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Full Length Research Paper

Differentiation of pork from beef, chicken, mutton and chevon according to their primary amino acids content for halal authentication

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The detection of pork in various food products has been an important subject of study in many countries. The current study was aimed to differentiate pork from selected meats of beef, mutton, chevon and chicken based on their primary amino acid contents using reverse phase-high performance liquid chromatography (RP-HPLC) with derivatization by o-phthalaldehyde (OPA) and ultraviolet (UV) detection. The results show that the most discriminative amino acids between pork and others were valine, histidine, serine, alanine and arginine. The findings here lay the ground work for the future research to develop a marker for halal meat authentication based on the amino acids content.

Key words: RP-HPLC, OPA, Amino acids, halal meat, pork.

INTRODUCTION

Food plays an important role in social, cultural and religious life style of every community throughout the world. Authentication of raw materials and finished products and the detection of various forms of food adulteration are of primary importance for both consumers and industries (Ahmad, 2006; Aida et al., 2005; Ozen et al., 2003). Due to increasing health concerns and sensitivity among the consumers over the food quality, there is currently a great need for food analysis and authentication.

According to Islam, an important factor for Muslim consumers is the halal (lawful) or haram (unlawful) status of the food. Demands for food products with halal authentication are increasing and this trend is expected to continue concurrently with the population growth (Ahmad, 2006). Therefore, detection of pork in various food products has been an important subject of study in many countries, especially where religious laws prohibit the consumption of pork products. Presently, numerous physicochemical and genetic techniques have been developed for pork involvement in food products such as Fourier transform infrared (FTIR) spectroscopy, chromatography techniques, electronic nose (EN), polymerase chain reaction (PCR) and restriction-enzyme fragment length polymorphism (RFLP).

FTIR is a common and proper technique for detection and quantification of pig derivatives (Lard and Gelatin) in some food products such as cake (Syahariza et al., 2005), chocolate products, biscuit, animal fats (Jaswir et al., 2003), some vegetable oils, cod liver oil and virgin coconut oil (Mansor et al., 2011).

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To analyze volatile flavor compositions in pork, gas chromatograpgy - mass spectrometry (GC - MS) has been used extensively. In some studies it has been identified that cooked pork contained substantial low concentration of alcohols compared with other muscles (cooked chicken and beef) (Farag et al., 2003).

From these techniques, electronic nose (EN) systems are able to recognize complex odors with the involvement of various types of electronic chemical gas sensors with partial specificity together with a suitable statistical method. Its application has been increased in the evaluation of volatile compounds in food products (Schaller et al., 1998). But the sensors used in this technique are not very selective for particular types of compounds in pig derivatives.

Molecular biology-based techniques such as PCR (Regenstein et al., 2003; Najiha et al., 2010) and RFLP (Kamm et al., 2001; Lizhi et al., 2010) are currently used to identify species-specific nucleotide sequences or variations within the mitochondrial DNA based on species recognition. In spite of high specificity and sensitivity of genetic techniques, they are expensive regarding laboratory equipments and high level of technical expertise needed. These techniques also undergo high degree of false-positive rates that come with their high sensitivity. The finding of species-specific sequences takes remarkable time and long validation process. In addition, one specific designed primer is particularly for only one species, which renders it more useful for ruling out or confirming the presence of meat from a single species.

Analytical tools like high performance liquid chromatography (HPLC) are widely used for characterization and detection of adulteration of different food products (Cordella et al., 2002) such as flavonoids in fruit juices (Kawaii et al., 1999), organic acids in apple juices (Blanco et al., 1996), phenolic pigments in black tea liquors (McDowell et al., 1995), proline isomers and amino acids in wines (Moreno-Arribas et al., 1998) and anthocyanins in jams (Garcıia-Viguera et al., 1997). Generally, the chromatography-based techniques offer quick and credible tools for the separation and quantitative analysis of lots of major and minor components with highly similar chemical structures in complex foods (Cserháti et al., 2005). However, to the best of our knowledge, there is no report on the amino acid composition of meat products aiming to detect halal authentication. Amino acids exist in foods either in free form or linked together as peptides, polypeptides or proteins. As amino acids do not appreciably absorb visible or UV radiation, they require either derivatization with a suitable chromophore or fluorophore (Cheung et al., 2007; Oguri et al., 1997) in RP-HPLC with pre-column derivatization. o-Phthalaldehyde (OPA) is commonly used as a derivatizing reagent for amino acids determination with fluorescence and UV detection (Ying et al., 2005). Thus, the objective of the present study was to differentiate the common meat types using their primary amino acid profile as possible markers of meat authentication.

MATERIALS AND METHODS

Chemicals and reagents

Acetonitrile, hydrochloric acid and methanol (HPLC grade) were obtained from Merck KGaA (Darmstadt, Germany). o-Phthaldialdehyde 3-mercaptopropionic acid (OPA/3-MPA) (PN 5061 to 3335), borate buffer (PN 5061 to 3339), amber wide-opening vials, glass conical inserts with polymer feet and screw caps and solutions of 17 amino acids standard (PN 5061 to 3330 through 5061 to 3334) in five concentrations (10, 25, 100, 250 and 1 nmol/µL) were obtained from Agilent (USA). Milli-Q water was used throughout the study.

Sample preparation

Six authentic samples of meat per animal types were collected for further investigations. Samples of beef, mutton, chevon and chicken (24 samples in total) were obtained from Department of Animal Science, Faculty of Agriculture, Universiti Putra Malaysia, Serdang, Malaysia. Six Pork samples were purchased from a local wet market in Malaysia. The meat samples (0.3 to 0.4 g) of chickens' breast and longissimus dorsi of beef, mutton, chevon and pork were hydrolyzed under nitrogen gas with 15 ml of 6 N HCl into a preheated oven at 110°C for 24 h. After cooling to room temperature, the volumes were top upped to 50 mL with Milli-Q water. The sample solutions were filtered through 0.45 µm pore size cellulose acetate membrane filters before injection into the HPLC.

HPLC analysis

Amino acids were determined using an Agilent 1100 HPLC system (Agilent Technologies, USA), equipped with a quaternary pump delivery system, robotic autosampler, thermostatted column compartment and a diode array detector (DAD). The samples were submitted to automatic pre-column derivatization with OPA-3MPA. After derivatization, an amount equivalent to 3.5 µL of each sample was injected on a Zorbax Eclipse-AAA column, 4.6 × 150 mm, 3.5 µm (PN 963400 to 902). Mobile phase A was 40 mM NaH_2PO_4, adjusted to pH 7.8 with NaOH, while mobile phase B contained 45 acetonitrile, 45 methanol, and 10% deionized water. The chromatographic column temperature was set at 40°C with a flow rate of 1.5 mL/min with a gradient program (Table 1) Detection wavelength was set at UV 338 nm, 10 nm bandwidth. The identity and quantity of the amino acids were assessed by comparison with the retention times and peak areas of standard amino acids.

Statistical analysis

One-way analysis of variance was done using the General Linear Model (PROC GLM), while principal component analysis (PCA) was performed using PROC PRINCOMP of Statistical Analysis System (SAS) computer package (SAS Institute Inc., 2005). Subsequently, the Duncan's New Multiple Range Test was used to separate means. Principal Component Analysis was performed to determine the main directions of variations among the meats. The samples were located in a graph based on the first three PCs.

RESULTS AND DISCUSSION

Up till now, several methods have been described for the

Table 1. Scheme of elution gradient used in the process.

Time (min)	Mobile phase A	Mobile phase B
0	100	0
1.9	100	0
18.1	43	57
18.6	0	100
22.3	0	100
23.2	100	0
26	100	0

determination of amino acids. The classical approach to amino acid analysis is separation on a sulphonate cation-exchange resin, followed by derivatization with ninhydrin and spectrophotometric detection (Fekkes et al., 1995; Qureshi and Qureshi, 1989). These methods are adequate but generally time consuming and in addition, they require substantial amounts of sample. The use of RP-HPLC permits amino acid determinations in a relatively short time on small samples and with good sensitivity and specificity (Buzzigolli et al., 1990; Qureshi et al., 1984). In this study, we selected a method that employs pre-column derivatization with OPA/ 3-MPA. OPA has been used to react with primary amino acids (De Bernardo et al., 1974; Lin and Lai, 1980; Schuster, 1988; Simons and Johnson, 1976). This chemical is more sensitive and easier to use than fluorescamine and 10 times more sensitive than ninhydrin (Antoine et al., 1999; Roth, 1971)

The composition of amino acids profile of different meat types are presented in Table 2. The results show that GLY have the highest concentration compared to other amino acids measured in all the meat types, although its concentration was not significantly different between different meat types. Similarly, Aristoy and Toldra (1991) reported that GLY had the highest amount compared to other amino acids studied in pork. The lowest content of amino acids was MET in beef, mutton, chevon and chicken. Similar results were reported by Eng et al. (1986), Hoffman et al. (2005) and Zyl and Ferreira (2003). The lowest concentration of the amino acid in pork was TYR. This phenomenon is in agreement with Flores et al. (2000). Interestingly, the amount of VAL was found to be significantly lower in pork than other meat types studied, thus making VAL a possible candidate marker for halal authentication in meat products. Aristoy and Toldra (1998) reported that the quantity of VAL was lower than other amino acids measured in pork meat while Gilka et al. (1989) and Webb et al. (2005) indicated high quantity of VAL in mutton and chevon. Two amino acids of GLU and HIS were shown to be significantly different between chevon and chicken. The quantity of ARG was found to be significantly different between mutton and those found in chevon and pork. In agreement, Gilka et al. (1989) reported the same results. Chicken meat appeared to be poor in ALA and SER compared to other meat types. Although PRO is difficult to detect, it was measured in all the meat types in our study. This could be due to the fact that OPA reacts preferentially with primary rather than secondary amino acids (Garcia et al., 2007). Chicken meats had significantly richer contents of PRO than mutton.

Results of simple correlation coefficients among the amino acids measured in beef, mutton, chevon, chicken and pork are presented in Table 3. VAL as the main marker for separating pork from the other meat types was found to be highly correlated ($p \leq 0.01$) with ASP, SER, HIS, THR and ALA with correlation coefficients of -0.63, -0.82, 0.68, 0.68 and -0.77, respectively. The direction of the correlation coefficients showed negative relationships between VAL and ASP, SER and ALA. This indicates that VAL concentration in the meats studied increased when concentration of ASP, SER and ALA decreased, and vice versa. In contrast, VAL concentration was found to increase with increasing HIS and THR, exhibiting positive relationships. In addition, VAL also revealed negative and significant relationships (at $p \leq 0.05$) with ILE, LYS and PRO with correlation coefficients of -0.59, -0.54 and -0.54, respectively.

SER was found to have highly significant correlation (at $p \leq 0.01$) with ASP, GLU, THR, VAL, LYS and PRO with correlation coefficients of 0.65, 0.66, -0.62, 0.82, 0.79 and 0.76, respectively. Positive and significant correlation ($p \leq 0.05$) was also obtained between SER and ALA ($r = 0.60$). It was found that reduction in concentration of THR in the meats evaluated resulted significant increase in SER concentration. Additionally, increase in concentrations of ALA and GLU in the meat studied was found to reduce HIS concentration ($r = -0.59$ and -0.51, respectively). Since there was a positive relationship between HIS and VAL, increasing ALA and GLU indirectly decreased VAL concentration.

In general, It can be concluded that SER, HIS, ALA as the main amino acids separating pork from the other meats studied were found to be significantly correlated with VAL as the key distinguisher for pork. This indicates that the changes in concentrations of the key amino acids for separating the meats studied were in the same direction. However, there was no significant correlation between VAL and ARG as the amino acid differentiating pork from mutton.

Table 2. Mean values for the amino acids measured from different raw meats.

Treatment	Amino Acid Mean Values (%)																	
	ASP	GLU	SER	HIS	GLY	THR	ARG	ALA	TYR	CYS	VAL	MET	PHE	ILE	LEU	LYS	PRO	CP
Beef	2.82^{a}	7.81^{ab}	4.06^{ab}	5.89^{bc}	20.18^{a}	3.03^{a}	8.66^{ab}	6.93^{a}	2.13^{a}	2.31^{b}	4.33^{b}	2.03^{a}	3.05^{a}	3.47^{a}	7.08^{a}	8.05^{a}	8.17^{ab}	57.58^{ab}
Mutton	2.92^{a}	7.65^{ab}	4.16^{a}	6.24^{abc}	18.00^{a}	2.88^{a}	9.95^{a}	6.50^{b}	2.36^{a}	2.63^{ab}	5.63^{a}	2.13^{a}	3.02^{a}	3.10^{a}	5.82^{a}	8.35^{a}	8.66^{a}	50.27^{b}
Chevon	2.98^{a}	8.26^{a}	4.20^{a}	5.28^{c}	18.47^{a}	3.42^{a}	8.37^{b}	6.55^{ab}	2.45^{a}	3.21^{a}	4.50^{ab}	1.77^{a}	3.25^{a}	3.64^{a}	7.13^{a}	8.42^{a}	8.46^{ab}	54.52^{ab}
Chicken	2.70^{a}	7.11^{b}	3.81^{b}	7.29^{a}	19.20^{a}	3.96^{a}	9.27^{ab}	6.11^{c}	2.40^{a}	2.92^{ab}	5.79^{a}	1.97^{a}	3.01^{a}	3.45^{a}	6.94^{a}	7.83^{a}	7.43^{b}	61.67^{a}
Pork	2.86^{a}	7.67^{ab}	4.15^{a}	6.74^{ab}	18.88^{a}	2.76^{a}	8.54^{b}	6.72^{ab}	2.27^{a}	2.49^{ab}	4.05^{c}	2.29^{a}	3.14^{a}	3.35^{a}	7.26^{a}	8.23^{a}	8.15^{ab}	52.07^{b}
Mean	2.86	7.70	4.08	6.29	18.98	3.18	8.96	6.56	2.27	2.71	4.54	2.05	3.14	3.43	6.85	8.21	8.17	55.22

Means followed by the same letter in the same column are not significantly different at p≤0.05 based on DNMRT, ASP= Aspartic acid, GLU=Glutamic acid, SER= Serine, HIS= Histidine, GLY= Glycine, THR= Threonine, ARG= Arginine, ALA= Alanine, TYR= Tyrosine, CYS= Cystine, VAL= Valine, MET= Methionine, PHE= Phenylalanine, ILE= Isoleucine, LEU= Leucine, LYS= Lysine and PRO= Proline

Table 3. Simple correlation coefficients among the amino acids measured on beef, mutton, chevon, chicken and pork.

Amino acid	ASP	GLU	SER	HIS	GLY	THR	ARG	ALA	TYR	CYS	VAL	MET	PHE	ILE	LEU	LYS
GLU	0.18															
SER	0.65**	0.66**														
HIS	-0.20	-0.51*	-0.42													
GLY	-0.53*	-0.15	-0.33	-0.31												
THR	-0.69**	0.01	-0.62**	0.19	0.18											
ARG	-0.42	-0.21	-0.20	0.03	0.01	0.10										
ALA	0.23	0.4	0.60*	-0.59*	0.24	-0.63	-0.13									
TYR	0.88**	-0.22	0.32	0.04	-0.53*	-0.52	-0.37	-0.05								
CYS	0.38	-0.32	-0.04	-0.06	-0.20	0.01	-0.13	-0.36	0.63**							
VAL	-0.63**	-0.48	-0.82**	0.68**	0.04	0.68**	0.33	-0.77**	-0.35	-0.10						
MET	-0.08	0.20	0.05	0.32	-0.29	0.08	-0.03	0.00	-0.2	-0.58*	0.29					
PHE	0.74**	-0.21	0.29	-0.11	-0.45	-0.62**	-0.03	0.09	0.81**	0.56*	-0.34	-0.36				
ILE	0.69**	0.06	0.41	-0.21	-0.43	-0.46	-0.32	0.19	0.68**	0.57*	-0.59*	-0.55*	0.72**			
LEU	0.14	-0.17	-0.15	0.01	-0.09	-0.10	-0.50	0.03	0.19	0.07	-0.21	-0.14	-0.01	0.25		
LYS	0.85**	0.34	0.79**	-0.07	-0.70**	-0.56*	-0.19	0.19	0.71**	0.20	-0.54*	0.08	0.58*	0.58*	-0.14	
PRO	0.47	0.66**	0.76**	-0.41	-0.45	-0.48	0.10	0.49	0.11	-0.23	-0.54*	0.21	0.35	0.33	-0.32	0.63**

*= significant at p≤0.05 and **=significant at p≤0.01.

Table 4. The first three principal components for the amino acids measured in beef, mutton, chevon, chicken and pork.

Amino acid	PC_1	PC_2	PC_3
ASP	0.346578	0.053971	0.017749
GLU	0.312288	0.044194	-0.221707
SER	0.339189	-0.106299	0.001137
HIS	-0.288534	-0.082267	0.204363
GLY	-0.160772	-0.237016	-0.328385
THR	-0.190464	0.404296	-0.104073
ARG	-0.071866	0.017028	0.466548
ALA	0.176706	-0.355396	-0.231927
TYR	0.298106	0.207436	0.113500
CYS	0.049320	0.481756	-0.015777
VAL	-0.214358	0.337270	0.159617
MET	-0.025466	-0.40167	0.265640
PHE	0.310891	0.101158	0.206980
ILE	0.187631	0.253066	-0.335123
LEU	-0.065059	-0.014108	-0.450169
LYS	0.322204	0.014480	0.196869
PRO	0.328239	-0.077111	0.113621

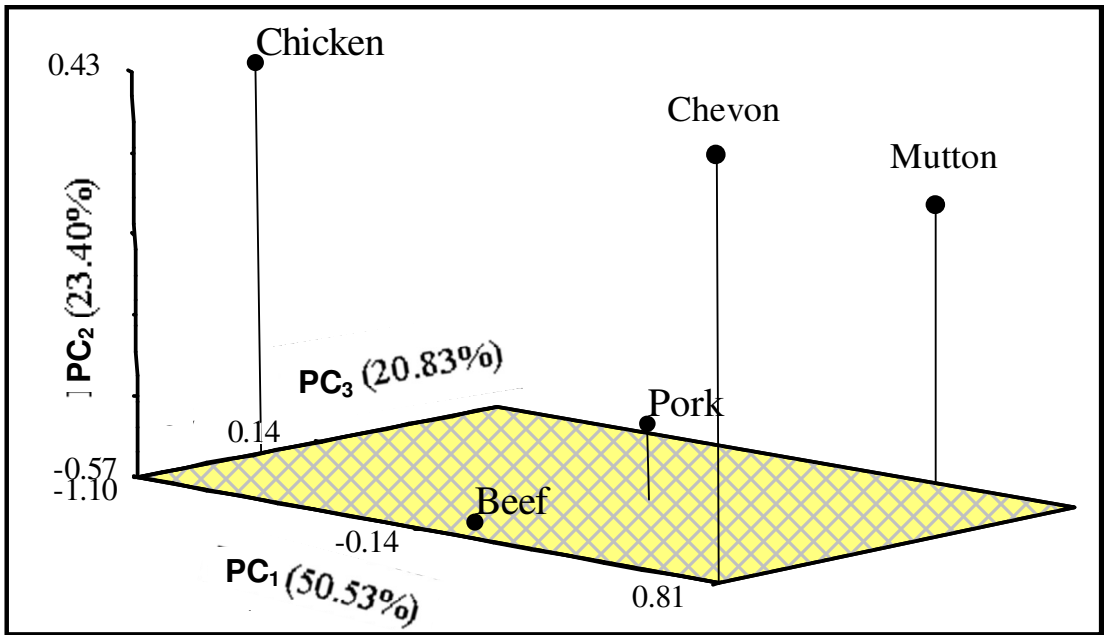

Figure 1. Three-dimensional graph showing relationships among the sample meats studied based on the first three principle components obtained from the quantities of the amino acids measured. PC1, PC2 and PC3: The three axes represent the first three principal components.

The PCA analysis revealed that the first three PCs accounted for 94.75% of the total variation. PC 1 data set accounted for 50.53% of the total variation, where ASP, SER, PRO, LYS, GLN and PHE were associated positively with PC_1, but negatively associated with HIS, VAL, THR and GLY (Table 4). Based on the ANOVA results, SER and HIS highly associated with PC 1 were found to be the main amino acids for separating the meat types studied. Chicken was located furthest from chevon, followed by mutton, pork and beef in PC_1 (Figure 1). Therefore, SER and HIS were identified as the main amino acids for differentiating chicken from the other meats studied. PC_2 accounted for 23.40% of the total morphological variability that was positively attributable to

differences in VAL while negatively associated with ALA. This indicates that PC_2 separated the different meat types based on the differences in the percentage of VAL and ALA where chicken had the most difference from mutton. PC_2 was also found to be able to separate pork from the other meat types studied (Figure 1). Based on the ANOVA table, pork was found to have the lowest percentage of VAL among the meats studied.

The main amino acids correlated with PC_3 accounted for 20.83% of the total variation were ARG and LEU. Since the meat types had similar percentage of LEU, ARG was found to be the amino acid differentiating the meat types in PC_3. Beef was clearly distinguished from the other meat types based on the differences in PC_3. However, PC_3 showed that pork was more similar to beef. This was due to insignificant difference in percentage of ARG between beef and pork. Therefore, beef and pork were best separated by PC_2. It can be concluded that the first three PCs were able to differentiate the meat types based on the variation in the percentage of SER, HIS, VAL, ALA and ARG. This was similar to the results obtained from Duncan mean comparison.

In this study, Eigenvectors and Eigenvalues were estimated among the meat types using all data obtained from all samples and replications, but not the sample means. This was to ensure that the Eigenvectors and Eigenvalues were precisely estimated using all the data available. However, the PCA result obtained using individual samples was found to be in agreement with those obtained after pooling all samples for each meat type. This was due to the fact that the variation among the samples was already considered in the analysis when eigenvectors and eigenvalues were estimated.

The findings of the current study elaborated that there is no close relationship among the meat types studied. Chevon and chicken meat were found to be the most different meat types, while beef and pork were the most similar. Pork could be distinguished from the other meat types using VAL as a marker which was significantly lower compared to chevon, chicken, beef and mutton. Moreover, it was observed that chicken appeared as an individual group at the positive scores of PC3 separated from other meat types (Figure 1). Also, chevon and mutton appeared as two different meat types at the right upper side of the plot at positive scores of PC1.

Conclusion

It can be concluded that SER, ALA and VAL might be a candidate markers to differentiate pork and chicken. Moreover, pork might be distinguished from the other red meat types using VAL, HIS and ARG as indicators. However, as for a single marker candidate, VAL merits further investigations. It appeared that RP-HPLC with pre-column derivatization could be used for fast, simple and cost effective technique for authentication of pork from chicken, beef, chevon and mutton.

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