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Qualitative and quantitative adulteration identification of milk powder using the DNA with novel extraction method

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ABSTRACT

The extraction of high-quality DNA from processed dairy products can often represent the crucial step in an authentication process by PCR-based methods. In this study, we optimized a novel DNA extraction method for milk powder and used the extracted DNA for identification of milk powder based on PCR analysis. The DNA quality was assessed by amplifying target sequences from mitochondrial genes, as well as by monitoring the yield, purity, and integrity of the extracted DNA. In addition, a laboratory adulteration model of milk powder was detected by PCR-based methods (PCR and real-time PCR) using primers targeting the mitochondrial 12S rRNA gene. Results showed that a sufficient amount and quality of DNA could be isolated from milk powder with this method. Both PCR and real-time PCR detection of cow milk compositions in goat milk powder further confirmed the DNA extracted with this extraction method could be widely used in addressing milk powder adulterant by a PCR-based method.

Key words: milk powder, DNA extraction, adulteration, PCR, DNA detection

INTRODUCTION

In recent years, goat milk powder has been preferred by consumers over cow milk powder because it has superior mineral composition, bioavailability, digestibility, and biological value (Raynal-Ljutovac et al., 2008). With increasing demand for goat milk powder, substitution or adulteration of goat milk powder with cow milk powder has been frequently reported. Adulteration is due to cow milk powder costing less and being more available than goat milk in many countries (Zachar et al., 2011). Each year in China, 16 to 18 million babies are born and 300,000 t of milk powder is consumed (Liu et al., 2012); thus, the authenticity of milk powder is a major issue in China and has been a concern of government authorities for decades. To avoid the possible fraudulent substitution of goat milk powder with cow milk, it is necessary to develop analytical procedures able to detect such frauds and protect consumers from misleading labels.

Existing methods for identification such as immunological, electrophoretic, and chromatographic methods (Enne et al., 2005; Mayer, 2005; López-Calleja et al., 2007) usually use lipids and proteins as a target analytes. These methods had been successfully applied to dairy products identification and played a significant role in the food industry, but they may not be practical for products containing lipids and proteins as target analytes because of their poor stability under high temperatures or pressures and chemical treatments during the processing of food products. More recently, DNA molecules have received much attention and been the target compounds for species identification based on PCR because they are thermally more stable than lipids and proteins. Therefore, PCR-based methods have been designed and applied to dairy products for authenticating goat milk, and these methods can detect very small amounts of cow milk in goat milk (Kotowicz et al., 2007; Hutu et al., 2013). Conventional PCR, however, cannot be used as an accurate quantitative tool for milk adulteration; thus, attempts to use real-time PCR as a quantitative tool for dairy products authentication are increasing, and the method has been applied to exclude the false positive of conventional PCR, to reduce contamination rates, and to improve the reliability of results (Madani et al., 2005). So far, real-time PCR assays have been proposed to detect and quantify bovine milk in cheese (Lopparelli et al., 2007; Mininni et al., 2009) and in goat milk (Jung et al., 2011). These studies showed that PCR-based methods have a potential in addressing food adulteration.

A successful PCR assay depends largely on the quality of extracted DNA; thus, extraction of high-quality DNA has been a crucial step in the authentication process. Numerous DNA-extraction methods have been used for the preparation of DNA from milk, including research protocols and commercial kits (Liu et al.,

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2014; Volk et al., 2014; Pokorska et al., 2016), but the DNA-extraction method for milk powder is still poorly defined. As adulteration of milk powder increases, it is necessary to develop an effective DNA extraction method suitable for PCR-based investigations of milk powder. The aim of our study was to develop a new and effective DNA extraction method for milk powder. In addition, both conventional PCR and real-time PCR were carried out for detecting adulteration of cow milk in commercial goat milk powders.

MATERIALS AND METHODS

Samples

Whole milk powder was sourced from a local supermarket and their species were authenticated by subjecting the samples to species typing through a PCR-based approach.

DNA Extraction and Quality Evaluation

DNA Extraction Method of Milk Powder. To separate milk fat and other impurities, 1 g of milk powder was washed with 9 mL of purified water and centrifuged for 15 min at 4,150 × *g* at 4°C. The top layer and middle layer were removed, leaving the bottom sediment, which was washed twice with 600 μL of PBS (pH 7.4, 4.8 μg of NaCl, 0.12 μg of KCl, 0.864 μg of Na_2HPO_4, 0.144 μg of KH_2PO_4, and 600 μL of double-distilled H_2O) before being centrifuged for 10 min at 10,625 × *g* at 4°C in a 1.5-mL centrifuge tube. After the washing stage, the sediment was mixed with 350 μL of extraction buffer (pH 8.0, 100 m*M* Tris Cl, 100 m*M* NaCl, and 5 m*M* EDTA), 50 μL of 20% (wt/vol) SDS, and 10 μL of proteinase K (20 mg/mL). These mixtures were then incubated at 56°C for 4 h before an equal volume of Tris-phenol was added to the digested cell concentrate followed by vortex mixer for 1 min at 1,600 rpm. The mixture was centrifuged for 10 min at 10,625 × *g* at 4°C to obtain supernatant. The supernatant was extracted once with an equal volume of phenol:chloroform:isoamyl alcohol (ratio of 25:24:1) and twice with an equal volume of chloroform:isoamyl alcohol (ratio of 24:1), followed by spinning in the vortex mixer for 1 min at 1,600 rpm before being centrifuged for 10 min at 10,625 × *g* at 4°C in a 1.5-mL centrifuge tube to obtain the supernatant. Finally, DNA was precipitated with ice-cold absolute ethanol and washed once with ethanol:water (ratio of 7:3). Twenty-five microliters of Tris-EDTA (pH = 8.0, 1 m*M* Tris-Cl and 0.5 m*M* EDTA) was added to dissolve DNA.

Evaluation of DNA Quality. The concentration and purity of total DNA were controlled by absorbance readings at 260 and 280 nm using the Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). The DNA integrity was detected with 1% agarose gel electrophoresis at 100 V for 40 min. After electrophoresis, the gel was observed under UV light imaging analyzer. In addition, the bovine *Cytb* gene and ovine *ATP6* gene (Table 1) were amplified by PCR to verify the presence of amplifiable mitochondrial DNA in all isolates. The PCR reactions of *Cytb* (*ATP6*) were performed under following thermal cycling conditions: initial denaturation at 95°C for 10 min followed by 30 cycles of 94°C for 30 s, 60°C (62°C) annealing for 30 s, 72°C for 30 s, and a final extension at 72°C for 10 min. Both of the *Cytb* and *ATP6* amplification reactions were carried out in a mixture (20 μL) comprising 6.8 μL of TaqMan Universal PCR Master Mixture (CWBIO, Shanghai, China), 0.6 μL (0.3 μ*M*) of each primer, and 2 μL of the template DNA; DNase/DNA-free water (CWBIO) was added to the reactions up to 20 μL. The PCR products were detected with 1% agarose gel electrophoresis at 100 V for 40 min and photographed under UV light.

Qualitative Detection of Cow Milk in Goat Milk Powders

Cow-specific primer *12SBT-REV* (Table 1) was selected for detection cow milk in goat milk powder by amplifying a 346-bp fragment in the 12S rRNA gene. The spiked model was prepared by admixtures compromising 0.1, 0.5, 1, 5, 10, 30, and 50% (wt/wt) cow milk powder in goat milk powder, with each sample repeated

Table 1. Sequences of the primers

Gene	Primer sequence (5′ to 3′)	Fragment length (bp)
Cytb	Forward: CGACCTTCCAGCCCCATCGA Reverse: AGCCGTAGTTCACGTCTCGGC	173
ATP6	Forward: TATTAGGCCTCCCCCTTGTT Reverse: CCCTGCTCATAAGGGAATAGCCC	294
12SBT-REV[1]	Forward: CTAGAGGAGCCTGTTCTATAATCGATAA Reverse: AAATAGGGTTAGATGCACTGAATCCAT	346

[1]Primer sequences adopted from López-Calleja et al. (2005).