A sensitivity-enhanced heterologous immunochromatographic assay based on monoclonal antibody for the rapid detection of histamine in saury samples

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Abstract: Histamine (HA) is an essential test item for fishery samples. However, the fast and effective determination of HA is difficult due to its simple structure and small molecule. In this study, a sensitive and specific monoclonal antibody against \( p \)-nitrobenzoylated histamine (NPHA), which can be easily obtained from the reaction of HA and \( p \)-nitrobenzoic acid N-hydroxysuccinimide ester (PNBA-OSu) under mild condition, was generated for the first time. Based on this mAb, an immunochromatographic assay strip (ICA strip) using colloidal gold nanoparticles-antibody (GNPs) probe for rapid detection of HA in saury samples was established. After screening the coating antigens and optimization of analytical parameters, a heterologous coating based ICA strip exhibited the most excellent detection ability with a visual detection limit (VDL) of 6.0 mg kg\(^{-1}\) in qualitative experiment and a detection of limit (by strip reader) of 1.0 mg kg\(^{-1}\) in semi-quantitative experiment for fish samples, with no cross-reactivity with HA analogs. Good correlation between the ICA strip with liquid chromatography-tandem mass spectrometry was achieved for spiked and naturally contaminated saury samples. Overall, this method is suitable for screening of HA residue for a large scale of fish samples in a quick, simple and low-cost manner.
1. Introduction

Histamine (HA) is one of the most important biogenic amines related to human health for HA poisoning characterized by headache, nausea, vomiting, diarrhea, itching, an oral burning sensation, red rash, and hypotension resulting from excessive intake of it. However, HA accumulation was commonly observed in tissues of fish and other seafoods when spoilage by bacteria commenced during storage, which doesn’t influence the seafood normal appearance and odor initially. Therefore, to avoid serious risk to human health, regulatory levels for HA in fishery products have been set by many countries and organizations thus far. China has regulated HA in mackerel and other marine fish should not exceed 1000 mg kg\(^{-1}\) and 300 mg kg\(^{-1}\), respectively. The United States Food and Drug Administration has set 50 mg kg\(^{-1}\) to be maximum residual level (MRL) for HA in fish tissue. The European Union (EU) has regulated that the marketing into EU of fish and fishery products belonging to the families of Coryphaenidae, Scombridae, Clupeidae, and Eugraulidae were authorized after a systematic check of the compliance to European histamine limits was performed. Normally, HA content in foods is determined by chromatography analysis which requires extensive sample cleanup, cost equipment and professional staff and not suitable for high-throughput and on-site analysis. Antibody-based immunoassays are well-established and received rapid methods which have been developed for antibiotics, pesticides and other harmful chemicals rapid screening for their high sensitivity, rapidity, low cost and applicability for large numbers of samples.
However, the production of sensitive and specific antibody against HA is a big challenge due to the simple structure of HA without sufficient epitome to elicit sensitive and specific antibody. Mita et al. attempted to produce a specific antibody against HA through immunizing with rabbit with three conjugates of different HA derivatives with BSA, but failed. Another strategy is to generate antibodies against the derivatives of HA, such as p-benzoquinone-histamine, succinyl-glycinamide-histamine, then developed immunoassays for HA via a pre-derivatization. However, all the reported derivatization of HA suffered from some drawbacks: long time, low yield, along with side reactions, and unstable of products, etc.

In this work, 4-((2-(1H-imidazol-4-yl) ethyl) carbamoyl) benzoic acid (hapten1, see Fig. 1)-BSA conjugate was used as an immunogen to prepare a monoclonal antibody which showed good sensitivity and specificity against a stable HA derivative, p-nitrobenzoylated histamine (NPHA, Fig. 1), which can be easily formed from the reaction of HA and p-nitrobenzoic acid N-hydroxysuccinimide ester (PNBA-OSu, Fig. 1) under mild condition. Furthermore, GNPs-based ICA, which is the most stable and classic ICA and ideally suited as on-site screening tool for large scale of samples for its low-cost, simplicity and rapid result judgment (within 3~5min), was developed for HA detection. Several heterologous coating haptens were synthesized and used as heterologous coating to study their effect on the assay’s sensitivity. In addition, portable strip reader was introduced when performing result judgment which can exclude the subjectivity arising from visual assessment. To our knowledge, this is the
first report of successful qualitative and semi-quantitative detection of HA by using ICA.

2. Material and Methods

2.1. Reagent and instrumentals

Ovalbumin (OVA), bovine serum albumin (BSA) and chloroauric acid (HAuCl₄·4H₂O), Sheep anti-mouse IgG were provided by Sigma Corporation (St. Louis, USA). Histamine dihydrochloride, L-histidine, L-tryptophan, tryptamine hydrochloride, tyramine hydrochloride, phenethylamine hydrochloride, 4-formyl benzoic acid, and benzoic acid were obtained from Heowns Biochem Technologies Co. Ltd. (Tianjin, China). Trisodium citrate, 1-ethyl-3-(3-dimethylaminopropyl)-carbodi-imide hydrochloride (EDC), sodium borohydride, Dimethylformamide (DMF), were obtained from Aladdin Chemical Technology Co., Ltd. (Shanghai, China).

PNBA-OSu and NPHA were prepared according to previous work. Nitrocellulose (NC) membranes, glass–fiber, sample pad, absorbent a pad and release pad were purchased from Millipore (USA). The HGS 510 dispensing platform, HGS 201 guillotine cutter were supplied by Autokun (Hangzhou, China). Membrane strip reader (DY6510) was supplied by Wanlian Biotechnologies Co. Ltd. (Guangzhou, China). The transmission electron microscope (JEM-2100F) was purchased from JEOL (Japan). The ultraviolet spectrophotometer (UV-3010) was purchased from Hitachi Corporation (Tokyo, Japan).

2.2. Preparation of HA derivatives and hapten-protein conjugates

Benzoic acid and five HA derivatives (Fig. 2, denoted as hapten1~6) with spacers
ending in a carboxyl group and were employed as haptens. The synthetic procedures of hapten1~5 were described in detail at supplemental information (See supplemental information Fig. S1). Hapten6 is commercial benzoic acid. Via the carboxyl group, these haptens were conjugated with the amino groups of BSA or OVA by the carbodiimide method according to previous work with some modifications. Briefly, a hapten (0.1 mmol) and carrier protein (10 mg) were added in the conjugation buffer (PBS, pH 6.0, containing 5% (v/v) DMF). The obtained solution was cooled to 0 °C at refrigerator, and EDC (32 mg) was added under agitation. The mixture was kept at 0 °C overnight under agitation, and then the conjugation mixture was dialyzed against 10 mM PBS (4×5 L) at 4 °C for 3 days, and finally stored at –20 °C until used.

2.3 Production of anti-NPHA mAb

The production of mAb was performed as previously described. The animal experiments were performed according to the Regulation Guideline for Experimental Animals issued by the Ministry of Science and Technology of China. Briefly, three BABL/c female mice aged 7 weeks were immunized with the immunogen (hapten1-BSA) on days 0, 28, 49 and 71. The mouse that exhibited the best titer for the immunogen was chosen as the donor of spleen cells for hybridoma production. Through cell fusion technology, the above spleen cells were fused with SP2/0 murine myeloma cells to form hybridomas. Then hybridomas secreting NPHA specific antibodies were subcloned five times by limiting dilution. The best antibody-producing clone (2G6/S5) was expanded and selected to produce ascetic antibodies. The obtained ascetics fluids were purified by caprylic acid-ammonium
method, and anti-NPHA mAb was obtained. Two ELISA formats, indirect ELISA (infELISA) and indirect competitive ELISA (ic-ELISA) as previously described were performed to screen target hybridoma and characterize the mAb’s binding with coating antigens.

2.4 Preparation of anti-NPHA-mAb-GNPs conjugate

GNPs with an average diameter of 35~40 nm was produced using the sodium citrate method. Briefly, 100 mL of 0.01% HAuCl₄ solution in ultra-purified water was boiled thoroughly for 1 min, and then 1.5 mL of 1% sodium citrate solution was rapidly added under continuous agitation, and then the color gradually changed from light yellow to black, and finally to brilliant red. After the color change, the solution was boiled for another 3 min, cooled and preserved at 4 °C with 0.05% sodium azide.

Transmission electron microscopy (TEM) was employed to characterize the GNPs. To conjugate the GNPs with anti-NPHA-mAb, the optimum mAb amount for labeling was investigated, and the results was present in Fig S2. As a result, 24 µL of mAb was added dropwise to 10 mL of GNPs (pH 8.5, adjusted by 0.1 M K₂CO₃) and kept at room temperature for 30 min with agitation. Then, 1.2 mL of 10% BSA was added dropwise to block residual surfaces of the GNPs. The mixture was further incubated for 30 min at room temperature and centrifuged for 15 min at 10000 rpm, and then the supernatant was discarded. The precipitate was resuspended in 1 mL of PB (0.05M, pH 7.4, containing 0.5 % BSA, 1% mycose, 6 % sucrose, and 0.1 % Tween-20) and stored at 4 °C until use.

2.5 Development of the GNPs-based ICA strip for HA
An ICA strip was assembled by five parts including PVC plate, three pads (sample, release, and absorbent pads) and one nitrocellulose (NC) membrane (Fig. 1). The coating antigen and sheep anti-mouse IgG were dispensed onto NC as the test (T) and control (C) line, respectively. The anti-NPHA-mAb-GNPs conjugate was dispensed by Biostrip dispenser HGS510 onto glass fiber pad to form the release pad. Then the release pad was dried over night at 37 °C. The sample pad was saturated with PB (0.05 M, pH 7.5, containing 0.1% PEG 20000, 0.1% Tween-20, 1% mycose, 6% sucrose) and dried at 37 °C overnight prior to use. For the absorbent pad, it was used directly without treatment. The assembly procedure was as follows: the NC membrane was pasted on the center of PVC plate, then the absorbent pad was pasted on it by over-crossing 1 mm on the top of the NC membrane, next, the release pad was pasted on it by over-crossing 1 mm on the bottom of the NC membrane, and then the sample pad was pasted by over-crossing 2 mm on the bottom of the release pad. At last, the whole assembled ICA strip was cut into 3 mm wide strips using HGS 201 guillotine cutter and stored in sealed plastic bags containing desiccant until use. Fig.1 shows the schematic illustration of the ICA strip.

**2.6 Sample preparation and test procedure**

Fish samples were purchased from local market in Guangzhou, China, and the preparation steps were as previously described with some modifications. Briefly, an equivalent weight of distill water (d.w.) was added to the muscles and homogenized, and then d.w. (8 mL) and the homogenate (2 g) were mixed and shaken vigorously for 1 min. The mixture was centrifuged at 3000 g for 10 min, and the supernatant (sample
extracts) was and subjected to the following derivatization steps: 200 µL of standard solution or sample extracts, 200 µL of borate buffer (pH 9.0) and 200 µL of PNBA-Osu solution (4 mg mL\(^{-1}\) in acetonitrile (ACN)) were mixed in a 1.5 mL conical tube and vortexed for 10 s. The reaction mixture was kept at room temperature for 20 min, and 100 mg of NaCl was added to separate the organic layer (ACN) from the aqueous layer. The organic layer was then diluted 20-fold in working solution (0.05 M PBS, pH 7.4). A 70 µL of the above diluted solution was added to sample pad. The result was judged by both naked eyes and D Y6510 strip reader within 3~5 min. As illustrated in Fig. 1, if the sample solution has the target analyte, the target analyte will compete the binding sites of mAb-GNPs with the coating antigen on T line, then the T line will be weak or not appeared, indicating the test result was weakly positive or positive; if no target analyte was contained in the sample solution, mAb-GNPs will fully bind to the coating antigen on T line, and the T line will display the strongest color, demonstrating the result was negative. While the C line must appear to indicate the test is valid.

2.7 Evaluation and validation of ICA strip

HA free fish matrices solution confirmed by previously proposed LC-MS/MS,\(^{18}\) was used to dilute HA standard stock solution (10 mg mL\(^{-1}\) in distill water) to form a serial concentrations (0~2000 µg L\(^{-1}\)) of HA containing matrices solutions. Then, these solutions were subjected to the above derivatization procedure and assayed by ICA strip to evaluate the sensitivity of the ICA strip for fish samples. Structure analogs (histidine, tyramine, phenethylamine and serotonin) at the concentration of 1, 5, 500
mg L\(^{-1}\) were used to test the specificity of the ICA strip. HA spiked fish samples and naturally contaminated fish samples were also assayed by the proposed ICA strip and confirmed by reference method (LC-MS/MS) to evaluate the accuracy and reliability of the ICA strip.

3. Results and discussion

3.1 Characterization of anti-NPHA mAb

Titer curves of the anti-NPHA mAb against six coating antigens were achieved by in-ELISA, and then dose-response curves for NPHA against theses effective coating antigens (titer>1.0×10\(^3\)) were obtained by ic-ELISA (Fig. S4A). As shown in Fig. S4A, the titer, which was defined as antibody dilution factor when the A\(_{450}\) value was at about 1.0, under homologous coating format (hapten1-OVA) was the highest (5.12×10\(^6\)). However, the titers under heterologus coating format exhibited degree of heterology dependent decrease compared to the titer under homologous coating format. From the viewpoint of geometry, hapten1, hapten2 and hapten3 share the same main structure, a 4-ethyl-1H-imidazole and a benzene ring, their difference only exists in the linking bond between benzene ring and 4-ethyl-1H-imidazole: the linking bond is amido bond (-CONH-) for hapten1, C-N double bond (-HC=N-) for hapten2, C-N single bond (-HC-NH-) for hapten3. Since amido bond (-CONH-) and C-N double bond (-HC=N-) are planar rigid bond, while C-N single bond (-HC-NH-) is a rotatable bond, therefore the structure of hapten3 is more flexible than hapten1 and hapten2, thus hapten3 was considered to be of higher degree of heterology with hapten1 than that of hapten2. Accordingly, higher titer (2.56×10\(^6\) versus 6.4×10\(^5\))
using hapten2-OVA coating than using hapten3-OVA was obtained. Meanwhile, the
anti-mAb NPHA shows no reactivity with hapten5-OVA and hapten6-OVA, and much
lower titer ($8 \times 10^3$) was achieved using hapten4-OVA coating (Fig. S4A). Thus, it
could be concluded that the benzene ring contained in hapten1, 2, 3 is conducive for
the antibody recognition, while the acylated HA moiety (Fig. 1 red part in
hapten1-OVA) is a more critical part for antibody recognition, which can be further
confirmed by the results of dose-response ic-ELISA curves (Fig. S4B). As shown in
Fig. S4B, the highest sensitivity was attained by using hapten3-OVA as coating
antigen with a $\text{IC}_{50}$ of 2.7 ng mL$^{-1}$, indicating the mAb has the lowest affinity to
hapten3-OVA. However, a relative higher $\text{IC}_{50}$ (3.1 ng mL$^{-1}$) was obtained by using
hapten4-OVA, which demonstrates that altering the acylated HA moiety of hapten1
imposes a more significant effect on antibody affinity decrease than altering benzene
ring moiety. In conclusion, hapten1, 2, 3, 4-OVA were effective coating antigens for
development of ELISA method for histamine based on the mAb, and the sensitivity of
the corresponding ELISA method on these coating antigens decreased in the
following order: hapten3-OVA, hapten4-OVA, hapten2-OVA, hapten1-OVA.
Furthermore, the specificity of the anti-NPHA mAb was also tested by ic-ELISA
under hapten4-OVA coating, and the results demonstrated that the anti-NPHA mAb
was highly specific to NPHA and can’t recognize histamine and p-nitrobenzoic acid
(Fig S4C).

3.2 Characterization of GNPs

GNPs of various sizes have been used to conjugate with antibody to prepare the ICA
probe in previous reports. However, according to several ICAs for small molecular
compound detection, diameter of 40 nm GNPs was the optimal particle size for its
trade-off between steric hindrance and required visibility. 24, 25, 26 As shown in Fig. S3,
the average diameter of the prepared GNPs was about 35–40 nm, which approximates
the optimal particle size. Meanwhile, the GNPs solution was stable by labeling with
optimal amount of anti-NPHA mAb and the immunoprobes present a clear, stable and
equally color on T with C line of NC membrane in the strip assay. These results
evidenced that the prepared GNPs was effective and could be applied in the following
experiments

3.3 Screening the coating antigen for ICA strip

Since immunoassays for small molecular compound (mono-epitope) are performed
based on competitive format, impairing the binding ability between antibody and
competitor antigen (coating antigen) usually improved assay sensitivity. Therefore,
four coating antigens, hapten1, 2, 3, 4-OVA, which showed reactivity with anit-NPHA
mAb in ELISA were coated on the T line, respectively, to study their effect on the
sensitivity of the ICA strip. Consequently, hapten3,4-OVA were failed to develop a
ICA strip, because faint color line (hapten3-OVA) or no visible line (hapten4-OVA)
were appeared in T line for negative simple (data not shown). However, for T line
coated with hapten1-OVA or hapten2-OVA, clear red color, which is of equably
intensity to that on C line, was appeared for negative simple. After optimization the
coating concentration (hapten1-OVA 0.5 mg mL⁻¹, hapten2-OVA 2 mg mL⁻¹), serial
concentrations of NPHA standard solutions (0–400 ng mL⁻¹ in PBS) were tested the
sensitivity under hapten1-OVA and hapten2-OVA coating. As shown in Fig. 3, the sensitivity under hapten2-OVA coating (heterologous) improved about 8-fold compared to that under hapten1-OVA coating (homologous). These results indicated that heterologous coating was also effective in enhancing sensitivity of ICA strip, while some coating haptens (hapten3, hapten4) having higher degree of heterology with immunogen hapten, which could be applied in developing highly sensitive ELISA can be ineffective for ICA strip, as the antibody’s binding ability may decline due to the steric hindrance after conjugated with GNPs.

3.4 Optimization of analytical parameters for ICA test
As an antibody-antigen reaction based immunoassay, ICA was largely influenced by ionic strength, pH and organic solvents concentration in test solution. Thus PBS of different PO₄³⁻ concentrations (0.01–0.1M) and pH values (6.2–10.0) containing different concentrations of Tween-20 (0–0.1%) were used as working solution to prepare a series of concentrations of NPHA standard solutions from NPHA stock solution (1 mg mL⁻¹ in methanol), respectively. Then, these standard solutions were tested by the ICA strip. As shown in Table S1, Tween-20 contained in working solution can decrease the sensitivity of ICA strip. Furthermore, it was found PBS (0.05M, pH7.4) results in the most sensitive ICA test (Table S2, Table S3). Thus, PBS (0.05M, pH7.4) was chosen as the working solution. To evaluate the organic solvent susceptibility of the ICA strip, the working solution (PBS, 0.05M, pH7.4) containing different concentrations of ACN (0–20%, v/v) was used to dilute NPHA stock solution (1 mg mL⁻¹ in methanol) to form a series concentration of NPHA standard
solutions, and then the obtained NPHA standard solutions were tested by the ICA strip.

As shown in Table S4, the sensitivity of the ICA strip was decreased significantly as the concentration of ACN increased, and the maximum ACN tolerance level for the ICA strip was 5% (Table. S4). Thus, the upper layer of derivatization reaction mixture was required to be diluted 20-fold by PBS (0.05M, pH7.4) for the ICA test.

3.5 Sensitivity and specificity of ICA

As shown in Fig. 4, the red color of T line became weaker as the concentration of HA increased. When HA concentration exceeded 600 ng mL$^{-1}$ (corresponding to 6.0 mg kg$^{-1}$ in fish sample according to the extraction procedure in sample preparation), the T line completely disappeared, thus, 600 ng/mL (corresponding to 6.0 mg/kg in fish sample according to the extraction procedure in sample preparation) was confirmed to be visual detection limit (VDL) of the ICA strip. Meanwhile, color intensity of T line ($A_T$) and C line ($A_C$) were measured by DY 6510 strip reader. The $A_T$/$A_C$ ratio of negative and positive samples were designated as $B_0$ and $B_x$. Calibration curve constructed by plotting the $B/B_0$ against logarithm of HA concentration (Fig. S5). As the color intensity of T line was significantly different ($B/B_0$ 0.5) with that of blank sample (HA free matrices solution), when HA concentration was at 100 ng mL$^{-1}$ (1 mg kg$^{-1}$ in fish sample). Thus, 1 mg kg$^{-1}$ was confirmed to be limit of detection (LOD) for the ICA strip in this study.

Since the MRLs for HA in fish varied from 50 mg kg$^{-1}$ to 1000 mg kg$^{-1}$, it seems that the proposed ICA strip is much too sensitive. However, it is necessary to develop a screening method having a LOD lower than MRLs. For one thing, to make the
working range of the method suitable for the varied MRLs set by different countries or districts, only suitable dilution factors are needed to be introduced in sample pretreatment; for another, larger dilution factor is required for sample pretreatment to remove matrices effect, when analyzing samples having more complex matrices. Several HA analogs were used to investigate the specificity of the ICA strip, while the color intensity of T line were almost identical with that of negative control (distill water), when these competitors at 1, 5, 500 mg L\(^{-1}\), indicating that the ICA strip was highly specific for HA with negligible cross-reactivity to these analogs (Table 1).

3.6 Analysis of HA in saury samples

Saury, a kind of fish belonging to family of Scombridae, which was reported to be potential histamine poisoning source, \(^2\) is commonly consumed in Guangzhou, China. Therefore, saury was chosen as a model matrices for the proposed ICA strip and a saury sample which initially contains 0.23 mg kg\(^{-1}\) of HA (confirmed by LC-MS/MS) was spiked with HA at the level of 2.0, 5.0 and 10.0 mg kg\(^{-1}\), and then the non-spiked and spiked samples were tested by the proposed ICA strip (Fig 5) and LC-MS/MS, respectively to evaluate the accuracy of the test strip. As shown Table 2, the results of the ICA strip correlate well with that of LC-MS/MS. Another saury sample was immediately pretreated (washing and removal of inedible part) after purchased from local supermarket, and its muscle was stored at 4°C for using as naturally contaminated sample. Then, it was subjected to HA analysis every two days by the proposed ICA strip and LC-MS/MS. As shown in Table 3, HA content in this saury was sharply increased to 224.5 mg kg\(^{-1}\) in the sixth days, which even surpasses the
regulated level set by FDA (50 mg kg\(^{-1}\)).

As a small molecular amine compound having strong polarity, HA is highly aqueous soluble, which is seriously adverse to the sample purification prior to analysis, since routine organic solvents cannot extract it from aqueous solution, thus, cation ion-exchange, which is a labor-intensive and time-consuming operation, was often involved in the sample purification for some instrumental methods for HA detection.

Although a derivatization step was required for the proposed ICA strip test, the whole sample preparation was simple and can be completed within 35 min, and all the used equipments can be portable, which benefit from a moderately polar compound, NPHA, was formed after HA interacted with PNBA-OSu (derivatizing reagent); since the NPHA is of bad water-solubility and good solubility in ACN, thus the formed NPHA was contained in the upper layer (ACN layer) and separated from most of interfering substances and can be tested directly via a pre-dilution by working solution after a certain amount of NaCl was added to the derivatization mixture solution (ACN-borate buffer mixtures). Thus, the proposed ICA strip can be used as an important tool to achieve rapid detection of HA in fish samples.

4. Conclusion

In this work, a stable hybridoma cell line generating mAb against NPHA was developed, which can be an unlimited supply of mAb for NPHA. Then, a sensitivity enhanced heterologous ICA based on this mAb for rapid HA detection was established and applied in assaying HA spiked saury samples and naturally contaminated saury samples. The ICA showed a VDL of 6.0 mg kg\(^{-1}\) and a LOD of 1.0 mg kg\(^{-1}\) (by strip
reader) for saury samples, with no cross-reactivity with other HA related compounds. Moreover, the results obtained from the ICA strip was in agreement with that from LC-MS/MS. Thus, the ICA strip was suited as a tool for rapid HA on-site screening, and ICA may also be developed for other biogenic amines by referring to the strategies involved in this study. In addition, the heterologous coating should be adopted as a potential general strategy for improving sensitivity when developing ICA based detection method for small molecules.

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Notes and references


**FIGURE CAPTIONS**

Fig. 1. Schematic diagram of the immunochromatographic assay for HA detection.
Fig. 2. Schematic diagram of coating antigens, hapten-1-OVA conjugate was homologous coating antigen; hapten-2, 3, 4, 5, 6-OVA conjugates were used as heterologous coating antigens.

Fig. 3. The NPHA sensing results of ICA strip under hapten-1-OVA (left) and hapten-2-OVA coating (right). The concentration of NPHA standard solutions (diluted from stock solution by PBS) were 0, 20, 50, 100, 200, 400 ng mL\(^{-1}\) from left to right.

Fig. 4. Sensitivity of ICA for fish samples. The test results of a serial concentrations of HA standard solution (prepared by HA free matrices solution). From left to right, the HA concentration was 0, 10, 20, 40, 60, 80, 100, 200, 400, 600, 800, 1000, 2000 ng mL\(^{-1}\).

Fig. 5. Results of spiked fish sample assayed by ICA strip. 0.23 mg kg\(^{-1}\) was confirmed to be the initial HA concentration of the fish sample by LC-MS/MS. Then the fish sample were spiked with 2.0, 5.0, 10.0 mg kg\(^{-1}\) of HA.
Figure 1
Figure 2

Coating antigen 1 (hapten 1-OVA)

Coating antigen 2 (hapten 2-OVA)

Coating antigen 3 (hapten 3-OVA)

Coating antigen 4 (hapten 4-OVA)

Coating antigen 5 (hapten 5-OVA)

Coating antigen 6 (hapten 6-OVA)
Figure 3
Figure 4
Figure 5
Table 1. Cross reactivity (CR) with histamine related compounds by the ICA strip

<table>
<thead>
<tr>
<th>compounds</th>
<th>structure</th>
<th>Concentration (mg/L)</th>
<th>CR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Histamine</td>
<td><img src="image" alt="Histamine structure" /></td>
<td>1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.03</td>
</tr>
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<td>Histidine</td>
<td><img src="image" alt="Histidine structure" /></td>
<td>1</td>
<td>0.97</td>
</tr>
<tr>
<td>Trytamine</td>
<td><img src="image" alt="Trytamine structure" /></td>
<td>1</td>
<td>0.98</td>
</tr>
<tr>
<td>Tryamine</td>
<td><img src="image" alt="Tryamine structure" /></td>
<td>1</td>
<td>0.98</td>
</tr>
<tr>
<td>Phenethylamine</td>
<td><img src="image" alt="Phenethylamine structure" /></td>
<td>1</td>
<td>0.96</td>
</tr>
</tbody>
</table>

<sup>a</sup> CR, cross-reactivity. The percentage of CR was calculated by the following equation:

\[
CR(\%) = \left(\frac{IC_{50}(\text{histamine}, \text{mmol/L})}{IC_{50}(\text{cross-reactant}, \text{mmol/L})}\right) \times 100.
\]

<sup>b</sup> Recorded B B<sub>0.01</sub> by strip reader
Table 2. Results of spiked fish sample analyzed by ICA strip and LC-MS/MS (n=3)

<table>
<thead>
<tr>
<th>Spiked (mg/kg)</th>
<th>ICA $^a$ (mg kg$^{-1}$)</th>
<th>LC-MS/MS (mg kg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>_ $^b$</td>
<td>0.23</td>
</tr>
<tr>
<td>2</td>
<td>2.0</td>
<td>2.30</td>
</tr>
<tr>
<td>5</td>
<td>6.2</td>
<td>5.27</td>
</tr>
<tr>
<td>10</td>
<td>11.8</td>
<td>10.30</td>
</tr>
</tbody>
</table>

$^a$ HA concentration was extrapolated from the calibration curve

$^b$ HA concentration lower than the LOD
Table 3. Results of monitoring HA formation in saury by ICA strip and LC-MS/MS (n=3)

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>ICA(^a) (mg kg(^{-1}))</th>
<th>LC-MS/MS (mg kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>(-)^ b</td>
<td>0.76</td>
</tr>
<tr>
<td>2</td>
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<td>4</td>
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<tr>
<td>6</td>
<td>260.0</td>
<td>224.52</td>
</tr>
</tbody>
</table>

\(^a\) HA concentration was extrapolated from the calibration curve

\(^b\) HA concentration lower than the LOD