

## Effect of dry-ageing with *Mucor flavus* on beef taste and aroma

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### Abstract

Okinawa Industrial Technology Centre succeeded in isolating the mould strain, *Mucor flavus*, creating savoury odours during beef ageing. Several companies already produce dry-aged beef products using the isolated mould strain in Okinawa. It will be a new food content as the Okinawan brand for tourists from all over the world. After trimming, each amino acid in the meat surface was analysed by LCMS and aroma volatile compounds from the headspace were analysed by solid-phase micro extraction followed by GCMS to reveal the effect of dry-ageing with *M. flavus* on beef taste and aroma. Each amino acid in the surface of dry-ageing beef with the mould increased. It was hypothesised that there is a gradient of moisture content from the surface of the edible part to the inner side of the dry-aged beef, where amino acids are easily generated from the protein degradation. Amino acids react with sugars by heating, resulting in a savoury flavour. Besides, the peak number of volatile compounds using GCMS was more detected in dry-aged beef with mould than that in dry-aged beef without mould. There is a high possibility that *M. flavus* can produce higher polar compounds in beef meat. Several aldehydes detected only in dry-aged beef with the mould were related to savoury flavours, such as nutty and boiled meat.

## 1. Introduction

Dry-aged beef has become a popular food in western countries and Asian countries (Dashmaa *et al.*, 2016; Hanagasaki and Asato, 2018a) and could be available globally. It is originally from New York and is usually made by ageing with mould (Hanagasaki and Asato, 2018a). Steaks using this dry-aged beef are served at high prices in upmarket steakhouses and exclusive restaurants (Neil, 2012). For example, 'mellow and intense' and 'earthy and nutty' are the types of phrases commonly used to describe the flavour characteristics of dry-aged beef (Savell, 2008), and these result from the type of mould used. Okinawa Industrial Technology Centre succeeded in isolating the mould strain, *Mucor flavus*, creating savoury odours (Hanagasaki and Asato, 2018a). Several companies produce dry-aged beef products in Okinawa, Japan, using the isolated mould strain. Okinawa had over 10 million tourists in 2018, and there is an urgent need to develop new food content. The mould strain has distinctive characteristics (Hanagasaki and Asato, 2018a) and then it is possible to create a new Okinawan brand using the mould, attracting tourists. By the way, regarding free amino acids inside meats, we did not draw definitive conclusions that the mould ageing

process can concentrate amino acids, although mould itself produced many kinds of amino acids (Hanagasaki and Asato, 2018a). However, the surface meat after ageing is expected to have more amino acids because the moisture content is relatively low compared to the interior meat. Therefore, amino acids in the meat surface should be analysed to confirm the increase. Besides, the mould strain can add a unique savoury flavour, not originally from meat, to dry-aged beef (Hanagasaki and Asato, 2018a), but the details of aroma compounds are yet unclear. The present study analysed volatile aroma compounds related to savoury flavour from dry-aged beef with the mould using gas chromatography-mass spectrometry with solid-phase microextraction.

## 2. Materials and methods

### 2.1 Beef material

The meat used for the experiments was beef sirloins imported from New Zealand. The cow beef was a crossbreed between Angus and Hereford, rose in pastures.

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## 2.2 Ageing environment

The ageing environment was established in a refrigerator (Showa Denko K.K., Japan) in the Okinawa Industrial Technology Centre. Accordingly, dry boxes were placed in the refrigerator. Further, three pieces of meat were placed in the boxes for 3 weeks under-maintained conditions of approximately 80% relative humidity for dry-aged beef with *M. flavus* or not. For dry-aged beef with the mould, the mould strain was cultured on potato dextrose agar (PDA) plates (Merck Ltd., Japan) for a week and then allowed to contact each piece of meat.

## 2.3 Quantitative analysis of amino acids

Samples from the surface of the edible part of the ageing beef were collected after being trimmed. Three samples were obtained in one meat block. These were cut and homogenised. Extract solutions were obtained from these homogenised samples after the protein was removed with acetonitrile and perchlorate, and the fat was removed with hexane. Sample solutions for LC/MS were prepared after filtered extract solutions. Sample solutions were injected into an Intrada Amino Acid column (3 × 100 mm, Imtakt Corp., Kyoto, Japan) at a 0.6 mL/min flow rate. The separation was performed with a two-pump gradient. Solvent A was acetonitrile/tetrahydrofuran/25 mM ammonium formate/formic acid (9/75/16/0.3, v/v/v/v). Solvent B was acetonitrile/100 mM ammonium formate (20/80, v/v). The gradient programme was as follows: 0 min, A 100%; 2.75 min, A 100%; 7.75 min, A 83%; and 7.76 min, A 0%. Analyses were monitored in the positive-ion mode using an ESI source at 350 °C and MRM. Three runs were performed for each measurement, and the average was calculated. Amino acids were sorted according to their features into four groups. Glycine, alanine, threonine, serine and proline were classified as sweet-tasting amino acids. Aspartic acid, glutamic acid, glutamine and asparagine were classified as umami-tasting amino acids. Methionine, lysine, isoleucine, leucine, phenylalanine, tyrosine, valine, histidine, arginine and cysteine were classified as bitter or flavour-tasting amino acids. Finally, carnosine, anserine, taurine, ornithine and GABA were classified as functional amino acids. The values were adjusted for the concentration of moisture content after ageing.

## 2.4 Volatile compound analysis

1.0 g of dry-aged beef samples were placed in 20 mL of glass vials sealed with a PTFE/silicone septum (GI sciences inc., Netherlands). The volatile compounds were prepared according to the modified method (Yu *et al.*, 2008) of the headspace solid-phase microextraction

(HS-SPME) using a divinylbenzene/carboxen/polydimethylsiloxane (50/30 µm thickness) SPME fibre (Supelco Co., Bellefonte, PA, USA). Volatile components were injected with split less mode onto a InertCap Pure WAX ProG 2 M column (0.25 mm × 60 m, df = 0.25 µm, GI sciences inc.) at a flow rate of 1.23 mL/min helium at 40°C (2 min) – (5.0°C/min) – 230°C (5 mins) in the GCMS-QP2010 Ultra (Shimadzu corp., Japan) system. As MS conditions, ion source and interface temperature were 230 °C and 240 °C, and the MS detector was operated in scan mode (20–600 m/z) using electron impact ionisation (70 eV.). Based on the data obtained from GCMS, the NIST11 Mass Spectrum Library (National institute of standards and technology, USA) and FFNSC 2 Flavour and Fragrance Natural & Synthetic Compounds GCMS Library (Chromaleont S.r.l., Italy) was used to identify the volatile compound in each peak with the highest similarity score of mass spectra. Identified compounds containing Si and Ni were removed for Figure 4 because they were not originally from beef. In addition, identified compounds below 85% of similarity were removed from Table 1.

## 3. Results and discussion

### 3.1 Amino acids content

After dry-ageing, *Mucor flavus* covered beef sirloin with white hyphae (Figure 1). Previously, the amino acid content of the interior meat samples did not increase stably during dry-ageing with the mould (Hanagasaki and Asato, 2018a). Regarding amino acids contained in beef meat, there are several steps of proteinogenic degradation with enzyme reaction (Hanagasaki and Asato, 2018b). The way to produce free proteinogenic amino acids assumes to be from short peptides and not directly from protein (Hanagasaki and Asato, 2018b). In fact, the sum of amino acids increased more as the temperature for ageing, meaning an acceleration of enzyme reaction depending on high temperature (Savell, 2008). The amino acid content of each beef meat varies according to which step of meat to be in, depending on how many days elapsed after slaughter before delivery. Therefore, molecular biological techniques make it difficult to elucidate the difference in the underlying mechanism of amino acid production among different ageing processes. This time, the surface after trimming was chosen as the sample to analyse amino acids. The surface was not where the hyphae of the mould extended. Amino acid contents depend on choosing the sampling point. No difference in amino acid increase in the interior meat samples of aged beef conducted under 80% and 100% relative humidity has been reported (Hanagasaki and Asato, 2018b). These sample points were over 1 cm depth from the surface after trimming, and the moisture contents were almost the same (about 74%). In this

result, both amino acid contents in dry- or wet-aged beef were similar. However, moisture contents were lower in the immediate sample inner of the trimming part (about 71%) than in the interior meat. It was hypothesised that there is a gradient of moisture content within about 1 cm from the surface of the edible part to the inner side (Figure 2), and there would also be a gradient of amino acid content. Also, that would lead to a more proteinogenic degradation process. The total amino acids significantly increased in the meat surface after dry-ageing with the mould ( $P < 0.01$ ) even though the moisture content was adjusted (Figure 3). That means each amino acid increased due to the proteinogenic degradation process. Although the trimming part becomes the cause of the burnt deposit turning out black, the surface of the edible part must be most concentrated and most tasty; releasing good odours after it is grilled because amino acids or other components are concentrated in part. Amino acids react with sugars by heating, resulting in a savoury flavour (Okumura, 1993), which also contributed to various smells on dry-aged beef.



Figure 1. The appearance of *M. flavus* covering dry-aged beef.

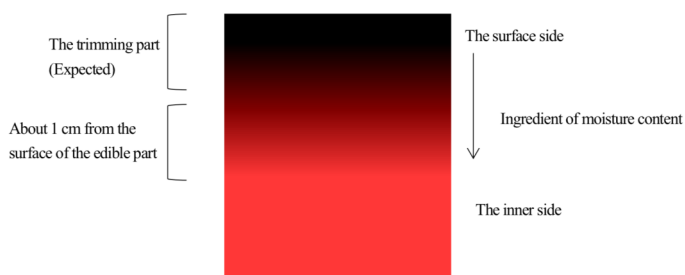


Figure 2. A cross-section from the surface to the inner side in dry-aged beef where an ingredient of moisture content presumably occurred.

### 3.2 GCMS chromatogram

In Okinawa, using a mould is becoming common for producing dry-aged beef. However, the effect of dry-ageing with mould is not obvious, such as *M. flavus* on aroma volatiles in beef. Accordingly, odour analysis should be conducted to discover volatile compounds that *M. flavus* can add to beef, and useful information about added value for dry-aged beef we produce in Okinawa. The mould strain must produce a savoury flavour that adds value to dry-aged beef companies in Okinawa produce. From the result of the GCMS chromatogram,

the distribution of total ion chromatogram of volatile compounds from dry-aged beef with the mould was considerably different from that of dry-aged beef without mould despite some peaks being identified as the same compounds in both types of dry-aged beef (Figure 4). And the number of peaks was higher in dry-aged beef with mould (75 peaks) than that without mould (67 peaks). Besides, peaks detected in dry-aged beef with the mould were scattered horizontally within 30 min. In contrast, relatively many peaks in dry-aged beef without mould were plotted within 20 min of retention time. Accordingly, higher polar compounds existed in dry-aged beef with the mould because the Wax column has a highly polar polyethylene glycol stationary phase. As a result, there is a high possibility that *M. flavus* can produce higher polar compounds in beef meat.

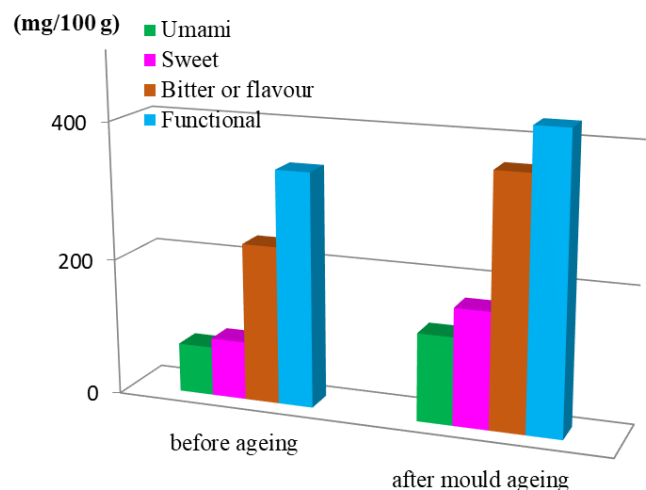


Figure 3. The levels of each amino acid group in the surface meat before and after dry-ageing with mould.

Amino acid groups are classified as functional, bitter or flavour-, sweet- or umami-tasting. Significant differences were detected in sweet ( $P < 0.05$ ) and bitter or flavour ( $P < 0.05$ ).

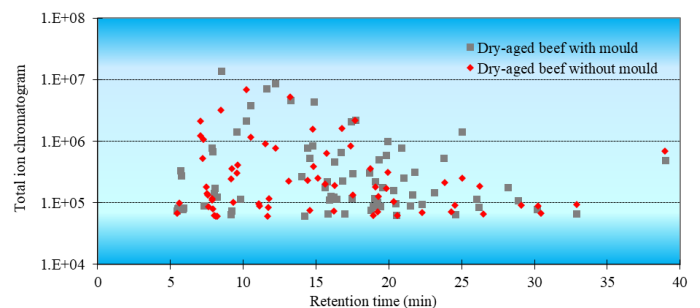


Figure 4. Total ion chromatogram of volatile compounds vs retention time by GCMS with dry-aged beef with and without mould.

### 3.3 Aroma volatile compounds

Table 1 shows the volatile compounds detected in dry-aged beef with *M. flavus*. Among acids detected in dry-aged beef with mould, two compounds were also detected in dry-aged beef without mould, such as acetic acid and 2-methyl-propanoic acid. However, three

Table 1. Identified volatile compounds from dry-aged beef with *M. flavus*.

Category		Volatile compounds	Aroma	Retention Index	Peak area
Acids		Acetic acid	sour, pungent, vinegar <sup>a, b, c</sup>	576	5.00E+05
		2-methyl-propanoic acid		711	3.00E+05
		<b>Heptanoic acid</b>		1136	1.00E+05
		<b>2-Oxo-octanoic acid</b>		1309	6.00E+04
		<b>Undecanoic acid</b>		1471	2.00E+05
Alcohols		1-Hexanol	popcorn-like <sup>d</sup>	860	9.00E+06
		1-Heptanol	woody, oily, corn-like <sup>d</sup>	960	4.00E+06
	Alkene	<b>cis-Hept-4-enol</b>		968	1.00E+05
		1-Octanol	green, citrus, roasted, meat-like <sup>d, e</sup>	1059	2.00E+06
	Alkene	trans-2-Octen-1-ol	grassy, plant-like <sup>d</sup>	1067	3.00E+05
	Thio	<b>3-(Methylthio)-1-propanol</b>		912	8.00E+05
	Alkene	trans-2-Nonen-1-ol		1167	3.00E+05
	Benzen	Phenylethyl Alcohol	rosy, floral <sup>b, c</sup>	1136	1.00E+06
Ketones	Pyrroline	<b>2-Acetyl-1-pyrroline</b>	<b>popcorn<sup>f</sup></b>	945	7.00E+06
Aldehydes		<b>Heptanal</b>	<b>fatty, green, floral<sup>g, f</sup></b>	905	8.00E+05
		Octanal	fatty, soapy, peel <sup>h, e</sup>	1005	4.00E+06
	Alkene	<b>trans-2-Octenal</b>	<b>fatty, green, citrus, nutty, cooked flour<sup>i, e, f</sup></b>	1013	3.00E+05
	Alkene	<b>trans-2-Nonenal</b>	<b>green, cucumber, fatty<sup>e</sup></b>	1112	7.00E+05
	Alkadien	<b>2,4-trans,trans-Nonadienal</b>	<b>nutty, fatty<sup>f</sup></b>	1120	6.00E+04
		<b>Decanal</b>	<b>rubber tubing, smokey<sup>j</sup></b>	1204	1.00E+05
	Alkene	<b>2-Undecenal</b>	<b>fatty, boiled meat<sup>j</sup></b>	1311	3.00E+05
Esters	Vinyl	n-Caproic acid vinyl ester		974	2.00E+05
		<b>1-Norbornanemethanol, acetate</b>		1181	1.00E+05
	Alkene	<b>cis-3-Octen-1-ol, acetate</b>		1191	1.00E+05
		<b>Decanoic acid, ethyl ester</b>		1381	9.00E+04
		<b>Ethyl 9-hexadecenoate</b>		1986	1.00E+05
	Benzen	Benzoic acid, 2-formyl-4,6-dimethoxy-, 8,8-dimethoxyoct-2-yl ester		2658	1.00E+05
Ether		<b>1-Ethoxy-cis-2-heptene,</b>		1000	4.00E+04
Heterocycle	Pyridine	<b>2,3,4,5-Tetrahydropyridine</b>		740	8.00E+05
	Furanone	<b>5-Ethylidihydro-2(3H)-furanone</b>		986	7.00E+04
	Thiazoline	<b>2-Acetyl-2-thiazoline</b>		1104	1.00E+05
Others	Alkene	3,5,5-Trimethyl-1-hexene		757	2.00E+05
	Cyclohexene	<b>3-Methylcyclohexene</b>		763	7.00E+04
	Cyclopenten	<b>5-Hexyl-3,3-dimethyl-1-cyclopentene</b>		1274	1.00E+05

Bold letter indicates volatile compounds detected only in dry-aged beef with *M. flavus*, not in that without mould. Aroma descriptions were obtained from the following publications: <sup>a</sup>Frauentorfer and Schieberle (2008); <sup>b</sup>Majcher *et al.* (2020); <sup>c</sup>Yu *et al.* (2019); <sup>d</sup>Migita *et al.* (2017); <sup>e</sup>Feng *et al.* (2019); <sup>f</sup>Yang *et al.* (2008); <sup>g</sup>Liu *et al.* (2019); <sup>h</sup>Vera *et al.* (2020); <sup>i</sup>Bi *et al.* (2020); <sup>j</sup>Song *et al.* (2010)

compounds were detected only in dry-aged beef with the mould. They are aliphatic higher acids and could be produced by the mould during ageing. It was reported that acids were almost not detected in dry-aged beef produced in the ageing room with airflow (Mikami *et al.*, 2021). That means acids could be volatilised by airflow. On the contrary, there was no airflow in the dry box where dry-ageing was conducted in the present study, and accordingly, acids could remain easily on the meat. As for alcohols, six compounds were also detected in dry-aged beef without mould. Among them, 1-hexanol, 1-

heptanol and 1-octanol were also detected in wet-aged beef (Mikami *et al.*, 2021). They are described as having malty, popcorn-like, corn-like, and citrus smells (Migita *et al.*, 2017; Feng *et al.*, 2019). As a result, they are supposed to be generated during the ageing process of meat, not related to the mould. On the contrary, three compounds, cis-hept-4-enol, 3-(methylthio)-1-propanol and trans-2-nonen-1-ol, were detected only in dry-aged beef with the mould. Unfortunately, it is not known what they smell, but there is a possibility that the mould can produce those compounds. As for aldehydes, one

compound was also detected in dry-aged beef without mould. Heptanal, trans-2-octenal and trans-2-nonenal were detected in dry- and wet-aged beef (Mikami *et al.*, 2021); however, in this study, trans-2-octenal smelled nutty only when found in dry-aged beef with mould. In addition, 2,4-trans,trans-nonadienal and 2-undecenal, aliphatic higher aldehydes were detected in dry-aged beef with the mould. As a result, they are unique compounds in dry-aged beef with the mould, *M. flavus*. Additionally, these smells are described as nutty and boiled meat, which can be a savoury flavour you smell. As for esters, two compounds were also detected in dry-aged beef without mould. Four ester compounds were detected only in dry-aged beef with the mould, but the smell they imparted was not investigated in detail. However, it is known that esters have a fruity aroma and they could be involved in contributing to the aroma and flavour of mould ageing beef. Interestingly, all heterocyclic compounds were detected only in dry-aged beef with the mould. Heterocyclic compounds could have a unique aroma. Heterocyclic compounds are of interest because of their varied occurrence in food flavours and their valuable organoleptic characteristics. Even though heterocyclic aroma chemicals are found only in minute amounts in foods, their powerful odours and low odour thresholds, as expressed by high values, make them key in boosting flavours and fragrances (Zviely, 2008). Accordingly, they also could play an important role in savoury flavour (perhaps ‘mellow and intense’) in dry-aged beef with *M. flavus*. As a result, the number of volatile compounds by GCMS in dry-aged beef with the mould was higher, and several peaks were related to the savoury flavour possessed only by dry-aged beef with the mould.

### 3.4 Next step

Lee *et al.* (2021) reported thirty-seven volatile compounds, including aldehydes, furans, ketones, etc. The method of sample gas collection was conducted by headspace without SPME and incubation for 10 mins at 80°C. Among detected compounds, only one compound, hexanoic acid, was detected in the present study. This time, the gas collection method was conducted using SPME and incubation for 10 mins at 60°C. The temperature difference must be why detected compounds are different between them. Of course, higher temperature makes it easier to detect more compounds because the higher temperature can volatilise more compounds in meat, and it is closer to actual beef restaurants serve. The next step is to analyse volatile compounds using GCMS with incubation for higher temperatures and research the composition of fatty acids and others during mould ageing.

## 4. Conclusion

Each amino acid in the surface of dry-ageing beef with the mould increased. It was hypothesised that there is a gradient of moisture content from the surface of the edible part to the inner side of the dry-aged beef, where amino acids are easily generated from the protein degradation. The peak number of volatile compounds by GCMS was more detected in dry-aged beef with mould than in dry-aged beef without mould. Additionally, 2,4-trans,trans-nonadienal and 2-undecenal, aliphatic higher aldehydes were detected in dry-aged beef with the mould. They are unique compounds in dry-aged beef with the mould *M. flavus*. These smells are described as nutty and boiled meat, which can be the savoury flavour you smell. As a result, *M. flavus* that we succeeded in isolating creates savoury odours. And the dry-aged beef with mould will be a new food content as an Okinawan brand for tourists from all over the world.

## Conflict of interest

The authors declare no conflict of interest.

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