SHIMADZU
Excellence in Science

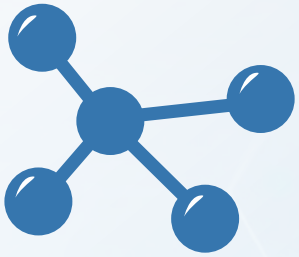
METABOLOMICS:

Applications for Food Safety and Quality Control

Metabolomics is an array of techniques used to comprehensively detect and analyze various metabolites formed in vivo during biological activity.

In the food industry, metabolomics is used to qualitatively and quantitatively analyze biological changes in samples. This technology plays a key role in improving food quality and safety, enhancing taste and developing functional foods designed to help promote health and reduce the risk of disease.

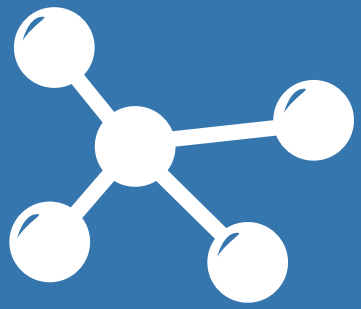
Shimadzu supports quantitative food metabolomics by providing solutions combining mass spectrometers, metabolite databases and data analysis software. This eBook shares ***3 examples of metabolic analysis*** using these high-precision instruments and advanced technology.



Aroma compounds

Geographical origin of agricultural food products

Deterioration characteristics of alcohol



ANALYSIS OF AROMA COMPOUNDS AND METABOLITES IN FOOD

One of the most effective ways to evaluate food quality is to analyze all of the various compounds present in the sample and use multivariate analysis to find trends in these compounds. For example, researchers can measure all aroma compounds and metabolites present in food. Then, they can use these results as an indicator of qualities such as flavor, functionality and deterioration.

Using a Shimadzu GCMS, we measured aroma compounds and metabolites present in sake and investigated which of these compounds could be used to distinguish between different sake types.

Three commercially available sakes were analyzed for aroma compounds and metabolites:

- Normal quality sake (futsu-shu)
- Sake made with rice and malted rice (junmai-shu)
- Sake made with the rice polished to at least 50% (daiginjo-shu)

The main compounds found in these sake samples were then identified and analyzed further. We were able to clearly separate a pattern of detected compounds in these three types of sake. Combining this method of quality control with conventional methods such as sensory evaluation will allow for the collection of more precise and revealing quality control data.

Analysis
of Aroma Compounds

We added ultrapure water and 1 mg/mL of an aqueous solution of 3-octanol to prepare the three sake samples that contained 10% ethanol and 0.5 mg/L of 3-octanol. We then added 1 mL of each prepared sample to a headspace sampler vial followed by 0.5 g of sodium chloride. The analytical conditions are listed in Table 1.

Table 1 – Analytical Conditions for Aroma Compound Analysis

Headspace sampler	HS-20
Triple quadrupole gas chromatograph mass spectrometer	GCMS
HS	
Mode	Trap
Trap tube	Tenax GR
Number of multi-injections	5
Oven temperature	70 °C
Sample line temperature	150 °C
Transfer line temperature	150 °C
Vial pressurization gas pressure	100 kPa
Vial warming time	10 min
Vial pressurization time	2 min
Pressurization equalization time	0.1 min
Loading time	1 min
Loading equalization time	0.1 min
Injection time	2 min
Needle flush time	5 min
Sample charged volume	1 mL
GC	
Column	HP-INNOWax (60 m × 0.25 mm I.D., 0.25 µm)
Carrier gas	He
Control mode	Linear velocity (25.5 cm/sec)
Injection method	Split
Split ratio	3
Oven temperature	From 40 °C (5 min) by (3 °C/min) to 240 °C (15 min)
MS (EI Method)	
Ion source temperature	200 °C
Interface temperature	200 °C
Tuning mode	Standard
Measurement mode	Scan (mlz 35 to 350)
Event time	0.3 sec

Results

Samples of the three sake types were labeled as futsu-shu, junmai-shu, and daiginjo-shu. Using the analysis results, we performed peak identification based on the NIST 14 library and quantitative ions, reference ions and retention indices. The numbers of compounds identified are shown in Table 2. The 86 compounds detected by this analysis are also listed in Table 3.

Principal component analysis (PCA) was performed for the 76 compounds detected in all samples. A score plot of this analysis is shown in Fig. 1. The three different sake types are clearly separated on the score plot. A loading plot of this analysis is shown in Fig. 2. From these results, we identified compounds characteristic to each sample.

The results suggest that performing a complete analysis of aroma compounds and subsequent multivariate analysis of identified compounds may be useful for food quality evaluation.

Table 2 – Numbers of Compounds Detected by Aroma Compound Analysis

	Futsu-shu	Junmai-shu	Daiginjo-shu
Detected compounds	78	76	86

Figure 1 – Score Plot of Aroma Compound Analysis

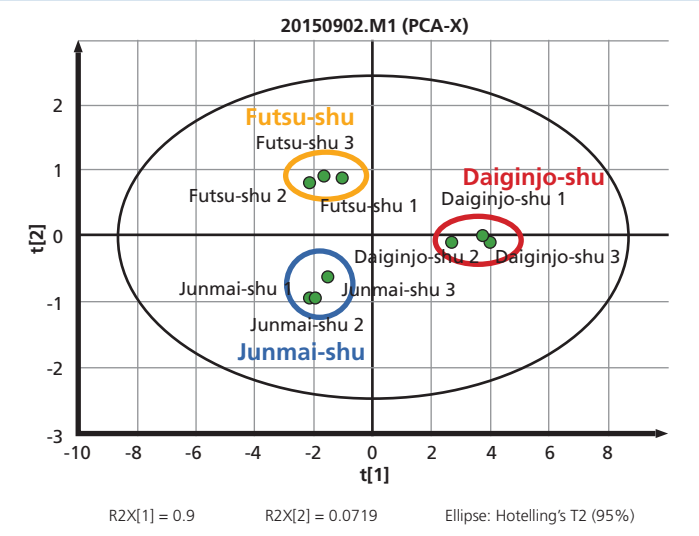


Figure 2 – Loading Plot of Aroma Compound Analysis

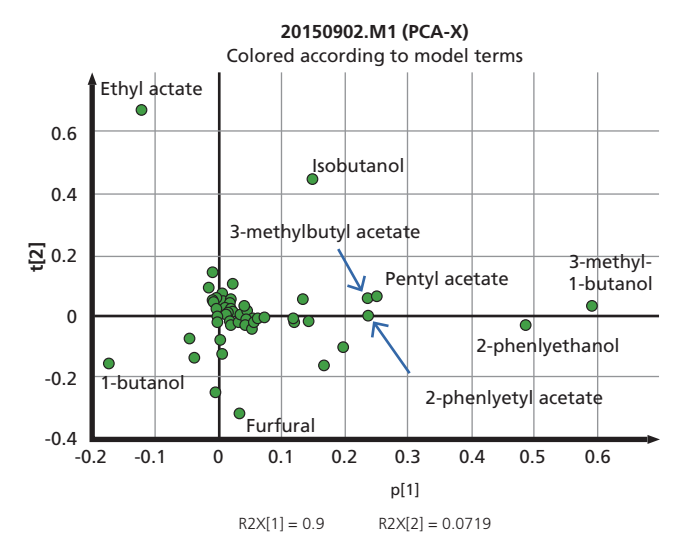


Table 3 – List of Compounds Detected by Aroma Compound Analysis (86 Compounds)

ethyl acetate	3-methylbutyl propanoate	2-ethyl-1-hexanol	1-decanol
3-methylbutanal	4-pentenyl acetate	decanal	β-citronellol
2, 4, 5-trimethyl-1,3-dioxolane	3-methyl-1-butanol	2-nonanol	diethyl pentanedioate
ethyl propanoate	ethyl hexanoate	ethyl 3-hydroxybutanoate	ethyl phenylacetate
ethyl 2-methylpropanoate	3-octanone	benzaldehyde	2-phenylethyl acetate
propyl acetate	styrene	ethyl 2-hydroxyhexanoate	2-(2-butoxyethoxy ethyl acetate
2, 3-butanedione	hexyl acetate	propanoic acid	hexanoic acid
isobutyl acetate	2-octanone	1-octanol	benzyl alcohol
ethyl butanoate	octanal	3-methylbutyl methoxyacetate	diethyl hexanedioate
1-propanol	acetoin	ethyl 3-methylthiopropanote	butylated hydroxytoluene
ethyl 2-methylbutanoate	2-heptanol	ethyl decanoate	2-phenylethanol
ethyl 3-methylbutanoate	3-methyl-1-pentanol	butyrolactone	heptanoic acid
butyl acetate	ethyl heptanoate	1-nonanol	phenol
DMDS	ethyl lactate	acetophenone	dehydromevalonic lactone
1-(1-ethoxyethoxy) pentane	1-hexanol	phenylacetaldehyde	octanoic acid
isobutanol	3-ethoxy-1-propanol	furanmethanol	ethyl hexadecanoate
3-methylbutyl acetate	2-nonanone	ethyl benzoate	decanoic acid
ethyl pentanoate	ethyl octanoate	diethyl succinate	2-phenylethyl octanoate
1-butanol	1-heptanol	(Z)-3-nonen-1-ol	benzoic acid
ethyl 2-butenoate	3-methylbutyl hexanoate	3-methylthio-1-propanol	dodecanoic acid
pentyl acetate	acetic acid	pentanoic acid	
2-heptanone	furfural	naphthalene	

Analysis
of Metabolites Present in Foods

Next, metabolites present in foods were extracted from each sake sample, derivatized and analyzed by GCMS. We took 20 µL of each sample, added 60 µL of an aqueous solution of ribitol (0.2 mg/mL) as an internal standard solution and dried this mixture thoroughly in a centrifugal concentration device. We added 100 µL of a methoxyamine hydrochloride/pyridine solution (20 mg/mL) to the dried residue. This mixture was shaken at 30 °C for 90 minutes. Subsequently, 50 µL of N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was added, and the mixture was shaken at 37 °C for 30 minutes. This sample was then added to a GCMS vial and used for analysis. The analytical conditions are listed in Table 4.

Table 4 – Analytical Conditions for Analysis of Metabolites Present in Foods

Triple quadrupole gas chromatograph mass spectrometer	GCMS
Option software	Smart Metabolites Database™
GC	
Column	BPX5 (30 m × 0.25 mm I.D., 0.25 µm)
Carrier gas	He
Control mode	Linear velocity (39.0 cm/sec)
Injection method	Split
Split ratio	30
Oven temperature	From 60 °C (2 min) by (15 °C/min) to 330 °C (3 min)
MS (EI method)	
Ion source temperature	200 °C
Interface temperature	280 °C
Tuning mode	Standard
Measurement mode	MRM
Loop time	0.25 sec

Results

Using the analysis results, we performed peak identification for compounds registered in the Smart Metabolites Database based on their quantitative ions, reference ions and retention indices. Table 5 shows the numbers of compounds identified. The 149 compounds detected by this analysis are also listed in Table 6.

Table 5 – Numbers of Compounds Detected by Aroma Compound Analysis

	Futsu-shu	Junmai-shu	Daiginjo-shu
Detected compounds	147	140	149

Table 6 – List of Compounds Detected by Analysis of Metabolites Present in Foods (149 Compound)

2-Aminobutyric acid	Aspartic acid	Histidine	Ornithine
2-Aminoethanol	Batyl alcohol	Homocysteine	Palmittic acid
2-Aminopielic acid	Benzoic acid	Homoserine	Pantothenic acid
2-Deoxy-glucose	Cadaverine	Hydroxylamine	Phenylacetic acid
2-Hydroxybutyric acid	Caproic acid	Hypotaurine	Phenylalanie
2-Hydroxyglutaric acid	Citramalic acid	Hypoxanthine	Phenylpyruvic acid
2-Hydroxlsocaproic acid	Citric acid	Indol-3-acetic acid	Phosphoric acid
2-Hydroxylsovaleric acid	Cystamine	Isocitric acid	Proline
2-Isopropylmalic acid	Cystathionine	Isoleucine	Psicose-meto
2-Ketoglutaric acid	Cysteine	Lacitc acid	Putrescine
3-Aminoglutaric acid	Cystine	Lactitol	Pyridoxamine-4TMS
3-Aminopropanoic acid	Cytidine	Lactose	Pyruvic acid
3-Hydroxy-3-methylglutaric acid	Cytosine	Lauric acid	Ribitol
3-Hydroxybutyric acid	Decanoic acid	Leucine	Ribose
3-Hydroxyglutaric acid	Dihydroxyacetibe phosphate	Lysine	Saccharopine
3-Hydroxylsobutyric acid	Dopamine	Lyxose	Serine
3-Hydroxypropionic acid	Elcosapentaenoic acid	Maleic acid	Stearic acid
3-Methoxy-4-hydroxybenzoic acid	Elaidic acid	Malic acid	Succinic acid
3-Phenyllactic acid	Fructose	Maltitol	Tagatose
4-Aminobutyric acid	Furnaric acid	Maltose	Threitol
4-Hydroxybenzoic acid	Galactose	Mannito	Threonic acid
4-Hydroxyphenylacetic acid	Galacturonic acid	Mannose 6-phosphate	Threonine

Continued on next page

4-Hydroxyproline	Glucose	Mannose	Thymine
5-Aminolevullnic acid	Glucuronic acid	Margaric acid	Trehalose
5-Methoxytryptamine	Glutamine	Methlonine	Tyramine
5-Methylthicadenosine	Glutaric acid	Methylsuccinic acid	Tyrosine
5-Oxoproline	Glyceric acid	Mevalonic lactone	Uracil
Acetylglycine	Glycerol 2-phosphate	Myristic acid	Urea
Aconitic acid	Glycerol 3-phosphate	N6-acetyllysine	Uridine
Adenine	Glycero	N-acetylmannosamin	Valine
Alanine	Glycine	Nicotinic acid	Xanthine
Allose	Glycolic acid	Nonanoic acid	Xylito
Arabinose	Glycyl=Glycine	Norvaline	Xylose
Arabitol	Glyoxylic acid	Octanoic acid	Xylulose
Arginine	Guanine	Octopamine-4TMS	
Ascorbic acid	Hexanoylglycine	Oleic acid	
Asparagine	Histamine	O-Phosphoethanolamine	

The next step was to perform principal component analysis (PCA) for the 138 compounds detected in all the sake samples. A score plot of this analysis is shown in Fig. 3. The three different sake types are clearly separated on the score plot. A loading plot of this analysis is shown in Fig. 4. Compounds characteristic to each sample were identified from these results.

Figure 3 – Score Plot of the Analysis of Metabolites Present in Foods

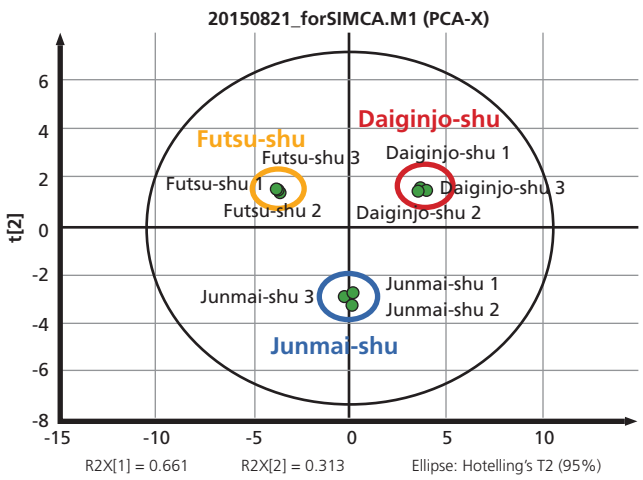
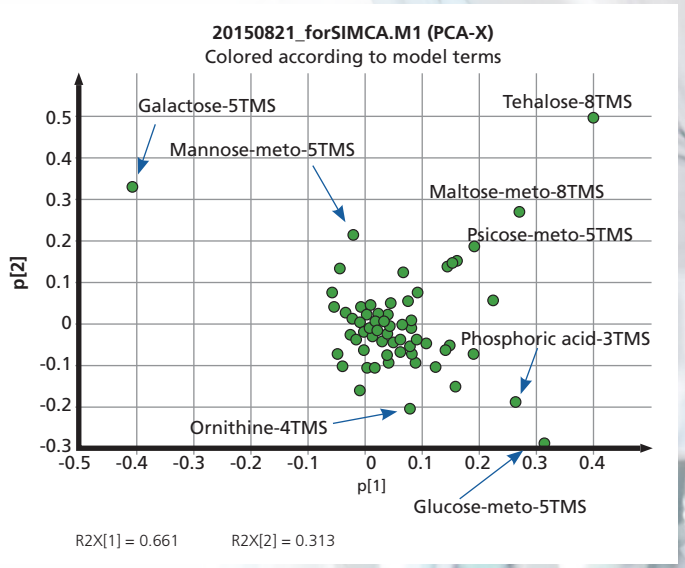


Figure 4 – Loading Plot of the Analysis of Metabolites Present in Foods



DETERMINATION OF GEOGRAPHICAL ORIGIN OF AGRICULTURAL PRODUCTS BY SMART METABOLITES DATABASE™

Accurate geographical labeling of agricultural products is important to help food producers and consumers verify their authenticity, quality and safety. However, the determination of geographical origin can be difficult based on genetic information and other bioinformation possessed innately by agricultural products. Instead, researchers use information about elements introduced into agricultural products from external sources.

Another technique to determine geographical origin is to analyze metabolites that are produced internally, such as amino acids, organic acids, fatty acids and sugars. Many common agricultural products contain these metabolites.

The concentration of these metabolites is thought to change dynamically over time as determined by the surrounding environment. This means it might be possible to discover patterns in the abundance ratio of the metabolites using comprehensive measurement. Analysts can then use those patterns to determine the geographical origin of products produced in different regions.

Using the Shimadzu Smart Metabolites Database, we measured the metabolites in 106 samples of domestic Japanese asparagus and asparagus produced in other countries. The database enables simultaneous measurement of the compositions of 337 hydrophilic metabolites. Then we prepared a model for the determination of domestic or foreign origin. The results of this experiment confirmed that determination with an accuracy of approximately 90% is possible.

Extraction and Derivatization
of Metabolites from Asparagus

For this experiment, we prepared 58 domestic Japanese samples and 48 samples of foreign origin. The cut asparagus was reduced and freeze-dried, and the samples were powdered. The powders were then extracted and derivatized by a pretreatment protocol based on the Bligh & Dyer method for metabolites analysis. We used the Ribitol internal standard.

Measurement
of Derivatized Hydrophilic Metabolites

After derivatization, the sample solutions were measured by GC-MS/MS. The analytical conditions conformed to those in Shimadzu's Smart Metabolites Database. Table 1 shows the detailed conditions.

Table 1 – Measurement Conditions

GCMS	GCMS-TQ™ 8040 NX
Column	BPX5 (30 m × 0.25 mm, 0.25 µm)
GC	
Injection mode	Split (30:1)
Vaporizing chamber temp.	250 °C
Column oven temp.	60 °C (2 min) by (15 °C/min) by 330 °C (3 min)
Control mode	Linear velocity (39.0 cm/sec)
Purge flow rate	5.0 mL/min
MS	
Measurement mode	MRM
Ion source temp.	200 °C
Interface temp.	280 °C
Event time	0.25 s

Detection
of Peaks

Peak detection work was done using Shimadzu's LabSolutions Insight™ analysis software, version 3.5. The following rules were set for peak detection.

RULE 1:
Compounds were considered to be "detected" if they met these conditions:

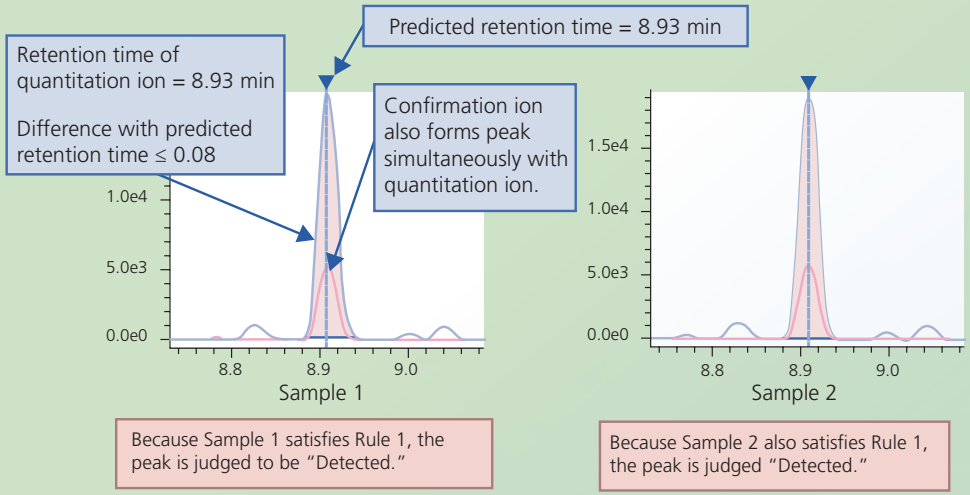
- The quantitation transition and the confirmation transition formed peaks simultaneously within the retention time of ±0.08 min predicted from the retention index.
- The height of the quantitation ion was 1,000 or more (Fig. 1).

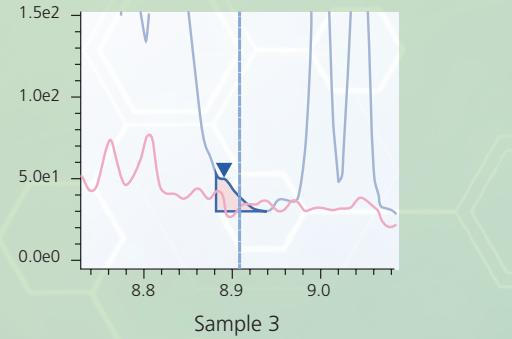
RULE 2:
In cases where the data did not satisfy Rule 1, compounds were judged to be "detected" if:

- At least half of the data was considered to be "detected."
- They resembled a peak close to the predicted retention time.

This was done to avoid cases where "undetected" data became a missing value or zero (Fig. 1).

Figure 1 – Example of Rules 1 and 2





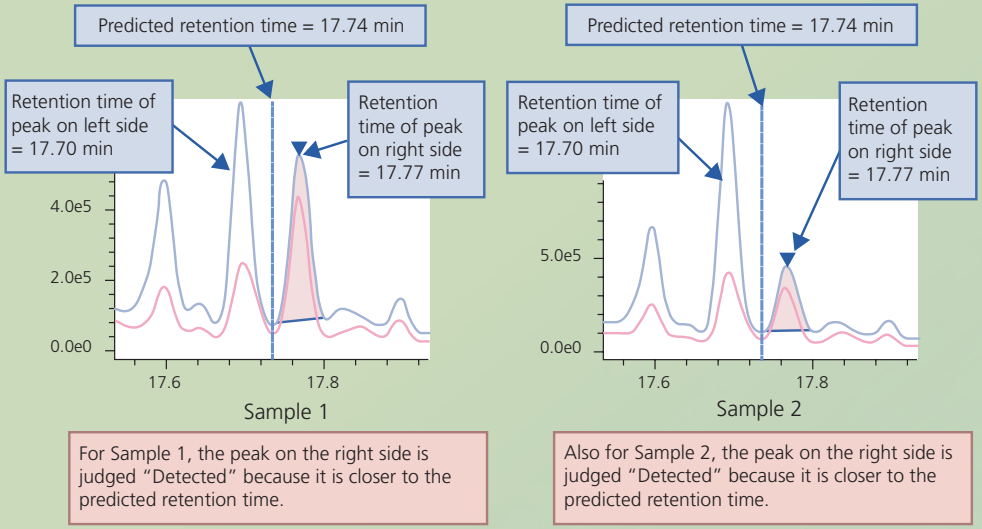
RULE 3:

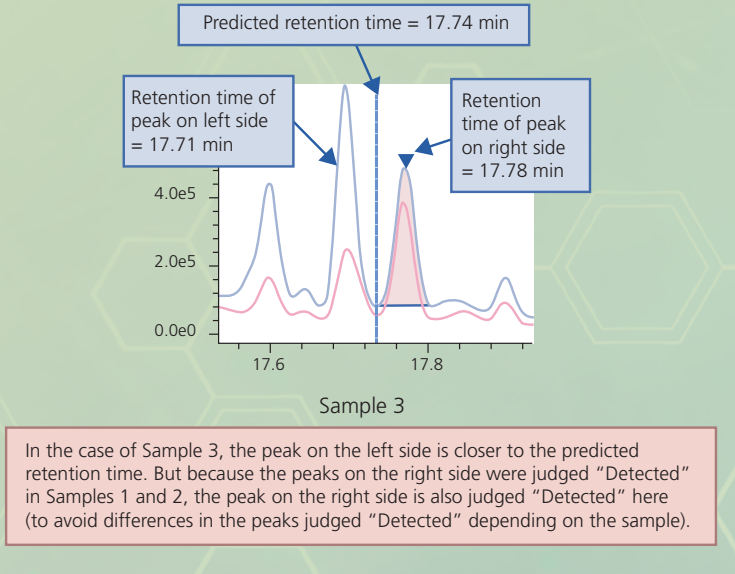
When there were two or more peaks "detected" near the predicted retention time, the closer peak was considered "detected."

However, if this differed depending on the sample, the peak that was "detected" in at least half of the samples was considered "detected." This was done to avoid cases where peaks that differed depending on the sample were considered "detected" (Fig. 2).

LabSolutions Insight supports this type of peak detection work in a short time, even with a large number of data. In this experiment, many peaks with stable shapes were obtained by GC-MS/MS measurement, enabling the detection of a large number of peaks (total of 217 components).

Figure 2 – Example of Rule 3





Creation of Model

for Determination of Geographical Origin

After peak detection work, the heights of all peaks were output as a data matrix. We omitted the treatment of missing values because no data rows containing missing values existed as a result of peak detection based on the rules. For some samples, the peak of the internal standard was no more than the standard value. These samples were regarded as anomalies caused by the derivatization process and were deleted from the data matrix.

For all other samples, the values obtained by dividing the respective peak area values by the peak area value of the internal solution were normalized by the z-score and used as data. After randomly dividing all samples into a training set and a test set, we checked the boxplots and distributions of outliers of the 217 components for which peaks were detected. We then selected 13 components that appeared to be effective for determination.

Using those 13 components, we created a model for the determination of geographical origin using a Random Forest algorithm. We also randomly replaced the training set and test set in the samples 50 times. An average model accuracy of 91.7% was calculated by applying each of the models generated in this process. Fig. 3 shows a representative confusion matrix and ROC curve obtained by this model.

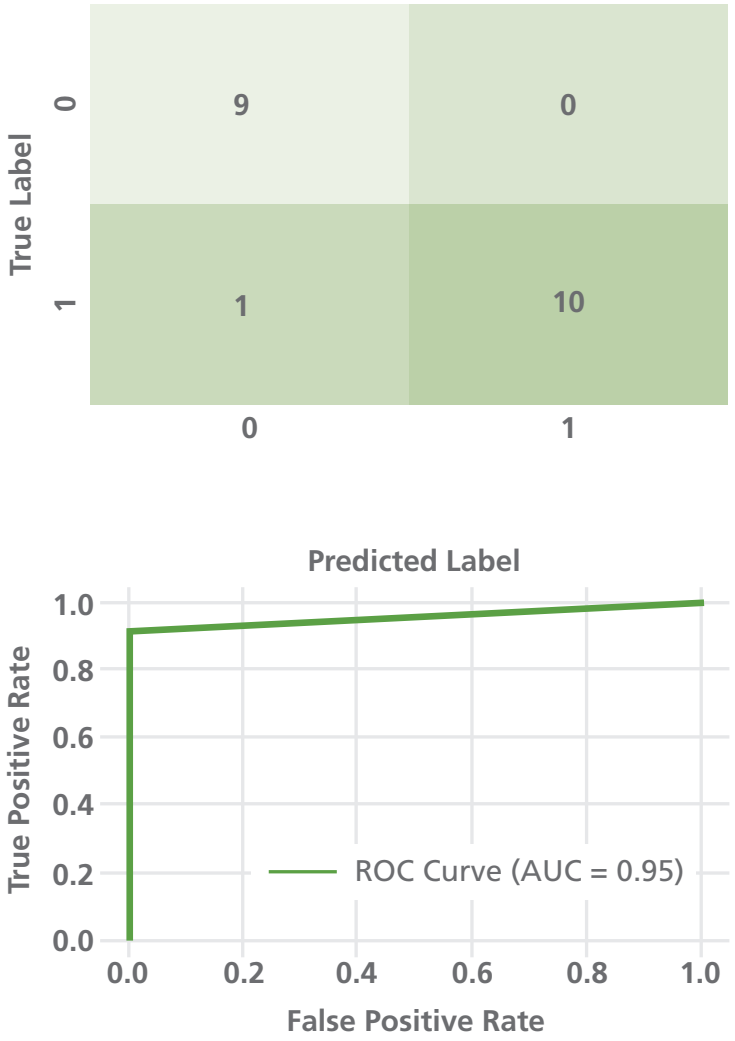


Figure 3 – Confusion Matrix (Top) and ROC Curve (Bottom) Obtained by Model for Determination of Geographical Origin of Asparagus

FOOD METABOLOMICS ANALYSIS OF DETERIORATION CHARACTERISTICS OF ALCOHOLIC DRINKS USING LC-MS/MS

The conventional method to assess food flavor, aroma, deliciousness and grade is to rely on the sensory analysis of human assessors. Food metabolomics is used to evaluate or predict the quality of food and explore functional ingredients using a more scientific method. This process comprehensively analyzes the metabolites in food and compares the findings against those from human sensory analysis.

We used an analysis method based on metabolomics to determine the deterioration characteristics of foods.

The samples, commercially available Japanese rice wine (sake) and white wine, were stored under adverse conditions and then separated by high performance liquid chromatography mass spectrometry (LC-MS/MS). Then we used multivariate analysis to comprehensively investigate the changes in hydrophilic metabolites, including amino acids, organic acids, nucleosides and nucleotides.

Samples and Deterioration

Experiment

Alcoholic beverages are distributed domestically and internationally, and large volumes are imported and exported. The ability to transport these beverages without a negative impact on quality is important to maintain the value of the products.

Our experiment was designed to reproduce the conditions under which the quality of the products might be adversely affected during transportation, including exposure to the sun, high temperatures and vibration.

Every sample stored under each of the specified conditions was separated by centrifugation at 12,000 rpm for 5 minutes. In addition, the supernatant was diluted 100-fold with ultrapure water, so it could be analyzed by LC-MS/MS.

The characteristics of the samples are shown in Table 1. To perform accelerated deterioration testing, the samples were stored under each of the test conditions shown in Table 2.

Table 1 – Characteristics of Test Samples

Samples	
Sake No. 1	Junmai-daiginjoshu, rice-polishing ratio = 50%, Alcohol by volume (ABV) = 15%
Sake No. 2	Ginjoshu, brewer's alcohol added, rice-polishing ratio = 50%, ABV = 15%
White wine	Produced in Australia, antioxidant (sulfite) added, ABV = 13%

Table 2 – Experimental Conditions for Accelerated Deterioration Testing

Storage Condition	
A	Stored in a refrigerator protected from light for 2 weeks
B	Stored at room temperature exposed to light for 2 weeks
C	Stored in a refrigerator protected from light for 2 weeks, followed by heating to 50 °C while protected from light for 24 hours
D	Stored in a refrigerator protected from light for 2 weeks, followed by shaking at room temperature while protected from light for 24 hours

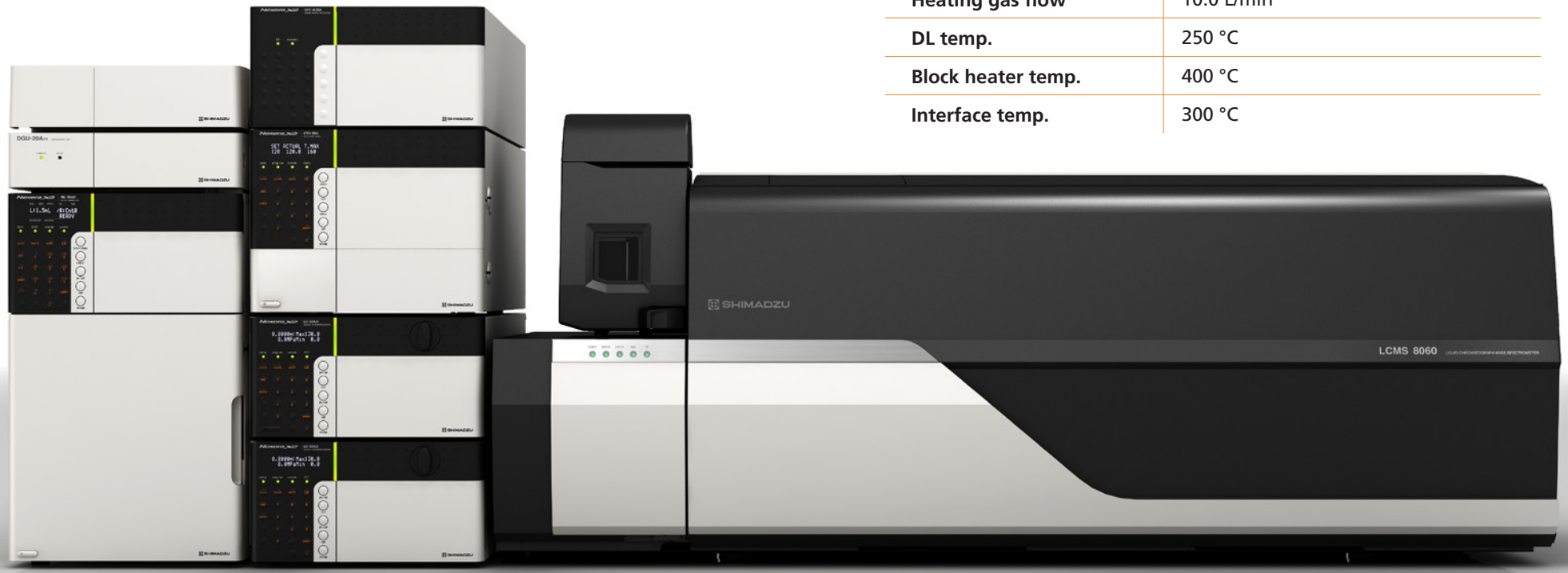
Analysis

Conditions

We conducted the analysis with Shimadzu's LCMS-8060 using the ion-pairing free LC-MS/MS method of the LC-MS/MS Method Package for Primary Metabolites Version 2. The analysis method included in the package enables the simultaneous analysis of the 97 hydrophilic metabolites, which are known to be important in metabolome analyses. The HPLC and MS analysis conditions are shown in Table 3.

Table 3 – Analysis Conditions

[HPLC conditions] Nexera™ X2)	
Column	Reversed-phase column
Mobile phases	A) 0.1% Formic acid in water B) 0.1% Formic acid in acetonitrile
Mode	Gradient elution
Flow rate	0.25 mL/min
Injection volume	3 µL
[MS conditions] (LCMS-8060)	
Ionization	ESI (Positive and negative mode)
Mode	MRM
Nebulizing gas flow	3.0 L/min
Drying gas flow	10.0 L/min
Heating gas flow	10.0 L/min
DL temp.	250 °C
Block heater temp.	400 °C
Interface temp.	300 °C



Metabolome

Analysis

Each sample was measured by LC-MS/MS, and then principal component analysis (PCA) and one-way analysis of variance (one-way ANOVA) were conducted using the areas of each component with Traverse MS software.

When PCA was performed, no apparent difference was observed between the samples stored under different conditions for any of the types of alcoholic drinks tested. In contrast, a detailed examination of ANOVA results revealed that some of the components increased or decreased according to the type of alcoholic drink and/or storage conditions.

Some of the components measured in the samples of sake No. 1 stored under condition B were significantly different from those of the samples stored under the other conditions. A similar trend was observed for sake No. 2 and the white wine. This shows that some of the experimental conditions, such as heating to 50 °C or shaking for about 24 hours, were not sufficient to have a significant impact on hydrophilic compounds, including amino acids and organic acids.

It is difficult to draw definitive conclusions based solely on the findings of this study. However, the results suggest that even if the products are accidentally exposed to conditions such as heating and shaking for a short period, this is unlikely to have a significant impact on the quality of the product.

In the samples of sake No. 1, cysteine, methionine sulfoxide and uric acid showed significant differences ($p < 0.05$) between the samples stored under condition B and those stored under the other conditions. Additionally, there were several other components that showed a different trend only in the white wine samples. The results of comparing these components in each alcoholic drink tested are shown in Figs. 1 to 4.

Methionine Sulfoxide

The results of statistical analysis for the methionine sulfoxide contained in each alcoholic drink tested are shown in Fig. 1. The level of this component in the samples stored under condition B was markedly higher than those stored under the other conditions, regardless of the type of alcoholic drink.

Methionine is known to be an amino acid residue that is more susceptible to aging-associated oxidation. It is considered to be a cause of the increased in vivo oxidative protein damage and is promptly oxidized to methionine sulfoxide under intracellular oxidative stress conditions. The results of this study suggest the possibility of using methionine sulfoxide as a marker of oxidation of the components of alcoholic drinks.

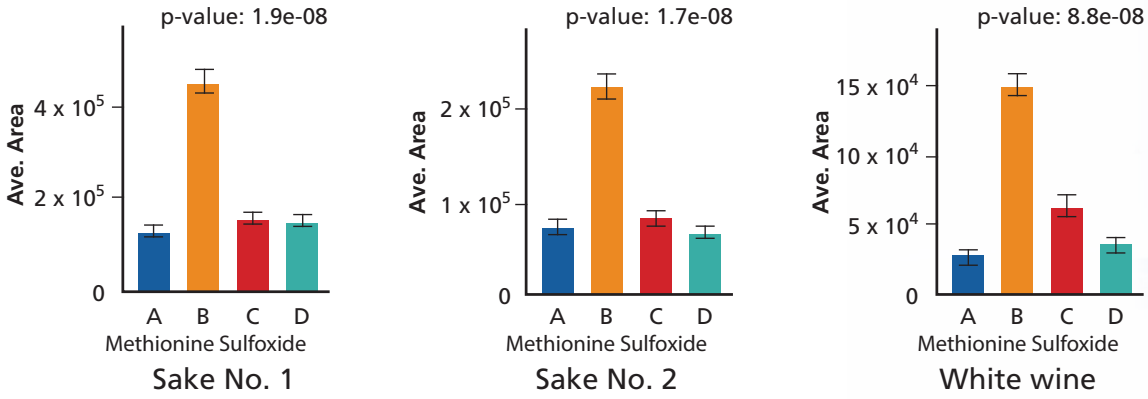
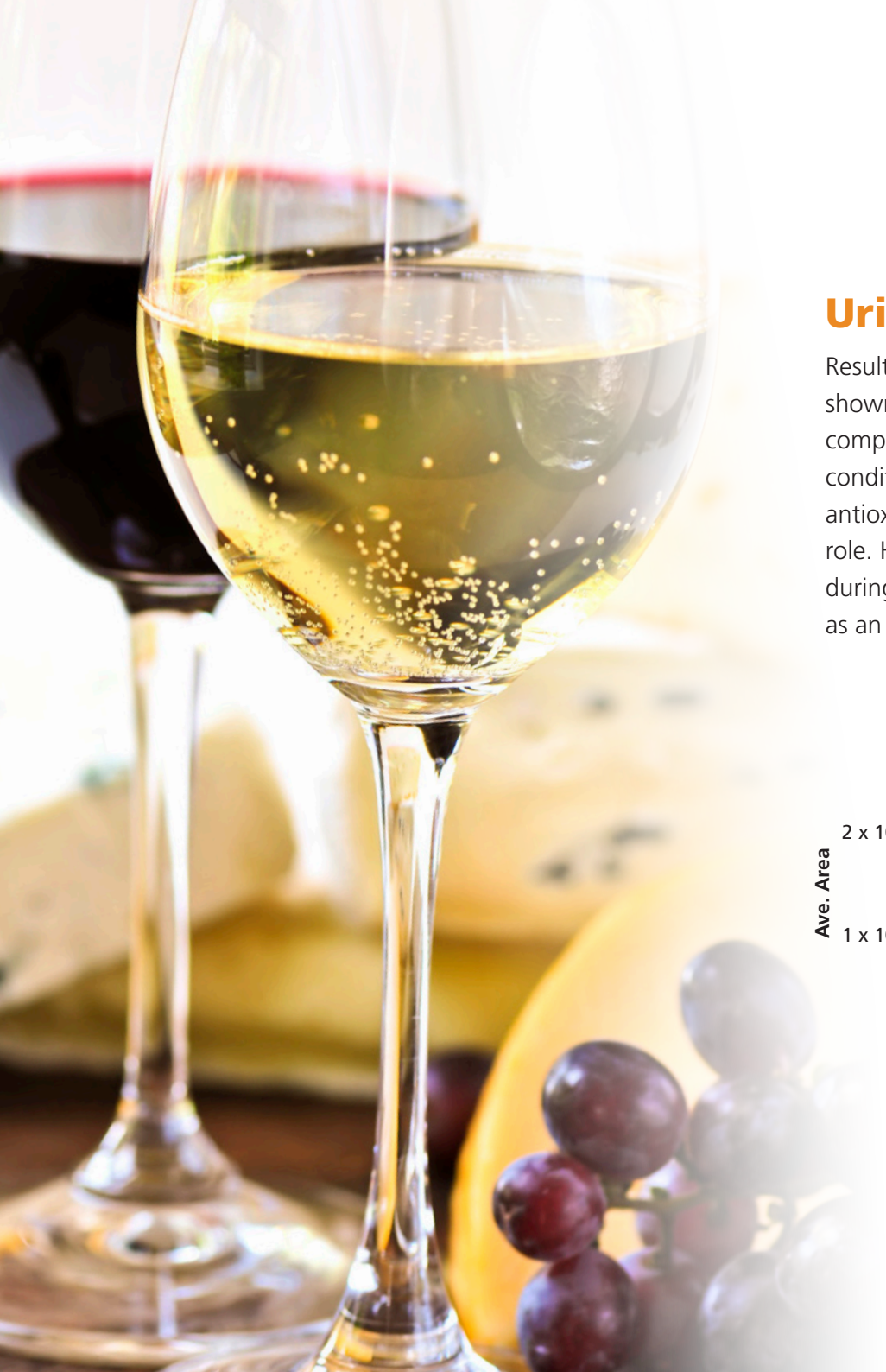


Figure 1 – Results of ANOVA for Methionine Sulfoxide



Uric Acid

Results of statistical analysis for uric acid contained in each alcoholic drink tested are shown in Fig. 2. The analysis revealed that only in the sake samples was the level of this component in those stored under condition B lower than those stored under the other conditions. Uric acid is highly susceptible to oxidation. That means it exerts a strong antioxidant effect comparable to that of ascorbic acid, which is a known physiological role. However, in this study, it was unlikely that the uric acid would undergo oxidation during the storage period because the white wine used in this study had sulfite added as an antioxidant.

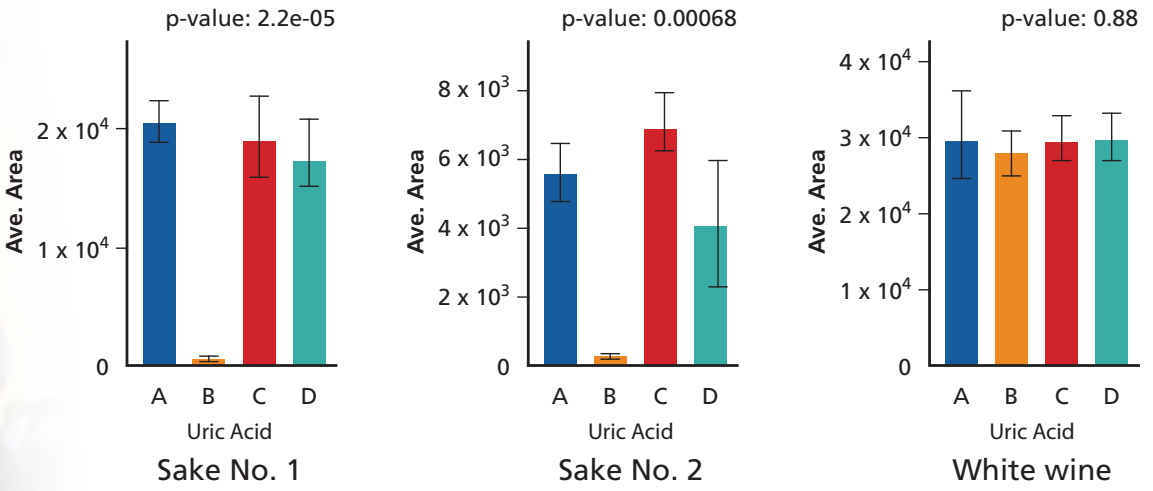


Figure 2 – Results of ANOVA for Uric Acid

Cysteine

Results of statistical analysis for cysteine contained in each alcoholic drink tested are shown in Fig. 3. The analysis revealed that only in sake No. 1 was the level of this component in the samples stored under condition B significantly lower than those stored under the other conditions.

Besides methionine, cysteine is known as a precursor of dimethyl trisulfide (DMTS), a major malodorous component of deteriorated sake. We associated the lowered level of cysteine with an increase in DMTS production. That means the addition of brewer's alcohol (a substantial difference between the samples of sake No. 1 and No. 2) may be a factor that affects the susceptibility of sake to deterioration.

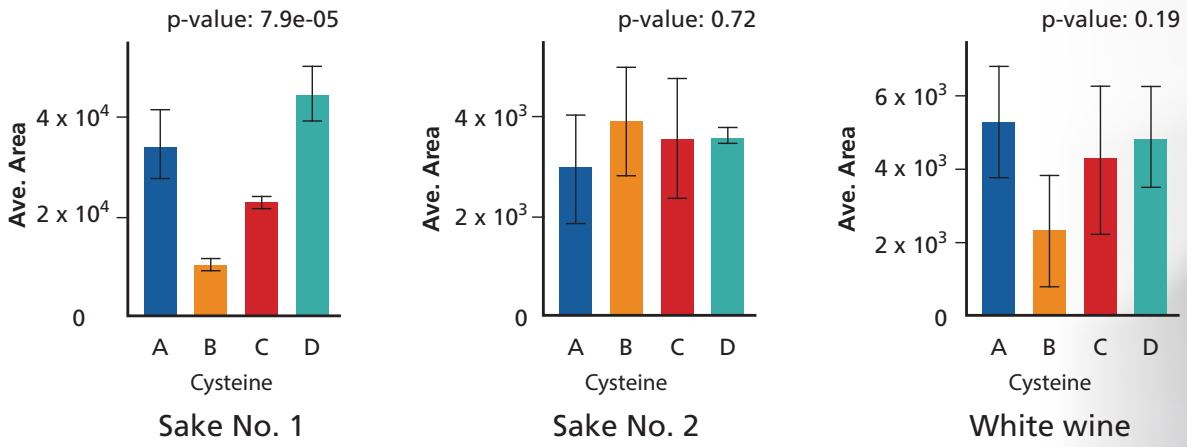


Figure 3 – Results of ANOVA for Cysteine

Tryptophan and Kynurenine

Results of statistical analysis for tryptophan and kynurenine in the white wine samples are shown in Fig. 4. The analysis revealed that the levels of tryptophan and kynurenine in the samples stored under condition B were lower and higher, respectively, than those stored under the other conditions.

A similar trend for tryptophan and kynurenine was observed in the samples stored under condition C, although the degree was small. Tryptophan is known to be metabolized to kynurenine through one of its known metabolic pathways, the kynurenine pathway (Fig. 5). Therefore, the changes in these components appear to correspond to the changes predicted from their relationship to this pathway.

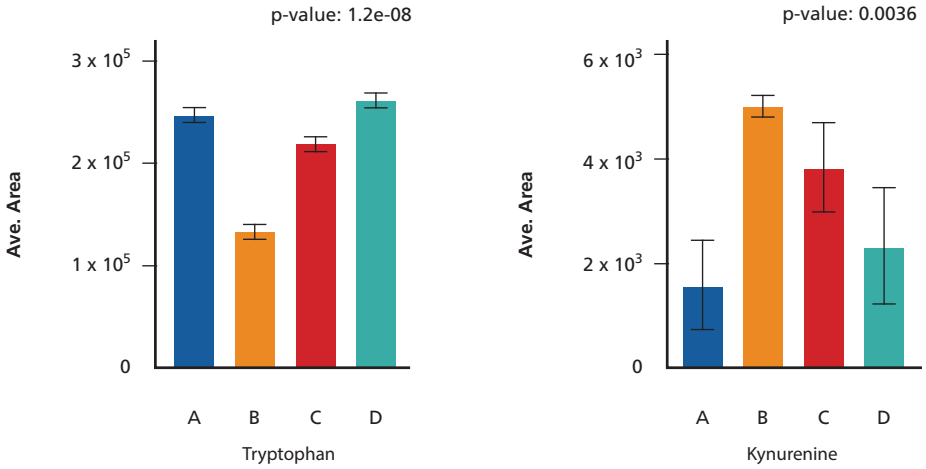
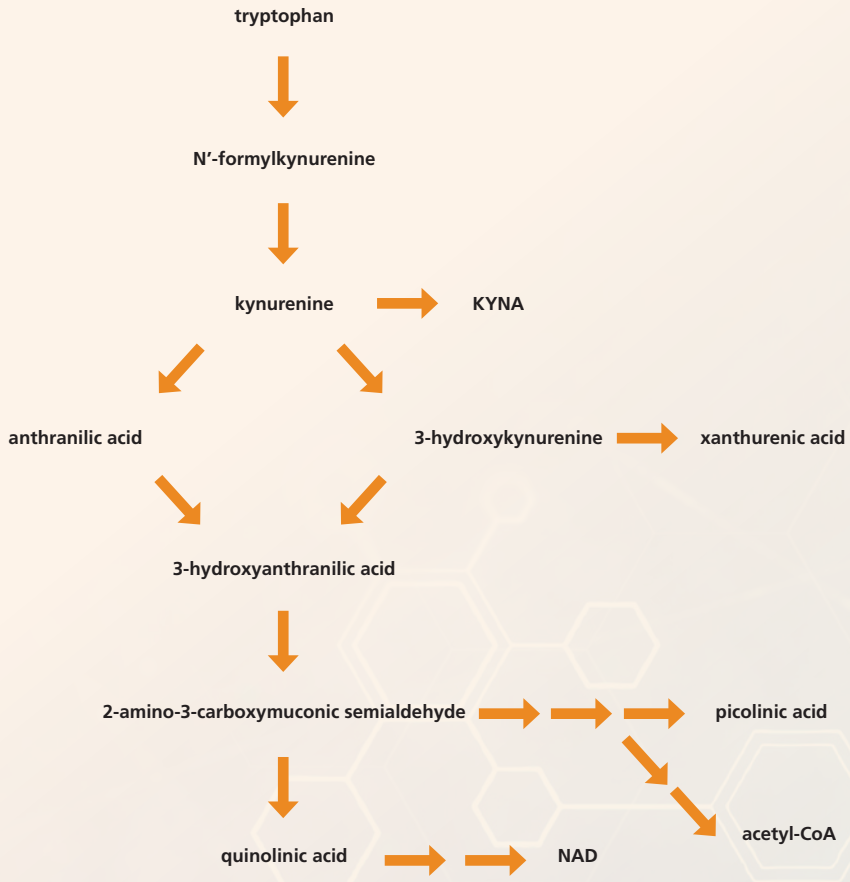


Figure 4 – Results of ANOVA for Tryptophan and Kynurenine in the White Wine Samples

Figure 5 – Kynurenine Pathway



Conclusion

Results from these three experiments demonstrate the effectiveness of metabolomic analysis of food and beverage samples for quality control research. Shimadzu's innovative instruments and technologies provide the most reliable and reproducible data, highest sensitivity and increased productivity to meet the demands for consistent and uncompromising food and beverage analysis.

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7102 Riverwood Drive, Columbia, MD 21046, USA
Phone: 800.477.1227 / 410.381.1227
www.ssi.shimadzu.com