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Quantitative PCR Analysis for Fruit Juice Authentication Using PCR and Laboratory-on-a-Chip Capillary Electrophoresis According to the Hardy−Weinberg Law

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DNA-based analysis for the authentication of fruit juices was evaluated using the Polymerase Chain Reaction (PCR) and laboratory-on-a-chip capillary electrophoresis (LOC). A PCR restriction fragment length polymorphism (RFLP) assay demonstrated the detection of grapefruit juice in orange juice, although the assay was relatively insensitive with a limit of detection of 10% v/v. A PCR heteroduplex assay for detecting mandarin juice in orange juice was successfully applied to the LOC system and demonstrated greater sensitivity with a limit of detection of 2.5% v/v. Results for both assays using authentic juice mixtures were consistent with that expected following the random reannealing of PCR-amplified DNA at PCR plateau according to the principles of the Hardy−Weinberg law. Calculations of theoretical and expected yields of homoduplex and heteroduplexes indicated that the heteroduplexes were underestimated by 1.5-fold on the LOC. Although the LOC can provide good quantitative end-point analytical data from PCR methods, care must be taken in data interpretation because different data interpretation applies dependent on the attainment of the PCR plateau.

KEYWORDS: Quantitative PCR; Hardy−Weinberg; fruit juice; laboratory-on-a-chip; electrophoresis; adulteration

INTRODUCTION

The ability to authenticate food products provides a means of monitoring and identifying products for consumer protection and regulatory compliance. The consumer rightly expects that product labeling represents the true identity of the product. However, in some cases either accidental or fraudulent substitution occurs. Accidental substitution may occur as a result of inadequate cleaning following the changeover of products during manufacture and may be symptomatic of poor manufacturing practices. Fraudulent substitution is also a serious matter, representing deliberate extension of products with cheaper additives. High-profile examples of fraud include water addition, meat and fish species substitution, milk substitution in dairy products, misrepresentation of rice varieties, and adulteration of fruit juices. Fraudulent substitution not only attempts to deceive the consumer but also may go hand in hand with dangerous practices. The ability to detect misrepresentation and deliberate adulteration is therefore essential to both prevent fraud and protect the safety and well-being of the consumer.

DNA-based approaches have been utilized to address a number of food authenticity issues related to processed food (*1*, *2*). These include the detection of plant ingredients derived from genetically modified plants (*3*), plant speciation (*4*), for example, rice (*5*), non-durum wheat in durum wheat (*6*), mandarin juice in orange juice (*7*, *8*), and authentication of locust bean and guar gums in processed foods (*9*). The techniques have also been applied extensively to issues in meat and fish adulteration (*10*−*23*). Although generally successful, the uptake by enforcement laboratories of Polymerase Chain Reaction (PCR) and real-time quantitative PCR has been limited due to the nature of the equipment and skill base needed to perform the analyses. However, more convenient technologies are bringing opportunities for more routine DNA analyses. Laboratory-on-a-chip capillary electrophoresis (LOC) technology represents a simple analytical platform for the analysis of DNA-based assays. The relatively low cost and ease of use of the system combined with reportedly accurate sizing and quantification of fragments suggest that it can be readily exploited for species profiling and quantification of PCR products for food authentication (*24*−*27*).

Previous studies carried out in this laboratory have resulted in the development of molecular assays for the differentiation of citrus species. This has included the development of a quantitative heteroduplex-based assay for detecting mandarin juice in orange juice and a restriction fragment length polymorphism (RFLP) based method for determining grapefruit juice in orange (*7*, *8*). Mandarin or grapefruit juice may be added to improve the quality of poor quality orange juice. Although such additions are not unlawful, they must be declared to protect the manufacturers and consumers from fraud.

The aim of this work was to determine if these two assays could be transferred to a LOC format and to investigate the quantitative potential of the assays. The LOC system was evaluated for the detection of grapefruit juice in orange juice using a PCR *Eco*R1

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RFLP assay that recognizes amplified grapefruit DNA and not amplified orange DNA. In this assay, PCR primers based on conserved sequences of the chloroplast DNA accD−psaI intergenic spacer region are used to amplify a 177 bp sequence. Amplified grapefruit DNA can be cleaved by *Eco*R1 to yield 125 and 52 bp fragments. Amplified orange DNA cannot be cleaved. This RFLP has been confirmed previously using amplified DNA from authentic juice samples and conventional nondenaturing polyacrylamide gel electrophoresis (PAGE) using 43 grapefruit and 27 orange authentic samples(*7*).

In any PCR that results in coamplification of similar targets or alleles, then heteroduplex is the predominant product following reannealing of denatured duplexes when the PCR plateau phase has been reached. This reannealing is analogous to the assortment of gametes at fertilization within a randomly mating population at equilibrium according to the Hardy−Weinberg law (H−W law) originally described 100 years ago (*28*−*30*). For a population at equilibrium the ratio of homozygous dominant/heterozygotes/homozygous recessive is given by the ratio p^2:2pq:q^2, where p and q represent the proportion of dominant (p) and recessive (q) alleles within the population, respectively. The sum total of $p + q$ is always equal to 1 because there are only two alleles.

For mixtures of two haploid genomes, for example, chloroplast or mitochondrial genomes (commonly utilized as markers for food authentication), the random reassortment of PCR products within a DNA population at PCR plateau is also expected to distribute heteroduplexes and homoduplexes according to the same formula, p^2:2pq:q^2, where p and q represent the concentrations of authentic and adulterant homoduplexes and pq represents that of each heteroduplex.

The application of the H−W law to PCR analysis requires four key assumptions: (1) that the PCR reaction reaches a plateau such that random reassortment of denatured duplexes results in random heteroduplex formation during reannealing; (2) that the PCR amplification efficiencies are equal for the two alleles (3) that there is no heteroduplex RFLP cleavage; and (4) that homoduplex and heteroduplex are bound by the fluorescent intercalator used for quantification with equal efficiency.

For the grapefruit RFLP assay quantification of adulterant grapefruit derived RFLPs at PCR plateau results in the measurement of the proportion of cleavable homoduplex within the population of amplified DNA that is equivalent to %q^2 homoduplex. Measurement of %q^2 underestimates the actual concentration of grapefruit DNA within the amplified DNA that is represented by %q. Although not widely recognized, consideration of heteroduplex formation is extremely important in quantitative PCR-RFLP analysis, because adulterant homoduplex is a minor product at PCR plateau compared with heteroduplex. The ability of restriction endonucleases to cleave mispaired heteroduplexes has been reported.

Although a number of restriction enzymes can cleave mismatches, they can do so at both slower and faster rates than homoduplex substrates, often cleaving single strands and resulting in partial cleavage. Most type II enzymes such as *Eco*R1 used in this study, without redundant recognition sites, do not cleave heteroduplexes. However, changes in assay conditions, for example, high glycerol concentrations, can result in relaxed specificity or "star" activity, allowing heteroduplex cleavage. It should not be assumed that any restriction enzyme either does or does not cleave mismatched target sequences without experimental data (*31*, *32*).

The second task of this study was to evaluate the LOC system for the detection of heteroduplexes between amplified orange and mandarin DNA. We have previously demonstrated that PCR amplification of a fragment of the chloroplast *trnt -trnl* intergenic spacer derived from mixtures of DNA extracted from orange and mandarin juice results in heteroduplex formation owing to the presence of an 8bp indel. Measurement of the percent heteroduplex formed (%2pq) following nondenaturing polyacrylamide gel electrophoresis (PAGE) allows calculation of the relative concentrations of the authentic orange (%p) and adulterant mandarin (%q) species (*8*). Although conformational-based separations and quantitative heteroduplex analysis can be achieved using conventional nondenaturing PAGE, no data were available demonstrating that the LOC could be used for conformational-based separation.

MATERIALS AND METHODS

Fruit Juice Samples. Authentic fruit juice samples used in this study are shown in **Table 1** (*33*) and were prepared by conventional domestic juice pressing. Authentic orange or mandarin juice mixtures were prepared by mixing equal quantities of the juices indicated in **Table 1**.

Table 1. Authentic Citrus Samples

sample	citrus type	cultivar	origin
88	grapefruit	Star Ruby	Cyprus
96	grapefruit	Marsh	Cyprus
222	grapefruit	Marsh	Swaziland
243	grapefruit	Marsh	South Africa
352	grapefruit	Valencia	Morocco
393	grapefruit	McCarty	Belize
92	mandarin	Nova	Cyprus[a]
94	mandarin	Clementine	Cyprus[a]
93	mandarin	Freemont	Cyprus[a]
131	mandarin	Clementine	Florida[a]
133	mandarin	Fairchild	Florida[a]
404	mandarin	Nobilis	Belize
184	mandarin	Freemont	California
181	mandarin	Dancy	California
189	mandarin	Fortune	California
144	orange	Pera	Florida[a]
164	orange	Rhode Red	Florida
271	orange	Pera C	Brazil
273	orange	Pera D	Brazil
275	orange	Bahia 7	Brazil
277	orange	Bahia Retiro	Brazil
278	orange	Bahianinha	Brazil[a]
290	orange	Parson Brown	Brazil[a]
299	orange	Double Fina	Brazil
300	orange	Tarocco	Brazil
350	orange	Valencia	Brazil
351	orange	Washington Naval	Morocco
352	orange	Valencia	Morocco[a]
355	orange	Pineapple	Morocco[a]
357	orange	Cadenera	Morocco
126	citrus hybrid	Osceola	Florida
135	citrus hybrid	Ortanique	Florida
136	citrus hybrid	Temple	Florida
137	citrus hybrid	Ugli	Florida
160	citrus hybrid	Mineola	Florida
175	citrus hybrid	Fairchild	California
183	citrus hybrid	Fairchild	California
215	citrus hybrid	Ellendale	Swaziland
282	citrus hybrid	Tangelo	Brazil
284	citrus hybrid	Page	Brazil
336	citrus hybrid	Kara	Morocco
184	citrus hybrid	Freemont	California
181	citrus hybrid	Dancy	California
189	citrus hybrid	Fortune	California
404	citrus hybrid	Nobilis	Belize

[a] Samples used for validation mixes.

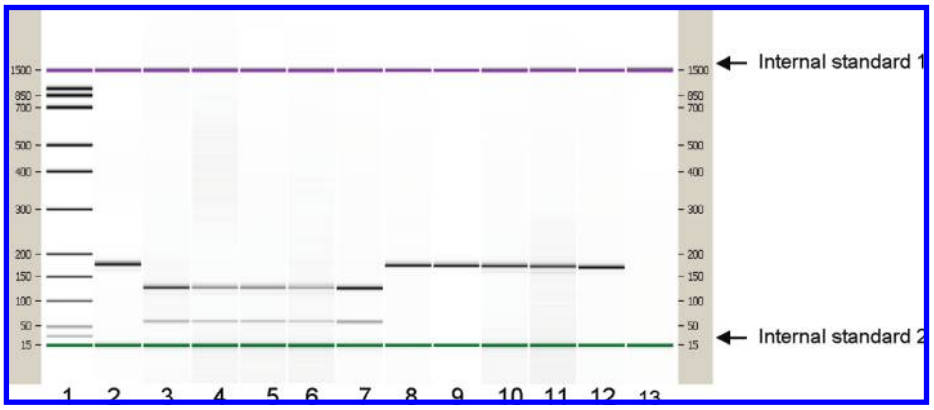

Figure 1. *Eco*R1 RFLP analysis of authentic grapefruit and orange samples: lane 1, LOC marker; lane 2, authentic grapefruit (88) uncut; lanes 3−7, *Eco*R1 digests of amplified DNA from different authentic grapefruit samples (88, 96, 222, 243, and 393); lanes 8−12, *Eco*R1 digests of amplified DNA from different authentic orange samples (142, 144, 278, 290, and 352); lane 13, negative control. Sizes are shown in bp. Positions of LOC internal standards 1 and 2 are also shown.

DNA Extraction and PCR. DNA was extracted from 100 μL of fruit juice following neutralization with 10 μL of 2 M Tris-HCl, pH 8.0, using a commercially available kit (Phytopure, GE Healthcare, Little Chalf-ont, U.K.) in accordance with the manufacturer's instructions and as previously described (*8*). Extracted DNA (2 μL) was used for PCR. PCR used "Ready To Go" PCR beads (GE Healthcare) or high-fidelity "Maximase" (Transgenomics, Paris, France) for heteroduplex studies. Reactions were carried out in thin-walled PCR tubes in a final volume of 25 μL in accordance with the manufacturer's instructions. PCR primers were synthesized by Applied Biosystems (Warrington, U.K.). Mandarin/orange heteroduplex primers were B_{FOR} 5′-AGAAAGATACAATCCCGCTAAACG-3′ and B_{REV} 5′-GTATCCGCAATTCAATATAGATGGA-3′ (*8*). Mandarin PCR used Maximase at 95 °C for 2 min, followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s, 68 °C for 30 s, and 1 cycle of 68 °C for 5 min before holding at 4 °C. Grapefruit/orange PCR primers were Chl-3 5′-GCCGGGCAAATAAAATGAATTTC-3′ and Chl-4 5′-GAAAAGAATTTCTTACAAATTCCC-3′ (*7*). PCR conditions were 95 °C for 4 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and 1 cycle of 72 °C for 1 min before holding at 4 °C.

RFLP Analysis. Restriction enzymes were obtained from Roche U.K. and used in accordance with the manufacturer's instructions. PCR product (5 μL) was digested in a total volume of 10 μL at 37 °C for 1 h, and 1 μL was analyzed using the LOC.

LOC Capillary Electrophoresis. All analyses were carried out using the Agilent LOC capillary electrophoresis 2100 Bioanalyzer and reagents obtained from Agilent U.K. Estimates of DNA fragment size and quantity were based on the LOC supplied standard within the Agilent DNA 1000 kit.

RESULTS

PCR-RFLP Analysis Using the LOC for Determining Grapefruit Juice in Orange Juice. Figure 1 shows the typical RFLP pattern resulting from amplification and cleavage of DNA from orange and grapefruit samples. The theoretical amplicon size was 177 bp, and the theoretical *Eco*R1 cleavage products were 125 and 52 bp. The LOC measured values varied from 179 to 183 bp, from 128 to 135 bp, and from 61 to 69 bp, respectively. A minor band was frequently visualized migrating below the 177 bp product in both orange and grapefruit PCR products. This band demonstrated similar *Eco*R1 cleavage to the major product, suggesting that it was not a nonspecific amplification product and most likely results from chloroplast heteroplasmy or PCR stutter. The reported sizing accuracy on the LOC system is ±10%, and the values obtained were in reasonable agreement.

Theoretical and Practical Considerations for Quantitative PCR-RFLP Analysis Using the LOC. According to the manufacturers, the reported limit of detection of the LOC system is 0.10 ng. To determine the theoretical yields of homoduplex and heteroduplex PCR products at PCR plateau, a self-calculating spreadsheet based on H−W law was constructed (**Table 2**). The spreadsheet allows calculation of anticipated levels of heteroduplex and homoduplex at different chip loadings and the theoretical limit of detection for different percent DNA mixtures when the quantity of PCR product is considered relative to the LOC limit of detection. For the grapefruit RFLP assay the 125 bp *Eco*R1 fragment represents 125/177 or 70% of the total PCR product. Because the minimum quantity of DNA for a detectable band using LOC is 0.10 ng, then this must represent 70% w/w of the grapefruit PCR product at the limit of detection. Therefore, a minimum of 0.1429 ng of grapefruit homoduplex is required for the detection of 0.10 ng of the 125 bp grapefruit *Eco*R1 RFLP band. Calculations showed that in order to reach a detection limit

Table 2. Calculation of the Theoretical Detection Limits at Different Chip Loadings for the Detection of Grapefruit Juice (*q*) in Orange Juice (*p*) and the Distribution of Different Duplexes According to H−W Law at Different Chip Loadings[a]

	chip sensitivity at differnent chip loadings		
	50.0000 ng of DNA	25.0000 ng of DNA	10.0000 ng of DNA
minimum quantity of adulterant homoduplex (ng)	0.1429	0.1429	0.1429
RFLP band quantity at LOD (0.7 × 01.1429) (ng)	0.1000	0.1000	0.1000
q^2 = adulterant homoduplex/total DNA	0.0029	0.0057	0.0143
$q = \sqrt{}$adulterant homoduplex/total DNA	0.0535	0.0756	0.1195
$p = 1 - q$	0.9465	0.9244	0.8805
authentic homoduplex (p^2)	0.8960	0.8545	0.7752
adulterant heteroduplex ($2pq$)	0.1012	0.1398	0.2105
adulterant homoduplex (q^2)	0.0029	0.0057	0.0143
amounts of duplex DNAs (ng)			
authentic homoduplex p^2 × DNA (ng)	44.7976	21.3632	7.7521
adulterant heteroduplex $2pq$ × DNA (ng)	5.0595	3.4939	2.1050
adulterant homoduplex q^2 × DNA (ng)	0.1429	0.1429	0.1429
total DNA (ng)	**50.0000**	**25.0000**	**10.0000**
detection limit			
% *q* (adulterant)	**5.3452**	**7.5593**	**11.9541**
% *p* (authentic)	94.6548	92.4407	88.0459

[a] Data assumes a limit of detection of 0.1 ng (Agilent data).

of approximately 5% w/w DNA by *Eco*R1 RFLP, a lane loading of 50 ng must be applied to the LOC. In practice, this means that the PCR product yield must reach 50 ng/μL so that the maximum gel loading (1 μL) can be applied. However, this can be difficult to achieve in practice (without DNA precipitation) because PCR yields are frequently 10−25 ng/μL, resulting in a theoretical RFLP based detection limit of 12.0−7.6% v/v juice. The small proportion of cleavable homoduplex resulting from reannealing at PCR plateau means that conventional RFLP analysis is an inherently insensitive approach for determining adulteration.

Quantitative PCR-RFLP Analysis Using the LOC for Detecting Grapefruit Juice in Orange Juice. Four different mixtures of authentic orange and grapefruit samples (243/352, 142/88, 278/96, and 355/392) were analyzed using the *Eco*R1 RFLP LOC method at four different juice concentrations (0, 10, 15, and 20% v/v grapefruit in orange juice). Results obtained (% w/w DNA) were analyzed in comparison to the original juice mixture concentrations (% v/v juice) using two different approaches. First, the 125 bp *Eco*R1 RFLP grapefruit homoduplex present was quantified using the LOC standard and software. Because the 125 bp band represents 70% of the total homoduplex that must be present, this allowed calculation of the total grapefruit homoduplex present. This value was then added to the value for the 177 bp band to give the total PCR product yield. This was more accurate than simply adding the value of the 52 bp band because this band was quantified less efficiently (data not shown). The quantity of grapefruit homoduplex present was expressed as % w/w homoduplex/total PCR product (%G RFLP or %q^2). This represents the conventional approach for calculating adulteration that does not consider heteroduplex formation.

Second, using the same method of homoduplex quantification, the predicted levels of homoduplex were calculated by assuming heteroduplex formation and H−W law (p^2:2pq:q^2), where p^2 and q^2 represent the measured quantities of orange and grapefruit homoduplex bands, respectively, and 2pq represents the quantity of heteroduplex. In this case the quantity of grapefruit adulterant (q) is represented by the square root of the calculated value for the fraction of adulterant homoduplex present. Because $p + q = 1$, the percent adulteration (%q) = $q/p + q \times 100$. In simple terms, according to H−W law, the percent adulteration is equal to the square root of the fraction of total adulterant homoduplex (q^2) in the total DNA present × 100. The data obtained from the fresh authentic juices mixtures are shown in **Figure 2**.

Quantitative PCR Heteroduplex Analysis Using the LOC for Detecting Mandarin Juice in Orange Juice. Figure 3 shows the results obtained from LOC analysis of PCR-amplified DNA extracted from authentic juice samples of orange juice and mandarin juice and 50% (v/v) mixtures. Resolution of the heteroduplex bands and partial resolution of the mandarin (208 bp) and orange (200 bp) homoduplex bands were obtained. The two heteroduplexes showed conformational-based migration, typical of electrophoresis under nondenaturing conditions and migrated at approximately 251 and 269 bp. The resolution of the heteroduplexes on the chip system was unexpected and demonstrates that the LOC used was a nondenaturing electrophoresis system. A faint band (band A, not shown) was often present migrating at approximately 246 bp in mandarin samples; this band was not previously resolved using conventional PAGE analysis (*8*). The origin of this band is unknown but probably results from the differential migration of an alternative heteroduplex form of the 251 bp band resulting from some low-level heteroplasmy with the mandarin chloroplast population. Heteroplasmy can contribute to a background heteroduplex measurement in mandarin samples. When present, this band increased with increasing adulteration and, when added to the quantity of the 251 bp band, provided an approximately equal quantity of product as the 269 band, consistent with the expected equal yield for each heteroduplex predicted by Hardy−Weinberg.

The LOC was capable of partially resolving the 8 bp size difference distinguishing orange and mandarin homoduplexes. This partial resolution is shown in the electropherogram in

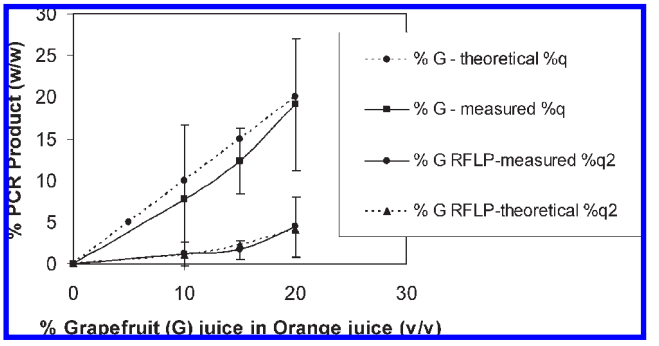

Figure 2. Comparison of the measured percent grapefruit RFLP (%G RFLP or % q^2) compared with the theoretical %G RFLP or %q^2 predicted from Hardy−Weinberg law. Data are also shown comparing the calculated grapefruit adulteration %G or %q compared with the theoretical H−W values. Theoretical values assume an equal contribution of PCR product from both orange and grapefruit juice DNA and random reassortment of single-stranded PCR products at PCR plateau. Error bars represent the standard deviation.

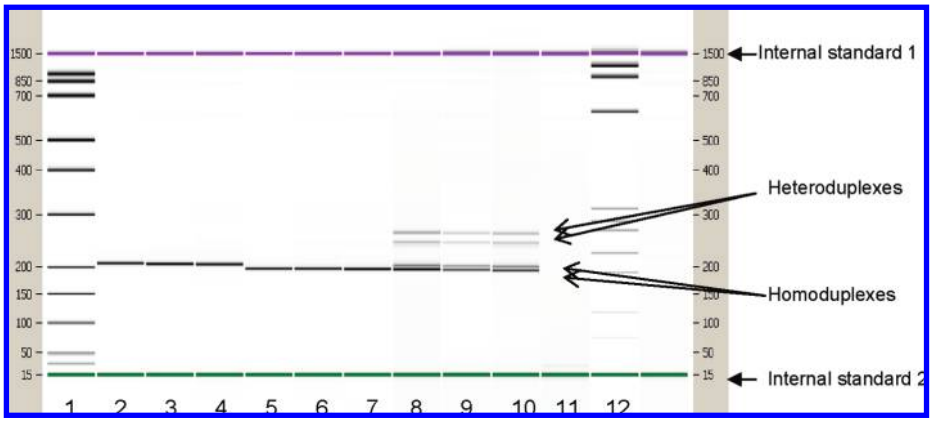

Figure 3. Heteroduplex analysis following LOC electrophoresis: lane 1, LOC marker; lanes 2−4, authentic mandarin samples (181, 184, and 189); lanes 5−7, authentic orange samples (164, 290, and 355); lanes 8−10, 50% mandarin in orange mixes (181/164, 181/290, and 184/355); lane 11, negative control; lane 12, LOC marker. Sizes are shown in bp. Positions of LOC internal standards 1 and 2 are also shown.

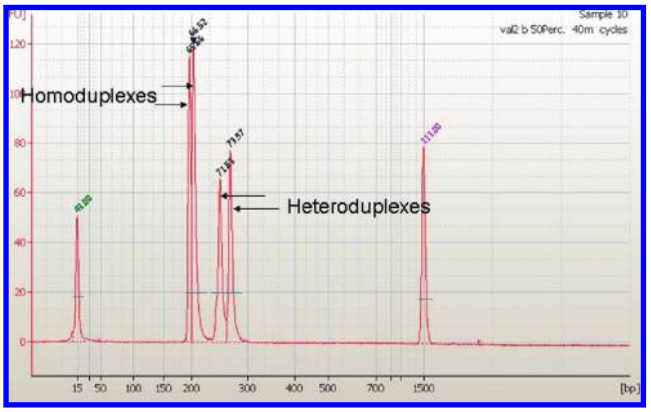

Figure 4. Example electropherogram obtained following LOC analysis of PCR product obtained from a 50% mandarin in orange juice mixture.

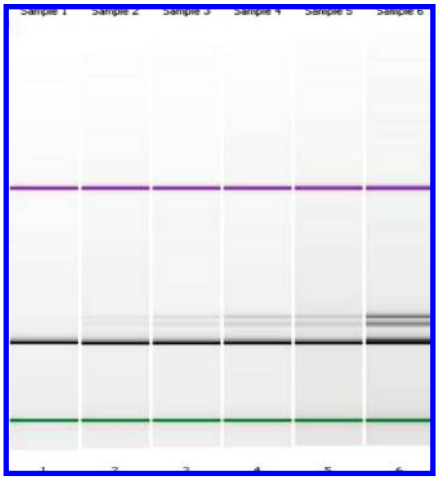

Figure 5. Detection of mandarin juice in orange juice using PCR and LOC heteroduplex analysis: lanes 1−6, 0, 2.5, 5, 10, 15, and 50% v/v mandarin in orange juice, respectively.

Figure 4 and enabled quantitative analysis of all four bands obtained from 50% (v/v) orange and mandarin juice mixtures. Although the resolution of the homoduplex peaks was poor, the peak heights were similar and allowed comparison of the observed and predicted heteroduplex band yields based on the quantity of homoduplex according to H−W law for 50% (v/v) mixtures. Analysis of homoduplexes at lower values (< 50% v/v) was not possible owing to the dilution of the adulterant homoduplex by the authentic homoduplex, resulting in heteroduplexes. Measured heteroduplex values were always less than the predicted values according to H−W law based on calculation from measured homoduplex.

The discrepancy between the quantities of observed and predicted total heteroduplex was further investigated by repeated (× 9) analysis of PCR products from 50% v/v juice mixtures to confirm the observed discrepancy and determine a correction factor. The mean value for predicted heteroduplex calculated from the measured homoduplex was 49.03% (w/w PCR product) (SD 1.27%); however, the mean measured heteroduplex was only 32.95% (SD 5.68%). Statistical analysis using Student's unpaired *t* test confirmed that the predicted and measured values for heteroduplex were significantly different ($P < 0.0001$) and that measured heteroduplex values were 1.49 times less than expected on the basis of the measured homoduplexes. Measured values for heteroduplex were therefore multiplied by a factor of 1.49 to correct for the observed differences.

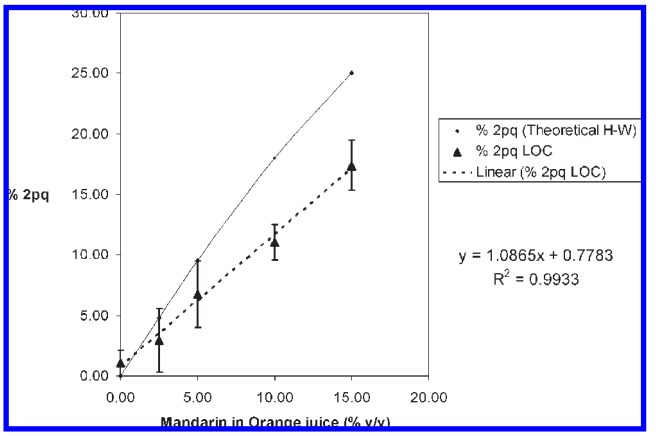

Figure 6. Expected yield of heteroduplex (%2*pq*) according to the Hardy−Weinberg law plotted against percent mandarin in orange juice. Data are shown for analysis using LOC. Theoretical data assume an equal contribution of PCR product from both orange and mandarin juice DNA and random reassortment of single-stranded PCR products at PCR plateau. Error bars represent the standard deviation.

The assay was further evaluated using mixtures of freshly squeezed authentic samples. We therefore prepared pooled mixtures of five different authentic mandarin and orange juices at different concentrations (% v/v). Typical results are shown in **Figure 5**. The assay readily detected 5% v/v mandarin juice in orange juice on the LOC. Duplicate PCR data for complete repetition of juice mixing, DNA extraction, and PCR were obtained, and the yield of heteroduplex was adjusted using the 1.49 correction factor. Results for the pooled data are shown in **Figure 6** compared with the theoretical %2*pq* (w/w) heteroduplex values predicted using the H−W law from the original juice mixtures (assuming equal concentrations of DNA are obtained from each species). Both theoretical H−W values and LOC H−W values approximated a linear fit over the 0−15% v/v juice range ($r^2 = 0.9961$ and 0.9933, respectively).

DISCUSSION

The data analysis shows how the principles of the Hardy−Weinberg law can be applied to interpretation of quantitative PCR data at PCR plateau. Results for the grapefruit RFLP assay demonstrated good agreement with values expected according to H−W law. Perhaps not surprisingly, the *Eco*R1-based RFLP analysis for grapefruit authentication was relatively insensitive because cleavable homoduplex can only form a relatively minor fraction of the PCR product. In contrast, the heteroduplex-based mandarin assay was more sensitive. Results for corrected values using LOC were very similar to those obtained previously for ethidium bromide stained PAGE gels (*8*). However, comparison of the two data sets is complicated because it is not known if the intercalator used for nondenaturing PAGE analysis (ethidium bromide) demonstrates a lesser affinity for heteroduplex than the proprietary intercalator used for LOC (for which a correction factor was applied). This question is difficult to resolve because the homoduplex bands are not readily separated by conventional PAGE. Overall, and similar to our previous data based on PAGE analysis and ethidium bromide staining, mandarin juice appeared to have less DNA/unit volume than orange juice, and this probably accounts for the observed deviance from H−W law.

Interpretation of data according to H−W law provided a much closer relationship with authentic juice mixtures than that based on a direct comparison of percentage PCR product yield. The failure to consider H−W in this type of PCR analysis may lead to significant errors in quantification. Accurate end-point

quantification of PCR must either ensure that the PCR plateau is reached so that duplexes are re-assorted according to H−W law or that the PCR plateau is not reached such that only homoduplexes are measured. Any failure to reach plateau prevents segregation according to H−W. If this occurs, then the resulting PCR product can only contain homoduplexes, and thus the results of the RFLP analysis appear to be more similar to the original juice mixtures.

The measured percent grapefruit RFLP, that is, $\%q^2$, was in agreement with the calculated theoretical H−W values. The data showed that the predicted values for adulteration ($\%q$) based on calculation using H−W were in agreement with the actual juice values and close to the anticipated values assuming an equal contribution of DNA from both orange and grapefruit juices (**Figure 2**). Although the analytical error associated with the measurement of $\%q^2$ for RFLP analysis may appear to be small, the errors are much greater in terms of the calculated percent adulterant ($\%q$) because q is always less than one. As predicted by H−W law for an RFLP assay without heteroduplex cleavage, the grapefruit assay was insensitive with a limit of detection of 10% v/v juice. Similar detection limits are likely for microsatellite- or indel-based markers if measurement occurs following non-denaturing LOC electrophoresis at PCR plateau and heteroduplexes are not recognized. Increased sensitivity may be achieved by measurement before plateau (where the H−W law does not apply); however, this would be difficult to ensure in practice. It is important that any end-point quantitative PCR analysis should consider if measurement has occurred pre- or post-PCR plateau because heteroduplex formation according to the principles of H−W law is expected to occur at PCR plateau. Although real-time quantitative PCR is often considered to be a superior analytical approach, there are no published data directly comparing heteroduplex analysis of coamplified alleles based on data interpretation according to H−W law. However, direct quantitative analysis at PCR plateau using alternative approaches such as pyrosequencing that measure relative quantities of SNPs has shown that such approaches can be used to obtain comparable quantitative data. A comparison of different technologies for allele frequency determination including RFLP, real-time pyrosequencing analysis, single-base extension (SBE), Taq man, and MALDI-TOF-based primer extension, revealed that all of these methods (if used appropriately) can provide reasonably accurate allele frequency estimation of SNPs in DNA pools (*34*, *35*). LOC analysis as described in this work may provide a simple screening tool for food authentication that is readily transferrable to official control laboratories.

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