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ARTICLE

qPCR Analysis of DNA Extracted from Adulterated Personal Care Products Using a Modified CTAB-based DNA Extraction Method

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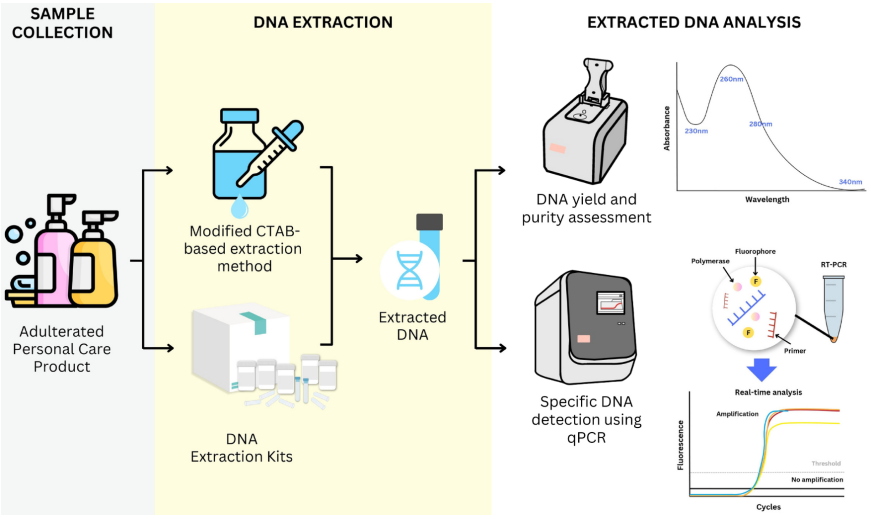

Personal care and cosmetic products are susceptible to adulteration due to the high market demand. Manufacturers tend to substitute plant-based materials with animal-derived alternatives as a cost-saving measure. The unlabelled adulterated ingredients arouse concern among consumers, especially the vegan community, as this fraudulent action can lead to adverse effects such as intoxication and allergy. To overcome this issue, Real-Time Polymerase Chain Reaction (qPCR) is an ideal technique for species identification based on specific primer design to detect targeted DNA from adulterated personal care products. In this work, cetyltrimethylammonium bromide (CTAB) method used for DNA extraction was modified to ensure successful detection and authentication of target species in personal care products. Robustness of the modified CTAB method was evaluated by comparing the DNA purity and yield across various samples. The developed CTAB method showed a promising result of DNA $A_{260/280}$ purity ratio ranging 1.15 – 2.12, whereas the commercial kits ranged 1.39 – 3.34 and 1.55 – 1.79 for the PowerPrep™ Gelatin DNA Extraction Kit and the PrimeWay Plant DNA Extraction Kit respectively. The extracted DNA were further analysed using qPCR, revealing detectable DNA in all tested products except for the shampoo sample used in this study, with concentration of porcine DNA ranging 2.94×10^{-4} – 1.46×10^{-2}. This modified CTAB method can be served as effective in-house method for species authentication in common personal care products.

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INTRODUCTION

Beauty and personal care product industries are experiencing a significant growth over the years. Personal care products and cosmetics help to improve quality of life by contributing the increase one's self-esteem and social interaction. Along with brisk modernity, consumers are becoming more health and wellness conscious. In line with the advancement of technology, different formulations are constantly researched and invented by manufacturers to enhance the quality of their products. The awareness regarding the ingredients contained in the products they consumed or applied routinely are highly crucial. Hence, the verification of authenticity and acceptability of product ingredients are necessary as the information concerning the identity and source of the ingredients processed are not always readily available.[1]

Personal care widely refers to a wide range of products that commonly can be found in the health and beauty sections in drug stores. The US Food and Drug Administration (FDA) defines personal care products and cosmetics as products that function to cleanse, protect the skin and enhance our appearance.[2] The Malaysia National Pharmaceutical Regulatory Agency (NPRA) has not stated any definition of personal care products. However, NPRA defines cosmetic products as substances or preparations intended to be in contact with external parts of the human body such as the epidermis, hair, nails, lips, and external genital organs, teeth, or mucous membranes of the oral cavity without ingestion of the products, mainly for cleaning them, perfuming them, changing their appearance and/or correcting body odours and/or keeping them in good condition.[3]

With the emergence of cosmetic industries and commercial demand, some personal care products such as body lotions, creams, and face packs are susceptible to substitution and adulteration, and contamination with undesired materials during the production process which can affect the quality of the product, which can leads to the adverse effects such as the increase of intoxication and allergy risks for consumers.[4] Commonly, manufacturers tend to substitute plant-based ingredients with compounds derived from animals such as fatty acid, glycerine, and collagen to reduce the production cost and to give false indications of the quality of the products.[5] Fatty acids were commonly utilised in personal care products due to its hydrating properties for the skin,[6] and pig collagen functioned to reduce the appearance of wrinkles on the skin.[7] Lard is also chosen by manufacturers as a substitute for oil since it is more economical and easily accessible.[8] Apart from confectionary, gelatine derived from skin and bone of the porcine is useful to be utilized as a gel-forming component in the production of cosmetic products and medical products.[9] In addition, it is also worth noting that the consumption of porcine derivatives is prohibited in certain religions like Islam.

Adulteration determination studies mainly focus on food products and drugs as these products are more susceptible for adulteration. Due to a very complex sample matrix, common chemistry-driven authentication techniques involve Fourier Transform Infrared (FTIR) spectroscopy, Gas Chromatography (GC), High-Performance Liquid Chromatography (HPLC), Liquid Chromatography (LC) and Tandem Mass Spectroscopy (MS/MS), often coupled with chemometric analysis such as Principal Components Analysis (PCA) and Hierarchical Cluster Analysis (HCA) for species discriminations.[10]

Proteins-based detection such as enzyme-linked immunosorbents assays (ELISA) are commonly applied in the bioanalytical industries.[11-13] However, personal care products are one of the very highly processed products. The protein-based approach is not a suitable authentication method for highly processed samples as protein can be easily denatured by heat, salt, and pressure.[14] Therefore, the protein and conventional chemistry-based analytical methods are hardly chosen, as sample matrices can contribute to the inevitable noise and interference during sample analysis. DNA-based approaches are preferred and widely used for species identification because DNA is more stable than RNA and proteins.[4] Furthermore, DNA is unique for every organism, making DNA detection techniques more efficient, convenient, and sensitive.[15]

This study was conducted to improve and optimise the cetyltrimethylammonium bromide (CTAB) method referenced from Doyle & Doyle[16] for DNA extraction and authentication analysis. CTAB was chosen for

its versatility and effectiveness in extracting DNA from complex and highly processed samples, providing reliable DNA quality.[17] In this study, personal care products were adulterated with porcine by spiking the samples with porcine DNA. A porcine-specific primer was developed for adulterants identification and detection in this study (porcine) using Real-Time Polymerase Chain Reaction (qPCR) analysis. The robustness of the developed method was compared with commercial DNA extraction kits. The developed method was applied to detect the presence of porcine-derived ingredients in commercial and laboratory synthesised soap samples.

MATERIALS AND METHODS

Reagents

Cetyltrimethylammonium bromide, CTAB (Sangon Biotech, China), NaCl (Emsure Denmark), Tris-HCl (J.T. Baker, USA), EDTA (Sigma Aldrich, Switzerland), 1,4- dithiothreitol (DTT) (Biochemistry grade) (Sigma Aldrich, Canada), RNase A (Invitrogen, USA), chloroform (Biotech grade) (Sigma Aldrich, USA), absolute ethanol (Analytical grade) (Emsure, Germany), nuclease free water (Sigma Aldrich, United Kingdom), Tris-EDTA buffer solution (Sigma Aldrich, USA), porcine genomic DNA (Novagen, USA).

Personal care product samples

Personal care products such as shampoo, body wash and baby wash were purchased from drug stores in Kuala Lumpur. These samples were chosen based on their popularity, demand and common availability in households. Different types of samples were selected to diversify the matrices used in their formulation. The samples were stored at room temperature before being analysed. About 180 mg of personal care product samples were spiked with 20 µL of porcine genomic DNA and mixed using a vortex. The adulterated samples were left for 2 days at 4 °C prior to extraction. Pig pancreas soap was purchased through an online store, and 60% lard adulterated soap that was synthesized at the University Malaya Halal Research Centre (UMHRC) laboratory was used as samples in this work.

Control samples

Tomato, onion, potato, rice, ginger, and fish were purchased from local supermarkets. Cow DNA (Integrated DNA Technologies, USA), chicken DNA (Integrated DNA Technologies, USA), and porcine DNA (Novagen, USA) were purchased from a distributor. The meat and plant samples were used to determine the specificity of the porcine-specific primer for qPCR analysis.

DNA Extraction

Extraction of DNA from spiked personal care products using a modified CTAB-based DNA method

1000 µL of CTAB buffer (20 g L^{-1} CTAB; 1.4 mol L^{-1} NaCl; 0.1 mol L^{-1} Tris-HCl; 20 mmol L^{-1} EDTA), 10 µL of RNase, and 10 µL of 1 M dithiothreitol (DTT) were added into a microcentrifuge tube containing ~180 mg of personal care product sample. The tube was then vortexed and incubated for 60 min at 65 °C in a shaking water bath prior centrifugation at 13,000 rpm for 10 min. The supernatant was transferred to a new 2 mL sterile microcentrifuge tube which contained 800 µL of chloroform. The samples were mixed with vortex before being centrifuged at 8000 rpm for 15 min. Two layers were formed, the upper aqueous layer and the organic layer at the bottom. The top aqueous layer was then transferred to a new sterilized 2 mL microcentrifuge tube containing 600 µL of chloroform. The mixture was mixed and centrifuged for 15 min at 8000 rpm. The top aqueous layer was transferred to a sterilized 1.5 mL microcentrifuge tube containing 900 µL of absolute ethanol, vortexed, and centrifuged at 13,000 rpm for 15 min. Ethanol was discarded leaving a precipitated white pellet at the bottom of the tube. 1000 µL of 80% ethanol was then added to wash the pellet. The tube was centrifuged at 13,000 rpm for 5 min. The ethanol was then discarded and the microcentrifuge tube cap was left open to dry out the remaining alcohol. The pellet was resuspended in 100 µL Tris-EDTA (TE) buffer at pH 8.0 and kept at 4 °C before further downstream analysis.

Extraction of DNA from spiked personal care products using extraction kits

DNA from samples spiked with 1, 5, and 10 ng μL^{-1} and bar soap samples were extracted using two commercially available extraction kits, PowerPrep™ Gelatin DNA Extraction kit (Kogene Biotech, Korea) and PrimeWay Plant DNA Extraction Kit (1st Base, Singapore), according to the manufacturer 's protocol. The experiment was done by extracting duplicate samples for each personal care product.

The yield and purity of extracted DNA

The concentration and purity of extracted DNA was determined using NanoDrop™ 2000c spectrophotometer (Thermo Fisher Scientific, United States). 1 µL of (Tris-EDTA) TE buffer / elution buffer for resuspension of extracted DNA pellet was used as a blank solution for the baseline correction of the instrument. 1 µL of the sample was used for the analysis.

Probe and Primer design

Porcine-specific primer

The porcine-specific primer used in this study is listed in Table I. The primer was designed using Primer3web tool to target the *Sus NDH5* gene, a mitochondrial DNA (mtDNA). The selected gene was referenced from a halal detection study of cosmetic products.[5] The TaqMan probe consists of FAM fluorescent dye at the 5' end and is attached to a non-fluorescent quencher with a minor groove binder (NFQ-MGB) at the 3' end of the probe's oligonucleotide sequence. mtDNA was chosen due to its higher copy number in cells and its tendency to degrade more slowly compared to nuclear DNA (ntDNA), as the double membrane of the mitochondria provides protection to the DNA molecules.[18]

Table I. Sequence of the Porcine-specific primers

Primer Assay		Sequence 5'-3'	Amplicon size (bp)
Porcine-specific primer	Forward	GCCTCACTCACATTAACCACACTG	139
	Reverse	AGGGGACTAGGCTGAGAGTGAA	
	Probe	CCCTAACTACGTAAAAAC	

Quantitative Polymerase Chain Reaction (qPCR)

Internal control

The genomic DNA of *Bacillus subtilis* DSM 5750 (Certified Reference Material IIRM-312) was implemented as an internal control solely used to ensure no false negative amplification occurred during the qPCR-specific detection analysis. 16 rRNA gene was chosen to ensure that only the targeted procaryotic DNA (*Bacillus subtilis*) was amplified, without interfering with the detection of the porcine DNA.

qPCR run program

Real-time PCR was performed with total aliquots of 20 µL, where the components of the PCR assays were shown in Table II. The DNA amplification was conducted using QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems, USA). The PCR procedures were programmed as follows: denaturation (95 °C, 20 seconds) for 1 cycle; annealing of primers (95 °C, 1 second) and elongation (60 °C, 20 seconds) repeated for 40 cycles.

Table II. Components for PCR reaction

Component	Volume of each component in 20 µL of PCR mixture (µL)	Final concentration
TaqMan™ Fast Advanced Master Mix (Applied Biosystems, USA) 2X	10	1X
Porcine specific primer/ probe 20X	1	1X
Internal control gene	1	N/A
Internal control Primer 20X	1	1X
Template DNA	5	-
Nuclease free water	2	-

N/A: not available

Designated primer validation tests

The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) was used as a guideline to develop a framework to elucidate the reliability of designated porcine-specific primers.[19,20] The validation of the porcine-specific primer was tested based on the mandatory information provided by the established MIQE guidelines.

RESULTS AND DISCUSSION

Porcine-specific primer validation

Specificity of the porcine-specific primer

The specificity test aims to ensure that the designated porcine-specific primer is highly sensitive and selective towards the target species only. If DNA from species other than the targeted was amplified, the designated primer is considered non-specific, defeating the purpose of this study. The specificity of designated porcine-specific primer was determined by amplifying diverse types of animals and plant species. DNA from plant and meat samples is easily extracted and purified due to the high abundance of DNA molecules and less complex sample matrices. In contrast, highly processed and manufactured samples often contain significant amounts of PCR inhibitors that are difficult to eliminate during the DNA extraction process.

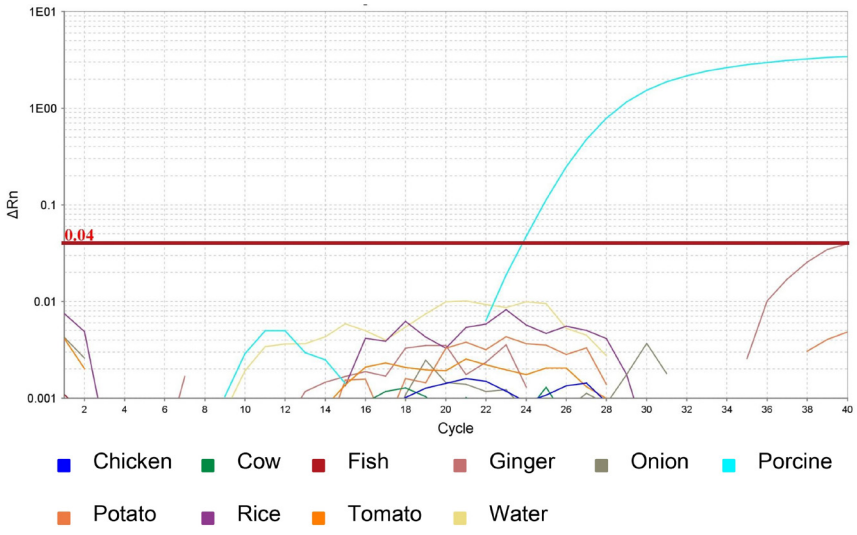


Figure 1. Amplification curves from qPCR testing for porcine-specific primer specificity across animals and plant species. The only amplification detected was from porcine DNA, with a C_t value of 26.64, confirming the primer's specificity.

Referenced to Figure 1, there was no amplification detected in all tested DNA assays except for the porcine DNA (C_t value of 26.64). Thus, this indicates the designated porcine-specific TaqMan primer is highly specific for porcine species only.

qPCR efficiency and Limit of Detection (LOD)

The reaction efficiency (E) of porcine-specific TaqMan real-time PCR primer assay was determined by generating a standard curve with a 10-fold dilution series (1.0, 0.1, 0.01, 0.001, and 0.0001 ng μL^{-1}) of porcine DNA.

The slope obtained, -3.1017 was then implemented into the formula, Efficiency (E) = $10^{-(1/slope)}$ - 1,[21] resulting the acceptable efficiency of PCR value which was E = 1.10. The range of acceptable efficiency is commonly between E = 0.90 - 1.10, and the maximal efficiency is at E = 1.00, which indicates the DNA templates doubles exponentially in each amplification cycle and the amplification of qPCR is adequately efficient.[22,23]

The sensitivity of the porcine-specific TaqMan primer was determined by amplifying the lowest detectable concentration of porcine DNA from the standard curve generated. 20 replicates of 0.0001 ng μL^{-1} of diluted porcine DNA were amplified, as shown in Figure 2. All the replicates were amplified, and the given mean C_t value is 32.13 ± 0.42.

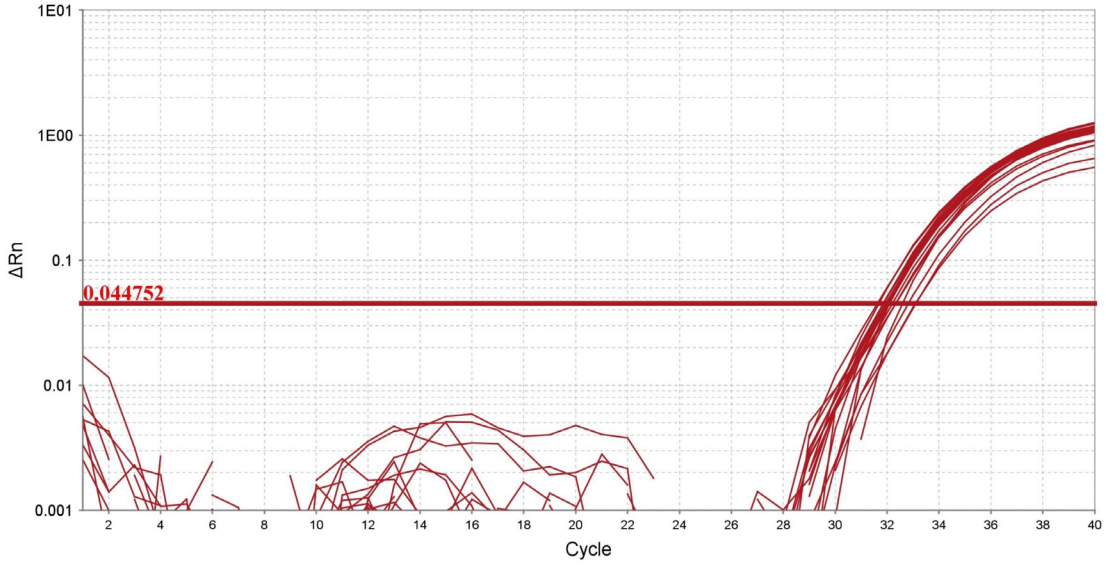


Figure 2. The sensitivity of the porcine-specific TaqMan primer was assessed by amplifying 20 replicates of 0.0001 ng μL^{-1} porcine DNA, the lowest detectable concentration. All replicates were successfully amplified, with a mean C_t value of 32.13 ± 0.42, confirming the primer's consistent limit of detection (LOD).

Analysis of DNA extracted from spiked personal care products

Modification of the CTAB-based DNA extraction method

The developed CTAB-based DNA extraction method was modified based on the established RNA extraction technique for plant samples by Doyle & Doyle.[16] Table III compares the modified CTAB extraction method with the conventional CTAB extraction method established by Doyle & Doyle.

Table III. Conventional and modified CTAB-based DNA extraction method comparisons

Extraction step	Conventional CTAB	Modified CTAB
Lysis (CTAB Buffer)	100 mM Tris-HCl (pH 8.0), 1.4 M NaCl, 20 mM EDTA, 2% (w/v) CTAB, 0.2% β-mercaptoethanol	100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, 2% (w/v) CTAB The buffer solution was adjusted to pH 8.0 1 M dithiothreitol (DTT)
Purification	Chloroform-isoamyl (23:1)	Chloroform
Precipitation	Isopropanol	Ethanol
Washing (wash buffer)	76% ethanol, 10 mM ammonium acetate	80% ethanol

The modified CTAB method reduced the use of chemicals and prevented β-mercaptoethanol from being incorporated into the lysis buffer. β-mercaptoethanol is a toxic, volatile, and highly pungent compound, that was replaced with a safer alternative, dithiothreitol (DTT). Both β-mercaptoethanol and DTT serve as reducing agents, to degrade RNase by deactivating the disulfide-bonded tertiary structure.[24] Isoamyl alcohol is commonly paired with chloroform to minimize emulsification and prevent the formation of carboning chloride ($COCl_2$) gas during DNA purification. The chloroform-isoamyl mixture creates a distinct separation between the aqueous and organic layers, reducing the risk of DNA lost during the extraction process.[25] However, isoamyl alcohol can inhibit RNase activity and lower DNA purity;[26] therefore, it was excluded from the modified CTAB-based DNA extraction method. Additionally, no frothing was observed when chloroform was added into the lysed sample, thus further eliminating the need for isoamyl alcohol in the modified procedure.

Before the modification of the CTAB-based DNA extraction method, personal care product samples were extracted using the conventional CTAB method by Doyle & Doyle,[16] and the extracted DNA was amplified using qPCR. Table IV shows that no C_t values were observed during the amplification of the extracted DNA, indicating the presence of PCR inhibitors that were not removed during the extraction process. Therefore, it is necessary to modify the conventional CTAB method to ensure successful detection and amplification of the target DNA.

Table IV. Average C_t values of extracted DNA assays using conventional CTAB-based DNA extraction method

Sample	Mean C_t value of target primers	
	Porcine-specific	**Internal control**
Body wash	UND	UND
Shampoo	UND	UND
Baby wash	UND	UND
Negative control (NTC)	UND	18.02
Positive control (porcine DNA)	23.73	18.01

UND: Undetected

Yield of extracted DNA

The NanoDrop UV-Vis spectrophotometer assesses the concentration and quality of nucleic acids and proteins by utilizing the Beer-Lambert law. The abundance of nucleic acids (DNA and RNA) is determined

by measuring the absorbance of the sample within the 260 nm region in the ultraviolet-visible (UV-Vis) range of the electromagnetic spectrum.[27] The maximum absorbance of nucleic acids at 260 nm is influenced by the presence of purines (thymine, cytosine, and uracil) and pyrimidines (adenine and guanine).[28,29]

The average DNA yield extracted from the samples spiked with three concentrations (1, 5, and 10 ng μL^{-1}) of porcine genomic DNA are represented in Figure 3. The bar chart illustrates that the various types of extraction method favor different types of personal care product samples, whereas the error bar represents the standard error of the data. PowerPrep™ Gelatin DNA Extraction Kit and the PrimeWay Plant DNA Extraction Kit were selected to evaluate the quality of DNA extracted compared to the modified CTAB-based DNA extraction method. A previous study on halal detection in cosmetic products found that PowerPrep™ DNA extraction kit yielded the best results among the methods tested,[5] which influenced its selection for this study. The PrimeWay Plant DNA extraction kit was chosen for its potential effectiveness in removing common compounds found in the personal care product matrices that can impede PCR detection and amplification.

Generally, the PrimeWay Plant DNA Extraction Kit yielded the highest concentration of DNA extracted from the body wash sample, while the concentration of DNA extracted from the shampoo sample using the modified CTAB extraction method was the highest compared to the two DNA extraction kits. However, the yield of DNA detected in certain samples exceeded the amount of porcine DNA spiked in the sample. A deduction that can be drawn from this observation is that there are contaminants present in the sample assay that absorb strongly near the 260 nm region. Nevertheless, the possible identity of these contaminants is beyond the focus of this study and hence, it will not be further discussed and explored.

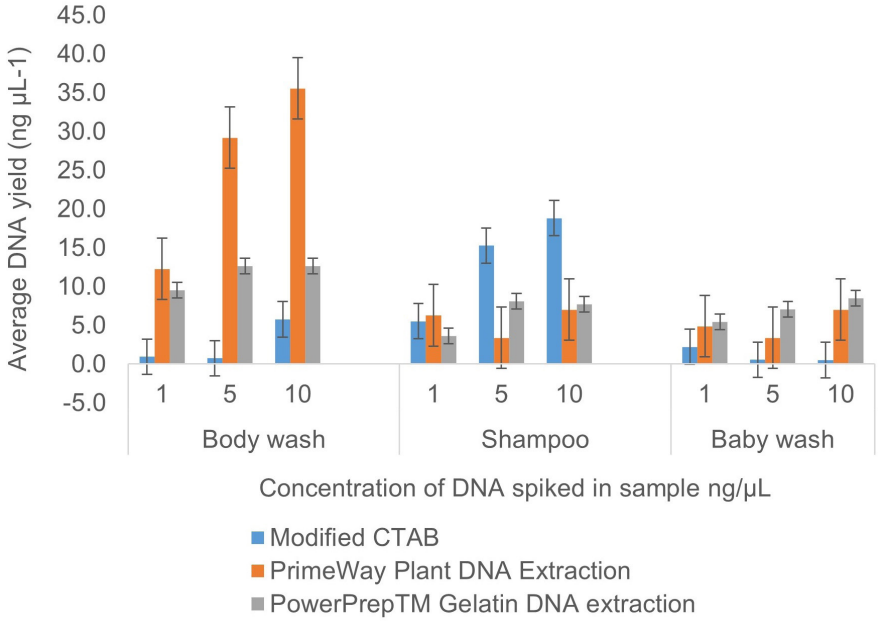


Figure 3. Average DNA yield from personal care products spiked with different concentrations of porcine DNA (1, 5, and 10 ng μL^{-1}) extracted using three different methods: Modified CTAB, PrimeWay Plant DNA Extraction Kit, and PowerPrep™ Gelatin DNA Extraction Kit.

Both extraction kits employed the solid phase nucleic acid extraction method for DNA isolation as both extraction kits utilised the spin columns for the DNA extraction procedures. The spin columns are commonly deployed in the commercialized nucleic acid extraction kit. The spin column is packed with solid phase materials such as silica membranes that act as solid sorbents, providing a surface for DNA binding. Subsequently, alcohol or low ionic strength solvent will then be used to break the sorbent-DNA interaction

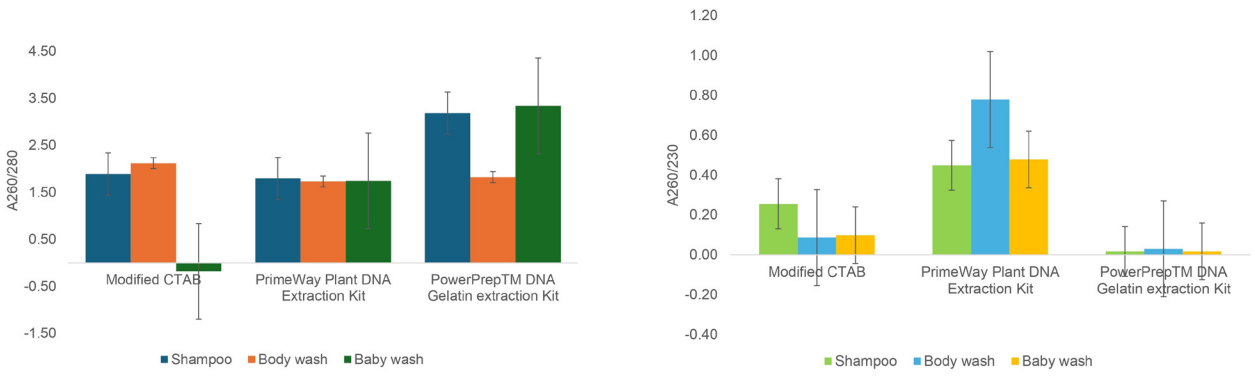
in the spin column.[30] The primary function of a spin column is to reduce the loss of low abundance and fragmented DNA in highly processed samples. However, its costly and unrecyclable properties contribute to the disadvantage of the spin column as one spin column can only be used once for a sample.

In contrast, the modified CTAB method mainly focused on liquid-liquid extraction as only solvents were used, and no sorbents were involved during the extraction process. Since a very low concentration of porcine DNA spiked in the sample, no DNA precipitation as white pellet was observed after the addition of absolute ethanol in the CTAB method procedures. The extraction process becomes tricky, and DNA might majorly be lost during the removal of ethanol after the centrifugation step. Nevertheless, the DNA isolated is still sufficient to be amplified and detected using qPCR.

Purity of extracted DNA

The quality of nucleic acids was assessed by determining the purity absorbance ratios of $A_{260/280}$ and $A_{260/230}$ to ensure high accuracy of the bioanalytical analysis.[27] The significance of these absorbance ratios lies in their applications for downstream analysis and the prediction of the potential types of inhibitors that are still present in the final product of the extracted sample. The absorbance ratios of $A_{260/230}$ are generally used to measure the suitability of the sample for microarrays while the $A_{260/280}$ are for PCR amplification.[28] DNA assay is highly pure if the $A_{260/280}$ is between the range of 1.8 – 2.0, while for $A_{260/230}$ is between 1.8 to 2.2.[28,31]

Personal care products contain very complex sample matrices, as various chemical compounds are incorporated into a single product. The main challenge of DNA purification is to remove the contaminants and inhibitors that can affect the downstream analytical procedure. The presence of inhibitors can interfere and hinder the amplification of DNA during the PCR process and can cause false-negative results. The purity of extracted DNA from all extraction methods is presented in Figure 4.



(A) (B)

Figure 4. Purity of DNA extracted from spiked personal care products (shampoo, body wash, and baby wash), measured by (A) $A_{260/280}$ and (B) $A_{260/230}$ absorbance ratios using NanoDrop UV-Vis spectrophotometer.

The PrimeWay Plant DNA Extraction Kit showed a consistent average purity of all extracted samples compared to the modified CTAB method and the PowerPrep™ DNA Gelatin Extraction Kit as illustrated in Figure 4(A). The DNA extracted using the PrimeWay Plant DNA Extraction Kit is considered to be pure to in almost all samples (ranging 1.73 – 1.79) compared to the other extraction methods. The purity ratio $A_{260/280}$ of some DNA essay extracted from the modified CTAB method is acceptably pure except for the baby wash. The very low value of the purity ratio indicates the possible presence of proteins, phenol, or other contaminants that absorb strongly at 280 nm.[32] The low yield of DNA that absorbs strongly at 260 nm region might also contribute to the low value of the ratio. The high value ratio of $A_{260/280}$ reflected by

some DNA extracted from PowerPrep™ DNA Gelatin Extraction Kit is deduced due to the presence of contaminants that absorb highly near the 260 nm region.

The absorbance ratio of $A_{260/230}$ is widely used as a secondary purity determinator for nucleic acid assays. All DNA extraction methods portrayed in Figure 4(B) does not give acceptable purity values. The DNA samples give a very low $A_{260/230}$ ratio due to the presence of chemical compounds that are unable to be removed or a carryover from chemicals used during the extraction process. Examples of these compounds may include guanidine, hydrochloric acid, ethylenediaminetetraacetic acid (EDTA), lipids, chaotropic salts, ethanol, or aromatic compounds absorb strongly at 230 nm region.[33] Hence, all of the extracted assays deemed to be not suitable for microarray analysis.

qPCR amplification of spiked porcine DNA

The concentration of porcine DNA detected using qPCR from the spiked personal care products was calculated from the C_t values and is shown in Table V and the C_t values of the internal control obtained were tabulated in Table VI.

Table V. Concentration of porcine DNA detected in spiked personal care products by qPCR

Concentration of porcine DNA detected by qPCR (ng μL^{-1})				
Sample	**Extraction method**	**Concentration of spiked porcine genomic DNA (ng μL^{-1})**		
		1	**5**	**10**
Body wash	Modified CTAB method	7.48×10^{-4}	4.86×10^{-4}	1.90×10^{-2}
	PowerPrep™ Gelatin DNA extraction Kit	5.18×10^{-1}	2.21	3.82
	PrimeWay Plant DNA Extraction Kit	6.80×10^{-2}	4.62×10^{-1}	1.29
Shampoo	Modified CTAB method	UND	UND	UND
	PowerPrep™ Gelatin DNA extraction Kit	5.45×10^{-5}	6.74×10^{-5}	4.92×10^{-4}
	PrimeWay Plant DNA Extraction Kit	1.56×10^{-3}	2.67×10^{-3}	7.23×10^{-5}
Baby wash	Modified CTAB method	2.94×10^{-4}	3.41×10^{-3}	1.46×10^{-2}
	PowerPrep™ Gelatin DNA extraction Kit	2.71×10^{-2}	8.97×10^{-1}	3.27
	PrimeWay Plant DNA Extraction Kit	5.93×10^{-1}	3.35×10^{-1}	9.88×10^{-3}

UND: Undetected

The common adulterants that are added to beauty products are commonly collagen, gelatine and lard from porcine as these substances can confer firmness, elasticity, and help moisturize the skin.[8] With the addition of various chemicals and harsh conditions for the production process, DNA can be easily degraded. Detection of personal care product samples has become a great challenge due to the high complexity of sample matrixes. Besides the effort to reduce DNA loss during the extraction procedure, it has become a great challenge to ensure that the DNA extract obtained from the extraction procedure is free from PCR inhibitors.

The modified CTAB method, PowerPrep™ Gelatin DNA extraction Kit, and PrimeWay Plant DNA Extraction Kit are effective and good enough to extract DNA from personal care product samples except for the shampoo samples used in this study. This is because the modified CTAB method was unable to remove the PCR inhibitor present in the shampoo sample, causing a false-negative result. This indicates that the extraction method might be not suitable to be used for shampoo products. The amplification of internal

control is shown in Table VI. The hindrance of PCR amplification is perhaps due to the unremoved inhibitor from the shampoo samples. However, the DNA extracted using PowerPrep™ Gelatin DNA extraction Kit and PrimeWay Plant DNA extraction Kit was detected by qPCR as shown in Table V, indicating the commercial extraction kits could remove inhibitors from the shampoo sample used in this study. However, the detection of the porcine-specific DNA came late with concentration between 5.45 x 10^{-5} to 2.67 x 10^{-3} ng μL^{-1} and was also shown to be inconsistent. The late detection of porcine DNA might be caused by cross-contamination during the extraction procedure.[34]

Table VI. C_t values of internal control for spiked personal care product samples

C_t mean value of Internal Control				
Sample	**Extraction method**	**Concentration of spiked porcine genomic DNA (ng μL^{-1})**		
		1	**5**	**10**
Body wash	Modified CTAB	16.77	19.70	19.15
	PowerPrep™ Gelatin DNA extraction Kit	17.58	17.67	18.39
	PrimeWay Plant DNA Extraction Kit	16.90	17.80	16.86
Shampoo	Modified CTAB	UND	UND	UND
	PowerPrep™ Gelatin DNA extraction Kit	18.04	17.76	17.99
	PrimeWay Plant DNA Extraction Kit	17.40	17.66	18.67
Baby wash	Modified CTAB	19.56	19.18	18.65
	PowerPrep™ Gelatin DNA extraction Kit	17.47	17.82	17.34
	PrimeWay Plant DNA Extraction Kit	16.38	16.60	17.61

UND: Undetected

The occurrence of this event may also be due to the hindrance of primer anneals to the DNA strands as many unwanted contaminants are still present in the sample assay. Nevertheless, based on the amplification results of internal control shown in Table VI, the C_t value for the shampoo sample extracted using extraction kits was consistent with other personal care product samples. Therefore, the result further suggests the poor efficiency of the modified CTAB method to purify the DNA from the shampoo sample which in turn hinders amplification. The other hypothesis to rationalize this event might be due to the presence of some contaminants in the shampoo sample such as preservative molecules that can degrade or impair DNA replication and result in the failure amplification of PCR.[35] Generally, preservatives such as parabens, triclosan, propionic acid, formaldehyde, and hexamidine are added to the personal care product formulation as a preventive measure for microbial contamination.[36] Chloroform that was implemented during the extraction procedure is not effective enough to remove all inhibitors such as phenol, sodium dodecyl sulphate (SDS), and proteinases[37] that may be present in the sample. It is also important to note that results may vary between different shampoo samples due to variations in sample formulation and sample matrix.

The possible type of inhibitor can be referred to the purity ratios measured using the NanoDrop UV-Vis. It can be predicted that the high inhibition might be caused by the high number of contaminants that absorb strongly at 230 nm as shown in Figure 4(B). Besides, it is possible that PCR inhibitors that are undetected at 230 nm and 280 nm regions could still be present in the shampoo sample matrix used in this study. The inhibition mechanisms may include interference with the DNA polymerase or nucleic acids during amplification reactions.[38]

Authentication analysis of adulterated personal care products

Yield of extracted DNA from adulterated soap samples

To detect the presence of porcine DNA from the adulterated personal care products, 200 mg of pig pancreas soap and 60% lard adulterated soap were extracted using the modified CTAB method, PowerPrep™ Gelatin DNA Extraction Kit, and PrimeWay Plant DNA Extraction Kit prior to DNA analysis.

Table VII shows the yield of extracted DNA from adulterated soap samples. The modified CTAB method is able to extract an extremely high concentration of DNA from the pig pancreas soap samples compared to the extraction kits and shows a good DNA assay purity which is $A_{260/280}$ = 1.81.

Table VII. Average yield of DNA extracted from adulterated soaps using three different extraction methods

Sample	Extraction Method	Average DNA yield (ng μL^{-1})
Pig pancreas bar soap	Modified CTAB method	1182.22 ± 141.24
	PowerPrep™ Gelatin DNA Extraction Kit	133.10 ± 15.34
	PrimeWay Plant DNA Extraction Kit	23.62 ± 3.65
Lard adulterated bar soap	Modified CTAB method	1.48 ± 1.11
	PowerPrep™ Gelatin DNA Extraction Kit	17.85 ± 12.26
	PrimeWay Plant DNA Extraction Kit	12.08 ± 4.56

However, the enormously high yield of DNA detected from pig pancreas soap samples may be due to deviations of absorbance measured due to the limitation of Beer-Lambert law itself. In addition, the extracted DNA from the pig pancreas soap samples does not appear to be colorless. The extracted sample clearly showed that a high number of contaminants were still unable to be separated and removed from the DNA. Theoretically, Beer-Lambert law describes the absorption characteristics of solutions with low concentrations (<10 mM) of analyte. High concentrations of analyte (>10 mM) will behave differently upon interactions with surrounding molecules in the solvent.[39] Thus, the NanoDrop UV-Vis is unable to provide consistent absorbance readings, which caused huge uncertainty and inconsistency of the absorbance readings for the pig pancreas soap.

In contrast, the DNA extracted from lard-adulterated bar soap using the modified CTAB method is very low compared to the other commercial extraction kits. During preparation of the adulterated soap, some DNA might be fragmented and degraded as heat was introduced to melt the lard before the soap-making process. The extraction kits show better extracted DNA yields because the spin columns in the extraction kits provide a surface for the short fragmented DNA to be absorbed at the surface of silica reducing DNA loss during extraction and allowing better DNA recovery.[40,41] Contrarily, extracted DNA as the final product using the modified CTAB method mainly depends on the precipitation of the DNA. Thus, short, and fragmented DNA are prone to be lost during extraction procedure.

Purity of extracted DNA from adulterated soap samples

Figure 5 shows the average purity ratios for pig pancreas soap samples extracted using the modified CTAB method. The DNA from the developed method is considered pure compared to the other extraction methods. Even though it was apparent that there are contaminants present in the final product of the extracted pig pancreas sample, it is safe to say that the modified CTAB method is able to remove contaminants that absorb strongly at 280 nm, such as proteins and phenol.[32]

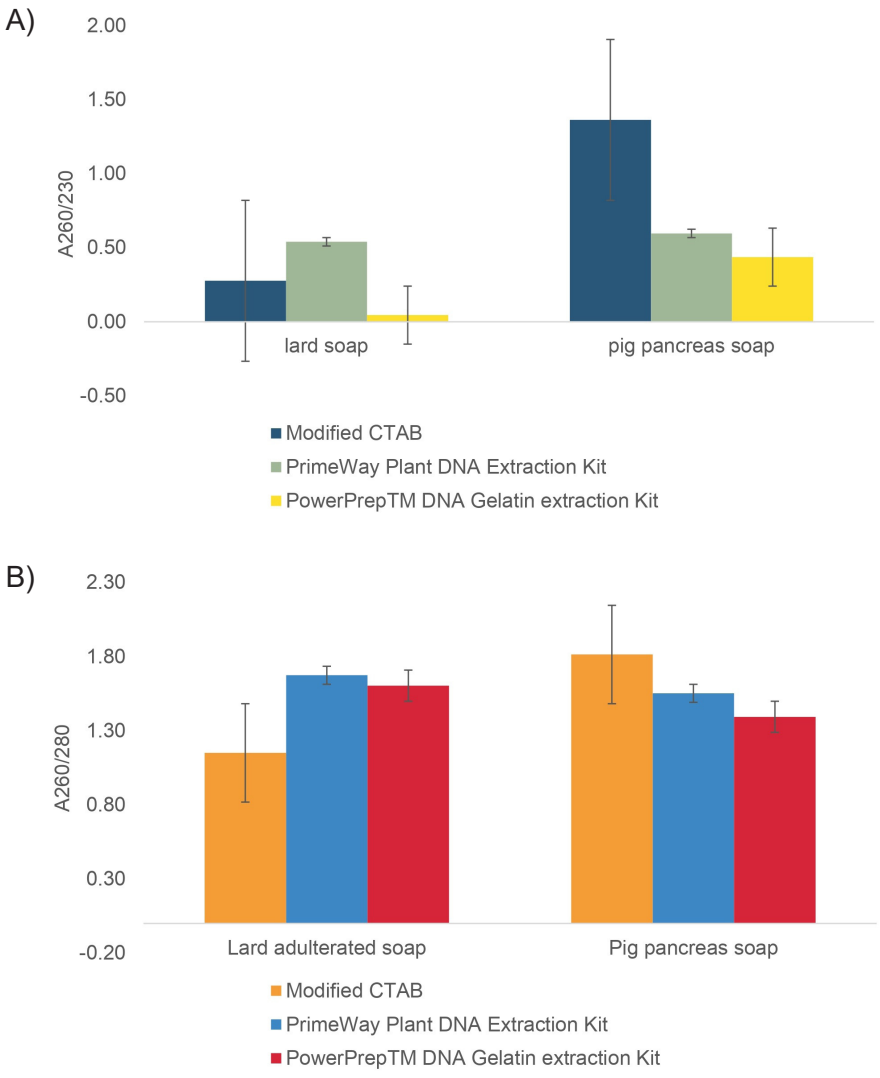


Figure 5. Purity of DNA extracted from adulterated soap samples (pig pancreas soap and lard adulterated soap), was determined by calculating the A) $A_{260/280}$ and B) $A_{260/230}$ absorbance ratios using NanoDrop UV-Vis spectrophotometer.

Unlike pig pancreas soap, the purity ratio of $A_{260/280}$ for lard adulterated soap shown in Figure 5(A) was the lowest compared to the other extraction methods. This might be due to the low amount of DNA was able to be extracted and the high amount of contaminants that absorb strongly at 280 nm region.[33]

The DNA purity shown in Figure 5(B) suggests the presence of a very high amount of contaminants that absorb strongly at the 230 nm region. These contaminants can come from the sample matrix itself and from the chemicals used during the extraction process such as hydrochloric acid, EDTA, lipids, chaotropic salts, ethanol or aromatic compounds.[33]

Even though the final product of extracted DNA was not as pure as intended, not all contaminants present together with the extracted DNA are PCR inhibitors. Thus, the detection of porcine DNA was carried out to confirm the amplifiability of the target DNA.

Detection of porcine DNA using qPCR

The amplification of porcine DNA from extracted adulterated soap samples were carried out using qPCR and the results are shown in Table VIII.

Table VIII. The concentration of porcine DNA extracted from adulterated personal care products and the average C_t value of the internal control

Sample	Extraction method	Concentration of porcine DNA (ng μL^{-1})	Average C_t value of internal control
Pig Pancreas Bar soap	Modified CTAB method	5.28	18.26
	PowerPrep™ Gelatin DNA extraction Kit	7.83×10^{-1}	17.61
	PrimeWay Plant DNA Extraction Kit	1.12	16.92
Lard adulterated bar soap	Modified CTAB method	4.51×10^{-5}	16.38
	PowerPrep™ Gelatin DNA extraction Kit	2.62	17.15
	PrimeWay Plant DNA Extraction Kit	5.56×10^{-5}	16.74

There is no denying that halal detection of personal care product samples has become a major challenge due to the high complexity of the sample matrix itself. Chloroform that is implemented during the modified CTAB method extraction procedure is not effective enough to remove all inhibitors such as phenol, sodium dodecyl sulphate (SDS), and proteinases[37] that may be present in the sample. Contrarily, both DNA extraction kits can be implemented to remove inhibitors from the shampoo sample.

Besides the presence of apparent contaminants in pig pancreas DNA extracted using all extraction methods, porcine-specific DNA is successfully detected in all pig pancreas soap samples. Chloroform implemented in the modified CTAB extraction method was powerful enough to minimize the PCR inhibition during the DNA extraction procedure from the lard adulterated soap and pig pancreas soap. qPCR can detect and amplify the target DNA despite a low purity of extracted DNA. The extracted DNA from the modified CTAB extraction method is sufficient for PCR amplification and specific species authentication. Hence, it can be concluded that the modified CTAB method can be used for the extraction and determination of porcine DNA from porcine derivatives adulterated personal care products.

In the past years, few studies on species authentication from cosmetics have been made. However, the researchers commonly use commercialized DNA extraction kits and are more focused on detection methods rather than the extraction method itself. The major disadvantage of the commercialized extraction kits is the limited amount of reaction that can be used for every sample in the study despite a very expensive price. The developed modified CTAB method can become a good alternative as an in-house method due to the low amount of chemicals used and relatively more cost-effective compared to the extraction kits for extraction of DNA from personal care products for species authentication.

CONCLUSIONS

The modified CTAB method developed proved to be successful in detecting the porcine-specific DNA in various personal care products used in this study such as body wash, baby soap, lard adulterated soap, and pig pancreas soap. Even though the developed modified CTAB extraction method was unable to ultimately eliminate some PCR inhibitors in the shampoo sample used in this study, the NanoDrop UV-Vis result indicated that the method is still able and effective in extracting DNA. Despite the complexity of sample matrixes, the modified CTAB method is sufficient to be considered as an alternative method for extraction and species authentication of DNA from personal care products.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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