Rapid determination of gizzerosine in fish meals using microchip capillary electrophoresis with laser-induced fluorescence detection

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ABSTRACT
Sensitive detection of gizzerosine, a causative agent for deadly gizzard erosion in chicken feeds, is very important to the poultry industry. In this work, a new method was developed based on microchip capillary electrophoresis (MCE) with laser-induced fluorescence (LIF) detection for rapid analysis of gizzerosine, a biogenic amine in fish meals. The MCE separation was performed on a glass microchip using sodium dodecyl sulfate (SDS) as dynamic coating modifier. Separation conditions, including running buffer pH and concentration, SDS concentration, and the separation voltage were investigated to achieve fast and sensitive quantification of gizzerosine. The assay proposed was very quick and could be completed within 65 s. A linear calibration curve was obtained in the range from 0.04 to 1.8 μg ml\textsuperscript{-1} gizzerosine. The detection limit was 0.025 μg ml\textsuperscript{-1} (0.025 mg kg\textsuperscript{-1}), which was far more sensitive than those previously reported. Gizzerosine was well separated from other endogenous components in fish meal samples. Recovery of gizzerosine from this sample matrix (n = 3) was determined to be 97.2–102.8%. The results from analysing fish meal samples indicated that the present MCE-LIF method might hold the potential for rapid detection of gizzerosine in poultry feeds.

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Introduction
Gizzerosine (2-amino-9-(4-imidazolyl)-7-azanonoic acid; Figure 1) was first found in brown fish meal and considered to be a causative agent for a deadly poultry disease characterised by gizzard erosion, ulceration or 'black vomit' disease (Mori et al. 1983; Okazaki et al. 1983). Gizzerosine-containing fish meals have caused serious economic loss in the poultry industry due to mortalities or poor productive performance (Fossum et al. 1988; Wessels & Post 1989). This biogenic amine is derived from the reaction between the amino group of lysine and the imidazole ring of histidine during an overheated fish meal manufacturing process (Okazaki et al. 1983). Gizzerosine, as well as histamine, is a histamine H\textsubscript{2}-receptor agonist and is about 10 times more capable of inducing gastric acid secretion in chicks than histamine, and a high level of gastric acid was found to cause gizzard erosion in chickens (Masumura et al. 1985; Hino et al. 1987; Ito et al. 1988). One of these studies also revealed that oral or intravenous administration of gizzerosine in young chicks lowered the pH of the gastric and duodenal contents and severe symptoms of gizzard erosion were observed (Masumura et al. 1985). In practical diets, a gizzerosine level of less than 0.4 mg kg\textsuperscript{-1} was recommended (Sugahara et al. 1988). Since fish meal is a major component of chicken feeds, timely and readily measurement of this unexpected by-product during feedstuff production is of vital significance for the poultry industry. Several analytical methods have been reported for the quantification of gizzerosine. Pre- or post-column derivatisation of gizzerosine with o-phthalaldehyde (OPA) coupled with HPLC detection has been reported (Ito et al. 1985; Ohta et al. 1988; Murakita & Gotoh 1990). Radioimmunoassays (RIA) with monoclonal or polyclonal antibody labelling are sensitive analytical methods for gizzerosine identification (Rosselot et al. 1996; Torres et al. 1999); however, specific antibody must be prepared through lengthy immunisation of rabbits and radioactive labels were harmful to both operators and the environment. ELISA has been...
Miniaturnised analytical platforms, also known as micro-total analysis systems (μTAS) or lab-on-a-chip (LoC), have been widely appreciated due to their unparalleled merits in reducing sample reagent, power consumption and waste, faster determination, higher sensitivity, and the ease of integrating diverse functional components into a miniaturised disposable system (Figeys & Pinto 2000; Andersson & Berg 2004; Hongbin et al. 2009). Among them, microchip capillary electrophoresis (MCE), a miniaturised version of capillary electrophoresis (CE) equipped with laser-induced fluorescence (LIF) detection, has been widely welcomed in the fields of biochemistry and analytical chemistry research (Nikcevic et al. 2010; Huang et al. 2011a, 2011b). MCE-LIF detection has exhibited outstanding performance characteristics compared with other detection systems with respect to high sensitivity, wide linearity and good stability for the analysis of complex biological samples.

In this work we developed a new method based on MCE-LIF to detect gizzerosine. Gizzerosine was fluorescently tagged with fluorescein isothiocyanate (FITC) before being subjected to MCE separation and detected by LIF. Effects of voltage applied on the reservoirs, pH and concentration of running buffer, and SDS concentration on the separation performance of MCE were investigated to obtain the optimised separation conditions. Assay linearity, LOD and precision were evaluated. This method was applied to determine gizzerosine in fish meal samples. To the best of our knowledge, this is the first report of using MCE-LIF to analyse quantitatively gizzerosine in poultry feeds.

Materials and methods

Chemicals and solutions

All chemicals used were of analytical grade or higher. All solutions were filtered through 0.22-μm membrane filters before use. Double-distilled water (ddH2O) (Ulupure, Chengdu, China) was used for aqueous solution preparation. Gizzerosine and fish meal samples were kindly provided by Tongwei Group Co., Ltd. (Chengdu, China). FITC was purchased from Sigma (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS), sodium borate, sodium hydroxide (NaOH) and hydrochloric acid (HCl) were purchased from Tianjin Damao Chemical Reagent Factory (Tianjin, China). A 10 mM FITC solution (dissolved in 95% acetone with 0.01% pyridine) was used as a derivatisation reagent. Standard stock solution (2.4 mg ml−1 gizzerosine) was prepared by dissolving an appropriate amount of analyte in ddH2O and storing in the dark at 4°C. Calibration standards were prepared by serial diluting standard stock solution. Borate buffer solution was used as an electrophoretic running buffer.

Apparatus and microfluidic chip

The assays were performed on a microchip electrophoresis-confocal LIF detection system with excitation wavelength at 473 nm and emission wavelength at 520 nm (Shandong Normal University, Jinan, China) and a six-path high-voltage power supply, variable in the range of 0–5000 V (Shandong Normal University, Jinan, China) was employed for sample loading and MCE separation. Figure 2 shows schematic layout and dimensions of the glass microchip. Channels were etched in the bottom glass substrate 70 μm in width and 25 μm in depth. The buffer waste reservoir (BW) was 45 mm downside from intersection and the other three were 5 mm from it. Four reservoirs in the upper glass substrate were drilled 3 mm in diameter and 1.5 mm in depth.

Figure 1. Chemical structure of gizzerosine.
Fish meal sample preparation

Since no certified gizzerosine-containing reference material is available, method validation had to be based on recovery tests for the assessment of accuracy and repeatability. Fish meal sample was treated with 6 M HCl in a 110°C incubator for 22 h, and then the pH adjusted to 7.0 by 3 M NaOH. For spike-recovery assays, serial diluted gizzerosine solutions were added into pH-adjusted lysates. ProElut™ Melamine Special Extraction Cartridge (Dikma Technologies Inc., Lake Forest, CA, USA) was rinsed with 3 ml of methanol and 3 ml of H$_2$O. The lysate was loaded at a flow rate of 1 ml min$^{-1}$. After rinsing with methanol and H$_2$O, the cartridge was eluted with 3 ml of 5% NH$_3$·H$_2$O. The eluent was concentrated to dryness under vacuum in a rotary evaporator at 50°C. The residues was redissolved in 1 ml of 20% methanol-H$_2$O solution and stored in the dark at 4°C.

Derivatisation procedure

Appropriate diluted standard solution and/or fish meal samples (10 μl) and FITC solution (10 μl) were added to 80 μl of borate buffer (50 mM). The reaction was performed overnight in the dark at RT. Derivatisation solutions were kept in the dark at 4°C.

Microchip electrophoresis

Chips were conditioned before use with 3 M NaOH for 60 min. Between runs, microchips were sequentially rinsed with 10 μl 1 M NaOH, H$_2$O, 50 mM SDS, H$_2$O, and running buffer for 3, 1, 1, 1 and 3 min, respectively. The sample reservoir (S) was then filled with sample solution and the other reservoirs with running buffer. Four platinum electrodes were inserted perpendicularly into four reservoirs. Sample was introduced into the intersection by pinched injection mode: 400 V at S reservoir, 250 V at buffer reservoir (B), 400 V at BW reservoir, with the sample waste reservoir (SW) grounded. Sample loading was for 30 s and then the potentials were switched into separation mode: 1000 V at S reservoir, 1500 V at B reservoir, 1000 V at SW reservoir and BW grounded (Table 1). Separation mode lasted for 120 s and the effective separation length was 35 mm.

Results and discussion

Surface modification of microfluidic glass channels

MCE separation was carried out using the combination of two electrokinetic effects: electro-osmosis and electrophoresis. When an external electric field is applied, the former plays a dominant role in propelling analytes towards the detection window and the latter discriminates molecules with different charges (cationic, neutral and anionic) (Landers 2007). In the microfluidic channels, a higher surface area-to-volume ratio (S/V) leads to more interactions between analytes and wall surface, which tends to cause uncontrolled electro-osmotic flow (EOF). As documented in many CE works, uncontrolled EOF is detrimental to a reproducible and reliable CE separation, and thus should be restrained. Covalent coverage or dynamic coating offer efficient approaches in the attempt to tailor EOF and enhance resolution (Belder & Ludwig 2003). Dynamic coating is much more widely used due to its simplicity; surface-active compounds such as surfactants or polymers are applied within a conditioning step before analysis and/or are added to the electrophoretic buffer. Our initial tests

Table 1. Optimisation of voltage applied in injection and separation modes.

| Voltage programme | S (V) | SW (V) | B (V) | BW (V) | Time (s) |
|-------------------|-------|--------|-------|--------|----------|
| Injection mode    | 400   | 0      | 250   | 400    | 30       |
| Separation mode   | 1000  | 1000   | 1500  | 0      | 90       |

Note: B, buffer reservoir; BW, buffer waste reservoir; S, sample reservoir; SW, sample waste reservoir.
performed without any surface treatment resulted in drastically varied migration time and peak area from run to run. Therefore, a commonly used anionic surfactant, SDS, was utilised to rinse the capillary channel for dynamic coating of the microchip prior to the analysis. The hydrophobic carbon chain of SDS is preferentially absorbed on the capillary wall, reducing interaction of analytes with the glass channels. Concentrations of SDS ranging from 10 to 100 mM were investigated, and each concentration was evaluated five times. The repeatabilities of migration time and peak areas are shown in Table 2. It is obvious that the increase in the SDS concentration contributed tremendously to obtaining reproducible migration time and peak area, and 50 mM of SDS provided the best result. However, when the SDS concentration was higher than 50 mM, decreased resolution and higher background noise were observed. Therefore, 50 mM was chosen for dynamic coating throughout the experiments.

**Optimisation of running buffer pH**

The pH and concentration of the buffer affect the thickness of the electrical double layer and zeta potentials on the channel wall, which determine the velocity of EOF (Harrison et al. 1993; Whitesides & Stroock 2001). The effects of concentration (10–100 mM) and pH of borate buffer (8.5–10.0) as a background electrolyte were evaluated. The results demonstrated that a 50 mM borate buffer with pH 9.3 was considered suitable for this analysis.

**Optimisation of separation voltage**

The effects of applied voltage on the separation performance were also investigated. Generally, higher voltage brings better resolution. Separation voltages from 1000 to 2000 V were evaluated in this work. It was shown that although a higher voltage led to a decrease in migration time, the results were unrepeatable. This was perhaps because under a higher electric field strength a larger joule heating was produced in the separation channel, resulting in the broadening of the zone and a decreasing of the separation efficiency. Taking migration time and resolution into account, a 1500 V separation voltage was chosen. Based on the optimised conditions mentioned above, the final separation parameters were decided as follows: an extra rinsing step with 50 mM SDS; a separation voltage of 1500 V; and a running buffer of 50 mM borate buffer with pH 9.3. A representative electropherogram is shown in Figure 3.

**Analytical figures of merit**

Assay linearity, LOD and precision were assessed. Five successive runs were performed and the RSDs of migration time and peak area were determined. A seven-point linear calibration curve was established by calculating the serial dilutions of gizzerosine standard stock solution ranging of 0.048–1.8 μgm l\(^{-1}\) (0.2–7.5 μM). Linear regression analysis of the tests yielded the following equation:

\[
y = 5.2262x + 0.8567, R^2 = 0.9917
\]

where \(y\) is the peak area (arbitrary unit, a.u.); and \(x\) is the gizzerosine concentration (μgm l\(^{-1}\)). The LOD was calculated to be 0.025 μgm l\(^{-1}\) based on \(S/N = 3\), which was comparable with a gizzerosine concentration of 0.025 mg kg\(^{-1}\). This limit was 10 times lower than that obtained by HILIC-MS/MS (0.25 mg kg\(^{-1}\)) and far more sensitive than HPLC methods (1 or 0.5 mg kg\(^{-1}\)) (Ohta et al. 1988; Murakita & Gotoh 1990; Uhlig et al. 2011). The LOQ was calculated as 0.083 μgm l\(^{-1}\). RSDs were used in the evaluation of migration time and peak area precision tests. Gizzerosine solutions (1.20 and

**Table 2.** Different concentrations of sodium dodecyl sulfate (SDS) on the repeatabilities of migration time and peak areas.

| SDS concentration (mM) | 0   | 10  | 25  | 50  | 75  | 100 |
|------------------------|-----|-----|-----|-----|-----|-----|
| Migration time (RSD)   | 11.7% | 6.6% | 5.7% | 4.5% | 4.8% | 5.1% |
| Peak areas (RSD)       | 14.5% | 5.8% | 6.4% | 4.7% | 5.3% | 5.2% |

**Figure 3.** Electropherogram obtained from separating a fluorescein isothiocyanate (FITC)-labelled gizzerosine solution (1 μM) on sodium dodecyl sulfate (SDS) surface-modified glass chips. Microchip capillary electrophoresis (MCE) separation conditions: 50 mM borate buffer (pH 9.3); separation voltage, 1500 V.
0.12 μg ml⁻¹) were analysed and the results suggested that RSDs of run-to-run migration time and peak area were less than 4.6% and 5.3%, respectively (Table 3). The LOD and precision results suggested that this method was capable of detecting a gizzerosine concentration as low as 0.4 mg kg⁻¹, the recommended maximum concentration of gizzerosine in poultry diets.

**Analysis of fish meal samples**

More accurate and rapid measurements of gizzerosine offer more thorough prevention of losses in poultry production. Since no certified gizzerosine-containing reference material is available, the method was applied to fish meal samples spiked with serial diluted gizzerosine solutions. Samples with or without gizzerosine spike were processed and inspected under the optimised conditions. Comparisons of representative electropherograms are presented in Figure 4(a,b). As can be seen in the electropherograms, a clear peak, corresponding to the gizzerosine concentration of 0.12 μg ml⁻¹ (0.12 mg kg⁻¹), was observed within 65 s (Figure 4(b)). The gizzerosine peak was well separated from those interference peaks. The precision of the detection results was evaluated by repeatedly analysing each fish meal sample in triplicate within one working day. The results of three representative spiked samples are summarised in Table 4. The RSDs were less than 4.8%, and the recoveries were within the range of 97.2–102.8% (n = 3).

**Table 3.** Repeatabilities of migration times and peak areas of gizzerosine.

| Gizzerosine concentration (μg ml⁻¹) | Migration time RSD (%) | Peak area RSD (%) |
|--------------------------------------|------------------------|-------------------|
| 1.20                                  | 4.6%                   | 4.8%              |
| 0.12                                  | 4.1%                   | 5.3%              |

**Table 4.** Determination of gizzerosine in fish meal samples.

| Sample | Gizzerosine added (mg kg⁻¹) | Gizzerosine found (mg kg⁻¹) | Recovery (%) | RSD (%,
|--------|-----------------------------|-----------------------------|--------------|--------------------------|
| #1     | 1.200                       | 1.167                       | 97.2%        | 2.7%                     |
| #2     | 0.600                       | 0.587                       | 97.9%        | 4.8%                     |
| #3     | 0.120                       | 0.123                       | 102.8%       | 4.2%                     |

Note: *No gizzerosine was detected in these fish meal samples.*

**Conclusions**

We have developed a quick and sensitive analytical method for detecting gizzerosine in poultry feeds. It is based on MCE with LIF detection. FITC derivatisation of gizzerosine prior to MCE separation is deployed to achieve high assay sensitivity. Dynamic coating of the microfluidic glass channels with SDS significantly suppressed analyte adsorption, and thus improved MCE reproducibility and resolution. The proposed assay is advantageous in terms of reagent and sample consumption, analysis time, assay sensitivity, and applicability to complex samples such as fish meals. By using the present MCE-LIF method, gizzerosine at trace levels in poultry feeds can be quantified without needing pre-assay enrichment. In addition, the quantitative analysis can be completed within 65 s, which is remarkably faster than any other gizzerosine assays previously reported. The proposed assay may become a powerful tool for fast detection of gizzerosine in poultry feeds.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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