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Comparison of Real-Time PCR and FTIR with Chemometrics Technique in Analysing *Halal* Supplement Capsules

Mohd Sukri Hassan, Ahlam Inayatullah Badrul Munir, M. Husaini A. Rahman

Abstract**—***Halal* authentication and verification in supplement capsules are highly required as the gelatine available in the market can be from halal or non-*halal* sources. It is an obligation for Muslim to consume and use the halal consumer goods. At present, real-time polymerase chain reaction (RT-PCR) is the most common technique being used for the detection of porcine and bovine DNA in gelatine due to high sensitivity of the technique and higher stability of DNA compared to protein. In this study, twenty samples of supplements capsules from different products with different *Halal* logos were analyzed for porcine and bovine DNA using RT-PCR. Standard bovine and porcine gelatine from eurofins at a range of concentration from 10^{-1} to 10^{-5} ng/µl were used to determine the linearity range, limit of detection and specificity on RT-PCR (SYBR Green method). RT-PCR detected porcine (two samples), bovine (four samples) and mixture of porcine and bovine (six samples). The samples were also tested using FT-IR technique where normalized peak of IR spectra were pre-processed using Savitsky Golay method before Principal Components Analysis (PCA) was performed on the database. Scores plot of PCA shows three clusters of samples; bovine, porcine and mixture (bovine and porcine). The RT-PCR and FT-IR with chemometrics technique were found to give same results for porcine gelatine samples which can be used for *Halal* authentication.

Keywords**—**Halal, real-time PCR, gelatin, FTIR and chemometrics.

I. Introduction

THE detection of any contamination of pig becomes essential to ensure the safety and also the *halalness* of a product. The percentage of gelatin production in the world in 2006, still dominated by pig gelatin, with 45.8% the materials is coming from pigskin and 28.4% from cowhide [1]. The analysis based on chemical had been done to analyze the animal source of gelatin in order to detect of *halalness* with Mass Spectrometry [2], Fourier Transform Infrared (FTIR) Spectroscopic [3], and Sodium dodecyl Sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) that combined with PCA [4] and all the techniques is considered not sufficiently effective in detecting the animal source of gelatin. The instrument of analysis could classify soft capsule shell (sigma-Aldrich) that was made from pig and cow and capsule shell from markets, based on the peak spectrum. However, PCA could not give any specific information for any differentiates about the peak spectrum obtained from previous instrument (especially commercials product). The weakness point of the method was caused by the protein analysis, where the protein is unstable in high temperature and pH extreme [5].

Method analysis for gelatin capsule is developed using RT-PCR technique with the assist of SYBR green as the fluorescent dye. For the detection of porcine DNA, one of the genes that can be used as a marker is a specific gene from mitochondria cytochrome b (CYT b). The gene is expected to support the development of DNA detection methods in gelatin capsule. Preparation of DNA isolates (before amplification) becomes the important steps to smoothly amplify the DNA. SYBR Green and TaqMan probe is a chemical compound that most widely used to monitor the amplification that occurs during the cycle of real time PCR. SYBR Green considered more convenient because it is not need to design a probe [6]. Soares et al. [7] has successfully developed a method of SYBR Green real-time PCR for the detection and quantization of porcine DNA in processed meat products.

The aim of this study was to validate analysis method for identification of gelatin sources using real-time PCR for analysis gelatin capsules in supplement product. The use of FT-IR and chemometrics in this study is to compare the result that was obtained using real-time PCR previously.

II. Materials and Methods

Samples were purchased in Nilai area. Gelatin capsule from supplement product with *Halal* logo from different countries were used in this study.

A. Materials

Genomic DNA cattle and pig (eurofins), Sso advanced SYBR green (Bio-rad), specific pairs of primer porcine and bovine [8], Distilled water.

B. Instrument

The real time PCR from applied biosystem and The Varian 640-IR from Varian.Inc

C. Validation of Real-Time PCR

The sample from the genomic DNA cattle (Bos Taurus) and the genomic DNA pig (Sus Scrofa) was tested with the different concentrations in real time PCR with the selected concentration was 10^{-1} to 10^{-5} ng/µl.

M. S. Hassan is with the Institute of Halal Research and Management (IHRAM) and Faculty of Science and Technology, Universiti Sains Islam Malaysia (USIM), Malaysia (e-mail: pakar_4ren6@hotmail.com).

A. I. Badrul Munir is with the Faculty of Science and Technology, USIM, Malaysia (e-mail: ahlaminayatullah@gmail.com).

M. H. A. Rahman is with Institute of IHRAM and Faculty of Science and Technology, USIM, Malaysia (e-mail: seinrahman182@gmail.com).

D.Analysis Gelatin Capsule with Real-Time PCR, FTIR and Chemometrics

PCR amplification was conducted with mixture of reagent and dilution concentration of both porcine and bovine DNA with the total volume of 20 µl. Amplification was carried out at 95°C for 10 minutes as pre-denaturation stage and continued with 95°C denaturation step for 10 seconds and 55°C for 45 seconds annealing stage and followed by 40 cycles to obtain melt curve. For FT-IR analysis, it was made on the frequency 4000-650 cm^{-1}. The all value of absorbance in the FT-IR spectrum would be selected for the cluster analysis using PCA.

III. Result and Discussion

A. Precision (Repeatibility)

Precision (repeatibility) is measured using relative standard deviation (RSD) in percent. Test repeatability was low in inter-test experiments (< 25%) (Table I). The calculation of test precision and test variability is based on the Ct variation from the Ct mean value.

B. Sensitivity and Efficiency

The equation of linear regression line was used to evaluate qPCR assay validation. The coefficient of determination (R^2) of standard curve represents whether or not the experimental data fits the regression line. The slopes with ± 10% efficiencies are accepted. However, the value close to 100% is considered to be better [9]. In this study, porcine primer represents R^2 value: 0.962; efficiency: 97.4%; slope: -3.389, bovine primer shows R^2 value: 0.995; efficiency: 90.6%, slope: -3.569 (as shown in Fig. 1).

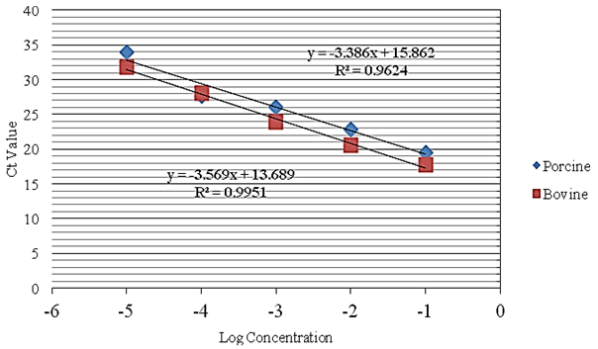
The threshold sensitivity of this method in targeting the presence of porcine and bovine DNA and also quantification is given by the lower DNA concentration detected in the linear zone (at least 95% of 6 tested replicates) and corresponds to the limit of detection (LOD) of porcine and bovine DNA of 10^{-5} ng/µl.

C. Specificity

Melt curve is used to measure the specificity which requires integration of a reporter dye such as SYBR green. The specificity of primers and fluorescent dye have been demonstrated here with the real-time PCR assays carried out on genomic DNA samples belongs to *S. Scrofa* and *B. Taurus*. The melting temperature of the amplified products were; 80.3 °C for bovine DNA and 81.6 °C for porcine DNA and for non-specific product that have a higher temperature (± 90 °C). Real-time PCR detection technology with the SYBR green method provides smelting curve analysis where fluorophore remains associated with the amplicon [2]. Melt curve analysis system can only be provide by SYBR green method. A probe detection system such as TaqMan is not compatible because the resulting signal changes have the properties of splitting and releasing fluorophore into solution during the PCR process [2].

TABLE I
MEAN, SD AND % RSD OF BOVINE AND PORCINE DNA

Concentration (ng/µl)	Porcine Primer		Bovine Primer	
	Mean ± SD	% RSD	Mean ± SD	% RSD
10^{-1}	18.14 ± 0.79	3.99	19.84 ± 1.58	8.69
10^{-2}	21.92 ± 1.07	4.60	23.22 ± 2.13	9.70
10^{-3}	24.77 ± 1.31	4.90	26.79 ± 1.14	4.60
10^{-4}	28.11 ± 2.07	7.12	29.02 ± 0.37	1.31
10^{-5}	31.94 ± 0.66	1.90	34.46 ± 0.75	2.36



A standard curve was generated using a 5-fold dilution of a template amplified on the StepOnePlus real-time system. Standard curve with the Ct plotted against the log of the starting quantity of template for each dilution. The equation for the regression line and the r value are shown above the graph.

Fig. 1 Standard Curves of 5-fold Dilutions of Porcine and Bovine genomic DNA

D.Gelatin Supplement with Real-Time PCR Analysis

The qPCR assays were further evaluated for species determination and quantization from gelatin capsules. The extracted DNA of isolated gelatin from 20 supplements was tested by three replicates of each sample and in addition to a negative control was tested in each run. Different concentrations of gelatin capsules were detected based on the threshold cycle (Ct) of each sample which is the cycle number where the samples fluorescent curve jumps sharply upward and corresponds to the initial concentration of DNA. Of 12 retail products purchased in Nilai area, 6 samples were found to contain porcine-bovine gelatin, 2 samples were found to contain only porcine gelatin, with the remaining 4 samples being bovine gelatin. Whilst this survey was very limited in scope, the clear discrimination between positive and negative samples of differing compositions shows its robustness. The detection of a gelatin capsule in supplement product, which was not *halal*, clearly shows the need for further surveillance of retail gelatin-containing foods and *possible* regulatory action by the authorities (Table II).

E. FTIR and Chemometrics Analysis

The spectra look very similar and show a typical absorption band of gelatin structure. Four regions involved are 3600-2300 cm^{-1} (Amide A), 1656-1644 cm^{-1} (Amide I), 1560-1335 cm^{-1} (Amide II) and 1240-670 cm^{-1} (Amide III). A typical gelatin capsule spectrum showed low intensities of Amides A, I, II and III bands, with the Amide III band almost non-existent of

a few samples (Fig. 2). This is consistent with changes expected because of denaturation of collagen to gelatin. A very low intensity showed for Amide III region is associated with loss of triple helix state during high temperature gelatin extraction [10].

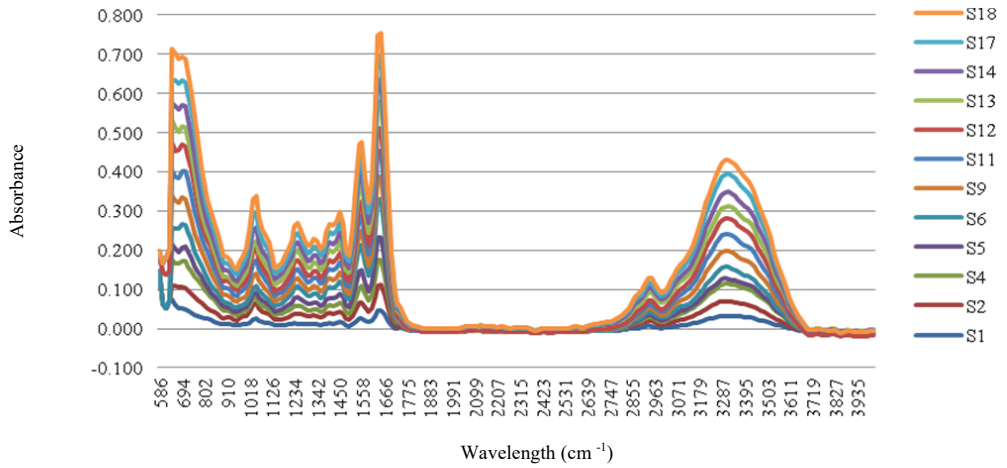
TABLE II
RESULT OF REAL-TIME PCR IN GELATIN CAPSULE OF SUPPLEMENT PRODUCT

Sample	Concentration (ng/µl)	Ct Value		Remarks
		Porcine	Bovine	
	Mean ± SD	Mean ± SD	Mean ± SD	
S1	4.85 ± 0.21	26.97 ± 0.38	20.54 ± 1.28	Mixture
S2	3.23 ± 0.34	-	26.20 ± 0.46	Bovine
S3	2.95 ± 0.05	-	-	-
S4	3.90 ± 0.02	26.41 ± 0.83	23.07 ± 0.76	Mixture
S5	5.08 ± 0.06	27.20 ± 0.74	-	Porcine
S6	6.14 ± 0.82	26.57 ± 0.55	-	Porcine
S7	4.61 ± 1.07	-	-	-
S8	4.41 ± 0.91	-	-	-
S9	2.50 ± 0.17	27.69 ± 0.81	20.52 ± 1.02	Mixture
S10	3.23 ± 0.01	-	-	-
S11	5.01 ± 0.14	27.73 ± 0.53	21.34 ± 0.68	Mixture
S12	4.72 ± 0.16	26.89 ± 0.47	16.67 ± 0.53	Mixture
S13	4.43 ± 0.01	-	24.23 ± 0.53	Bovine
S14	4.67 ± 0.01	-	31.49 ± 0.75	Bovine
S15	3.05 ± 0.41	-	-	-
S16	2.16 ± 0.18	-	-	-
S17	1.94 ± 0.07	-	31.38 ± 1.35	Bovine
S18	3.78 ± 0.54	27.8 ± 0.28	27.81 ± 0.29	Mixture
S19	4.82 ± 0.33	-	-	-
S20	4.30 ± 1.02	-	-	-
-CT	1.00	-	-	-

The Amide A (3600-2300 cm^{-1}) region is donated by N–H bond-stretching mode of hydrogen bonded amide groups. The absorption is polarized parallel to N–H bond, which is parallel to the helix axis in a-helical structures and perpendicular to the polypeptide chain in b-sheets. The band might be shifted to lower frequency when the hydrogen bonding strength increases [11]. The carbonyl C=O double bond-stretching mode, with contributions from in-phase bending of the N–H bond and stretching of the C–N bond, occurs in frequency range 1660-1620 cm^{-1} region which is often referred to as Amide I band. The frequency range 1660–1650 cm^{-1} was known as a-helical and 1640-1620 cm^{-1} as b-sheets structures.

The frequency range of 1550-1520 cm^{-1} is due to Amide II with a-helical structure between 1550-1540 cm^{-1} and b-sheets at 1525-1520 cm^{-1}. The Amide II vibration is caused by deformation of the N–H bonds. Fischer et al. (2005) and Lagant et al. (1983) attributed 1500-1200 cm^{-1} to CH_2 deformation. It is known that this region contains vibrations corresponding to groups present in fatty acids, proteins, polysaccharides and phosphate derivatives [12], [13].

Normalization and smoothing of the FT-IR spectrum of gelatin capsule was shown in Fig. 3. PCA manipulates the data of variables (peak height of amino acids) in the way that these variables can be displayed on an x, y coordinate system. PCA did this by calculating principal components (PC1) which are linear combination of original variables. PC1 (first principal component) explained the most variation among data, while PC2 or second principal component described the second largest variation among data. PC1 was orthogonal to PC2.



The figure showed the enlarged FTIR spectra at fingerprint regions. The different peaks in terms of peak intensity were used for selecting the spectral regions for the quantification and classification of gelatins capsule in supplement product.

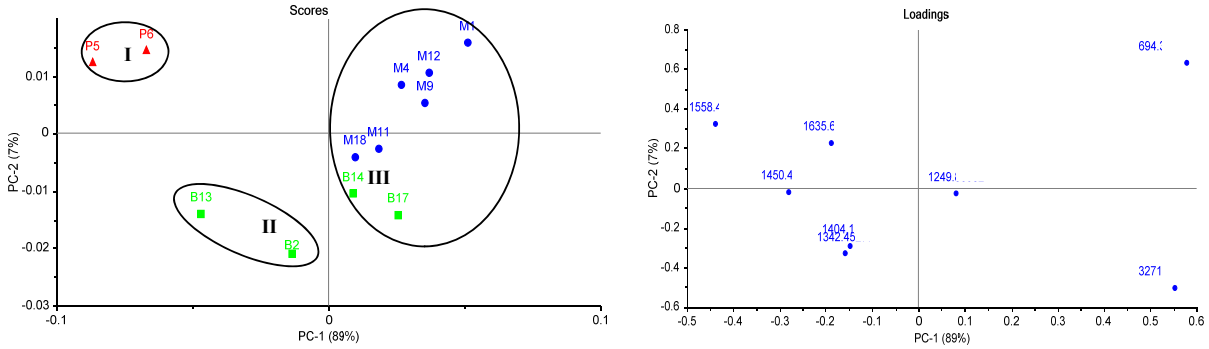
Fig. 2 FTIR Spectra in Gelatin Capsule of Supplement Product

Fig. 3 exhibited the PCA Scores plot (PC1 versus PC2) of same samples of capsule shells tested with PCR in Table I. The labeling was changed based on results from PCR where P referred for porcine, B for bovine and M for mixture of porcine and bovine. PC1 described 89% variation of data, while PC2 and PC3 account for 7% and 3% variations, respectively. Therefore, more than 90% of variation can be described only by three PCs. In the figure it was observed that porcine gelatin in group I were clearly separated from others. Two of bovine samples are observed in group II, however

other two samples labelled B14 and B17 are located with all mixture samples in group III. This might be due to the percentage composition of bovine is higher than porcine in B14 and B17. The right side of the Fig. 3 illustrated the Loadings plot for the determination of variables (wavenumber) contributing to the differentiation and separation of the samples.

The PCA Loadings plot described the projection of variables in the same plane as the Scores plot. From Fig. 3, it was known that 694 and 3271 cm^{-1} were the variables giving the most contribution toward PC1, while 1558 and 1635 cm^{-1} were more influencing on PC2. Based on Loadings plots in Fig. 3, it was seen that profiles were significantly different in the region 694, 3271, 1558 and 1635 cm^{-1}.

The frequencies 1558 cm^{-1} and 1635 cm^{-1} are mapped to P5 and P6 in Scores plot that indicates the significant frequencies for porcine FT-IR. Both frequencies indicate a C-N-H bending and C=O stretching of the peptide bonds [14], [15]. Frequency 3271 cm^{-1} in PC1 indicate the presence of aliphatic N-H stretching region in peptide bond.



PC1 vs. PC2. PCA was pre-treatment with Savitsky-golay and peak normalization and the S code would be changed to the specific classification where: P is code for porcine; B is code for bovine; and M is coded for mixture

Fig. 3 The PCA Scores and Loadings Table

IV. Conclusion

The use of PCA and infrared spectroscopy was investigated to confirm the presence of porcine, bovine and the mixture of porcine and bovine material from sample of PCR result. From this method, an attempt to assign vibrational frequencies at each stage of thermal decomposition was made. Wavenumber profiles in combination with principal components analysis can clearly classify all capsule shells made from porcine gelatine, mixture and only two of bovine gelatin. PCA was not successful for classification of other bovine gelatin in capsule shells due to the diverse of organs of animal used for gelatin preparation.

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Mohd Sukri Hassan holds a B.Sc. (Hons) from Universiti Kebangsaan

Malaysia (UKM) in 1987. After graduation, he worked as Government Chemist at the Department of Chemistry Malaysia. Then in 2005 he completed his PhD in forensic toxicology at the University of Bristol, United Kingdom. He serves as a lecturer at the Faculty of Science and Technology, USIM since 2012 and in he was a director of the Institute of Halal Research and Management (IHRAM) from 2015 to 2016. He has been actively involved in writing articles in newspaper, refereed journal and proceeding.

Ahlam Inayatullah Badrul Munir obtained a Bachelor of Chemistry Education from the Faculty of Mathematics and Science from Universitas Negri Medan of (UNIMED). Currently she is pursuing her masters in Food Technology at Universiti Sains Islam Malaysia (USIM).

Muhammad Husaini Abdul Rahman holds a B.Sc. (Hons) in industrial chemical technology from Universiti Sains Islam Malaysia (USIM). Currently he is pursuing his masters in Chemistry at Universiti Sains Islam Malaysia (USIM).