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Summary of Analytical Solution Development by ADSC Shimadzu

Analytical Solutions for Halal Authentication

Preface

Modern food technologies provide consumers a variety of choices of processed food with different tastes, nutrition balances and qualities. However, on the other hand, there are also new challenges in ensuring the food to be free of unpermitted ingredients and adulteration. Ascertaining halal status of processed food is particularly difficult, because our senses are incapable of identifying the presence of pork components and alcohol in various food matrices. In recent years, the latest analytical techniques have been used in food analyses for inspection of components such as pork meat, pork gelatin, lard and alcohol in processed food. Halal authentication is moving to scientific approaches. This analytical solution book summarizes several important analyses that are applicable to Halal authentication in food laboratories. Advanced LC/MS/MS methods developed and used in recent years for detection of pork meat and gelatin in processed food with high sensitivity and selectivity are described in this book. Detection of pork DNA fragment by PCR is a well-known technique and a method on a PCR-MultiNA platform is introduced. In addition, analysis methods for lard in edible oils by FTIR-ATR and alcohol in beverage and food by HS-GC-FID are also included.

Topics

An introduction - the scientific approach in Halal authentication

In recent years, food adulteration has emerged as a global concern. From spiking melamine into milk powder and mixing horse meat into burger, this issue violates customers' right from the economical as well as ethics/religion point of view (Figure 1).

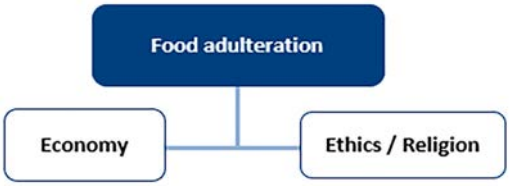

Figure 1. Global effect of food adulteration

Particularly for Muslim, dietary restriction concerning the consumption of foods containing non Halal materials has led to the development of Halal testing. According to Islamic law, Halal is defined as permissible and Haram as not permissible. "Halal" applies broadly to various consumer products from food, cosmetics to personal care product (PCP). Products containing any following materials are considered as non-Halal or Haram including pork and its by-products, alcohol, blood, and improperly slaughtered animal (Figure 2).

Figure 2. Halal testing (left) and definition of Halal (right)

Halal testing of food, cosmetics, and PCP are challenging due to the matrix complexity and lack of specific markers (Figure 2). In some cases, the adulterant is also present at such low amount and thus very difficult to trace. It is therefore essential to develop instrumental based Halal testing with remarkable detection limit.


Figure 3. Shimadzu instrumentations for Halal testing (ICP, FTIR, MultiNA, HPLC, GC, LCMS)

Instrumental-based Halal testing has been generally categorized into two approaches: targeted and non targeted. Non-targeted analysis acquires general profile of sample and performs qualitative comparison between adulterated and non-adulterated forms. Meanwhile, targeted analysis selects and targets specific marker(s) for sample authentication. The latter approach is more feasible for method validation and routine use.

With the rapid development in advanced analytical instrumentation and technology, Halal testing is currently leaning towards biomarker discovery specifically for pork and its by-products like gelatin and lard. DNA and peptides have been recently cited as the species-specific markers and applied in analysis for Halal authentication. In this application solution book, few key analytical methods for Halal testing of processed food, beverages, edible oils and PCP using various instrument from Shimadzu (Figure 3).

Advanced LC/MS/MS methods for detection of pork meat and gelatin

Background:

A number of analytical instruments have been utilized to develop Halal authentication methods from the standpoint of pork material detection (Figure 1). For targeted analysis, SDS-PAGE is the pioneering technique for Halal testing. However, the limit of detection (LOD) of the method is rather high. ELISA-based approach suffers from specificity of antibody used. Specific antibody can be only utilized for particular meat marker and thereby increase the cost.

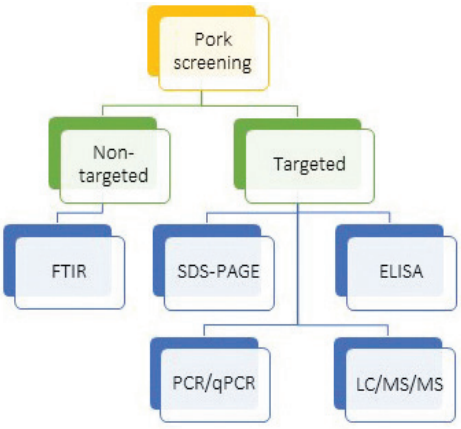

Figure 1. Technologies for pork material detection

Species-specific DNA fragment has been used as the conventional target for Halal testing. It is highly specific target and the PCR/qPCR is among the most commonly used instruments in Halal research and testing laboratories. However, the disadvantage of DNA marker is its proneness to thermal degradation in high temperature food processing (cooking).

In contrary, the primary structure of protein is rather stable against heat treatment. Therefore, there is a recent shift in the focus of target marker for Halal testing from DNA to peptides [1,2]. New method on LC/MS/MS platform with targeting peptide markers can result in highly sensitive outcome albeit its sample preparation is as tedious as DNA. Additionally, the establishment of multiple reaction monitoring (MRM) analysis in LC/MS/MS facilitates highly selective and accurate authentication. Development of Halal testing based upon peptide marker is carried out employing Shimadzu LCMS-8060, high sensitive ultra fast LCMS system.

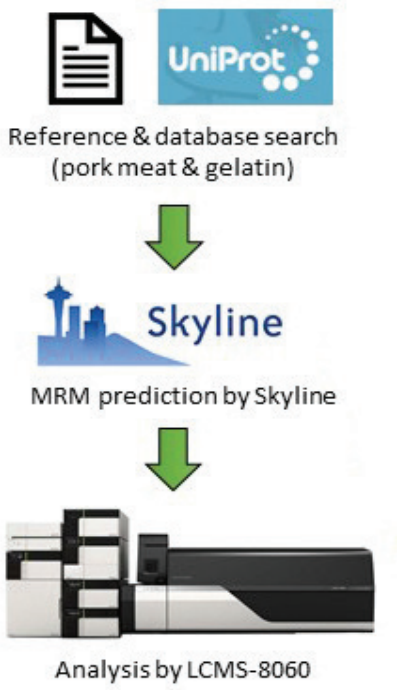

Figure 2. Workflow of technical approach

Technical approach:

At initial step, reference and database searching is conducted to search for pork/gelatin-specific peptide marker(s) (Figure 2). To establish MRM method for validation of species-specific markers, Skyline program is used as the tool for initial setting up of MRM parameters based on FASTA format of targeted proteins or peptides (Figure 3). After successful verification of peptide markers using raw meat and in-house gelatin standards, typical protein sample preparation is carried out for consumer products (Figure 4).

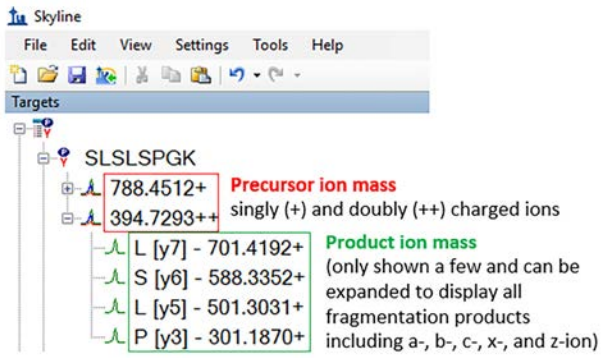

Figure 3. MRM prediction of peptide sequence by Skyline

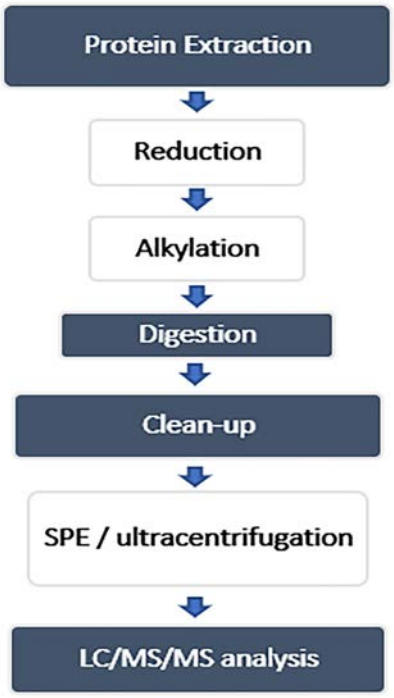

Figure 4. Sample preparation method

(I) LC/MS/MS method for pork meat detection [3]

Seven pork-specific peptide markers (Table 1, Figure 5) are selected and employed to verify the Halal status of processed food. The results show that the pork-specific peptides are able to verify the Halal status of commercial products (Table 2, Figure 6).

Table 1. Pork-specific peptides and MRM based method on LCMS-8060

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

Table 2. Results of screening analysis of peptide markers in processed food

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 samples were heated at 200°C for 30 min.

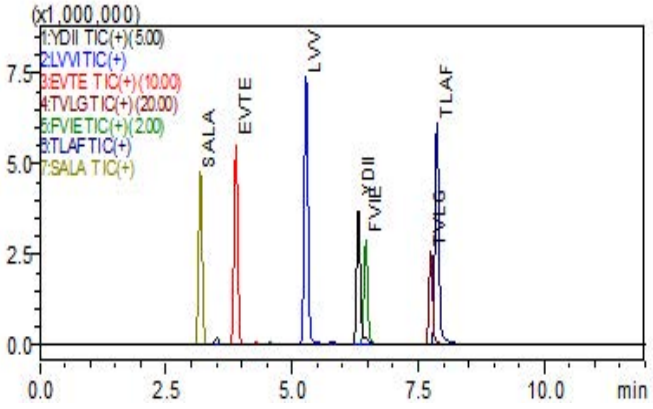

Figure 5. TIC of 7 pork-specific peptide markers

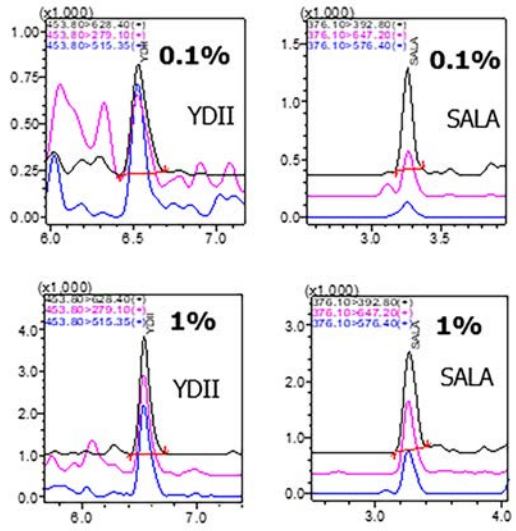

Figure 6. Chromatogram of Halal certified food spiked with 0.1% and 1% pork meat

(II) LC/MS/MS method for pork and beef gelatin detection [4]

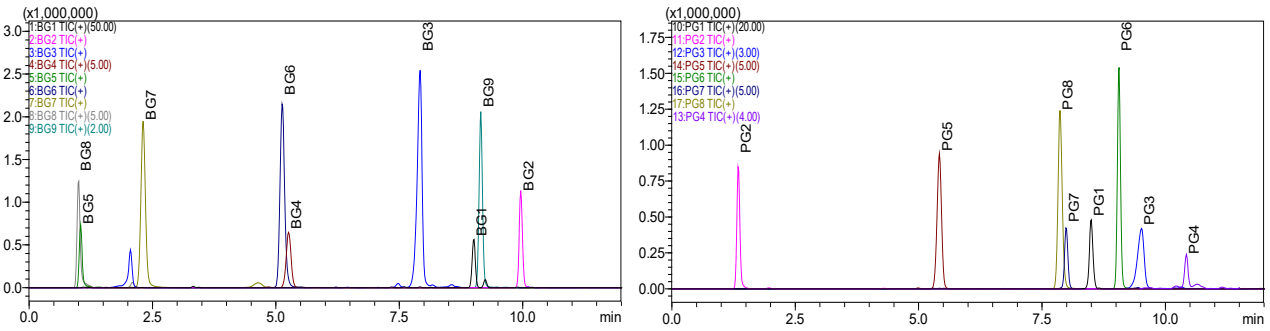

Figure 7. MRM chromatograms of 9 beef-specific peptides (BG) and 8 pork-specific peptides (PG)

General approval of animal-based gelatin is restricted due to incongruity with several religious customs and individual dietary preference which require the gelatin to be free from porcine (Muslim), bovine (Hindu) or any animals-by-products.

A total of 17 gelatin specific markers (9 bovine and 8 porcine-specific peptides) are selected using the same approach to pinpoint marker of pork meat (Figure 7). These markers are then utilized to verify gelatin content in 19 food, pharma capsule and PCP samples (Table 3).

Featured with high sensitivity and a high number of specific peptide markers, this LC/MS/MS method on LCMS-8060 offers a reliable approach for detection and speciation of animal-based gelatins (bovine and porcine) in commercial goods as low as 0.1% adulteration (Figure 8).

Table 3. Results of screening analysis of gelatin markers in food and PCP

No	Sample	Gelatin label & source	Detection	
			Bovine	Porcine
1	Gummy bear I	Yes, beef	✓	ND
2	Marshmallows I	Yes, pork	ND	✓
3	Chewing gum	Yes, undeclared	ND	✓
4	Marshmallows II	Yes, undeclared	✓	ND
5	Gummy bear II	Yes, undeclared	✓	ND
6	Vanilla cookie	Undeclared	ND	ND
7	Chocolate cookie	Undeclared	ND	ND
8	Chocolate bar I	Undeclared	ND	ND
9	Chocolate bar II	Undeclared	ND	ND
10	Chocolate bar III	Undeclared	ND	ND
11	Pharma capsule I	Yes, undeclared	✓	ND
12	Pharma capsule II	Yes, undeclared	✓	✓
13	Pharma capsule III	Yes, undeclared	✓	✓
14	Hand cream I	Hydrolysed collagen	ND	✓
15	Hand cream II	Hydrolysed collagen	ND	ND
16	Face cream I	Hydrolysed collagen	ND	✓
17	Face cream II	Hydrolysed collagen	ND	✓
18	Face cream III	Hydrolysed collagen	ND	ND*
19	Hair conditioner	Hydrolysed collagen	ND	ND*

(*) lower than LOD

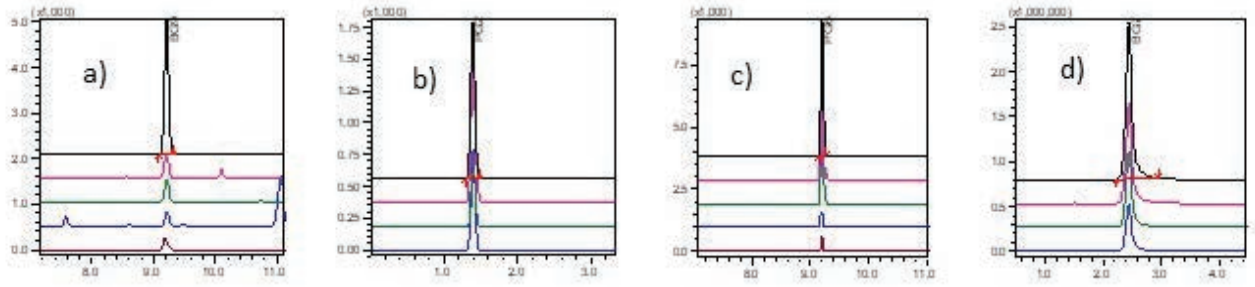

Figure 8. (a) 0.1% beef gelatin in pork matrix, (b) 0.1% pork gelatin in beef matrix, (c) pork gelatin in hand body cream, (d) beef gelatin in pharma capsule

Reference:

[1] von Bargen, C., Dojahn, J., Waidelich, D., Humpf, H.U., Brockmeyer, J., J Agric Food Chem (2013) 61: 11986−11994.

[2] G. Zhang, T. Liu, Q. Wang, L. Chen, J. Lei, J. Luo, G. Ma, Z. Su, Food Hydrocolloids (2009) 23: 2001-2007

[3] Udi Jumhawan, Jie Xing & Zhaoqi Zhan, "Highly-Sensitive Detection of Multiple Porcine-Specific Peptides in Processed Foods by LC/MS/MS Method", Application News AD-0153 (Shimadzu) 2017

[4] Udi Jumhawan, Jie Xing & Zhaoqi Zhan, "Detection and Differentiation of Bovine and Porcine Gelatins in Food and Pharmaceutical Products By LC/MS/MS Method", Application News AD-0164 (Shimadzu) 2017

PCR-MultiNA platform for pork meat detection in processed food

Background:

Species-specific DNA fragments are used in pork and other meat identification for inspection of adulteration and Halal authentication [1, 2]. Due to the excellent specificity and selectivity, DNA based PCR method and screening kits have been widely used in pork detection and screening. The PCR-MultiNA approach described here is used for pork detection with a singleplex primer of pork DNA. This PCR-MultiNA platform provides highly sensitive and specific analytical tool in identification of pork-DNA in processed food.

Technical approach and solution:

The analysis workflow for detection of pork DNA in processed food such as chicken sausage consists of three steps [3]: (1) DNA extraction from a meat sample, (2) PCR amplification of pork-specific DNA (using BioKits PCR, pork specific Mastermix from Neogen), (3) detection of targeted DNA fragment (314 bp) on MultiNA via microchip electrophoresis.

Specificity: The PCR Mastermix amplified the pork DNA fragment 313 bp (target size: 314 bp) and the housekeeping fragment 396 bp (target size: 380-420 bp) in pork sausage sample.

Selectively: In the DNA extract of chicken sausage, pork DNA fragment was not detected. The only observed fragment was the housekeeping DNA at 418 bp (Figure 1).

Sensitivity: Pork sausage was spiked into the halal certified chicken sausage to obtain 1wt%, 0.5wt% and 0.1wt% mixtures before DNA extraction. The results are shown in Figure 2 and Table 1. The PCR-MultiNA method is able to detect pork DNA fragment (314 bp) in chicken sausage spiked with pork sausage at level as low as 0.1%.

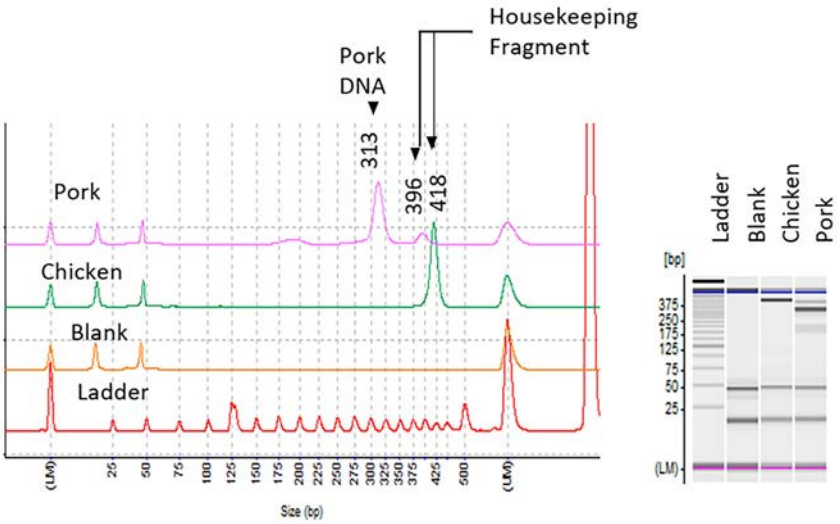

Figure 1. Electropherograms of PCR product of food samples. Detected peaks are pork DNA fragment (~313bp), housekeeping fragments (pork~395bp, chicken~418bp) and DNA ladders.

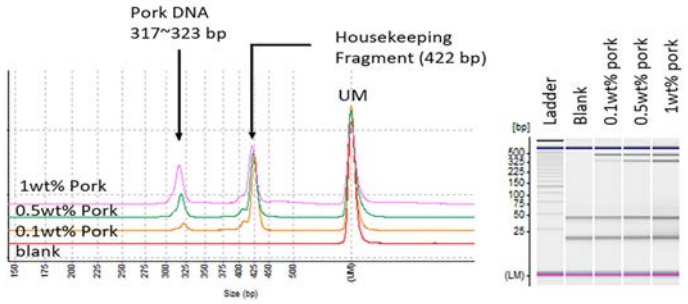

Figure 2. Detection of pork DNA fragment in chicken sausage samples spiked with 0% (blank), 0.1%, 0.5% and 1% of pork sausage (from bottom to top).

Table 1. Accuracy and reproducibility of PCR-MultiNA procedure for detection of pork DNA

Pork Content	Repeat No. (n)	Measured Ave (bp)	Accuracy, Ave (%)	RSD (%)
100%	4	317.7	101.2	0.74
3% & 5%	3	316.3	100.7	1.02
1%	3	316.7	100.8	0.18
0.5%	4	320.3	102.0	0.95
0.1%	5	322.0	102.5	0.54

Conclusions:

A highly sensitive and specific method for detection of pork in processed food was established on PCR-MultiNA platform. The microchip electrophoresis (MCE) and fluorescence detection of MultiNA exhibits the advantages of high sensitivity, reliability and easiness in operation. The method can detect as low as 0.1wt% of pork sausage spiked in halal certified chicken sausage sample.

Reference:

[1] M. Eaqub Ali & M. Kashif & Kamal Uddin & U. Hashim & S. Mustafa & Yaakob Bin Che Man, Food Anal. Methods, "Species Authentication Methods in Food and Feeds: the Present, Past, and Future of Halal Forensics (ISSN 1936-9751) Springer (2012)

[2] Application Notes 11, Rapid Identification of Meat Species with MCE-202 “MultiNA”, Shimadzu.

[3] D. Kesuma, L. G S Lim & Z. Zhan, “Sensitive Detection of Pork DNA in Processed Meat products on PCR-MultiNA Platform”, Application News AD-0120 (Shimadzu) 2016.

FTIR method for detection of lard in palm oil and coconut oils

Background:

Lard adulteration in edible oils is an example of food fraud for commercial profit. It also poses a serious concern in Halal authentication, because the Islamic law prohibits Muslims from consuming pork in any form, including lard in food products. Here we describe a rapid FTIR-ATR method for quantitative detection of lard adulteration in virgin coconut oil (VCO) and vegetable palm oil.

Technical approach and solution:

Infrared spectroscopy is a very useful technique in identification of organic components based on characteristic absorptions in the infrared region (650~4000 cm^{-1}). However, direct identification of lard present in edible oils is extremely difficult due to very small difference between pure oil and lard (Figure 1) [1]. With using chemometrics data analysis, i.e., partial least squares (PLS) regression, the small differences of IR absorption in the region 1500~1000 cm^{-1} enables qualitative and quantitative analysis of lard in various edible oils [1-3].

Two PSL calibration models were established using virgin coconut oil (VCO) and palm oil as matrices. Details of the PSL parameters used are shown in Table 2. The lowest contents of lard in the reference samples are as low as 0.5% for VCO and 1% for Palm oil. Both ATR and transmission methods were used in these experiments. Since a Specac Pearl™ accessory was adopted, IR measurements by transmission method was as fast and easy as ATR, with requiring only a drop of sample by micropipette [3].

Table 1. PSL calibration models and parameters for lard in edible oils

Oil Matrix	Virgin Coconut Oil (Ref)	Palm oil (Ref)
Number of sample	17 (3 meas. per sample)	11 (3 meas. Per sample)
Lard spiked (w/w)	0.5% ~ 30%	1% ~ 90%
Range (cm^{-1})	900 ~ 1500; 2750 ~ 3050	1000~1490
Pre-process	Derivative, Order = 2, Points = 5	Derivative, Order = 2, Points = 15
Number of factors	4	5
Coefficient (R^2)	0.9999	0.9993
MSEP	0.0001	0.0007
SEP	0.0098	0.0258

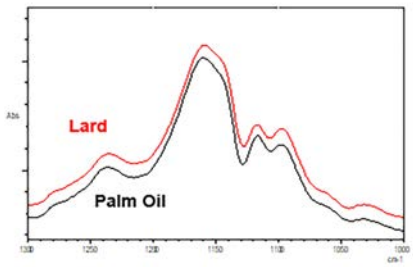

Figure 1. IR spectra of Lard and palm oil (1300~1000 cm^{-1})

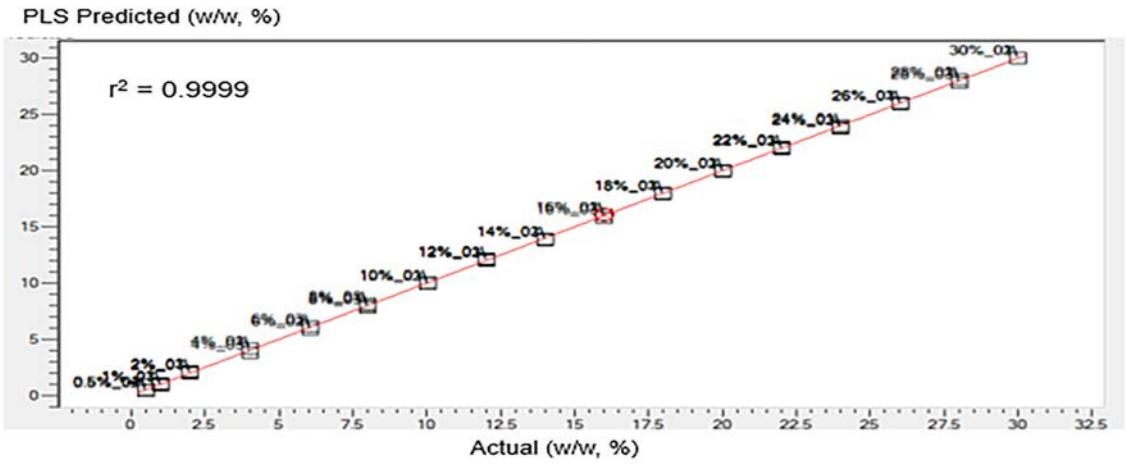

Figure 2. PSL calibration for lard in VCO predicted values versus actual values

Table 2. Evaluation results of FTIR-ATR with PSL methods for determination of lard in different edible oils samples

PLS model		**Virgin Coconut Oil (Ref)**				**Palm oil (Ref)**					
Oil Matrix		VCO (Brand A)		VOC (Brand B)		Palm Oil (Brand C)		Palm Oil (Brand D)		Olive Oil	Palm Soy Oil
Lard (% w/w), spiked		2.5	5	2.5	5	8.0	25.0	8.0	25.0	25.0	25.0
Lard (% w/w), measured	1	2.36	4.93	2.53	5.33	8.9	24.1	9.1	26.1	64.4	32.3
	2	2.44	5.31	2.62	5.29	8.2	23.5	7.6	25.8	64.6	32.1
	3	2.55	5.37	2.62	5.23	8.6	24.4	8.4	26.2	63.0	33.7
	Mean	2.45	5.2	2.59	5.28	8.6	24.0	8.3	26.0	64.0	32.7
Recovery (%)		98	104.1	103.6	105.7	107.2	96.1	104.2	104.1	255.9	130.8

Table 2 shows the results of method performance evaluation for detection and quantitation of lard in edible oils. The spiked lard was determined accurately in two different VCO samples at 2.5% and 5% and two different palm oil samples at 8% and 25%, respectively. However, the results of olive oil and palm soy oil are significantly higher than the expected values. This indicates that PSL models must be established and used for the same oil matrix to ensure accuracy and reliability.

Conclusions:

FTIR-ATR in combination with PLS data analysis provides a rapid technique for quantitative detection of lard adulteration in edible oils. Linear PLS calibrations were obtained for the range of lard in VCO from 0.5% to 30% and in palm oil from 1% to 90%. The good linearity ($r^2 > 0.9993$) indicates the possibility of the methods in detection of low level of lard adulteration. However, the accuracy and reliability of the methods rely on the conditions of PLS models, which require not only use of same type oil matrix, but also validation for the allowed variation of oils to be tested routinely.

Reference:

[1] A. Rohman; Yaakob B. Che Man; P. Hashim; A. Ismail; CyTA - Journal of Food 2011, 9, 96-101.

[2] Z. H Lee, J. S Kuek, J. Lim and A. M Chua, "Quantitative Determination of Lard Adulteration by FTIR Spectroscopy with Chemometrics Method-Vegetable Palm Oil", Application News AD-0162 (Shimadzu) 2017

[3] Z. H Lee, J. S Kuek, J. Lim and A. M Chua, "Quantitative Determination of Lard Adulteration by FTIR Spectroscopy with Chemometrics Method-Virgin Coconut Oil", Application News AD-0167 (Shimadzu) 2017

HS-GC-FID quantitative detection of alcohol in beverage and food

Background:

One of the most prominent prohibitions by the Islamic laws is alcohol (ethanol) [1]. Muslims are forbidden to ingest alcoholic content in all kinds of food and beverage (Table 1). Therefore, there is a need to develop easy and fast analytical method for quantitative detection of ethanol present in beverages, dairy products, vinegars and sauces for Halal Authentication testing.

Technical approach and solution:

Gas chromatography GC-FID is an appropriate method of choice for detecting ethanol with high sensitivity. However, aqueous samples could be damaging to the GC capillary column and cause backflash problem [2]. Headspace (HS) sampler provides a solution for injection of volatile components in aqueous samples. Here we describe a HS-GC-FID method for fast, sensitive and quantitative determination of ethanol in various aqueous food samples. The advantage of HS is its minimal sample preparation required [3].

Table 1. Maximum allowable contents of ethanol according to Halal administrative bodies in Southeast Asia [1]

Islamic Statutory Body	Max. allowable industrial ethanol	Max. allowable naturally-produced ethanol
MUIS (Singapore)	0.5% in additives 0.1% in final product	Unspecified
JAKIM (Malaysia)	0.5% in final product	1%
MUI (Indonesia)	Can be used, but has to be 0% in final product	1%
BIRC (Brunei)	prohibited	2%

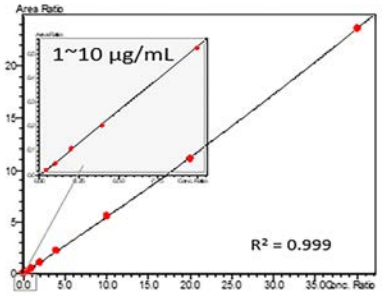

Fig 1. EtOH calibration curve for a range of 1~1000 µg/mL for liquid samples with IPA (25 µg/mL) as internal standard (IS). A zoomed portion of lowest levels 1~10 µg/mL is inserted.

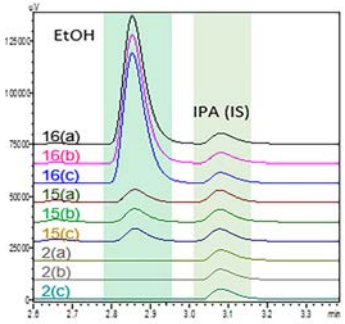

Fig 2. Triplicate HS-GC-FID results of liquid samples: energy drink (a), soy bean milk (b) and ginger ale (c).

Table 2. HS-GC-FID and conditions for quantitative determination of ethanol in aqueous food samples

Headspace Sampler	**HS-10**
Oven Temp.	80°C
Sample Line Temp.	100°C
Transfer Line Temp.	110°C
Pressurize Gas Pressure	60kPa
Gas Chromatograph	**GC-2014**
Column	SH-Rxi-5Sil MS, 30m x 0.25mm x 0.25µm
Carrier Gas	Helium, 99.9997% purity
Oven Temp. Programming	30°C (4min) →40°C/min to 250°C (2.5min)
Detector	**FID-2014**
FID Temperature	250°C
Gas Flow Condition	Hydrogen flow 30mL/min Air flow 400mL/min Makeup gas flow (nitrogen) 40mL/min

Table 3. Quantitative results of ethanol detected in 16 Halal -labelled samples for local super market using HS-GC-FID method (triplicate injections)

No.	Samples from local market (Halal labelled)	Concentration (µg/mL)	EtOH Content (%, w/w)
1	Carbonated drink	ND	Zero
2	Ginger ale	ND	Zero
3	Green tea	ND	Zero
4	Isotonic drink	ND	Zero
5	Milk	ND	Zero
6	Probiotic dairy drink	ND	Zero
7	Sparkling water	ND	Zero
8	Sweetened drink	ND	Zero
9	Vinegar 1	ND	Zero
10	Wintermelon tea	1.4	0.0001
11	Dipping sauce	4.1	0.0004
12	Honey drink	4.9	0.0005
13	Vinegar 2	10.9	0.0011
14	Grape juice	37.1	0.0037
15	Soy bean milk	43.7	0.0044
16	Energy drink	497.8	0.0498

A fast GC-FID method was employed with IPA as internal standard. A Linear calibration curve of ethanol (Figure 1) was obtained with correlation coefficient (R^2) of 0.999 across the range from 1 µg/mL to 1000 µg/mL. The relative standard deviation (RSD) for each level was less than 2% (n=3) except the lowest level (1 µg/mL, RSD 6%). The calibration curve is shown in Figure 1, depicting excellent linearity.

Sixteen food samples were analysed using the fast HS-GC-FID method described above. The quantitative results of the samples are summarized in Table 3, and chromatograms of three selected samples are displayed in Figure 2. The remark of ND (Not Detected) refers to the lowest calibration level of 1 µg/mL, which corresponds to 0.0001% (w/w).

Conclusions:

A fast and highly sensitive HS-GC-FID method was established for quantitative determination of ethanol present in beverages, vinegars, milk, soybean milk and sauce. With use of Head Space sampler, minimal or no sample preparation is required. The detection sensitivity of the method could be at or lower than 1 µg/mL or 0.0001% (w/w).

Reference:

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