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ORIGINAL RESEARCH

Morphometric and DNA Fingerprinting Strategies for the Detection of Rice Flour Adulteration in Chilli Powder

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Abstract

Chilli powder is an essential ingredient in worldwide cuisine and is a vital business commodity. In Sri Lanka chilli powder is reportedly adulterated with rice flour. To regain the lost red colour due to adulteration, blending of carcinogenic dyes such as Sudan III have been reported. Yet no strategies are available to detect these adulterations. Therefore the objective of this study was to identify the adulteration of chili powder with rice flour, using morphometric and molecular methods.

Pure chilli powder and rice flour were custom-made and an adulteration gradient at 10% increments was made by mixing chilli powder and rice flour. Subsamples of adulterated mixtures were blended with Sudan III. Ten commercial chilli powder samples were also collected. The pungency of all the samples, except the ones with Sudan III was ranked having a panel of ten tasters. The density, sedimentation in water and microscopic structure were recorded for the same set. Suspension in ethanol was observed for adulterant mixtures with and without Sudan III. DNA was extracted from pure chilli powder, rice flour, 20% adulterated mixture and chili cultivar MI-2 and PCR was performed using chili marker*CAM451* and rice marker *K20*.

Colour appearance, density, microscopy and suspension could not be used to detect the adulterations with Sudan III and rice flour. The pungency of chili powder remained stable up to about 20% adulterations with rice flour, allowing adequate room for fraudulent practices. Though marker *CAM451* was not informative, marker *K20* provided sharp bands to completely discriminate the adulteration of chili powder with rice flour.

Key Words: *Capsicum annum*, Food Adulterations, Chilli Powder

Introduction

Chilli (*Capsicum annum*) powder is an important ingredient in food, which provides a characteristic hotness known as pungency[1]. Economically, chilli is a very important crop[2,3] and 3.5 million tons of dried chilli is annually consumed in the world[4]. Sri Lanka imports about 50% of its dried chilli requirement from India[3]. Dried chilli is mainly used as a powder (fine or coarse) in food preparations. Grinding,

packing and selling of dried chilli are big businesses everywhere in the world. Unfortunately, adulterations of chilli powder with cheap and unacceptable substances to increase the profit, can be seen in countries like India and Sri Lanka.

Adulterations of chilli powder with biological and other material are well documented. In India, adulteration of chilli powder is a serious issue and the frequent adulterants are red beet pulp, almond shell dust [5] and brick powder[6].To improve the colour of adulterated chilli powder mixtures, mixing with carcinogenic Sudan dyes[7,8] have been observed. Though not many reports are available, adulteration of chilli powder with cheap substances such as rice flour is very frequent in Sri Lanka. The other types of adulterants to powders of chilli, turmeric, pepper and condiments are wheat flour, corn flour, fine brick particles, rice husk, saw dust and dried poonac[9]. Adulterations of chilli powder is a serious issue. Foreign matter in chilli powder usually goes undetected due to its intense colour[10]. The intentional adulterants are entirely for financial gains[11], which can lead to serious health hazards such as cancers[7], allergies and intoxications[12].

Numerous strategies have been proposed to detect food adulterations. The adulterants in chili powder could be detected by using microscopic examinations[9,13], measurement of total ash content[9] and sedimentation in water[11]. Chemical methods such as Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS) could be used to detect the colouring agents such as Sudan dyes[14].Molecular markers like Randomly Amplified Polymorphic DNA (RAPD) and Sequence Characterized Amplified Region (SCAR)[15] and DNA barcoding [16] can be used to detect the presence of DNA that belong to biological adulterants. However, the employment of molecular tools is often hampered due to the difficulty of extracting good quality genomic DNA from chili powder samples[17]. But, improved DNA isolation methods have recently been established[5,18].

The delinquency of adulterating chilli powder with rice flour is two fold. Firstly, it clearly violates the rights of consumer by partly replacing a portion of chilli powder with rice flour and secondly and more importantly, to improve the color of adulterated mixture, the addition of carcinogenic Sudan dyes lead to consumer health concerns[7, 8]. Thus the detection of adulterations of rice flour is extremely important. According to present knowledge, no studies have been conducted to detect the adulterants of chili powder in Sri Lanka. Therefore, the objectives of the present study were to assess the suitability of morphometric methods and to establish a robust DNA fingerprinting strategy to accurately detect the rice flour adulterations in chilli powder to safeguard the rights of the cor sumer.

Materials and Methods

Sampling

Dried chilli and white rice samples were collected and ground into fine powders (custom-made) using a commercial scale heavy duty grinder (Grinding Mill Brand,2AJIW, Sri Lanka). A series of chilli powder to rice flour adulteration gradient was prepared as shown in Table 1. Five samples of commercially available chilli powder, were purchased from supermarkets (which were branded, labeled and packed in sealed envelopes)

and five samples were purchased from road side grocery venders (not branded and sold as loose quantities). The market prices of the samples were also recorded. The colour appearance of pure chilli powder, rice flour, adulterated mixtures and commercial (LIDE100 Scanner, Canon Inc.,Japan) and the densities of all the samples were measured in five replicates. The microscopic views were taken under both low and high powers. A total of 0.01 g of Sudan III dye was added to 5 g each of adulterated mixtures, blended well, observed and photographed.

Organoleptic measurement of pungency

A total of ten participants were selected randomly and were requested to rank the pungency(the hotness felt by the tasters) of pure chilli powder, rice flour, adulterated mixtures (chilli powder with rice flour as in Table 1) and commercial chilli samples. Initially, and in between each tasting event, 25 ml of carbonated sweet cold drink was provided to relieve from the pungency effect of previous tasting. The scale of 1 to 4 was used for pungency ranking in which 1 was the least level of pungency and 4 was the highest.

Suspension in water and ethanol

The sedimentation of pure chilli powder, rice flour, adulterated mixtures, commercial chilli samples and adulterated mixtures blended with Sudan III dye were observed.

A total of 5 g from each sample was thoroughly mixed with distilled water and allowed to settle for 24 hours. The appearance of the solutions was noted. The mixtures adulterated with Sudan III dye were also observed for sedimentation, in comparison to pure chilli powder by dissolving them in distilled water and in 100% ethanol.

DNA fingerprinting

DNA was extracted from 0.1 g each of rice flour, pure chilli powder and the adulterated mixtures of chilli powder and rice flour (20% adulterated mixture in Table 1) using DNAEasy Plant Minikit (QIAGEN, Solna, Sweden) and stored at -20 °C. For comparison purposes DNA was also extracted in the same manner from leaves of MI-2, a commonly grown commercial chilli cultivar in Sri Lanka.

PCR

Rice genome specific microsatellite marker *K20* (forward primer: 5'CTGGACTTGACCCCAATGTA3' and reverse primer 5'TCTGATGGAGTGTTCGGAGT3')[19] and *Capsicum* genome specific marker *CAM451* (forward primer: 5'GCTCTTGACACAACCCCAAT3' and reverse primer 5'GCTCTTGACACAACCCCAAT3')[20] were used for PCR amplification.

PCR was carried out in 15 µl reaction mixtures. Amplifications were conducted in a Thermal Cycler (Takara, Japan) using the PCR cycle: Initial denaturation: 5 min at 94 °C; 35 cycles of 30 sec at 94 °C, 1.5 min at 53 ° C (for both markers), 2.5 min at 72 °C; and a final extension step of 10 min at 72 °C. PCR products were size separated using Ethidium Bromide stained 1% Agarose gel electrophoresis and 6 % denaturing Polyacrylamide Gel Electrophoresis (PAGE) followed by silver staining.

Data Analysis

The association between the degree of adulteration and the pungency rank (an organoleptic property) was analyzed using the Freqprocedure in SAS 9.3 (SAS Inc., Cary, NC, USA).The variation of the density of adulterated and commercial samples was analyzed using ANOVA procedure and Least Significant Difference (LSD) of means in SAS. The DNA fingerprints (*i.e.* DNA banding profiles of the samples) were compared to identify the specific bands to confirm the adulteration of chilli powder with rice flour.

Results

The colour appearance of adulterated mixtures (chilli powder to rice flour) in comparison to pure chilli powder and pure rice flour indicated a gradient of decreasing redness from 100% (pure) chilli powder to 10% chilli powder (in adulterated mixtures) (Figure 1).However, the chilli powder samples obtained from open market did not show any apparent variation of red colour when compared to pure custom-made chilli powder (Figure 2). There was a significant association (P<0.001) between pungency and the degree of adulteration. Strength of the relationship was also strong as indicated by Crammer'sV coefficient (0.81). Adulteration of chilli powder with 10% or 20% of rice flour did not cause any reduction in pungency as all respondents ranked pungency as the highest (Table 1).

This indicated that the counterfeit manufactures or sellers can add up to 20% of rice flour without affecting pungency; though the redness decreased, accordingly (Figure 1).

Figure 3, shows the effect of Sudan III dye (Figure 3A) on the improvement of colour in adulterated chilli powder samples. When the adulterated mixtures were blended with Sudan III dye, 40% adulterated sample (Figure 3B) or samples with even higher levels of adulterations showed marked similarity to the colour of pure chillli powder (Figure 3C).

The colour of 40% adulterated sample before adding Sudan III dye is given in Figure 3D.

The densities of pure chilli powder, pure rice flour and adulterated mixtures were not significantly different (P<0.05) from each other (Table 1). The mean densities of rice flour, 10% adulterated chilli mixture and pure chilli powder were 0.518 g/cm^{-3}, 0.514 g/cm^{-3} and 0.470 g/cm^{-3},respectively (Table 1). The microscopic observations did not yield any significantly different particle structures either.

The density of the commercial samples (0.21-0.30 g/cm^{-3})was lower compared to that of pure chilli powder samples, custom-made for the study (Tables 1 and 2). However, the mean pungency ranking (i.e. the hotness expressed by the tasters) was always less than 4.0 for commercial samples and it was in the range of 2.2 to 3.4 (Table 2).

Observation of suspension pattern revealed that adulterated samples had less red colouration in the supernatant liquid, compared to that of custom-made pure chilli (Figure 4). The red colouration of the supernatants of commercial samples was always similar to each other and also similar to Figures 4B, 4C and4D (the photos for sedimentation of commercial chili powder samples are not shown). When Sudan III dye-blended adulterated chilli

powder samples were suspended in water (Figure 5A), sedimentation was not different, and this was because Sudan III dye is not water soluble (Figure 5B). When adulterated chilli powder sample and Sudan III dye blended adulterated chilli powder mixtures were mixed with ethanol, characteristic red colour supernatant was observed for Sudan III dye blended adulterated chilli powder mixture (Figures 5C and 5D).

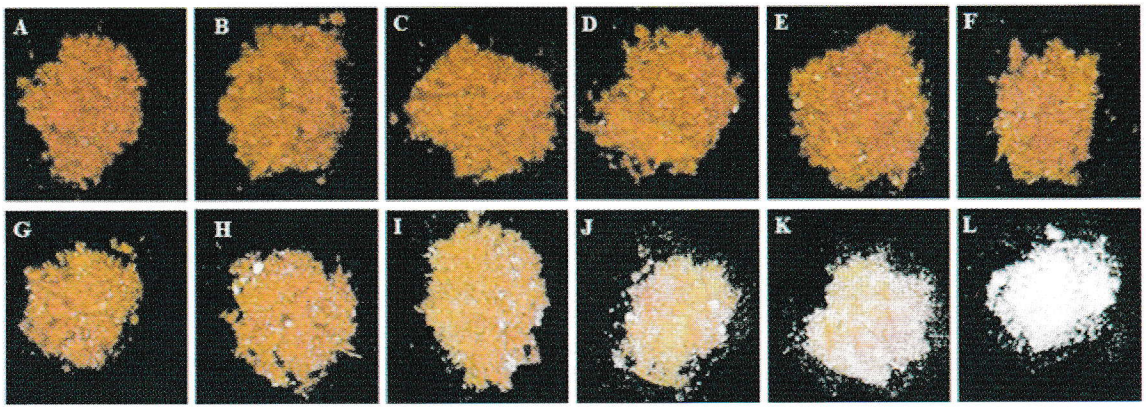

Figure 1. Colour appearance of chilli powder samples adulterated with rice flour. A: Sample collected from the market; B to K represent different levels of adulterations with rice flour; B:0%, C: 10%, D: 20%, E: 30%, F: 40%, G: 50%, H: 60%, I: 70%, J: 80%, K: 90%, L: 100% (as in Table 1)

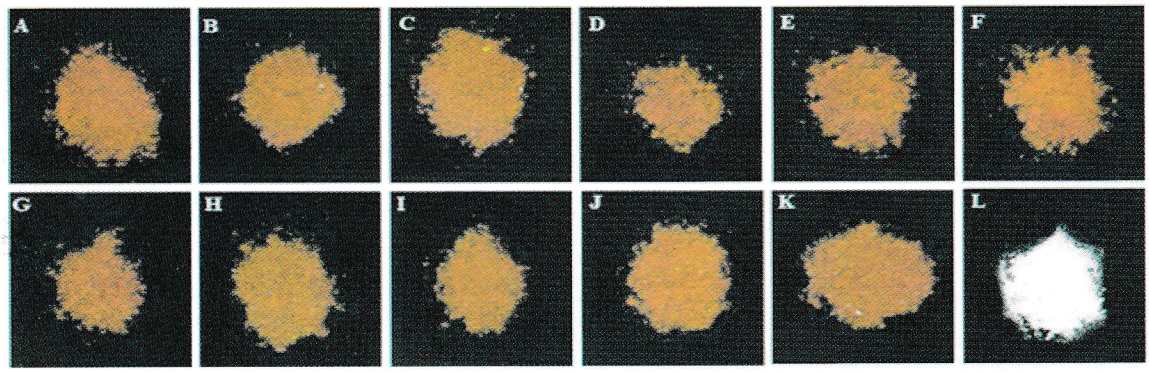

Figure 2 Colour appearance of the chilli powder samples obtained from the market. Samples A to E were proprietary products and were available in labeled and sealed packets; Samples F to J were non-proprietary products sold as loose quantities (as in Table 3). Sample K:home made pure chilli powder; Sample L: Pure rice flour.

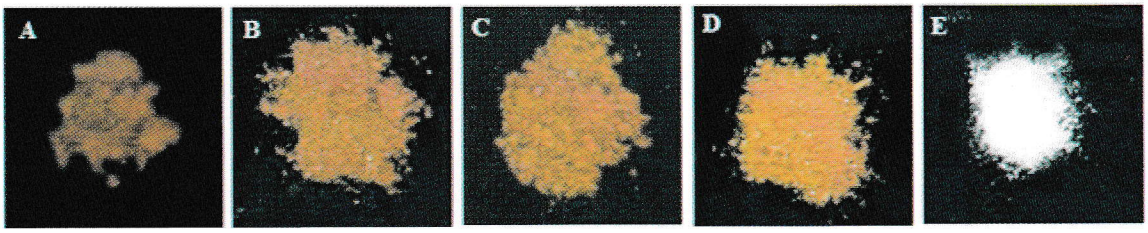

Figure 3 Colour appearance of the adulterated chilli powder sample stained with Sudan III dye. A: Sudan III dye; B:Chilli powder adulterated with rice flour (40%) and blended with Sudan III; C: Pure homemade 100% chilli powder; D: Chilli powder adulterated with rice flour (40%) and without Sudan III; E: Pure rice flour.

Table 1. Levels of adulteration of chilli powder, pungency rank and density of samples

%Adulteration	ID*	Pungency rank#					Density g/cm³
		% Respondents				Mean rank	
		1	2	3	4		
0	B	-	-	-	100	4.0	0.470^{abcd}
10	C	-	-	-	100	4.0	0.447^{cde}
20	D	-	-	-	100	4.0	0.449^{cde}
30	E	-	-	70	30	3.3	0.428^{de}
40	F	-	20	80	-	2.8	0.411^{de}
50	G	-	40	60	-	2.6	0.413^{de}
60	H	-	80	20	-	2.2	0.409^{e}
70	I	20	70	10	-	1.9	0.456^{bcde}
80	J	40	60	-	-	1.6	0.495^{abc}
90	K	100	-	-	-	1.0	0.514^{ab}
100	L	100	-	-	-	1.0	0.518^{a}
Chi-Square Value		195.39 (P<0.001)					
Cramer's V Coefficient		0.81					

Mean density values denoted by same letters are not significantly different (P<0.05)

*ID letters are indicated as shown in Figure 1
#The hotness felt by the tasters is given as the pungency rank. The scale of 1 to 4 was used for the pungency ranking in which 1 was the least level of pungency and 4 was the highest.

Table 2 Mean pungency and density of commercially available chilli powder samples

ID*	Authenticity of the sample	Mean pungency rank	Density g/cm3
A	Proprietary products, available in sealed packets	2.6	0.21^{a}
B		3.2	0.29^{a}
C		3.4	0.30^{a}
D		2.8	0.25^{a}
E		3.0	0.29^{a}
F	Non-proprietary products available in loose quantities	2.4	0.23^{a}
G		2.4	0.21^{a}
H		2.6	0.27^{a}
I		2.4	0.27^{a}
J		2.2	0.30^{a}

Mean density values in a column, followed by same letters are not significantly different (P<0.05)
* ID letters are indicated as shown in Figure 2

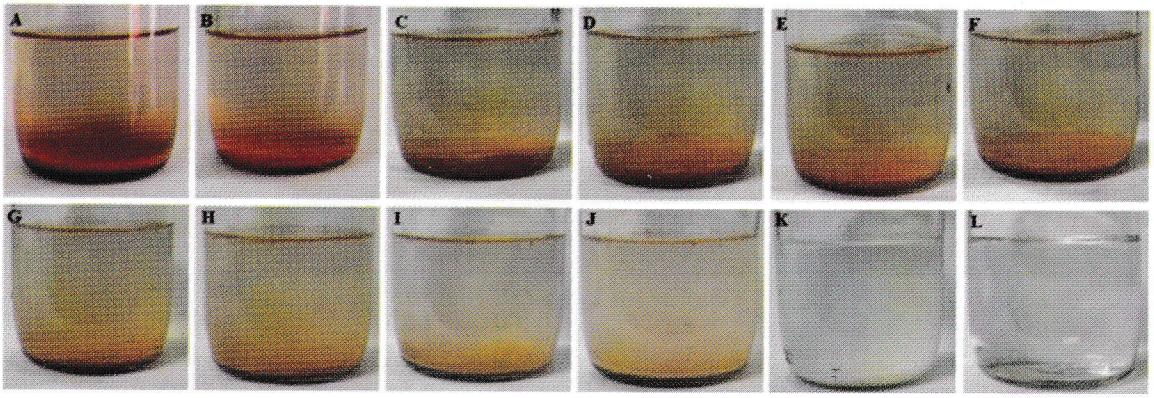

Figure 4. The appearance of the suspensions of adulterated mixtures (chilli powder with rice flour) in comparison to pure chilli powder and rice flour; 24 hrs after suspension in distilled water. A: 0% adulteration, B: 10%, C: 20%, D: 30%, E: 40%, F: 50%, G: 60%, H: 70%, I: 80%, J: 90%, K: 100%, L: control without any additions.

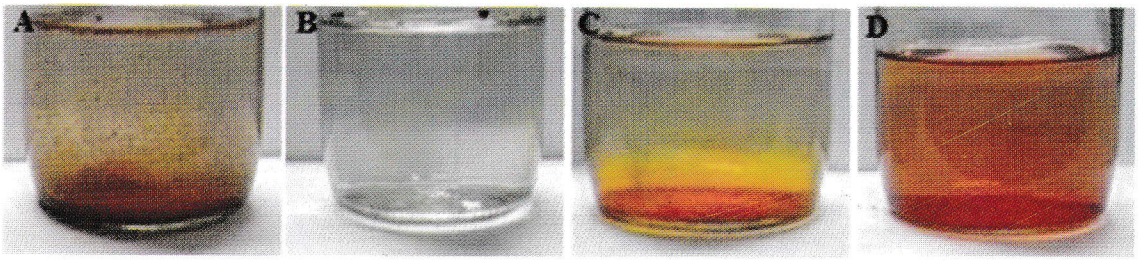

Figure 5 The comparison of the adulterated mixtures stained with Sudan III; 24 hrs after suspension in distilled water and 100% ethanol. A: 40% adulterated mixture with Sudan III dye in water, B: Sudan III dye in water, C: 40% adulterated mixture in ethanol, D: 40% adulterated mixture blended with Sudan III dye in ethanol.

DNA extracted from rice flour, custom-made chilli powder and adulterated mixture of chilli powder with rice flour (20% adulterated mixture) yielded measureable quantities of good quality DNA, which was comparable with the DNA extracted from the leaves of chilli cultivar MI-2 (Figure 6A). The PCR products of DNA templates from rice flour, chilli powder, rice flour and chilli powder adulterated sample and from MI-2for the marker *CAM451* yielded a complex banding pattern making it not possible for clear diagnosis of adulteration. PCR products for the same templates for the marker *K20* yielded a monomorphic band with a clear dosage (band intensity) difference (Figure 6B). Same *K20* PCR products when subjected to 6% PAGE, followed by silver staining resulted in very clear and non-ambiguous bands. For rice DNA two bands having the sizes of 195 and 50 bp were observed and for chilli DNA only 195 bp band was observed (Figure 6C).

According to the market prices of chilli powder in Sri Lanka (as at June, 2015) the mean price of 1 Kg of chilli powder was 470.25 Sri Lankan Rupees (LKR) (equivalent to 3.50 USD) and the mean price of 1 Kg of rice flour was 67.18 LKR (0.50 USD). The price of chilli powder was thus seven times higher than that of the rice flour. Hence, adulteration of chilli powder with 20% of rice flour provides a very high

profit margin for the large scale counterfeit chilli powder manufactures and sellers. When there is 20% adulteration with rice flour, the price of an equivalent chilli powder mixture would be 376.20LKR (2.80 USD) (given by 470.25 × 80%) plus 13.44 LKR (0.10 USD) (given by 67.18 × 20%) and is added up to 389.64 LKR (2.90 USD),thus assuring an illegal profit of80.61 LKR (0.60 USD) per Kg (given by 470.25 - 380.63 of LKR or 3.50 - 2.90 of USD) (excluding the minimal cost for Sudan dyes).

Discussion

Adulteration of food with biological or physical material is clearly a misdemeanor practice which goes against humanity and methods must be established to detect these adulterations. Such methods have been setup for adulterations of meat[21], herbal medicine[22] and basmati rice[23]. The robustness of the proposed molecular strategy relies on the ability of extracting good quality DNA and DNAEasy Plant Minikit (QIAGEN) was found to be very efficient. Employment of commercial kits for DNA based detection of adulterants in food makes the test more valid and quick as compared to the use of basic laboratory DNA extraction methods. In the present study, 20% adulterated mixture was selected for molecular analysis as it provided the highest level of adulteration with no effect on the pungency of the pure custom-made chilli powder (Table 1).

The major reported molecular techniques to identify the biological adulterations were PCR based DNA fingerprinting using RAPD[24],SSR[25]and DNA barcoding[26]. For molecular detection of biological material in chilli powder, a RAPD marker was identified[5]. However, because of the low repeatability and complexity of the banding patterns, the usefulness of the RAPD markers has often been questioned[27]. Therefore, in the present study, we focused on more robust DNA based markers as they provide repeatable and consistent bands.

However, finding a good DNA based marker is difficult and the marker in consideration should give very clear and distinct bands. The marker specific for chilli genome was not able to produce bands that could be used to distinguish rice flour from chilli powder. The marker *K20* is applicable for any suspected case of adulterated chilli powder with rice flour in Sri Lanka. Because, the marker *K20,* which is monomorphic to all the cultivated rice varieties in Sri Lanka[28] produce polymorphic bands to separate the adulterant rice flour from chilli powder. It is also evident that agarose gel electrophoresis is not able to resolve the polymorphic bands and that 6% PAGE must be employed. It is very useful to note that marker *K20* gives bands for chilli genome. The marker *K20* is from the phosphate uptake (*PUP*) locus in rice genome[19]and the present results imply that there should be an orthologous *PUP* region in the chilli genome as well.

Sudan dyes are problematic as they do not dissolve in water, thus preventing the users from recognizing any artificial red colouration even while cooking. But Sudan dyes are soluble in ethanol and when Sudan dye blended adulterated chilli powder is mixed with ethanol, a characteristic red colour can be seen compared to that of adulterated chilli powder without Sudan dye (Figure 5). This could be used as a quick test in the kitchen for a qualitative idea whether the commercial chilli samples are adulterated or not (Figures 5C and 5D). Though the testing of chemical adulterants such as

Sudan dyes was not within the scope of this study, pretty stable and tested protocols are available to check them[14]. These protocols, therefore, together could be employed to check chemical and other substituted adulterants.

The present finding of *K20* DNA marker polymorphism in 6% PAGE could be used to check the adulteration of chilli powder with cheaper rice flour (followed by colour improvisation with Sudan dyes). This may even be extended to check the adulterations in other important food items such as turmeric powder, black pepper powder and other spices. The organizations such as Consumer Protection Agency, Universities, Ministries of Health and Trade must be partnered to run a country-wide survey using our proposed strategy to bring in regulations. A centralized authoritative testing center must be equipped with the required technology for routine authentication of food (certified free from adulterations) and hence to protect the consumer rights.

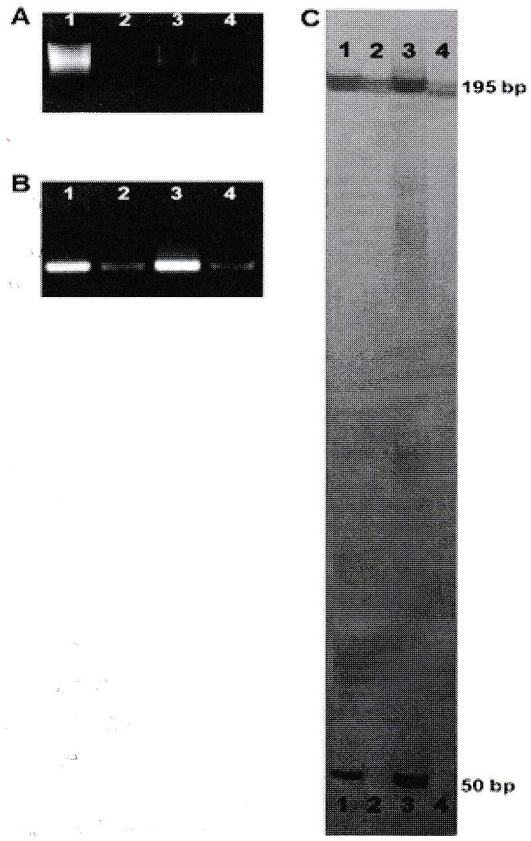

Figure 6 DNA fingerprinting analysis of chilli powder adulterated with rice flour. 1: Rice flour, 2: Custom-made pure chilli powder, 3: Adulterated chilli powder -80% Chilli powder 20% rice flour, 4: MI-2, a popular chilli variety grown in Sri Lanka. A: Genomic DNA samples (extracted from QIAGEN Plant DNA minikit) electrophoresed on 1% Agarose Gel; B: PCR products of rice DNA marker *K20* on 1% Agarose Gel (though there is an intensity difference, no length polymorphism was detected); C: 6% denaturing polyacrylamide gel. Only relevant portions of the gels are shown. Note that there are two alleles, which can be clearly used to separate chilli from rice. Sample 4 (MI-2) was used as the positive control.

Conclusion

The assessment of the adulteration of chilli powder with rice flour, using morphometric and molecular methods, reveals that the external colour appearance due to possible addition of dyes like Sudan III, density measurements and sedimentation can not be used to detect the adulterations. Microscopic observations are also inconclusive in detecting these adulterations. Good quality DNA can be extracted from chilli powder, rice flour and adulterated chilli powder samples with rice flour using QIAGEN DNAeasy plant mini kit. DNA marker K20 polymorphism in chilli and rice can be effectively used to determine the adulteration of chilli powder with rice flour.

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