

# Aging of Japanese Anchovies (*Engraulis Japonicus*) during Their Salting and Marinating Periods

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Japanese anchovy fillets were preserved in salt at 4°C for various periods and then stored at 20°C in olive oil for varying durations after being bottled to produce so-called anchovies. The salting durations were 0 (no salting), 1.5, 3, 7, 17, 28, 42, 56, 70 and 388 days. The marinating periods were 0 (no marinating), 7, 14, 28 and 365 days, and all of the fish were marinated except for those that were salted for 388 days. The condition of the anchovies during the aging process and the extent of putrefaction were determined through the monitoring of water activity ( $A_w$ ) and volatile basic nitrogen (VBN) levels, and the extent of protein decomposition was determined by means of SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins in the fillets were found to have decomposed into smaller molecules during salting without undergoing putrefaction, and the decomposition progressed during the 388-day salting period. Protein decomposition continued during the 365-day marinating period and was accompanied by a slight rise in VBN levels (which weren't evaluated as putrefaction-related), and the decomposition was less in fillets that were salted for a long time before being marinated. In contrast to the fillets that were salted before marination, those that were marinated without having been salted exhibited rapid protein decomposition to smaller molecules—changing steadily from big to small—with VBN levels evaluated as being clearly associated with putrefaction.

**Keywords:** Japanese anchovy, salting, marinating, water activity, volatile basic nitrogen

## 1. Introduction

The Japanese anchovy—a small marine fish—is a predominant fish resource in Japan. Some anchovies are consumed fresh, but most are boiled or processed in a variety of ways. A major problem with anchovy storage is the rapid autolytic degradation of their abdominal tissue—a phenomenon often referred to as belly bursting<sup>1)</sup>—which may be mainly caused by the high proteolytic activities of intestinal and pyloric caeca<sup>1, 2)</sup>. Myofibrils in the fish are also readily deteriorated by autolysis. However, these properties can be advantageously used in the aging of fish. The present study was undertaken to clarify the effects of salting and marinating on the aging of fish kept in storage, in order to highlight the differences between aging on the one hand and putrefaction caused by the above-mentioned processes on the other.

## 2. Materials and Methods

### Anchovy production

The Japanese anchovies used in the study were bought from an ordinary market in Hiroshima Prefecture. The fish weighed between 8.4 and 16.2 g each, and their body lengths ranged from 9.8 to 11.6 cm. The fish were cut into fillets with a binding string measuring approximately 10 cm in length (Fig. 1), and the fillets were placed at small distances from one another in plastic containers filled with salt. The fillets were stored at 4°C in a refrigerator for



**Fig. 1** Producing fillet of anchovy.

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**Table. 1** Storage period of fillets

Sample Set	Salting period (days)	Marinating period (days)
No. 1 <sup>*1</sup>	0	→ 7, 14, 28, 365
No. 2	1.5	→ 7, 14, 28, 365
No. 3	3	→ 7, 14, 28, 365
No. 4	7	→ 7, 14, 28, 365
No. 5	17	→ 7, 14, 28, 365
No. 6	28	→ 7, 14, 28, 365
No. 7	42	→ 7, 14, 28, 365
No. 8	56	→ 7, 14, 28, 365
No. 9	70	→ 7, 14, 28, 365
No. 10 <sup>*2</sup>	388	

<sup>\*1</sup> The fillets which were marinated directly without salting.

<sup>\*2</sup> The fillets which were salted for 388 days.

either 1.5, 3, 7, 17, 28, 42, 56, 70 or 388 days. The salt was then removed from the fillets by shaking them in a basket until no more crystals were observed on their surfaces. The salted fillets (except those salted for 388 days) were then placed into glass jars with adequate space for oil to permeate the spaces between them. Olive oil was then poured into the jars to a level just below their tops. The air between the fillets was removed by gently stirring them with chopsticks, and lids were screwed onto the jars. The sealed jars containing the oil-marinated fillets were stored in an incubator at 20°C for 7, 14, 28 or 365 days (Table 1).

#### Determination of volatile basic nitrogen value

The Conway method<sup>3)</sup> was used to detect the volatile basic nitrogen (VBN) value, which enabled evaluation of the extent of putrefaction of the preserved fish. First, each of the storage fillets was minced into small particles, and 3 g of the particles were homogenized at 25,000 rpm for 1 minute with 24 mL of distilled water. Then 3 mL of 20 w/v% trichloroacetic acid was added, and the mixture was stirred in a mixer and then left for 10 minutes. Finally the mixture was centrifuged at 3,000 rpm for 10 minutes. The filtrate of the supernatant was used as a sample solution. The inner section of a Conway microdiffusion unit (Shibata, Japan) was filled with 1 mL of boric acid solution in which 3.3 µg of bromocresol green, 3.3 µg of methyl red, 210 µL of ethanol and 10 mg of boric acid were dissolved. The outer section was filled with 1 mL of the sample solution, mixed with 1 mL of 33 w/w% potassium carbonate. The unit with its contents was incubated at 37°C for 80 minutes. After the incubation period, the boric acid solution

containing the VBN compounds was titrated with 0.02 mol/L of hydrochloric acid. The VBN value was calculated using the following equation, where “V” is the consumed volume of 0.02 mol/L hydrochloric acid, “f” is a titration factor of 0.02 mol/L hydrochloric acid and “B” is the consumed volume of a blank test:

$$\text{VBN value (mg\%)} = 14 \times 0.02 \times (V-B) \times f \times 1000$$

#### Monitoring of water activity

After the salt crystals and olive oil were removed from the samples (by gently wiping them with kitchen paper), each sample was minced into small particles, and water activity (Aw) was monitored using a Pawkit Water Activity Meter (Ainex, Tokyo, Japan).

#### Electrophoretic analysis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to determine the characteristics of fish protein decomposition. The Laemmli method<sup>4)</sup> was used, and 5–20% polyacrylamide gels (e-PAGEL HR, Atto, Japan) at room temperature were employed. Approximately 20 µg (wet weight) of white muscle was dissolved in the sample solution (EzApply, Atto, Japan) for each lane. (The purpose of this was to enable comparison of the differing states of the stored fish.) Correlation of molecular weight with relative mobility was obtained using the following standard proteins (EzStandard, Atto, Japan): Phosphorylase B (molecular weight 97,200), albumin (66,400), ovalbumin (45,000), carbonic anhydrase (29,000), trypsin inhibitor (21,100) and α-lactalbumin (14,300). After being run in an electrode buffer (EzRun MOPS, Atto, Japan) for about 30 minutes, the gels were stained with Coomassie brilliant blue (EzStain Aqua, Atto, Japan). Then the JustTLC program (Sweday, Sweden) was used to determine the distribution characteristics of the various molecular weights of the proteins in the stained gels.

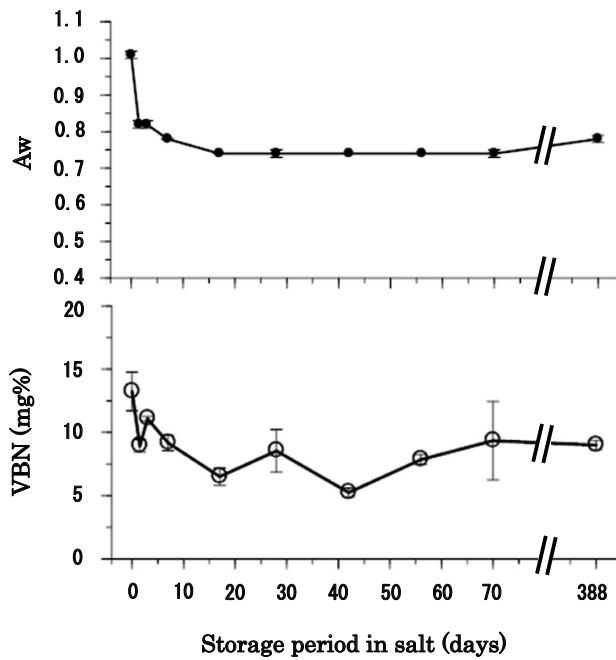
#### Chemicals

The analytical-grade trichloroacetic acid, bromocresol green, methyl red, ethanol, boric acid, potassium carbonate and 0.02 and 0.1 mol/L hydrochloric acid were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

### 3. Results and Discussion

#### Aw and VBN values of the fillets in salting periods

Variations of Aw and VBN in the fillets during the salting period are shown in Figure 2. Aw values of the fillets immediately decreased at the outset and then remained at around 0.80 (in which state growth and propagation of

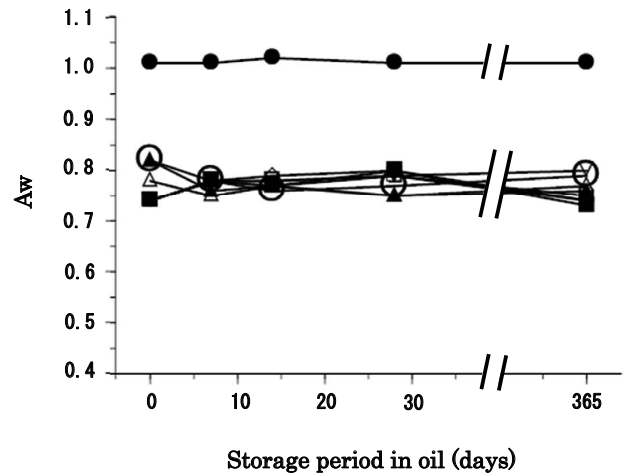


**Fig. 2** Variations of  $A_w$  and VBN in the fillets during salting period. Each symbol in figure indicates the mean value of 3 to 5 samples.

most bacteria that cause putrefaction are prevented) until the end of the period. By contrast, VBN values of the fillets were within half of the evaluated value considered to be the initial stage of putrefaction (30 mg%) while in the fresh state (0 days), and then the values smoothly decreased and remained at around one-third of the evaluated value (30 mg%) until the end. These results suggest that the ratio of free water in the fillets decreased in the early stage through dehydration (which resulted from high osmotic pressure) of the fillets by means of the salt and hydration with the ions from the salt. The growth and propagation of putrefactive bacteria seemed to be prevented by the salt throughout the salting period.

#### **$A_w$ values of the fillets in marinating periods**

Variations of  $A_w$  in the salted and non-salted fillets during the marinating period are shown in Figure 3. The values of the non-salted fillets remained at about 1.0 (a state similar to pure water in terms of the ratio of free water in the whole water molecules, including the bound water) throughout the marinating period. Measured values sometimes exceeded 1.0, with slight measurement errors. By contrast, the values of all the other fillets (those salted for 1.5 to 70 days before marinating) remained at around 0.80 throughout the marinating period. This suggests that once  $A_w$  decreases to the low values at which putrefactive bacteria cannot grow and propagate, the ratio of free water in fillets will remain



**Fig. 3** Variations of  $A_w$  in the fillets during marinating period. Fillets were salted for ●: 0 (no salting), ○: 1.5, ▲: 3, △: 7, ■: 17, □: 28, ▼: 42, ▽: 56 and ◇: 70 days before marinating. Each symbol in figure indicates the mean value of 3 to 5 samples.

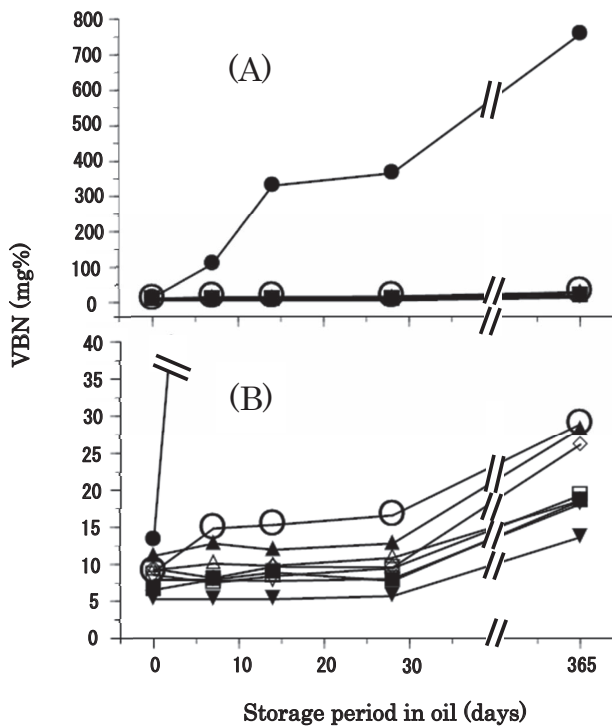
constant for at least a year if the fillets remain in oil. The variations in the graph also indicate that salting for several days is as effective as doing so for over month in terms of preventing putrefaction during the marinating period.

#### **VBN values of the fillets in marinating periods**

As with the low  $A_w$  values observed during marination in previously salted fillets (Fig. 3), the VBN values of previously salted fillets during marination were also below 30 mg% (Fig. 4(B)), which is considered the lower limit for the initial stage of putrefaction. Continuously increasing VBN values below 30 mg% were observed in previously salted fillets during marination (Fig. 4(B)), while a rapid increase in values toward approximately 25 times the 30 mg% amount (758 mg%) was observed in non-salted fillets during the same period (Fig. 4(A)). These results also demonstrate the effectiveness of salting for at least several days to prevent putrefaction during marination.

#### **Decomposition of fillet protein during salting and storage periods**

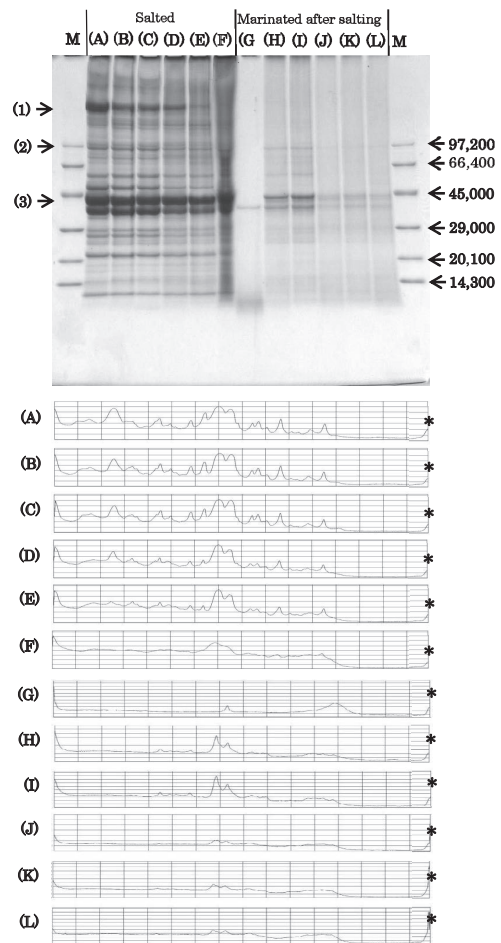
SDS electrophoretic patterns and related quantitative images of salted fillets (A to F) and subsequent 365-day marinated fillets (G to L) are shown in Fig. 5. The upper photograph shows the results of electrophoresis, with fish proteins in the fillets separated in the gel from top to bottom in line with molecular weight. The lower graphs show quantitative images of each lane from left to right in line with molecular weight. In the salted fillet lanes (A to F), protein decomposition with time elapsed ((A): 0; (B): 1.5;



**Fig. 4** Variations of VBN in the fillets during marinating period. Fillets were salted for ●: 0 (no salting), ○: 1.5, ▲: 3, △: 7, ■: 17, □: 28, ▼: 42, ▽: 56 and ◇: 70 days before marinating. The difference between (A) and (B) is the scale of the vertical axis. Each symbol in figure indicates the mean value of 3 to 5 samples.

(C): 3; (D): 28; (E): 56; (F): 388 days) is particularly observed in the (1) to (3) bands in the gel, and a real decrease is seen in the quantitative images in the lower graphs from (A) to (F). After 388 days of salting the protein was decomposed, and demonstrated consecutive molecular weight distribution from big to small as observed in lane (F). The fillet VBN values were below 15 mg% (not evaluated as putrefaction-related), and it was suggested that the decomposition of protein without putrefactive bacteria activity progressed in the fillets during the salting period.

Lane (G) shows the results of the fillets marinated for 365 days with no salting period, and lanes (H) to (L) show the results of the 365-day marinated fillets salted for each duration in advance. The total storage periods (i.e., salting and marination) in days were: (H): 366.5; (I): 368; (J): 393; (K): 421; (L): 435. Judging from the distributions of the various protein molecular sizes in lanes (H) to (L), the extent of protein decomposition appears to largely correspond to the length of the storage period. The images of the lanes are similar to that of lane (G), where the VBN value was 758 mg% (evaluated as putrefaction) in line with



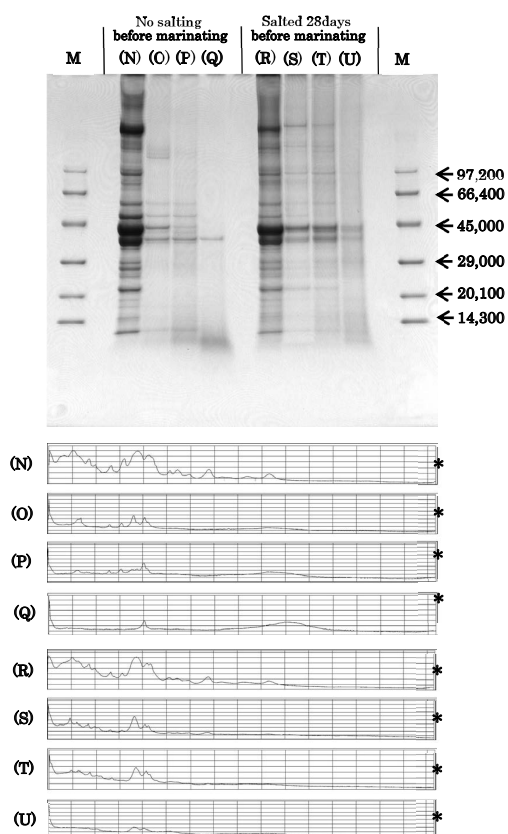
**Fig. 5** SDS electrophoretic patterns and related quantitative images (JustTLC) of salted fillets (A: 0; B: 1.5; C: 3; D: 28; E: 56; F: 388 days) and of 365-day marinated fillets after salting for each duration (G: 0; H: 1.5; I: 3; J: 28; K: 56; L: 70 days). Marker proteins: M is shown on each side of the gel as reference. Asterisks indicate measured values of 28,000 on the vertical axis with JustTLC.

the length of the marination period. However, the related VBN values were all below 30 mg%, which is not evaluated as putrefaction-related. It can be inferred that low-molecular-weight protein or peptides had accumulated in the fillets rather than VBN compounds derived from fish protein.

#### Difference between putrefaction and aging in terms of fish protein decomposition

SDS electrophoretic patterns and related quantitative images of marinated fillets without salting (N to Q) and marinated fillets after 28 days of salting (R to U) are shown in Fig. 6. The upper photograph shows the results of electrophoresis, with fish proteins in the fillets separated in the gel from top to bottom in line with molecular weight. The lower graphs show quantitative images of each lane from left to right in line with molecular weight. The





**Fig. 6** SDS electrophoretic patterns and related quantitative images (JustTLC) of marinated fillets (N: 0; O: 14; P: 28; Q: 365 days) not salted beforehand, and of marinated (R: 0; S: 14; T: 28; U: 365 days) fillets after 28 days of salting. Marker proteins: M is shown on each side of the gel as reference. Asterisks indicate measured values of 28,000 on the vertical axis with JustTLC.

marination periods and related VBN values were: (N): 0 days, 13 mg%; (O): 14 days, 331 mg%; (P): 28 days, 366 mg%; (Q): 365 days, 758 mg%. The values for the marinated fillets after 28 days of salting were: (R): 0 days, 9 mg%; (S): 14 days, 8 mg%; (T): 28 days, 9 mg%; (U): 365 days, 19 mg%. While the difference in VBN values between the sample sets from (N) to (Q) and from (R) to (U) are obvious, and evaluated as putrefaction and non-putrefaction, the variation patterns of both (changing from high to low molecular weight as observed in the gel and quantitative images) are quite alike. These results suggest that the representative difference between putrefied fillets and aging fillets might be the ratio of compounds generated by putrefactive bacteria in low-molecular-weight compounds, although fish proteins were decomposed in both cases.

Some researchers have reported decomposition of fish protein from high- to low-molecular-weight compounds during the aging period (wahoo meat cured in sake lees<sup>5</sup>,

Spanish mackerel meat cured in sake lees<sup>6</sup> or miso<sup>7</sup>, sardine meat cured in rice bran<sup>8</sup>, and sablefish meat cured in miso or sake lees<sup>9</sup>). In sardine meat cured in rice bran<sup>8</sup> in particular, most free amino acid content increased in line with the length of the aging period. It may be inferred that a variety of low-molecular-weight compounds such as bioactive peptides might accumulate over time during aging in various ways without putrefaction.

#### 4. Acknowledgements

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〔研究ノート〕

## カタクチイワシの塩漬および油漬期間における熟成

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## 要 旨

カタクチイワシは、内臓の消化酵素活性が高く、その鮮度落ちは外観からも腹部の穿孔に確認される。また、魚肉の脆弱化も速やかであり、これにはタンパク質分解酵素活性の高さが関与している。鮮度が良いうちの活用を考えると、カタクチイワシは扱いにくい魚であるが、筋肉タンパク質の分解による熟成が、品質にとって重要な要因となる発酵食品の原料としては、この魚の特性は優れている可能性がある。本研究では、カタクチイワシの発酵食品であるアンチョビに焦点を当て、様々な塩漬および油漬期間のサンプルを調製し、塩漬、油漬それぞれの意義を調べるとともに、状態を化学分析し評価することを目的とした。

広島県内で購入したカタクチイワシをフィレーにし、その一片一片の表面が食塩に接するようにプラスチック容器に入れ、そのまま4℃で一定期間(0, 1.5, 3, 7, 17, 28, 42, 56, 70および388日間)塩漬し、その後、388日間塩漬分以外のものについては、食塩を振るい落としした後、ジャム瓶に詰めオリーブ油を満たし、20℃で一定期間(7, 14, 28および365日間)油漬した。各段階の試料につき、(1)水分活性( $A_w$ )、(2)微量拡散法による揮発性塩基態窒素量(VBN)を測定し、併せてSDS-ポリアクリルアミドゲル電気泳動を行なった。その結果から、各試料の保持具合や、熟成に伴うタンパク質の分解の程度を推定し、アンチョビの熟成に及ぼす塩漬と油漬の効果を評価した。

フィレーの数日以上塩漬により、 $A_w$ は一般的な細菌が増殖できない0.80前後に低下し、その状態は塩漬最終日の388日まで持続した。また、腐敗細菌によるタンパク質分解産物の生成量を示すVBN値は、塩漬処理を施すと、初期腐敗と判断される値(30 mg%)の約1/3量を維持した。塩漬後の油漬期間においても、数日以上塩漬処理を施したフィレーには、 $A_w$ 、VBN両値において塩漬終了時の低い値( $A_w$ : 0.80前後、VBN: 30 mg%未満)の保持が確認された。一方、フィレーの電気泳動結果からは、塩漬、油漬両期間におけるタンパク質の分解が確認された。タンパク質の分解は、塩漬せず油漬処理のみを施したサンプルにおいて最も進行しており、初期腐敗値の約25倍ものVBN(758 mg%)が検出されたが、1.5日以上塩漬処理群においては、油漬期間中のタンパク質の分解は同様に進行していたものの、 $A_w$ は0.80前後に維持され、VBNの著しい増加は認められなかった。このことから、数日以上塩漬後に油漬すると、内部の自由水が制御された状態で熟成が進み、魚肉中には低分子化したタンパク質やペプチド等が蓄積されていくものと推察された。

**キーワード:** カタクチイワシ、塩漬、油漬、水分活性、揮発性塩基態窒素