nova cultivar, which is a hybrid between *C. reticulata* and Tangelo, which in turn is a hybrid between *C. reticulata* and *C. maxima* (grapefruit). The hybrid Fortune cultivar, also included in this study, comes from the crossing of two *C. reticulata* varieties and also maintains the same mandarin consensus sequence in this region. This simplifies the usability of this polymorphism for mandarin detection, as no differences in would be expected between the different cultivars. Thence, for this report, the G/T 51542 polymorphism was chosen to demonstrate its capacity to be used as target for MGB TaqMan[®] probes for the detection of the mandarin cultivars included in mixed orange/mandarin juice.

3.2. Dual probe qPCR assay

Based on this unique sequence marker, a qPCR genotyping system consisting of two PCR primers and a pair of MGB TaqMan[®] probes labeled with FAM (orange) and VIC (mandarin) was devised. Several preliminary tests using different combinations of primers and probes revealed that the “orange probes” displayed some cross-hybridization with mandarin DNA (data not shown). To minimize the resulting unspecificity, the T_m of the MGB-probe for orange was lowered by shortening the sequence to only 13 nucleotides, while the length of common primers and the mandarin probe was extended to allow the use of a relatively high (59 °C) temperature at the annealing step of PCR. The resulting dual-probe system, composed of primers CTR02F/CTR02R and Sin3FAM/Ret2VIC MGB-TaqMan[®] probes, was tested in single-reaction assays with three orange and three mandarin cultivars DNA. Plots of fluorescence increment (ΔRn) vs. cycle number did not show Ret2VIC fluorescence when orange DNA was present in the sample (Fig. 1), assuring that no false positives may appear when detecting mandarin in orange juice samples. Conversely, some residual signal could be detected for Sin3FAM when mandarin DNA was used as template. This may be relevant if the dual-probe detector is used for detection of orange in mandarin samples, which would require the accurate adjustment of the threshold to avoid false positives.

The efficiency and linearity of the PCR assay response were estimated from C_T vs. log DNA standard curves obtained with different dilutions of orange and mandarin DNA. The parameters of the standard curves obtained, shown in Table 2, confirmed each probe yielded a highly efficient and linear response in the 0.32–200 pg range tested. The limit of detection for mandarin DNA was established at 0.5 pg, yielding a C_T less than three times the standard deviation of the blank control (Long & Winefordner, 1983). Furthermore, the near overlapping of the standard curves for orange and mandarin suggests that the copy number of the target sequence per gram of total DNA obtained from leaf is identical in both species.

3.3. Mandarin detection by C_{Tc} method

The similarity of PCR efficiencies, denoted by the slopes of the curves, allowed estimation of the ratio of orange/mandarin DNA from the difference in fluorescence from Sin3FAM and Ret2VIC probes, C_{Tsin} and C_{Tret} , respectively. Thus, C_{Tsin} was used as internal calibrator, and the presence of mandarin was assessed as the difference $\Delta C_{Tc} = C_{Tret} - C_{Tsin}$ recorded by the dual-probe assay. To test this approach, different binary mixtures of mandarin and

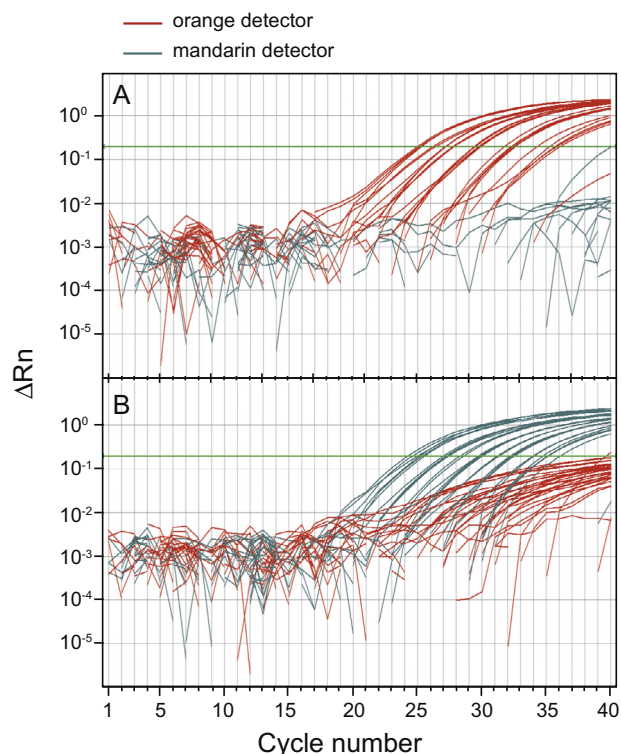


Fig. 1. Specificity of the dual-probe orange/mandarin assay. Kinetics of fluorescence signal in qPCR reactions using the TaqMan MGB orange and mandarin probes on DNA extracted from leaf tissue of A: orange #1, #2 and #6 cultivars; and B: mandarin #17, #18 and #19 cultivars. Results are shown for five 1:5 serial dilutions ranging from 200 to 0.32 pg of DNA per reaction. The threshold line (green) was set at $\Delta Rn = 0.2$.

orange DNA were examined (Table 3). As expected, ΔC_{Tc} showed an inverse correlation with the proportion of mandarin in the mixture. It can be observed that DNA mixtures containing less than 1% mandarin did not produce measurable signal with the mandarin probe after 40 cycles, while juices containing 2% or 5% yielded ΔC_T values of 9 and 6 cycles, respectively, which were significantly different from mixtures containing 1% mandarin ($p < 0.001$). Thus, a detection cut-off can be set at 10 cycles, meaning that samples showing a $\Delta C_{Tc} \leq 10$ cycles can be considered as positive for mandarin.

3.4. Detection of mandarin in fruit juice

Current foodstuffs regulations enforce the declaration of fruit species present in the product (European Council, 2001). Analytical methods for the detection of alternate species in single-fruit labeled products should be able to detect the added species at the 3–5% range, which may be considered as a reasonable limit to discern between intentional addition and possible contamination because the same processing equipment was used for different juices. To test the ability of the orange-mandarin dual-probe assay to de-

Table 2
Linearity and efficiency of the qPCR assay using the orange and mandarin detectors.

DNA	Slope	Intersection	R^2	Efficiency
Orange	-3.8 ± 0.3	32 ± 2	0.98	1.8
Mandarin	-3.6 ± 0.4	32 ± 2	0.98	1.9

Mean values $\pm$ SD from standard curves obtained for orange #1, #2 and #6 and mandarin #17, #18 and #19 cultivar DNAs.

Table 3

Observed C_T differences for mandarin and orange detectors in qPCR assays using different proportions of mandarin in DNA binary mixtures.

% Mandarin DNA	C_{Tret}	C_{Tsin}	ΔC_{Tc}
0	ND	22.7 ± 0.8	>16
0.5	ND	23 ± 0.5	>16
1	36 ± 1	23.4 ± 0.2	13 ± 1
2	32	23.3 ± 0.2	9.2 ± 0.8
5	30 ± 1	22.9 ± 0.2	6 ± 1
10	27	23	3.7 ± 0.4
20	25	23.3	1.8
50	23	24.6	-1.3
100	22	ND	<-18

qPCR reactions performed using 200 pg of mandarin + orange DNA mixtures. Mean values from 4 independent assays.

tect low proportions of mandarin in orange juice, a set of reference fruit juices containing different proportions of mandarin were prepared using concentrate (FC) and not-from-concentrate (NFC) industrial methods. DNA obtained from samples of these juices were tested using the dual-probe qPCR assay and the ΔC_{Tc} was calculated for each sample. Table 4 shows the results obtained using the $\Delta C_{Tc} = 10$ cycles cut-off to yield a positive result. This set up allowed positive detection in all samples containing $>5\%$ mandarin from both FC and NFC juices. Thus, the limit of detection for mandarin using the dual-probe method can be set at 5%, which would be adequate for the majority of applications where the aim is to identify the use of mandarin as ingredient in juice manufacturing, and is lower than the 10% achieved using other methods for apple/pear fruit juice also based on qPCR (Han et al., 2012). A 5% limit of detection has also been shown for orange/mandarin mixed juices analyzed by the relatively more complex assay based on heteroduplex formation of PCR products (Scott & Knight, 2009).

3.5. Detection of mandarin on commercial samples

Detection of DNA in commercial juices can be further complicated by varying fruit content, decreased PCR targets caused by thermal treatments, and possible hindering of PCR reactions caused by the presence of inhibitors in the DNA extract. It has been reported that standard industrial treatments performed to maintain the organoleptic properties and avoid spoilage, which range between 75 °C and 85 °C heating from 1 to 3 min to heating to 99 °C twice for 15 s with intermittent cooling to 4 °C (Ingallinera, Barbagallo, Spagna, Palmeri, & Todaro, 2005; Ros-Chumillas, Belisario, Iguaz, & Lopez, 2007), leads to a significant fragmentation of *C. sinensis* genomic DNA, although 250 bp fragments could be still

Table 4

Sensitivity of the dual-probe test for mandarin in orange juice using 10 cycles as ΔC_{Tc} cut-off.

Sample	% Mandarin	$C_{Tret} - C_{Tsin}$	% Positives
Fruit juice NFC	0	>10	0
	1	>10	0
	3	10 ± 1	60
	5	8.5 ± 0.8	100
	10	7.5 ± 0.2	100
	20	6.2 ± 0.6	100
Fruit juice FC	50	2.6 ± 0.3	100
	0	>11	0
	3	9.7 ± 0.8	80
	5	8.9 ± 0.9	100
	10	7 ± 1	100
	20	4 ± 1	100
	50	1 ± 1	100

qPCR reactions performed using 50 pg of DNA extracted from juice. Mean values from 4 independent assays.

amplified by PCR (Weiss, Ros-Churnillas, Pena, & Egea-Cortines, 2007). The detectors used in the dual-probe system require the integrity of the 77 bp encompassed by the CTR02F and CTR02R primers that would be expected, in principle, to be retained even after the most severe treatment. To test the behavior of the dual-probe system in commercial juices, PCR reactions were run in two different, brick-packaged commercial multi-fruit juices brands: NFC product A containing a mixture of orange/grapefruit/blood orange, and FC product B containing a mixture of 10 fruits, including 28% orange and 6% mandarin declared on the nutrition label. DNA was purified from both juices, and the amplification of orange/mandarin DNA was tested using the dual-probe assay. Reactions loaded with 50 pg of DNA yielded $C_{Tsin} = 27$ and 32 cycles for products A and B, respectively, elicited no signal for mandarin. To assess the possible inhibition of PCR in DNA samples obtained from the commercial juices, reactions were run using 2 μL of 50 pg μL^{-1} sample id#1 DNA mixed with 2 μL of product obtained from juices A or B. No inhibition was observed in these tests, suggesting that both C_T registered correspond with the amplifiable orange (or orange + grapefruit in A) DNA present in the extract. It is worth noting that the possible presence of mandarin in product B could not be assessed using the $\Delta C_T \leq 10$ cycles approach, as the recorded C_{Tsin} value was only 8 cycles before the last cycle (40th) of the PCR assay. In spite of the 0.5 pg detection limit for orange DNA, it can be reasoned that juices containing >5% mandarin DNA would yield a positive signal with the RET2Vic probe in reactions loaded with 50 pg of juice total DNA (regardless of orange DNA content) and, therefore, the content of mandarin product B was less than 5%.

3.6. Use of the mandarin dual-probe assay for quantitative measurements

Although initially not developed as a quantitative method, the present mandarin dual-probe assay might also be used for the determination of proportions of mandarin or orange in mixed juices. To test this approach, the C_T values for orange and mandarin collected from NFC and FC juice samples were interpolated on the standard curves defined in Table 2, and the DNA ratios obtained were compared to the actual ratios of orange/mandarin present in the juices. The results showed a linear correlation between the real and calculated proportions. However, a systematic underestimation of nearly 50% of mandarin content was also observed (not shown). Such bias may be explained if equal volumes of the orange and mandarin juices/concentrates used to prepare the mixtures contained different amounts of orange and mandarin DNA. Remarkably, a similar discrepancy between expected and measured proportions of mandarin in juices was also observed using DNA heteroduplex assay (Scott and Knight, 2009). To get a corrected measure of mandarin proportions in juices, a single matrix-adapted reference can be used to normalize the results. We have recently shown that such an approach may allow the quantification of pork meat in heat-treated beef/pork mixtures (López-Andreo et al., 2012). In the present orange/mandarin assay, the juice NFC mixture containing 50% mandarin was used as reference to calculate the number of cycles that needed to be subtracted from the experimental C_{Tret} to obtain the same amount of DNA as detected for orange. The C_T difference observed using this approach was 1.4 cycles. The difference observed suggests that a given volume of mandarin juice contains approximately 2.6-fold more *trnL-trnF* DNA copy number than the same volume of orange juice. This difference was not observed in DNA obtained from leaf tissue and may be due to different ratios of endocarp/juice in both fruits, which would release proportionally more mandarin cells than orange cells during juice processing. This observation stresses the importance of using matrix-adapted standards to calibrate

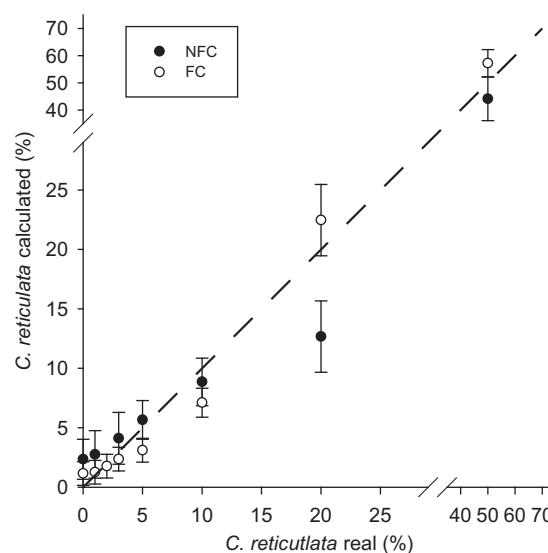


Fig. 2. Quantification of mandarin in orange/mandarin juice mixtures. The amounts of orange and mandarin in juice from concentrate (FC) and not from concentrate (NFC) were calculated by qPCR from the corresponding calibration curves and normalization to the 50% NFC juice. Plots display real vs. measured proportions of mandarin. Results are the mean of 4 independent assays $\pm$ SD. Discontinuous line shows the expected values.

quantitative results in food products. To obtain the adjusted orange/mandarin ratio, 1.4 cycles were subtracted from C_{Tret} (see Eq. (2)) and interpolated on the standard curve, allowing calculation with the matrix-normalized data. Proportions of mandarin in the sample juices calculated after normalization with the 50% sample and Eq. (2) are depicted in Fig. 2. The average experimental error for samples containing >3% mandarin was 22%, which may be acceptable for most practical applications. However, the relative high standard deviations, nearing 30% at the 5% mandarin range, compromises the accuracy of the quantitative measurements by this assay. Nonetheless, this quantitative approach may allow differentiation of high (>10%) from low (<10%) mandarin content in the sample, which can be useful information after detection of mandarin by the ΔC_{Tc} method.

The assay presented here may be easily adapted to other food applications requiring the differentiation of closely related species or cultivars, for which few species-informative sequence polymorphisms are available. It would be expected that, due to the genetic similarity among the target sequences, PCR reactions would display very similar efficiencies, allowing the use of a ΔC_{Tc} method for detection applications. A second-step quantitative assay after normalization with a matrix-adapted reference sample may be performed to ascertain the approximate proportion of the target in the product. When applied to species for which a large number of sub-species, varieties or hybrids are in the market, the target polymorphism chosen as target should be previously validated by sequencing among the expected materials to be found in the food products, and only this validated varieties should be included in the scope of the assay.

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