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Effect of bitterns and constituent salts on the inosinate phosphatase activity in horse mackerel muscle

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Abstract

Japanese horse mackerel (*Trachurus japonicus*) is a widely consumed commercial fish in Japan. Inosinic acid is one of the taste components generated by the degradation of ATP in fish muscle. In this study, we investigated the efficacy of bitterns for the preservation of fish taste by monitoring inhibition of inosinate phosphatase (IMPase) activity in horse mackerel muscles. The effects of two different kinds of bitterns produced from seawater (SW) or the ion exchange membrane (IEM) method were used. The relative activity of inosinate phosphatase was measured at different pH values and at different bitterns concentrations. We found that both SW and IEM bitterns significantly inhibited the enzyme when used at concentrations of 8% and 17%, respectively; interestingly, however, with the use of 1.7% bitterns, there was a profound increase in the activity of inosinate phosphatase. To further understand this phenomenon, the effects of different salts commonly present in bitterns were also investigated. We found that each salt (NaCl, $MgCl_2$, $MgSO_4$ and $CaCl_2$) had significantly different pH dependences and different effects on IMPase activity. $MgCl_2$ and $MgSO_4$ were found to be major constituents of bitterns that affected IMPase activity. These results indicated that with the proper concentration and pH, bitterns could effectively inhibit IMPase activity and therefore have the potential to be used as effective additives for the preservation of fish taste.

Introduction

Horse mackerel (*Trachurus japonicus*), classified within the Carangidae family in the order Perciformes, is an important saltwater fish distributed throughout the shores of the northwest Pacific Ocean. As a food source, horse mackerel is eaten fried, boiled, dried, or prepared as sashimi. Due to its popularity as a food source, many methods have been investigated for the storage and preservation of horse mackerel. Salt is one of the primary preservatives used for this purpose and has been reported to suppress microbial activity (Hwang *et al*., 2009) and help maintain the taste component of the fish (Tomioka and Endo, 1988; Oba and Niwa, 1993; Ooizumi, 2010). Maintaining the taste component is important because the flavor of fish needs to be maintained.

One of the main taste components of fish is inosinic acid (IMP). IMP is formed by the degradation of ATP (ATP → ADP → AMP → IMP) in fish muscle and is degraded to uric acid (IMP → HxR → Hx → uric acid) by inosinate phosphatase (IMPase) (Aubourg, 2001; Huidobro *et al*., 2001; Tejada *et al*., 2006). In fish, IMP accumulates at a steady rate because the degradation from ATP to IMP proceeds rapidly (Srirangsan *et al.*, 2010). Therefore, it is necessary to suppress the activity of IMPase to maintain IMP levels.

Bitterns are concentrated seawater solutions left over after sodium chloride precipitation. Bitterns can fall into two categories depending on the method of salt precipitation: seawater bitterns and bitterns obtained by the ion exchange membrane (IEM) method. While both seawater bitterns and bitterns obtained from IEM have $MgCl_2$ as the major component, seawater bitterns has a high amount of $MgSO_4$ and little $CaCl_2$ and bitterns obtained by IEM has a high amount of $CaCl_2$ and little $MgSO_4$ (Hashimoto and Murakami, 2003; Sawayama, 2010). These bitterns have been used as chemicals and coagulating agents in the production of tofu (Li *et al*., 2013). Recently, bitterns have been used as food additives, and many products containing bitterns are now sold to the general public (Haga *et al*., 2005). However, there are few scientific reports investigating the use and functionality of bitterns as food additives, and further functional studies are required to support the use of bitterns (Sato *et al*., 2011). Because bitterns are liquids, they are easy to mix and seep. In addition, we can easily calculate the salt concentration of the bitterns because salt is concentrated during the preparation of bitterns.

Therefore, in this study, we sought to investigate whether bitterns could be used to maintain fish taste. We also studied the effects of the two types of bitterns

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on IMPase activity in horse mackerel muscles at different pH values and concentrations. Finally, we monitored the effects of individual salts, namely NaCl, $MgCl_2$, $MgSO_4$ and $CaCl_2$, on the inhibition of IMP degradation. We expected that this study would provide important insights into the use of bitterns for the maintenance of IMP.

Materials and Methods

Sample preparation

We purchased fresh horse mackerel from Chiba at a supermarket in Tokyo between July and December 2012. We used "Siotakijiinotennennigari" (made by Yuriya-seienjyo Ltd., Nagasaki, Japan) and "Sesanonigari" (made by Nihon-kaisui Co., Ltd. Tokyo, Japan) as bitterns (Haga et al., 2005). "Siotakijiinotennennigari" was seawater bitterns, and "Sesanonigari" was bitterns obtained by the IEM method. The components of "Siotakijiinotennennigari" were as follows: NaCl, 8.01%; $MgCl_2$, 13.4%; $MgSO_4$, 5.6% (major salt components), and the components of "Sesanonigari" were as follows: NaCl, 2.4%; $MgCl_2$, 17.8%; $CaCl_2$, 7.5% (major salt components) (Haga *et al*., 2005). We used NaCl, $MgCl_2 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, and $MgSO_4 \cdot 7H_2O$ as reagent salts (Kokusan Chemical Co., Ltd.)

Examination of the extraction method and investigation of IMPase properties in horse mackerel

The dorsal meat from horse mackerel was collected, homogenized in 3 volumes of water, and minced using a homogenizer. Fish flesh homogenate and supernatants after centrifugation at 9000× *g* for 15 min were dialyzed against water for 2-2.5 days (Nedachi and Hirota, 1991). Dialysates were then filtered (No. 1, Toyo Roshi Kaisha, Ltd.), diluted twice with water at 10°C, and used as enzyme solutions.

The standard reaction mixture was composed of 75 mM buffer at pH 4–8, 1 mM IMP, and 8.3% enzyme solution in a total volume of 4 mL. We used 50 mM succinic acid/NaOH at pH 4-6 and 50 mM maleic acid/Tris/NaOH at pH 6-8 as buffer (Perrin and Dempsey, 1981). After incubation of the reaction mixture at 25°C for 24 h, the reaction was stopped by the addition of 10% perchloric acid (final concentration, 3.3%). The precipitate was separated by centrifugation, and free inorganic phosphate was determined using the molybdenum blue method (The Salt Industry Center of Japan, 2007). Samples without addition of any bitterns were prepared in a similar manner and used as a control.

Effects of bitterns on IMPase activity in horse mackerel

Enzyme solutions were prepared as described above. The standard reaction mixture was composed of 75 mM buffer at pH 4–8, 1 mM IMP, 1.7%–17% of each type of bitterns, and 8.3% enzyme solution in a total volume of 4 mL. The salt concentration of the dried fish was usually around 0.3–0.5 M NaCl (Ooizumi, 2010). After incubation of the reaction mixture at 25°C for 24 h, the reaction was stopped by the addition of 10% perchloric acid (final concentration of 3.3%). The precipitate was separated by centrifugation, and free inorganic phosphate was determined using the molybdenum blue method (The Salt Industry Center of Japan, 2007). We calculated the relative activity by assuming that the enzyme activity of the no salt sample was 100%, or by taking the highest activity as 100% when measuring the effects of bitterns concentrations (1.7%, 8.3%, and 17% bitterns).

Effects of the component of bitterns on IMPase activity in horse mackerel muscles

Enzyme solutions were extracted as described above. The standard reaction mixture was composed of 75 mM buffer at pH 4–8, 1 mM IMP, 1.7% NaCl, $MgCl_2$, $CaCl_2$, or $MgSO_4$ and 8.3% enzyme solution in a total volume of 4 mL. After incubation of the reaction mixture at 25°C for 24 h, the reaction was stopped by the addition of 10% perchloric acid (final concentration of 3.3%). The precipitate was separated by centrifugation, and free inorganic phosphate was determined using the molybdenum blue method (The Salt Industry Center of Japan, 2007). We calculated the relative activity by taking the highest activity as 100% when measuring the effects of various kinds of salts at a concentration of 1.7%.

Results and Discussion

Examination of the extraction method and investigation of IMPase properties in horse mackerel

The IMPase activity in homogenate filtrates and supernatants after centrifugal separation is shown in Figure 1. The activity increased at pH 5–6 and decreased at pH 7, but increased again at pH 8 in the homogenate. In the supernatant, the activity increased at pH 5–6, but decreased at higher pH values. IMPase activity in the supernatant resembled that reported by Tomioka and Endo (1988). The results of the homogenate analysis revealed the presence of at least two types of IMPases, exhibiting high activity at pH 5–6 and at pH 8, consistent with a report on IMPase

Table 1. The influence of changes in the concentration of bitterns on IMP-degrading enzyme activity at each pH

		Relative activity (%)					
Bitterns concentration		1.7%		8.3%		17%	
Kind of bitterns		A	B	A	B	A	B
None		100					
pH	4	90 (0.95)	43 (0.61)	30 (0.48)	12 (0.37)	8.9 (0.73)	5.3 (0.18)
	5	96 (0.19)	56 (0.40)	40 (0.20)	20 (0.89)	16 (0.35)	11 (0.28)
	6	471 (193)	420 (200)	44 (13)	31 (2.7)	15 (5.7)	12 (1.8)
	7	346 (11)	340 (5.6)	5.4 (0.10)	49 (21)	3.0 (0.34)	4.5 (0.040)
	8	131 (3.3)	153 (1.0)	1.6 (0.16)	9 (1.3)	1.1 (0.35)	0 (0.76)

A: Bitterns obtained by ion exchange membrances method; B: Seawater bitterns

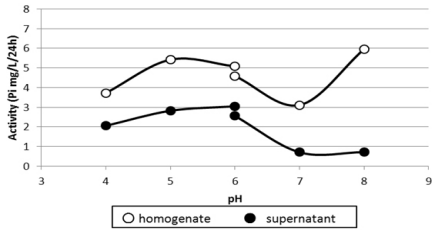

Figure 1. The influence of different extraction methods on IMP-degrading enzyme activity.

activity in horse mackerel by Ooizumi (2010). On the other hand, this high activity was not observed at pH 8 in the supernatant after centrifugal separation. These data demonstrated that IMPase activity different in the supernatant and homogenate; therefore, the enzyme showing the highest activity around pH 8 was not very soluble in water. Separate reports by Nedachi and Hirota (1991) and Obatake *et al.* (1988) showed similar results in the New Zealand Golden snapper and mackerel. Based on these results, and because we assumed that our experiments reflected the enzyme activity of the entire fish flesh, we decided to use homogenates and not supernatants in subsequent experiments in this study.

Effect of bitterns on IMPase activity

We next examined the IMPase activity in solutions prepared with different final concentrations of bitterns (1.7%, 8.3%, and 17%) (Table 1). IMPase activity varied widely, measuring 43%–471%, 1.6%–49%, and 0%–16% at bitterns concentrations of 1.7%, 8.3%, and 17%, respectively. Because the IMPase activity decreased as bitterns concentrations increased, these data suggested that bitterns suppressed IMPase activity. At a bitterns final concentration of 1.7%, the IMPase activity was 340%–471% at pH 6–7, a number that was quite high, suggesting that low concentrations of bitterns strongly promoted IMPase activity at pH 6–7. These results may be influenced by $MgCl_2$, which is a major component of bitterns. Indeed, Nedachi and Hirota (1991) reported that approximately 0.02% $MgCl_2$ is sufficient to activate IMPase in the New Zealand Golden snapper. In their report, Mg^{2+} activated IMPase at pH 6, and a marked increase was observed at pH 8. In addition, Hirota (1973) reported that bonito has IMPase activity at about pH 5 and pH

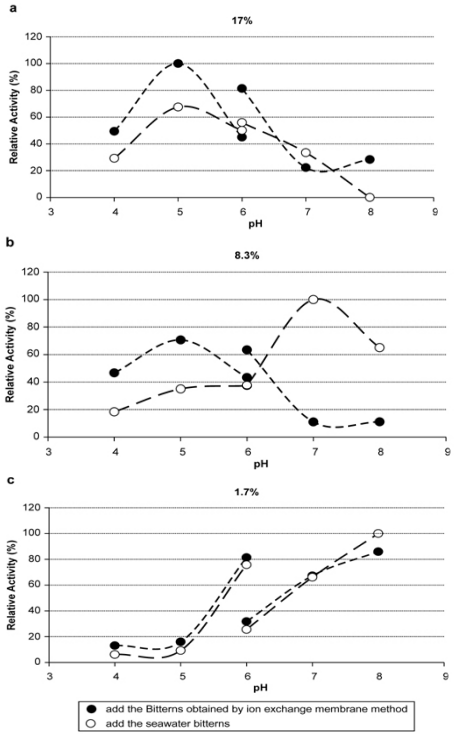

Figure 2. Effects of various concentrations and types of bitterns on IMP-degrading enzymatic activity.

9.5 and that IMPase activity is activated by 0.1% $MgCl_2$ at pH 6, similar to the results of our study. Moreover, in this study, $MgCl_2$ was added at a very low concentration (0.2%–0.3%), and the reaction mixture contained bitterns at a final concentration of 1.7% (Li *et al*., 2013); $MgCl_2$ was able to activate the enzyme at this concentration. However, IMPase activity (3.0%–49%) did not increase substantially when the concentration of bitterns was above 8.3%. When the bitterns concentration was 8.3%, $MgCl_2$ was added at 1.1%–1.5% (Li *et al*., 2013). In a study by Oba and Niwa (1993), IMPase activity was found to be suppressed in two types of fish at high $MgCl_2$ concentrations (approximately 5%). In our study, because the IMPase activity decreased in mixtures with bitterns concentrations above 8.3%, our data suggested that the $MgCl_2$ concentration had a great influence on enzyme activity. However, because the type of bitterns also affected IMPase activity at pH 4–5, it was possible that both $MgCl_2$ and other components in bitterns influenced IMPase activity.

Figure 2 shows the relationships between IMPase activity and pH. IMPase activity was lower in mixtures prepared with seawater bitterns than in mixtures prepared with bitterns obtained by the IEM method at pH values ranging from 4 to 6 when the final concentration of bitterns was 17%. Both solutions showed high activity at pH 5 and exhibited a tendency to decrease as the pH increased. Compared to the activity curve shown in Figure 1, these solutions exhibited the same tendency for high

activity at pH 5; however, the activity was strongly suppressed at pH 8 by the addition of bitterns. The relative activity was 0%–16% (Table 1) regardless of the pH or type of bitterns, and IMPase activity was suppressed when bitterns was added at a final concentration of 17%, suggesting that enzyme activity decreased in response to bitterns, but showed high activity at pH 8. At a final bitterns concentration of 8.3%, high activity was observed in the pH range of 5–6 following the addition of bitterns obtained by the IEM method, similar to the tendency observed by the addition of bitterns at a final concentration of 17%. On the other hand, IMPase activity was not high at pH 5–6, but was activated at pH 7 by the addition of seawater bitterns. At a final bitterns concentration of 1.7%, solutions prepared with seawater bitterns and bitterns from the IEM method both tended to exhibit low enzyme activity in the pH range of 4–5, and the activity increased as the pH rose. We did not observe a difference in activity based on the type of bitterns at this concentration.

These results demonstrated a difference in the enzyme activity according to the type and concentration of bitterns, suggesting the possibility that variations in the composition of bitterns influenced IMPase activity. With the exception of high $MgCl_2$ content, the two types of bitterns do exhibit different components. To date, one report has shown that NaCl suppresses IMPase activity in specific types of fish (Tomioka and Endo, 1988) and that $MgCl_2$ and $CaCl_2$ suppress IMPase activity in the walleye pollack and silver whiting (Oba and Niwa, 1993). However, these studies were performed at a constant pH, and no reports have described these effects in the horse mackerel.

Effect of bitterns components on IMPase activity

Next, we investigated influence of the individual salts found in bitterns on IMPase activity (Figure 3). Addition of NaCl revealed that IMPase activity was highest at pH 5 and decreased to around 10% at pH 7, but increased back to 50% that of its highest value at pH 8. The addition of $MgCl_2$ revealed that IMPase activity was highest at pH 5, similar to the effects of NaCl, and IMPase activity then decreased as the pH increased, reaching only around 25% activity at pH 8. Following $MgSO_4$ addition, IMPase activity was 0% at pH 4 and increased with increasing pH values. In particular, IMPase activity rose at pH 6–8, showing it highest activity at pH 8. $CaCl_2$ addition revealed that IMPase activity was highest at pH 4, decreased with increasing pH, and reached about 7% at pH 7. These results demonstrated that the individual salts exerted differing effects on IMPase activity.

When considering these results and the results presented in Figure 2, several patterns can be observed. IMPase activity in solutions prepared with bitterns obtained by the IEM method at final concentrations of 17% and 8.3% or by seawater bitterns at a final concentration of 17% showed high activity at pH 5, reflecting the results obtained without the addition of salts (Figure 1) or with the addition of NaCl and $MgCl_2$. IMPase activity at pH 6–8 was consistent with the results obtained following $MgCl_2$ and $CaCl_2$ addition. At a final bitterns concentration of 8.3%, IMPase activity was low at pH 4–5 and reached its highest at pH 7 in samples prepared with seawater bitterns. These results did not reflect any of the results obtained following the addition of various salts. At a final bitterns concentration of 1.7%, IMPase activity increased with increasing pH. Although this was in accordance with the activity curve created following $MgSO_4$ addition, these results could not reflect the addition of $MgSO_4$ because $MgSO_4$ was not found in bitterns obtained by the IEM method. Ooizumi (2010) reported that IMPase activity changes with variations in the NaCl concentration and that differences in salt concentrations may influence IMPase activity because salt concentrations were altered with changes in bitterns concentrations, as was the case in our study. Therefore, we calculated the salt concentrations for each sample having different final concentrations of bitterns, as shown in Figure 2, and investigated IMPase activity at each concentration of bitterns in order to identify which salts influenced IMPase activity.

For samples prepared using 17% bitterns obtained by the IEM method, NaCl was approximately 0.4%, $MgCl_2$ was approximately 3.0%, and $CaCl_2$ was approximately 1.3% in the reaction mixture. In addition, for samples prepared using 17% seawater bitterns, the reaction mixture contained 1.4% NaCl, 2.3% $MgCl_2$, and 1% $MgSO_4$. Because the content of $MgCl_2$ was the highest for both types of bitterns, these data suggested that $MgCl_2$ influenced IMPase activity greatly. Therefore, we investigated IMPase activity at an $MgCl_2$ concentration of 2.5%, which was similar to the concentration of $MgCl_2$ in both bitterns. The activity was high at pH 5 and decreased as the pH increased. Because this result was consistent with IMPase activity in samples prepared using 17% bitterns (Figure 2(a)), these data demonstrated that $MgCl_2$ influenced IMPase activity.

In addition, compared to samples without the addition of salts (Figure 1), IMPase activity was significantly decreased at pH 8. Therefore, we investigated changes in IMPase activity resulting from variations in the concentration of $MgCl_2$ at pH

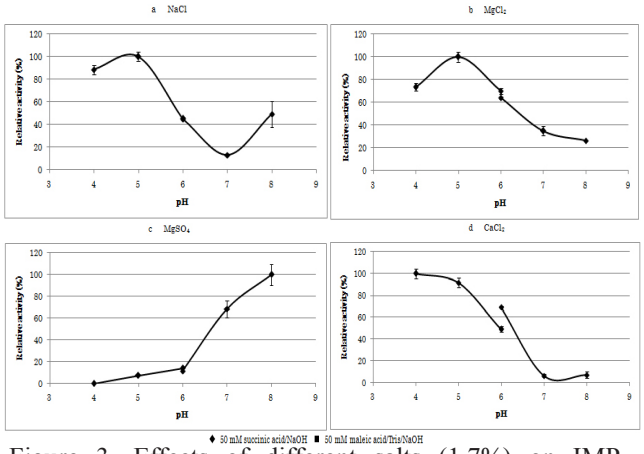

Figure 3. Effects of different salts (1.7%) on IMP-degrading enzymatic activity. The highest activity was defined as 100%.

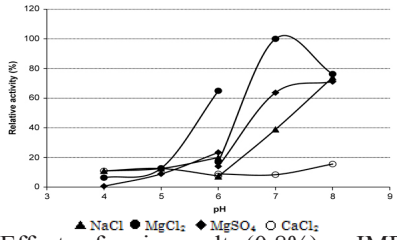

Figure 4. Effects of various salts (0.8%) on IMP-degrading enzymatic activity. The highest activity was defined as 100%.

8. When the activity on no salt sample was defined as 100%, relative activity was 9% at 2.5% $MgCl_2$ and 10% at 1.7% $MgCl_2$ and 293% at 0.83% $MgCl_2$. The activity decreased as $MgCl_2$ concentration increased; in particular, IMPase activity suddenly decreased when the final concentration of $MgCl_2$ varied from 1% to 1.5%. Interestingly, in samples prepared with 17% bitterns obtained by the IEM method, the relative activity was almost 0% when the $MgCl_2$ concentration was 3.0%. In contrast, in samples prepared with 17% seawater bitterns, the relative activity was approximately 5% when the $MgCl_2$ concentration was 2.3%. These results demonstrated that the IMPase showing high activity at pH 8 was greatly affected by the $MgCl_2$ concentration. Moreover, these results also suggested that IMPase activity was suppressed at high concentrations of bitterns due to the high levels of $MgCl_2$ present in the sample.

In reaction mixtures prepared with 8.3% bitterns obtained by the IEM method, the concentrations of NaCl, $MgCl_2$, and $CaCl_2$ were approximately 0.2%, 1.5%, and 0.6%. The $MgCl_2$ concentration was very similar to the concentration obtained using 1.7% $MgCl_2$ and IMPase activity in samples prepared with 8.3% bitterns obtained by the IEM method (Figure 2 (b)) was consistent with that shown for $MgCl_2$ with 1.7% bitterns in Figure 3. Therefore, these data suggested that IMPase activity was affected by $MgCl_2$.

On the other hand, in samples prepared with 8.3% seawater bitterns, reaction mixtures contained approximately 0.7% NaCl, 1.1% $MgCl_2$, and 0.5% $MgSO_4$. All of these salt concentrations were lower than those measured in samples prepared with 1.7% seawater bitterns (Figure 3). Therefore, we investigated IMPase activity in samples with 0.8% of each salt, which was the average concentration of NaCl, $MgCl_2$, and $MgSO_4$ (Figure 4). The activity increased as the pH increased for 0.8% NaCl, $MgCl_2$, and $MgSO_4$, and the activity curves were different from those observed in Figure 3. IMPase activity was the highest at pH 7 in samples with added $MgCl_2$ (Figure 4), and this agreed with our results of 8.3% seawater bitterns (Figure 2(b)). Together, these results supported that differences in salt concentrations influenced IMPase activity.

As shown in Figure 2(a,b), IMPase activity decreased following the addition of seawater bitterns in comparison with bitterns obtained by the IEM method at pH 4–6 for both 8.3% and 17% bitterns concentrations. It had been suggested that $MgSO_4$, which is found primarily in seawater bitterns, influenced IMPase activity. However, because $MgSO_4$ exists as Mg and SO_4^{2-} ions in solution, one of these ions must have reduced IMPase activity. Therefore, we added $MgSO_4$ and Na_2SO_4 and investigated the activity to confirm the influence of the SO_4^{2-} ion at pH 4 and 5. In comparison to samples without added salts, addition of $MgSO_4$ or Na_2SO_4 nearly completely abolished activity at pH 4 and reduced activity to 40%–80% at pH 5, demonstrating that IMPase activity was suppressed by the SO_4 ion at pH 4 and 5.

In general, 1.7% bitterns permitted high activity of IMPase, especially at pH 6 (Table 1). In this case, the concentration of bitterns was low, and therefore, the relatively high levels of $MgCl_2$ would likely have a large influence on IMPase activity. In bitterns obtained by the IEM method, $MgCl_2$ had a final concentration of approximately 0.3%, while that in 1.7% seawater bitterns was approximately 0.2%. In addition, as we discussed above, low concentrations of $MgCl_2$ can be used to activate IMPase activity. Therefore, we suggest that the low concentration of $MgCl_2$ in 1.7% bitterns acted as an activating agent, promoting IMPase activity.

Conclusion

Bitterns caused a significant inhibition of IMPase activity when used at optimal concentrations and pH values. Bitterns prepared from seawater and by the IEM method were both effective; however,

their inhibition efficacies were different. Moreover, different salts present in the bitterns showed various pH effects and IMPase inhibition activities, highlighting the complex biochemistry involved in this process. Based on these results, we concluded that pH 4–8 and a concentration of more than 8% bitterns should be used for taste component preservation in horse mackerel. Further studies are needed to monitor other taste and quality parameters in order to fully understand the use of bitterns for this application and to develop appropriate recommendations. Nevertheless, the results of this study highlight that bitterns can be used as an effective additive for fish taste preservation.

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