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Rapid screening of flonicamid residues in environmental and agricultural samples by a sensitive enzyme immunoassay

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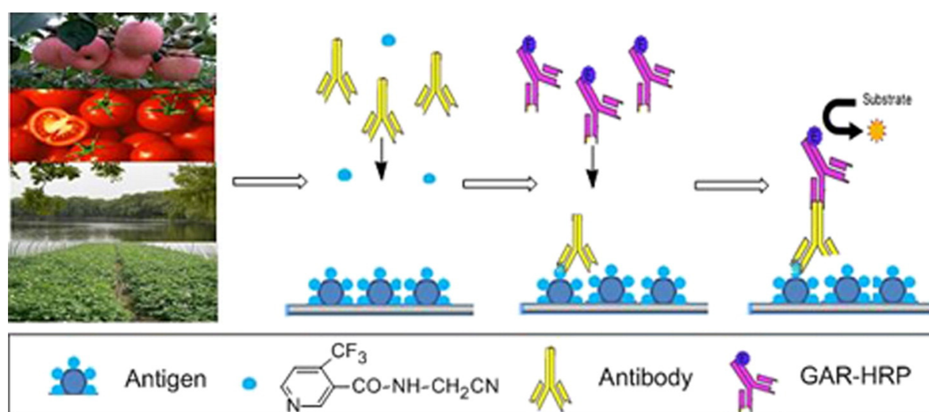
HIGHLIGHTS

- The antibody of flonicamid was obtained.
- A high-throughput, selective and simple ELISA for flonicamid was developed.
- The results of ELISA for the spiked samples were largely consistent with the gas chromatography method.
- This methodology appeared to be useful as a screening method prior to flonicamid analysis.

GRAPHICAL ABSTRACT



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ABSTRACT

A fast and sensitive polyclonal antibody-based enzyme-linked immunosorbent assay (ELISA) was developed for the analysis of flonicamid in environmental and agricultural samples. Two haptens of flonicamid differing in spacer arm length were synthesized and conjugated to proteins to be used as immunogens for the production of polyclonal antibodies. To obtain most sensitive combination of antibody/coating antigen, two antibodies were separately screened by homologous and heterologous assays. After optimization, the flonicamid ELISA showed that the 50% inhibitory concentration (IC_{50} value) was 3.86 mg L^{-1}, and the limit of detection (IC_{20} value) was 0.032 mg L^{-1}. There was no cross-reactivity to similar tested compounds. The recoveries obtained after the addition of standard flonicamid to the samples, including water, soil, carrot, apple and tomato, ranged from 79.3% to 116.4%. Moreover, the results of the ELISA for the spiked samples were largely consistent with the gas chromatography ($R^2 = 0.9891$). The data showed that the proposed ELISA is an alternative tool for rapid, sensitive and accurate monitoring of flonicamid in environmental and agricultural samples.

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1. Introduction

Flonicamid (*N*-cyanomethyl-4-trifluoromethylnicotinamide) is a novel selective systemic pesticide (Tomlin, 2003), which has been widely applied to rice, leafy vegetables, tomato and tea to control

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noxious insects, with high effectiveness against aphids and other sucking insects (Morita et al., 2000; Hengel and Miller, 2007). As a result, flonicamid is present in river water, soil and agricultural products. To protect consumers from risks related to flonicamid residue, maximum residue limits (MRLs) of flonicamid in agricultural samples have been established by the USA (0.45 mg kg^{-1} for carrot) and Japan (0.4 mg kg^{-1} for tomato and 1 mg kg^{-1} for apple) (Xie et al., 2011). There are no suggested MRLs for flonicamid in China.

Several instrument-based detection methods for flonicamid have been developed, such as gas chromatography (GC) (Xie et al., 2011; Shi et al., 2015), high-performance liquid chromatography (HPLC) (Ma et al., 2015) and high-performance liquid chromatography–mass spectrometry (HPLC–MS) (Chen, 2002; Zywitz et al., 2003; Hengel and Miller, 2007; Chen et al., 2012; Ko et al., 2014). Although these methods are characterized by low limits of detection and high precision and sensitivity, they cannot meet the needs of the high-throughput, rapid, screening of large numbers of environmental and agricultural samples. Enzyme-linked immunosorbent assay (ELISA) fulfills these requirements and has become a reliable analytical tool for rapid screening analysis (Liu et al., 2013). ELISA has been successfully used to detect contaminates, including toxins (Sheng et al., 2012; Kawatsu et al., 2014), antibiotics (Adrian et al., 2012; Peng et al., 2012; Sheng et al., 2013; Wang et al., 2015) and pesticides (Gurbuz et al., 2009; Liu et al., 2011; Watanabe et al., 2011; Liu et al., 2013; Navarro et al., 2013; Abad-Fuentes et al., 2014; Hua et al., 2015). However, ELISA of flonicamid has not been reported. In this paper, a rapid and sensitive ELISA was developed for the detection of flonicamid residues in environmental and agricultural samples based on polyclonal antibodies. Furthermore, the ELISA performance was evaluated with conventional GC-NPD in terms of precision and accuracy using spiked samples.

2. Materials and methods

2.1. Reagents

Pesticide-grade flonicamid with a purity of 98.5% was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). 4-Trifluoromethylnicotinic acid (TFNA), 4-trifluoromethylnicotinamide (TFNA-AM) and *N*-(4-trifluoromethylnicotinoyl) glycine (TFNG) were purchased from Hayashi Pure Chemical Ind., Ltd (Osaka, Japan). Bovine serum albumin (BSA), ovalbumin (OVA), Freund's complete and incomplete adjuvants, goat *anti*-rabbit IgG-horseradish peroxidase (GAR-HRP), polyoxyethylene sorbitan monolaurate (Tween-20), 3,3′,5,5′-tetramethylbenzidine.

(TMB), *N*-hydroxysuccinimide (NHS), N,N′-dicyclohexylcarbodiimide (DCC) and isobutyl chloroformate were purchased from Sigma Chemical Co. (Shanghai, China). Aminoacetic acid and 4-aminobutyric acid were purchased from Aldrich (Milwaukee, USA). Acetone, acetonitrile, toluene, ethyl acetate, petroleum ether, $SOCl_2$, $NaHCO_3$, NaCl, Na_2CO_3 and so on were purchased from Beijing Chemical Reagent Co., Ltd (Beijing, China). All reagents and solvents were analytical grade.

2.2. Instruments

Nuclear magnetic resonance (NMR) spectra were recorded on a DRX 500 spectrometer (Bruker, Germany). Mass spectral (MS) data were obtained with a LC-MS^{QDECA} (Finnigan, USA). Ultraviolet spectra were recorded on a DU 800 spectrophotometer (Beckman, USA). The 96-well polystyrene microplates (Maxisorp) were purchased from Nunc (Roskilde, Denmark). Absorbance was read with an Infinite M200 microtiter plate reader (Tecan, Switzerland) at 450 nm, and the ELISA plates were washed with a Wellwash Plus (Thermo, USA). The flonicamid ELISA was confirmed with an Agilent 7890A gas chromatograph (Agilent, USA).

2.3. Buffers and solutions

The following buffers were used: (A) coating buffer, 0.05 mol L^{-1} carbonate-buffered saline (CBS), pH 9.6; (B) blocking buffer, 5 g skim milk in 100 mL of phosphate-buffered saline (PBS, 0.01 mol L^{-1}, pH 7.4); (C) washing buffer, PBS containing 0.05% Tween-20 (PBST); (D) the TMB solution contained 0.4 mmol L^{-1} TMB and 3 mmol L^{-1} H_2O_2 in citrate buffer (pH 5.0).

2.4. Hapten synthesis

The structure and synthesis route of 4-(trifluoromethyl)nicotinoyl chloride (**A**), Hapten 1 and Hapten 2 are shown in Fig. 1.

Compound **A:** 4-(trifluoromethyl)nicotinic acid [2 g (9.7 mmol)] and toluene (30 mL) were added to a 100-mL round-bottomed flask and were cooled in an ice bath. Then, 5.3 g (44.6 mmol) of $SOCl_2$ was slowly added to the solution. After the addition, the temperature was increased to 45 °C for 2 h and was then gradually increased to 80 °C for 1 h. Finally, unreacted reagents were removed by a rotary evaporator, and brown liquid **A** (1.87 g) was obtained.

Hapten 1: A mixture of 1.3 g (175 mmol) of aminoacetic acid and 17.65 g (175 mmol) of triethylamine in 200 mL of toluene was stirred and cooled in an ice bath. Then, liquid **A** was added to the solution slowly, and the reaction mixture was stirred at room temperature for 2 h. The reaction mixture was moved into a separating funnel, 30 mL water was added, and the organic phase was separated, dried over anhydrous sodium sulfate and evaporated under reduced pressure. This residue was subjected to column chromatography [silica gel, ethyl acetate:petroleum ether (8:1, v/v)]. Yield: 38%. The product was characterized by ESI-MS and NMR: ESI-MS, m/z: 339 $[M + Na]^+$. ^1H NMR (400 MHz, DMSO-d_6), δ: 4.3 (s, 2H, CH2), 7.58–7.60 (d, 1H, CH), 8.66–8.68 (m, H, CH), 8.77 (s, H, CH).

Hapten 2: This hapten was synthesized using **A** and 4-aminobutyric acid. Yield: 48%. The product was characterized by ESI-MS and NMR: ESI-MS, m/z: 367 $[M + Na]^+$. ^1H NMR (400 MHz, DMSO-d_6), δ: 1.73–1.78 (m, 2H, CH2), 2.29–2.51 (m, 2H, CH2), 3.26–3.37 (m, 2H, CH2), 7.81–7.83 (d, 1H, NH), 8.74–8.91 (m, 3H, CH × 3), 12.1 (s, 1H, COOH).

2.5. Synthesis and identification of hapten–protein conjugates

Two flonicamid haptens were coupled with BSA using the active ester method to produce an immunogen and were conjugated with OVA by the mixed anhydride method to produce a coating antigen (Li et al., 2014). The conjugates were dialyzed against PBS for 72 h at 4 °C and were stored at −20 °C. The conjugates were confirmed by UV–Vis spectroscopy. The number of hapten molecules per molecule of protein (hapten density) of the conjugate was estimated directly by the molar absorbance at 280 nm (Liu et al., 2011).

$$\text{Hapten density} = (\varepsilon_{\text{conjugate}} - \varepsilon_{\text{protein}})/\varepsilon_{\text{hapten}}$$

2.6. Immunization and antibody preparation

According to the method described by Shan et al. (1999), four male New Zealand white rabbits (approximately 2 kg per rabbit) were divided into two groups and were immunized for Hapten 1-BSA and Hapten 2-BSA via intraperitoneal injection. The rabbits had free access to drinking water and commercial standard laboratory diet (CZZ, Nanjing, China) and were housed according to the EEC 609/86 Directives regulating the welfare of experimental animals. The immunogen (1 mg kg^{-1}) dissolved in physiological saline was emulsified with an equal volume of Freund's complete adjuvant and was injected intradermally at multiple sites on the back of each rabbit. After the first injection, four boosting injections were given at 2 week intervals with the immunogen (1.5 mg kg^{-1}) in Freund's incomplete adjuvant (1:1 v/v). The rabbits

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