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Application News

No. AD-0153

Halal Authentication Analysis / LCMS-8060

Highly-Sensitive Detection of Multiple Porcine-Specific Peptides in Processed Foods by LC/MS/MS Method

Udi Jumhawan, Jie Xing & Zhaoqi Zhan
Application Development & Support Centre, Shimadzu (Asia Pacific) Pte Ltd, Singapore

❑ Introduction

In recent years, unauthorized blending of undeclared materials into food products has been a major concern among consumers. Such action violates customer rights based upon economical and safety values and it is also a critical problem for communities with ethical and religious beliefs [1,2]. Both accidental and intentional adulteration of pork meat into food products are significant issue affecting Muslims and Jews as they have dietary restrictions on foods containing pork and its-by-product, such as gelatin. Particularly for Muslims, those restrictions are part of Islamic law concerning Halal (permissible) and Haram (non-permissible) foods. With an estimated 1.6 billion Muslims worldwide, development of sensitive method for detection of porcine materials in food products is indispensable. Various approaches and targets have been utilized to trace porcine materials in processed foods including pork DNA by qPCR [3] and PCR-MCE [4]. However, DNA is prone to thermal degradation thus its viability remains dubious after food processing (cooking). In recent years, porcine-specific peptide markers were discovered and utilized for detection of pork in food [5-8]. In this Application News, a highly sensitive LC/MS/MS method is described for ensuring halal food integrity by targeting more heat-stable porcine-specific peptide markers in processed foods.

❑ Experimental

Preparation of meat and processed food samples

The experimental procedure is modified based upon previous reports [5-8] and consists of four steps as follows: protein extraction, trypsin digestion, SPE clean-up and analysis of porcine-specific peptide markers on LC/MS/MS. The workflow of protein extraction, reduction, alkylation and digestion is displayed in **Figure 1**. Raw meats of pork, chicken and beef were used in method development to confirm the porcine-specific peptides and obtain optimized MRMs and retentions. The targeted samples are processed foods. Nine processed food products were obtained from local supermarket. It is to note that the supplementary addition of 20 µL dithiothreitol is to consume the unreacted iodoacetamide. The obtained tryptic digestion solution is cleaned up using SPE (solid phase extraction) approach prior to LC/MS/MS analysis. The details of the procedure are shown in **Figure 2**.

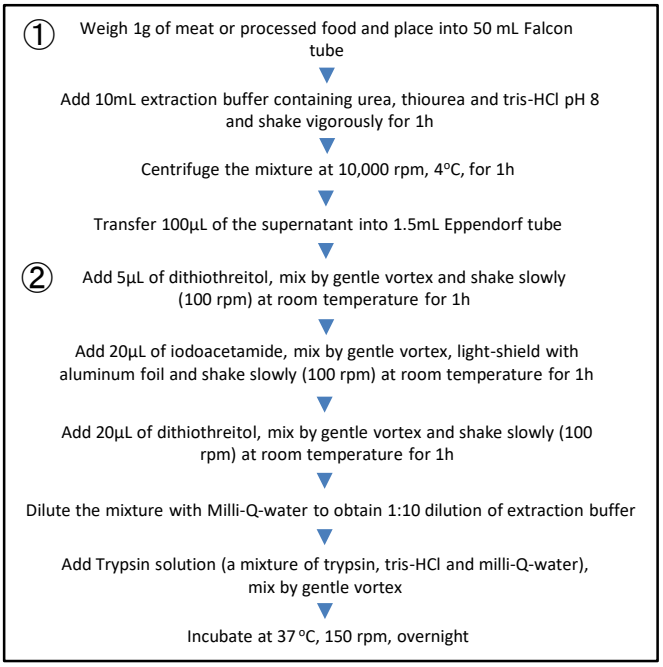

Figure 1. Workflow of protein extraction ①, reduction, alkylation, and digestion ②

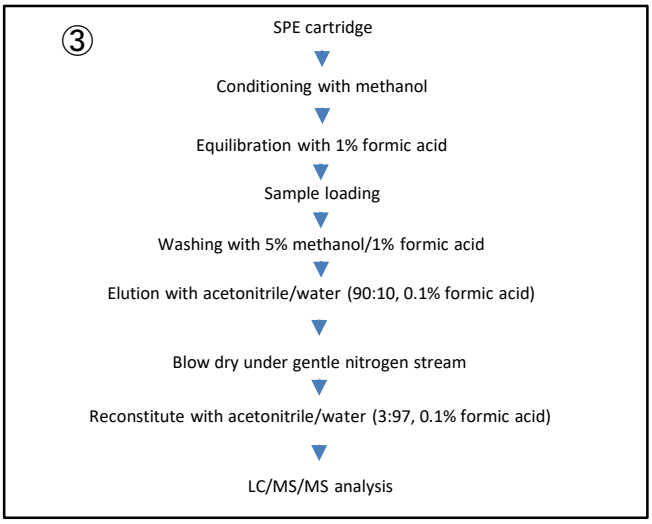

Figure 2. Workflow of SPE clean up procedure of trypsin digested proteins extracted from processed food or raw meat

LC/MS/MS analytical conditions

A high sensitive triple quadrupole system, LCMS-8060, coupled with a Nexera X2 UHPLC system was utilized to establish detection and semi-quantitation of porcine-specific peptides in processed food. A reversed phase Phenomenex column which has been described suitable for peptide mapping was used. The details of the UHPLC and MS/MS parameters are summarized in **Table 1**.

Table 1. Analytical conditions for detection and semi quantitation of porcine-specific peptides on LCMS-8060

Column	Aeris Peptide 1.7 µm XB-C18 100Å (150 mm x 2.1 mm I.D.)
Mobile Phase	A: Water with 0.1% formic acid B: Acetonitrile with 0.1% formic acid
Elution Program	Gradient elution, 3%B (0min) ➔ 45%B (12min) ➔70%B (12.01 –14min) ➔ 3%B (14.50 – 18min)
Flow Rate	0.3 mL/min
Oven Temp.	50 ºC
Injection	5 µL

Interface	Heated ESI
MS Mode	MRM, Positive mode
Block Temp.	400 ºC
DL Temp.	250 ºC
Interface Temp.	300 ºC
Nebulizing gas	N_2, 3 L/min
Drying gas	N_2, 10 L/min
Heating Gas	Zero air, 10 L/min

❑ Results and discussion

MRM-based method for detection of porcine-specific peptides

A number of porcine-specific peptides has been discovered by several researchers in past years [5-8]. The peptide markers for pork as well as other meat species were adopted in establishment of MRM based method on LC/MS/MS to achieve high sensitivity for inspection of blending pork or concerned meat species in Halal food. To enhance the detection reliability, this study incorporates all the known peptide markers reported previously [5-8] into a single LC/MS/MS method. A total of seven peptide markers was targeted and utilized**.**

Due to the unavailability of authentic peptide standards, development of MRM method was conducted according to the strategy shown in **Figure 3**. The targeted peptide sequences in FASTA format was retrieved from UniProt protein database and then submitted into Skyline software to obtain predicted MRM transitions and collision energies (CE). This simplified and speed it up greatly method development on LC/MS/MS. The predicted MRM parameters were imported into the LabSolutions, where MRM optimization were performed using the auto MRM optimization program. **Table 2** and **Figure 4** show the seven porcine-specific peptides and MRM method established on LCMS-8060. The peptide markers were found with good sensitivity using 5µL injection volume compared to that of higher volumes applied in previous reports (40-50 µL) [5-6].

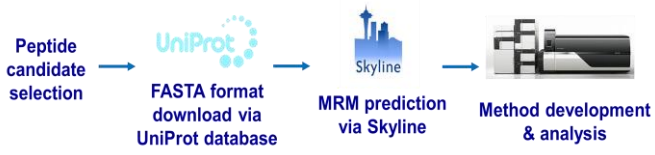

Figure 3. Schematic procedure of MRM method development of peptides from digested proteins with assistance of Skyline program.

Table 2. Porcine-specific peptides and MRM based method on LCMS-8060

Protein	UniProt accession No.	Peptide marker (short sequence)	Precursor ion & charges	Number of MRM	RT (min)
Troponin T	Q75NG7	YDII	453.8++	5	6.52
Myosin-1 & Myosin-4	Q9TV61/62	SALA	376.1+++	6	3.25
Myosin-4	Q9TV62	TLAF	534.3++	6	8.14
L-lactate dehydrogenase A chain	P00339	LVVI	450.3++	4	5.44
Serum albumin	P08835	EVTE	412.2++	4	3.99
		FVIE	388.8++	5	6.68
		TVLG	647.9++	3	8.01

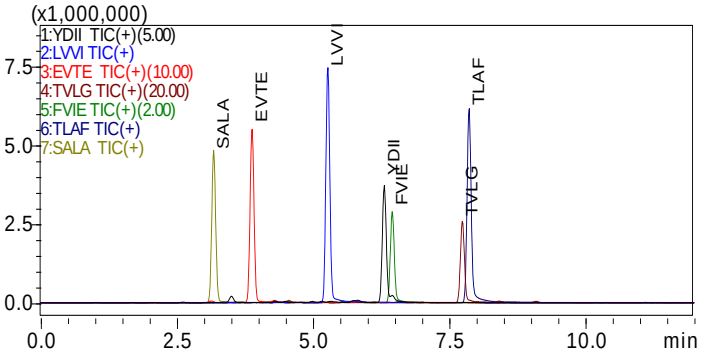

Figure 4. Total MRM chromatograms of seven porcine-specific marker peptides detected in trypsin-digested extract of raw pork meat.

Specificity of porcine-specific peptide markers

The specificity of the seven porcine-specific peptides were verified with beef and chicken meats following the exact same sample preparation and analysis conditions. The criteria included at least three MRM transitions, their ratios and RT. All the seven peptide markers were not detected in beef sample. However, as shown in **Figure 5**, one peptide peak in chicken meat was overlapped with porcine peptide which identified as LVVITAGAR. This particular peptide from L-lactate dehydrogenase protein in pork has identical amino acid composition to that of the same peptide in chicken (LVIVTAGAR) as shown with the same ion mass (*m/z*) [7]. However, based on Skyline MRM prediction, the difference in amino acid sequence among these peptides resulted in one unique product ion: 588.35 for pork and 574.35 for chicken**.**

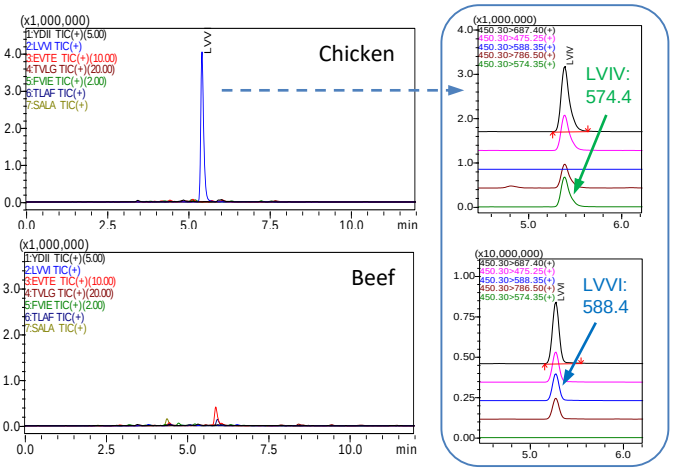

Figure 5. Specificity of porcine peptide markers in chicken and pork (Left). The sequence of LVVI marker peptide (LVIVTAGAR) in chicken is actually different from pork (LVVITAGAR), which could be differentiated from MRM (right)

The prediction was confirmed (**Figure 5 right**) and thereby verified method selectivity to pinpoint species-specific peptide marker.

Thermolability test of porcine-specific peptide markers

To investigate viability of the seven peptide markers after food processing (cooking), raw pork meat was subjected to heat treatment at 200˚C for 30 minutes before sample pre-treatment. The results indicated that these porcine-specific peptide markers exhibited certain depleted levels upon heat treatment but all of the peptide markers could be detected firmly. Furthermore, processed food products were also treated at 200˚C for 30 minutes and the results showed that the levels of the peptide markers remained in the samples were feasible for detection of pork by the established LC/MS/MS method (**Table 3**).

Screening analysis of porcine-specific peptides in processed foods

Table 3 shows the results of screening analysis of five non-halal and four halal-certified processed food products using the established method aiming for halal testing application. All the samples were pre-treated at 200˚C for 30 minutes before extraction, digestion and purification. This is to verify the feasibility of the method for cooked and processed food.

Table 3. Results of screening analysis of porcine-specific peptides in processed food products (Note: the peptide markers are indicated with first four letters of amino acid composition)

Halal label		Processed food	Porcine-specific peptide markers (*n*=2)						
			YDII	LVVI	EVTE	TVLG	FVIE	TLAF	SALA
HALAL	1	Chicken-Beef sausage	ND	ND	ND	ND	ND	ND	ND
	2	Lamb-Chicken sausage	ND	ND	ND	ND	ND	ND	ND
	3	Canned corned beef	ND	ND	ND	ND	ND	ND	ND
	4	Canned mutton curry	ND	ND	ND	ND	ND	ND	ND
NON HALAL	5	Chicken sausage	+	+	+	ND	+	ND	+
	6	Pork sausage	+	+	+	+	+	+	+
	7	Canned corned pork	+	+	+	+	+	+	+
	8	Pork meatball	+	+	+	+	+	+	+
	9	Noodle seasoning	+	+	+	+	+	+	+

(+) detected based on 3 MRM transitions RT matching; ND, not detected.

All the seven porcine-specific peptide markers were detected in all the processed samples with pork material (samples No. 5-9). None of the seven porcine-specific peptide markers was detected in the Halal certified samples (sample No. 1-4). However, five peptide markers were detected in chicken sausage (sample No. 5), which was not a Halal certified product. Following the confirmation criteria (RT matching with positive control, signal-to-ratio (S/N)>3, and at least 3xMRM transitions) the detection of the five porcine-specific peptides were confirmed. This may be resulted from cross-contamination due to non-halal sample handling during product manufacturing.

Detection limit of pork in processed foods

To evaluate the sensitivity of the method for screening analysis, it is important to know the detection limit in terms of content level of pork materials in processed food. To estimate the detection limit and confirmation reliability of the method, cooked pork meat (200ºC, 30 minutes) was pre-spiked into halal-certified mutton curry (sample No. 4) in four spiking percentages from 0.1% to 5.0% (wt) before sample preparation. The results of the spiked samples are summarized in **Table 4**.

Three porcine-specific peptide markers YDII, LVVI and SALA were detected for all spiking levels including the 0.1% (wt) with S/N >/= 3. The results were reproducible among four separate sample preparations and analyses in different days (inter-day). Peptide FVIE and EVTE had detection limits of about 0.5% and 1%, respectively. Peptide EVTE could be detected at only 5% spiking level whereas peptide TVLG could not be detected. This suggests that the detection sensitivity of the seven porcine-specific peptide markers are very different in processed food. It was found in thermolability tests that peptide YDII is most thermostable while the other six peptides are less stable at 200ºC.

Table 4. Detection limit of porcine-specific peptides in spiked Halal food sample

Peptide marker	Spiking percentage of cooked pork (%, wt) (*n*=4)			
	0.1	0.5	1.0	5.0
YDII	+	+	+	+
LVVI	+	+	+	+
EVTE	ND	ND	ND	+
TVLG	ND	ND	ND	ND
FVIE	ND	+	+	+
TLAF	ND	ND	+	+
SALA	+	+	+	+

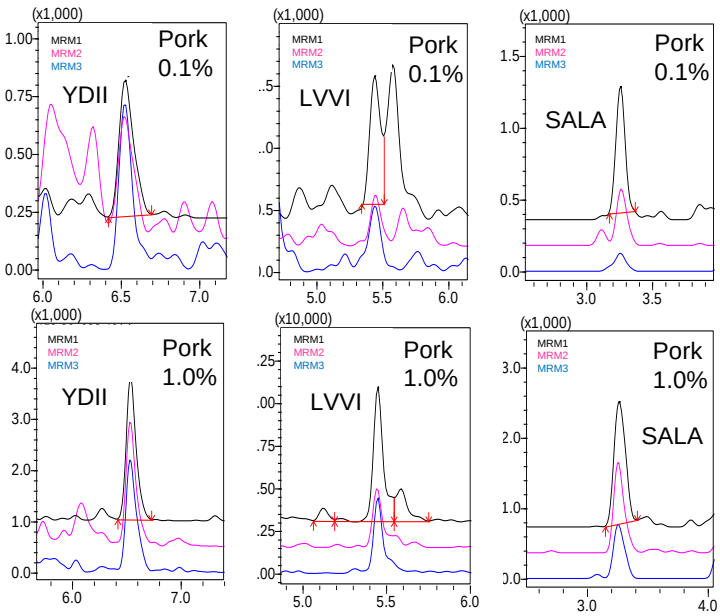

Figure 6. Detection of porcine-specific peptide markers YDII, LVVI and SALA in certified Halal food matrix spiked with cooked (200ºC) pork at 0.1% and 1% (wt).

The detection sensitivities of the seven peptide markers are different depending on the food processing and cooking conditions. The additional heating treatment of all raw meats and processed foods at 200°C under dry air in this study is considerably a severe condition which lead to degradation of proteins. The detection results under this experiment condition indicate the method is applicable and feasible for halal testing of various processed and cooked foods.

Semi-quantitative screening of pork blended in processed food

The four porcine-specific peptides YDII, LVVI, SALA and FVIE, which are more sensitive for detection of pork in processed food, are further evaluated for semi-quantitative screening. As shown in **Figure 7** and **Table 5,** linear relationship exists between peak area and content level of spiked pork (cooked at 200°C) in processed canned mutton curry (Halal certified). The linearity (R^2) is equal to or greater than 0.995 for the four peptide markers in the testing range. The quantitative results also exhibit acceptable repeatability with RSD of both peak area and concentration (spiking percentage) within acceptable value which is under 16%.

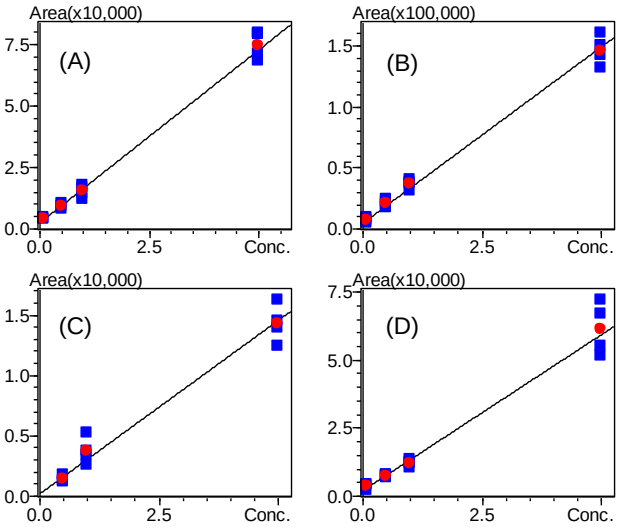

Figure 7. Linear relationship between peak area of four porcine-specific peptides and percentage (%) of cooked pork spiked in canned mutton curry. (A) YDII, (B) LVVI, (C) FVIE, (D) SALA. The blue and red dots represent repeated and average values, respectively.

In summary, the established MRM-based method on a LC/MS/MS platform offers a highly sensitive and selective approach to detect and monitor the presence of porcine-specific markers for semi-quantitation of the amount of pork in processed foods. The established method from sample preparation to LC/MS/MS analysis exhibits excellent reliable sensitivity, which is potentially able to support halal testing in processed food products. Analysis of various food matrices will be performed in the future.

Table 5. Calibration curves of four peptide markers in spiked samples

Peptide marker	Range (%)	Linearity	Repeatability at 1% (*n*=6)	
		(R^2)	Area RSD (%)	Conc. RSD (%)
YDII	0.1-5	0.997	11.3	13.6
LVVI	0.1-5	0.998	9.3	11.0
FVIE	0.5-5	0.995	14.4	15.3
SALA	0.1-5	0.998	8.3	10.1

❑ Conclusions

Despite its tedious sample preparation procedure, porcine-specific peptides are far more prominent targets for developing halal testing method, since peptides are more heat-stable than DNA, the common target for detection of pork in foods. With remarkable sensitivity and selectivity, this LC/MS/MS method provides a more robust approach to detect as low as 0.1% (wt) of porcine material blended in processed food.

❑ References

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Application Development & Support Centre, SHIMADZU (Asia Pacific) Pte. Ltd, 79 Science Park Drive, #02-01/08 Cintech IV, Singapore 118264, www.shimadzu.com.sg; Tel: +65-6778 6280 Fax: +65-6778 2050