Analysis of ethanol in soy sauce using electronic nose for halal food certification

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Abstract The purpose of this study was to analyze ethanol content in soy sauce using mass spectrometry (MS) with electronic nose (e-nose) to determine if MS e-nose can replace gas chromatographic analysis for halal certification. Gas chromatography–flame ionization detector (GC-FID), the standard method of ethanol content, was used to analyze 24 different kinds of soy sauce. Ethanol was detected from 13 soy sauce samples in the concentration range of 0.0004–1.7wt%. The MS e-nose data were analyzed by discriminant function analysis (DFA). Based on an addition method, the results were more than 96.6% accurate when the ethanol concentrations were greater than 0.5%. A high correlation between the first score of the DFA plot and the ethanol concentration was observed. Thus, mass spectrometry based on e-nose is an efficient method for determining ethanol as a primary screening tool for halal certification.

Keywords: halal food, soy sauce, ethanol, electronic nose, discrimination

Introduction

Halal refers to any object that is permissible to use according to Islamic law. To receive a halal certification, halal food should not contain ingredients such as pork, dog meat, animal blood, raptors, and alcohol (1). Because of the rigorous screening and selection process, halal food is regarded as safe and is rising in popularity among non-Muslims (2).

To access the halal market, it is necessary to obtain halal certification. An international halal standard has not yet been established, so there was a difference of the standards for alcohol contents depending on Islamic schools and local customs (3). As halal has strict alcohol regulations, it can be difficult to obtain halal certification for soy sauce due to the small amount of alcohol naturally produced by fermentation (4). In this regard, it is necessary to establish an exact method for measuring alcohol in soy sauce.

Gas chromatography–flame ionization detector (GC–FID) has been used as an analytical method for halal certification analyzing non-alcoholic malt beverages, fruits, vegetables, vinegar, and fermented glutinous rice for alcohol content (5,6). The alcohol contents of various wines have been analyzed by comprehensive two-dimensional gas chromatography–time-of-flight mass spectrometry (GC×GC–TOF-MS) (7). However, these methods have only been applied to a few

beverages and are ineffective when analyzing a large number of samples for halal certification due to the need for sample pretreatment and the long analysis time. Therefore, a method for alcohol analysis without pretreatment is required to simultaneously analyze multiple samples.

Electronic nose (e-nose) has the potential to conveniently and quickly analyze multiple samples. As a nondestructive analysis method, it has been widely used in various fields such as quality control (8,9), discrimination of fake food (10,11), and origin of food (12,13).

Moreover, the quantitative and differential analysis between samples is possible in the case of an e-nose coupled with mass spectrometry (MS). E-nose coupled with MS (MS e-nose) can also identify components of the expected ion fragments of samples using existing MS library data (14).

Hence, due to the growing number of companies seeking halal certification, this study attempted to use the data obtained from e-nose for pattern recognition in order to discriminate efficiently multiple samples, verifying if these samples can be halal objects through a speedy primary screening. The Korean soy sauce has not received halal certification yet (15,16). The objective of this study was to confirm whether MS e-nose can be used as a primary screening to certify soy sauce as halal or not. The e-nose data were

compared to the data obtained from an authorized GC–FID method to determine if MS e-nose can evaluate ethanol content effectively.

Materials and Methods

Samples Commercial soy sauce samples were purchased from a local market (Nowon-gu, Seoul, Korea) and named unknown-1 and unknown-2. Conventional soy sauces were purchased from Sunchang-gun (Jeollabuk-do, Korea). Ethanol (99.5%) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and used with three times distilled water depending on the experimental conditions. The three times distilled water was filtered by a Milli-Q System (Millipore, Billerica, MA, USA). Methanol (99.9%) was used as a standard material, 99.9% n-hexanol was used as an internal standard and dimethyl sulfoxide was used to manufacture standard solution. All these reagents were purchased from Sigma-Aldrich. A twister purchased from GESTEL-Twister™ (Gerstel, Mullheim an der Ruhr, Germany) was used for well blending samples.

GC–FID analysis GC-2010 Series gas chromatograph (Shimadzu, Kyoto, Japan) with an Agilent DB-WAX column (320 µm I.D.×60 m, 0.25 µm; Agilent, Santa Clara, CA, USA) was used for GC–FID analysis. The oven temperature was programmed as follows: maintained at 40°C for 5 min, ramped at 10°C/min to 240°C for 9 min holding time, and maintained for 9 min. Helium was used as a carrier gas with a flow rate of 1 mL/min.

A 0.5 g sample, 1.0 mL of n-hexanol (internal standard; concentration =2,500 mg/kg), and 8.5 mL of dimethyl sulfoxide were placed in a 20-mL gas-tight vial (Pharma Fix, Chemmea, Bratislava, Slovakia) containing a stir bar and sealed using a cap. This vial was agitated at 1,500xg for 60 min at 25°C before extraction, followed by another centrifugation at 1,300 rpm and 25°C for 10 min. The obtained supernatant was filtered by a 0.45-µm membrane filter and analyzed by GC–FID.

MS E-nose analysis A 0.5-g soy sauce sample was taken in a 10-mL vial (La-Pha-Pack™, GmbH, Langerwehe, Germany) and this was sealed hermetically using caps with PTFE/silicone septums (Shimadzu, Kyoto, Japan). This vial was agitated at 350 rpm for 10 min at 80°C. This was injected to injection port at 130°C. For the headspace analysis, 2.5 mL of volatile components was taken with a syringe placed on a temperature-controlled tray holder maintained at 110°C (CombiPAL, CTC Analytics, Industriestrasse, Zwingen, Switzerland) and were injected to injection of electronic nose (Smart Nose 300; Smart Nose Inc., Marin-Epagnier, Switzerland) at 130°C by an auto-sampler (CombiPAL; CTC Analytics, Zwingen, Switzerland). The headspace system was then used to analyze the gas components of the sample using an e-nose system (Smart Nose300; Smart Nose Inc., Marin-Epagnier, Switzerland) and a mass spectrometer (ThermoStar™ GSD 320 T2, Balzers Instruments, Masin-Epagnier, Switzerland). The volatile substances were ionized at 70 eV. The ionized substances

formed over a period of 3 min were passed through a quadrupole mass filter. The substances having the major ion fragments of the ethanol mass spectrum were used as the number of channels. An air sample was used as a control, and the test was repeated three times for each sample. The statistical program used in this analysis was Smart Nose™ statistical analysis software (version 1.51, THOPAS Soft Creation, Marin-Epagnier, Switzerland).

Discriminant function analysis The independent variables were the main ion fragments of ethanol or methanol in the range of 10–200 atomic mass units (amu). With the values of selected independent variable, discriminant function analysis (DFA) was conducted. The effect of an independent variable on the dependent variable was tested using the following equation:

$$DFA=B_0+B_1X_1+B_2X_2+B_3X_3\cdots+B_nX_n,$$

where B_0 is a constant, B_1 is a coefficient, and X is the sensitivity of each value in amu. DF1 and DF2 are the values of the discriminant function, which were selected by highly influential dependent variable. The discriminant function of the independent variables had a two-dimensional plot with DF1 and DF2 along the x- and y-axis, respectively.

Results and Discussion

Analysis of ethanol content in conventional soy sauces by GC-FID

GC–FID was used to analyze 24 different kinds of conventional soy sauce (Sunchang, Korea) for ethanol content (Table 1). Among the results, 13 soy sauce samples except #13 sample, all contained less than 0.13wt% ethanol.

Lee *et al.* (17) reported that conventional soy sauces generally contained a small amount of ethanol (approximately 0.18%), whereas soy sauces that didn't include yeast contained trace amounts (approximately 0.03%) (18). The average ethanol content of the 24 conventional soy sauces tested in this study was 0.08%, which is similar to the previously reported data (17).

Soy sauce generally produces various contents of alcohols by yeast fermentation. However, conventional soy sauce exclusively prepared from soybeans produces only a small amount of alcohols. There is a lack of salt inhibiting yeast growth (18). It is feasible to obtain halal certification for conventional soy sauce due to a small amount of alcohols produced by fermentation.

GC–FID can accurately measure a small amount of alcohols. However, it takes a long time to analyze a large number of samples due to the need for sample pretreatment and the selection of a column and separation conditions (19,20). The loss of volatile compounds during sample pretreatment is also another problem because alcohol standard for halal certification is strict (approximately 0–1.0%). Therefore, in this study, MS e-nose was used to analyze ethanol in soy sauce as a potential replacement for the conventional

GC–FID method.

Determination of ethanol by MS E-nose Samples containing 0.001 to 0.25% ethanol in distilled water were prepared based on a previous study that reported alcohol contents on the range of 0.001 to 3.5% in beverages sold in the Malaysian market (7). The prepared samples were analyzed by MS e-nose with the major ethanol ion fragments as the independent variable. *F* value indicated the discriminant power of ion fragments. High discriminant power values of ion fragments were used for DFA plots. (Fig. 1A)

As seen in Fig. 1A, the *F* value of DF1 was 6452.60, and that of DF2 was 53.74, indicating that the ratio of DF1 to DF2 was differentiated in a proportion of approximately 120:1. Thus, the samples can be divided mainly by DF1.

Most of all samples showed the lower DF1 score than that of air. These were located in the relatively left direction of quadrants depending upon the amount of volatile compounds. The samples with high ethanol concentration were located on the further left direction of air. Also, a little difference of low ethanol concentration could be discriminated by DF1.

Figure 1B illustrates the correlation between the DF1 score and ethanol concentration. The value of r^2 was approximately 0.98, indicating a high correlation, i.e., DF1 decreased with increasing ethanol concentration. It is considered that ethanol concentration can be predicted through the relationship between DF1 score and its concentration.

Han *et al.* (21) analyzed off-flavors from a polyethylene terephthalate water bottles and caps by MS e-nose and compared the sensitivity values of the suspected major amu of three elements as 2,4-di-tert-butylphenol, nonanal, and decanal. Son *et al.* (22) discriminated sesame oil mixed with perilla oil using MS e-nose. When the content of perilla oil in the mixed oil increased, relatively high mass ion fragments (amu) including 53 amu (like as pyrazines) were selected. An intensity of the selected ion fragments was processed to statistics of DFA. The results of statistics showed the mixed oil can be discriminated by a mixing ratio. Hong *et al.* (23) analyzed the degree of binding between rice-washing water and trimethylamine using MS e-nose based on an intensity of 59 amu, major ion fragment of trimethylamine.

In this regard, it was possible to predict ethanol content of soy sauce using MS e-nose with the major ion fragment of ethanol as the independent variable. MS e-nose analysis discriminated samples based on visualized patterns after DFA based on the sensitivity of ion fragments without pretreatment. One of the important things was to select the high discriminant power value of ion fragments for analysis of ethanol. Otherwise, the good differentiation among the samples could not be found. (24,25).

The major ion fragments of ethanol (31, 29, 45, 46, 27, 26, and 43 amu) were identified using an MS library and used to discriminate samples to be influenced only by ethanol compounds during MS e-nose analysis. The intensity values of these were used for statistics of

Table 1. Ethanol content in conventional soy sauce samples determined by gas chromatography–flame ionization detection (GC–FID)

Sample No.	Ethanol (mg/kg)
1	76.15
2	ND ¹⁾
3	ND
4	199.71
5	55.62
6	94.86
7	36.54
8	1223.57
9	137.54
10	ND
11	ND
12	568.59
13	17030.42
14	419.60
15	81.37
16	71.92
17	ND
18	ND
19	76.19
20	ND
21	ND
22	ND
23	ND
24	ND

¹⁾ND indicates that the ethanol content is estimated to be less than 5.40 mg/kg.

DFA. The DF1 and DF2 ratio resulting from DFA had a two-dimensional plot along the x- and y-axis by influential order. In this result ethanol contents of the samples could be accurately confirmed by MS e-nose analysis and DFA plot depending on their concentration. However, in soy sauce matrix, ethanol may be influenced by other components in soy sauce.

Thus, we examined if the ethanol content in soy sauce was influenced by other components in the matrix.

A primary screening of ethanol in soy sauce Ethanol was added to #20 sample, in which did not have ethanol content (Table 1), according to ethanol concentrations. Distilled water instead of ethanol was added to #20 sample as control. Each volatile compound was then analyzed by pattern recognition using MS e-nose. The results are shown in Fig. 2A. The *F* values of DF1 and DF2 were 310.37 and 104.48, respectively, indicating that the DF1 and DF2 ratio was differentiated in a proportion of approximately 3:1. Thus, the samples can be discriminated mainly by DF1. The samples containing relatively high alcohol concentration showed the lower DF1 score (left side) than control. The samples containing high concentration alcohol were located on the further left direction of quadrants according to ethanol concentration. It meant that ethanol content in soy sauce was not influenced by other components in the soy sauce matrix in the analysis of ethanol.

Fig. 1. (A) Discriminant function analysis of the obtained data by electronic nose for various concentrations of ethanol, (B) Relationship between discriminant function first score and various concentration of ethanol (DF1: $r^2=0.9997$, $F=6452.60$ and DF2: $r^2=0.9598$, $F=53.74$)

Determination of ethanol in soy sauce Six samples of the 24 soy sauces were selected based on the results seen in Table 1 and classified based on ethanol content. Group L (#20 and #21) comprised non-ethanol soy sauces, Group M (#4 and #15) comprised soy sauces with relatively low ethanol concentrations, and Group H (#8 and #13) comprised soy sauces with the highest ethanol concentrations. To confirm that these groups comprised soy sauces with similar ethanol contents, each of the six samples was analyzed using MS e-nose, and the results are shown in Fig. 3. The F values of DF1 and DF2 were 3938.50 and 325.13, respectively. DF1 was 12 times larger than DF2, indicating that the samples were mainly able to be discriminated by DF1.

All samples were positioned to the left direction of the air that didn't contain any other volatile component. When the samples increased the amount of volatile component, the location of DF1 score for the samples moved to the left direction against air. The samples were located on the left side of quadrants with high ethanol concentration. An increase of volatile compounds might be due to the increase of ethanol concentration.

The group H (1.70 and 0.12%) was located in the left side of

quadrants and showed the lower DF1 score. The groups of M (0.008 and 0.02%) and L (0 and 0%) were located in the right side of quadrants and showed the higher DF1 score. Therefore, it was found that soy sauce containing high ethanol concentration was located in the left direction of quadrants depending upon DF1 score.

As seen in Fig. 4, the ethanol contents of the six conventional soy sauces and commercial soy sauces along with unknown-1 and unknown-2 were determined. The unknown-1 and -2 samples were expected to contain added spirits. As seen in Fig. 4, the F values of DF1 and DF2 were 4293.10 and 322.51, respectively. DF1 was 13 times larger than DF2, indicating that the samples were mainly able to be discriminated by DF1.

Based on DF1, it was expected that the commercial soy sauces named as unknown-1 and unknown-2 which contained more than 1.70% ethanol because all unknown samples were plotted farther left than the H2 soy sauce containing 1.70% ethanol. Also unknown-1 plotted farther left than unknown-2, indication that unknown-1 contained more ethanol than unknown-2. In conclusion, both products are unable to obtain halal certification.

When major ion fragments of ethanol were used for MS e-nose

Fig. 2. (A) Discriminant function analysis of the obtained data by electronic nose for soy sauce contained various concentrations of ethanol, (B) Relationship between discriminant function first score (DF1) and soy sauce contained various concentrations of ethanol (DF1: $r^2=0.9920$, $F=310.37$ and DF2: $r^2=0.9766$, $F=104.48$).

Fig. 3. Discriminant function analysis of the obtained data by electronic nose for various kinds of soy sauce that produced at Sunchang (DF1: $r^2=0.9993$, $F=3938.50$ and DF2: $r^2=0.9914$, $F=325.13$)

Fig. 4. Discriminant function analysis of the obtained data by electronic nose for various kinds of soy sauce that were produced at Sunchang and commercial products (unknown-1 and unknown-2) (DF1: $r^2=0.9994$, $F=4293.10$ and DF2: $r^2=0.9927$, $F=322.51$)

Table 2. Ethanol concentration determined by addition method and electronic nose (e-nose) analysis

Added ethanol concentration (%)	Ethanol concentration obtained by e-nose (%)	Yield (%)
0.25	0.375	150.168
0.50	0.497	99.466
1.00	0.966	96.596
1.50	1.503	100.179

analysis, the samples containing high ethanol concentration were located in the left side of quadrants. However, Groups L and M were not clearly discriminated (Table 2). In this sense, each manufacturing company needs to secure the calibration curve between the ethanol concentration of soy sauce and the DF1 score to obtain halal certification.

Therefore, commercial soy sauces prepared from defatted soybean and wheat might be problematic in terms of obtaining halal certification because these soy sauces have added spirits to promote flavor and extend shelf life. And active enzyme activity during fermentation could make higher alcohol concentration rather than conventional soy sauce (19).

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