Development of indirect competitive ELISA for determination of dehydroabietic acid in duck skin and comparison with the HPLC method

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ABSTRACT Defeathering with rosin results in rosin residue in duck skin, which may present as potential risk to human health. Dehydroabietic acid (DHAA) is a major component of rosin. An indirect competitive enzyme-linked immunosorbent assay (ELISA) was developed for determination of DHAA in duck skin. A set of parameters was optimized, including coating antigen concentration, dilution of antiserum, dilution of HRP-IgG antibody, incubation time, and temperature for antigen reaction with antiserum. The indirect competitive ELISA yielded an excellent specificity against DHAA with low cross-reactivity toward other resin acids. The limit of detection and the working concentration range of DHAA in duck skin were 16.4 ng/g and from 40 to 8,060 ng/g, respectively. The indirect competitive ELISA was applied to the determination of DHAA in duck skin samples spiked with DHAA at different contents, and recoveries were found between 78.2 and 97.2%. Finally, DHAA contents in 32 duck samples were quantified by the indirect competitive ELISA and high performance liquid chromatography-fluorescence detector (HPLC-FLD) method. No significant difference was found between DHAA concentrations from indirect competitive ELISA and HPLC-FLD method for all samples, which indicated the indirect competitive ELISA established in this article was of the same accuracy as the HPLC-FLD method. The indirect competitive ELISA was simple, rapid, and reliable, which could be used to identify the duck carcasses defeathered with rosin in the market.

Key words: ELISA, dehydroabietic acid, rosin, duck, HPLC

INTRODUCTION Rosin is a natural substance from pines and is widely used in manufacturing of commercial products, for example, paper, soap, paint, adhesive, and so on. Rosin contains a series of resin acids, among which abietic acid (AA) and dehydroabietic acid (DHAA) are the major components. AA and DHAA were reported to inhibit growth of the crustacean Daphnia magna (Kamaya et al., 2005), affect reproduction of the zebrafish (Christianson-Heiska et al., 2008), and result in lysis of human alveolar epithelial cells (Ayars et al., 1989). Exposure to AA and DHAA might cause pulmonary and skin allergy (Smith et al., 1997). Abietic acid and DHAA could be found in paper mill wastewater (Liss et al., 1997), river (McMartin et al., 2002), and could also be detected in wide range of consumer products, such as medicaments (Lee et al., 1997), cosmetics (Nilsson et al., 2008), and food packaging materials (Mitani et al., 2007).

China is the largest producer and consumer of duck meat in the world (Han et al., 2019). Owing to its excellent adhesiveness, rosin was once widely used to get rid of feathers in duck processing in China. During defeathering with rosin, some of the rosin permeates through the duck skin and remains in the skin after the defeathering process. The rosin residue cannot be removed completely even after further processing and cooking, for example, dry-curing, roasting, and water-boiling, which may present as potential risk to human health. Since 2009, using rosin has not been allowed in duck processing in China; however, there have been reports of defeathering with rosin in duck processing in recent years.
To distinguish the duck carcasses defeathered with rosin from those without rosin, it is quite urgent to develop analytical methods with high sensitivity and accuracy for residual rosin in duck skin.

For analysis of rosin residue in various samples, methods based on gas chromatography and high-performance liquid chromatography (HPLC) are commonly used with AA and DHAA as the major residual markers. Before gas chromatography analysis, AA and DHAA usually have to undergo derivatization and analyzed by using a flame ionization detector (Peng and Roberts, 2000) or mass spectrometer (MS) (Volkman et al., 1993; Gao et al., 2015). Relatively speaking, HPLC coupled with a ultraviolet/photodiode array detector (UV/PAD) (Smith et al., 1996; Lee et al., 1997; Sadhra et al., 1997; Kersten et al., 2006; Nilsson et al., 2008), fluorescence detector (FLD) (Lee et al., 1997), and MS (Sadhra et al., 1997; McMartin et al., 2002; Mitani et al., 2007; Runoooka, 2008; Liu et al., 2014) appears to be more frequently used for determination of AA and DHAA. Although HPLC analysis can be performed without derivatization, some efforts still need to be made in sample preparation, for example, enrichment, cleanup, and so on.

Immunochemical analyses, such as enzyme-linked immunosorbent assay (ELISA), are regarded as alternatives to the instrumental methods. Owing to their high sensitivity, high throughput, and low cost, immunochemical analyses have been developed as semiquantitative or quantitative screening tools for detecting a wide range of analytes, for example, pesticides, veterinary drugs, toxin, and so on. There is a lack of immunochemical analytical methods dedicated to the determination of rosin residue in consumer products including foods. In the previous work, we established a HPLC-PAD-FLD method for simultaneous determination of AA and DHAA in duck skin (Zhu et al., 2014b). At the same time, we tried to develop a simple approach for the determination of rosin residue that was sensitive and cost effective, and we successfully prepared polyclonal antibody against DHAA by immunizing rabbits with an artificial antigen (Bian et al., 2018). Therefore, as an extension of the previous research, objectives of the present study were 1) to build an indirect competitive ELISA and apply it to determination of DHAA residue in duck skin and 2) to evaluate the ELISA performance by comparison with a high performance liquid chromatography-fluorescence detector (HPLC-FLD) method.

**MATERIALS AND METHODS**

**Reagents and Materials**

Abietic acid (95%) and DHAA (99%) were obtained from Helix Biotech (Vancouver, Canada). Palustric acid (PaA), pimaric acid (PiA), and isopimaric acid (iPiA) were provided by Bailingwei Biotechnology (Beijing, China). Goat anti-rabbit IgG–horseradish peroxidase (HRP) conjugate, bovine serum albumin (BSA), and keyhole limpet hemocyanin (KLH) were purchased from Bailingwei Biotechnology (Beijing, China). Acetonitrile and methanol (HPLC-grade) were supplied by Merck (Darmstadt, Germany). Water was purified with a Milli-Q system (Millipore, USA). Phosphate buffer saline (PBS, pH 7.5), phosphate buffer saline with Tween-20 (PBST, pH 7.5), and other chemicals were of analytical grade.

Polystyrene microtiter plates were obtained from Greiner Bio-One (Frickenhausen, Germany). Sep-Pak C18 (500 mg/3 mL) SPE cartridges were supplied by Waters (Milford, USA). Syringe filters (0.22 μm, polytetrafluoroethylene) were supplied by Anpel (Shanghai, China). New Zealand white rabbits were supplied by the Institute of Veterinary Immunology and Engineering, Jiangsu Academy of Agriculture Sciences.

Thirty-two whole duck carcasses, stored in polyethylene bags at −20°C with an average weight of around 2.0 kg, were purchased from farm product markets in Nanjing, China. These raw ducks were from poultry processing enterprises in Jiangsu, Shandong, Anhui, and Henan provinces, the major provinces of duck producing and processing in China. For determination of DHAA, skin (including subcutaneous fat) of the ducks was collected from the breast and the thigh, followed by mincing and mixing. A portion of each sample (100 g) was kept in the polyethylene bag at −20°C until ELISA and HPLC analysis.

**Instruments**

Absorbance in ELISA analysis was recorded with an Epoch 2 micro plate reader (Bio Tek, USA). HPLC analyses were performed on an e2695 HPLC system (Waters, USA), coupled with a FLD.

**Synthesis of Antigen and Preparation of Antibody Against DHAA**

Syntheses of antigens and preparation of antibody against DHAA were described in our earlier article (Bian et al., 2018). In brief, dehydroabietylamine (DHAM) was used as a hapten and was first transformed to succinylated DHAM (DHAM-SUC) by reaction with succinic anhydride. Succinylated DHAM was characterized by infrared spectrum, 1H nuclear magnetic resonance and MS. Succinylated DHAM further reacted with BSA and KLH, respectively, and antigens, that is, DHAM-SUC-BSA and DHANM-SUC-KLH, were produced, respectively. The hapten densities (hapten/protein ratios) were found to be 12 for DHAM-SUC-BSA and 35 for DHANM-SUC-KLH. Antisera were collected from 4 New Zealand rabbits immunized by intramuscular injection of DHAM-SUC-KLH and the titer was examined using indirect ELISA with DHAM-SUC-BSA as a coating antigen. At the end of immunization, the titer of antiserum from rabbit 4 was found to be 1:12,800, which was used to develop the indirect competitive ELISA in this study.
**Indirect Competitive ELISA Protocol**

Standard DHAA was diluted to various concentrations with PBS before the ELISA assay. For indirect competitive ELISA, 100 μL of the coating antigen (1.0 μg/mL in PBST) was piped into an enzyme plate and incubated at 4°C overnight. The excess binding sites were blocked with 2% milk in PBS for 2 h at 37°C. Plates were washed 3 times with 200 μL per well of PBST to remove the blocking solution; 50 μL antibody solution (diluted with PBS at 1:6,400) and 50 μL DHAA were added to each well. Unbound compounds were removed by washing after incubation for 1 h at 37°C. Hundred microliters of HRP-IgG solution (diluted with PBS at 1:10,000) were added to each well and incubated for 5 min at 37°C, then washed 3 times with PBST. Hundred microliters of substrate solution were then added to each well, and the enzymatic reaction was stopped after 10 min incubation at 37°C by addition of 50 μL stopping solution to each well. Absorbance values were measured at 450 nm using an ELISA plate reader.

The calibration curve of ELISA was determined by plotting inhibition (%) against the logarithm of the standard concentration using the following formula:

\[
\text{Inhibition} \, (\%) = \frac{A_o - A}{A_o} \times 100
\]

where \(A\) and \(A_o\) are the absorbance with and without DHAA, respectively.

**Cross-Reactivity**

The specificity of the ELISA was evaluated by determining the cross-reactivity (CR) toward other resin acids. Cross-reactivity was calculated as:

\[
\text{CR (\%) } = \frac{\text{IC}_{50} \, (\text{DHAA})}{\text{IC}_{50} \, (\text{cross reactant})} \times 100
\]

**Sample Preparation**

The sample was prepared as per the method described by Zhu et al. (2014a), with some modifications. In brief, 5 g of sample was weighed into a 20-mL centrifuge tube, followed by addition of 10 mL methanol. The tube was capped and shaken in an ultrasonic shaker for 15 min, followed by centrifugation at 4,000 rpm for 15 min. For ELISA analysis, 1 mL of the supernatant was collected and mixed with 1.5 mL of PBS in a 10-mL centrifuge tube. For HPLC analysis, 4 mL of supernatant was collected and mixed with 4 mL of water, followed by loading onto and passing through a C18-SPE cartridge, which was activated with methanol and water successively. The analyte was eluted with 2 mL of methanol and was dried under a gentle stream of nitrogen at 40°C. The residue was dissolved in 500 to 2,000 μL of the mobile phase (depending on the levels of analytes), followed by filtration through a 0.22-μm syringe filter before HPLC analysis.

**Conditions for HPLC-FLD Analysis of DHAA in Duck Skin**

Conditions for HPLC-FLD analysis was set up as per the method described by Zhu et al. (2014a), with some modifications. In brief, DHAA was separated on an Xbridge C18 column (4.6*250 mm, 5 μm, Waters, USA) by methanol/2 mM phosphoric acid (86/14, v/v) and was detected by a FLD with an excitation wavelength at 225 nm and emission wavelength at 287 nm.
**Statistical Analysis**

Determinations of DHAA in samples were carried out in triplicates by ELISA and HPLC-FLD, and statistical analysis was conducted by using SPSS software, version 17.0 for Windows (Chicago, USA).

**RESULTS AND DISCUSSION**

**Optimization of Indirect Competitive ELISA Conditions**

In the previous study, a polyclonal antibody was prepared by immunization of rabbits with an artificial antigen derived from DHAM and was found to be of good reactivity against DHAA (Bian et al., 2018). To establish the indirect competitive ELISA, a set of parameters, including coating antigen (DHAM-SUC-BSA) concentration, dilution of antiserum, dilution of HRP-IgG antibody, incubation time, and temperature for antigen reaction with antiserum, was investigated with DHAA as the competitor analyte in this study. A three-dimensional titration was used to determine the coating antigen concentration and dilution of antiserum, with DHAM-SUC-BSA concentration and dilution of antiserum varying from 0 to 4.0 μg/mL and from 1:800 to 1:12,800, respectively (Figure 1). The optimal coating antigen concentration and the optimal dilution of antiserum were found to be 1.0 μg/mL and 1:6,400, respectively, whereas the optimal dilution of HRP-IgG antibody was set at 1:10,000. The optimal incubation time and temperature for antigen reaction with antiserum were 1 h and 37°C, respectively. Under these optimized conditions, the recognition of free DHAA by the polyclonal antibody was evaluated in the indirect competitive ELISA. The inhibition curve with free DHAA concentration ranging from 10 to 5,000 ng/mL was displayed in Figure 2, and the linear regression equation was obtained:

\[
\text{Inhibition(%) = 26.03 Log[DHAA] - 13.87} \quad (R^2 = 0.992)
\]

For ELISA, the limit of detection (LOD) was commonly defined as the analyte concentration for yielding 10% inhibition, whereas the working concentration range was defined as the range of analyte concentration for yielding inhibition from 20% (IC20) to 80% (IC80) (Hennion and Barcelo, 1998). From the aforementioned linear regression equation, the LOD and the working concentration range of the present ELISA were calculated and summarized in Table 1. The precision of indirect competitive ELISA was evaluated by the intraday and interday determination of DHAA in 3 representative duck skin samples, and the corresponding resin acids as inhibitors, and IC50, CR, IC20, and IC80 were calculated and summarized in Table 1. The CR of the polyclonal antibody against other resins, that is, AA, PaA, PiA, and iPiA, was investigated.

The inhibition curves of these resin acids were obtained by indirect competitive ELISA with the 4 corresponding resin acids as inhibitors, and IC50, CR, IC20, and IC80 were calculated and summarized in Table 1. The CR of the polyclonal antibody against other 4 resin acids were found to be lower than 3.0%, which indicated these resin acids had a negligible effect on quantification of DHAA, and the indirect competitive ELISA developed in this study was of high specificity toward DHAA.

**Specificity of Indirect Competitive ELISA**

As a complex mixture of resin acids, rosin contains some resin acids other than AA and DHAA, for example, PaA, PiA, iPiA, and so on, which are smaller components of rosin but share the similar structure with AA and DHAA (Sadhra et al., 1997). To evaluate the specificity of the indirect competitive ELISA developed for determination of DHAA in this study, CR of the polyclonal antibody against other resins, that is, AA, PaA, PiA, and iPiA, was investigated.

The inhibition curves of these resin acids were obtained by indirect competitive ELISA with the 4 corresponding resin acids as inhibitors, and IC50, CR, IC20, and IC80 were calculated and summarized in Table 1. The CR of the polyclonal antibody against other 4 resin acids were found to be lower than 3.0%, which indicated these resin acids had a negligible effect on quantification of DHAA, and the indirect competitive ELISA developed in this study was of high specificity toward DHAA.

**Precision and Accuracy of Indirect Competitive ELISA**

The precision of indirect competitive ELISA was evaluated by the intraday and interday determination of DHAA in 3 representative duck skin samples, and the developed in this study would meet the demand of quantification of DHAA in duck skin.

**Table 1. Cross-reactivity (CR) of the polyclonal antibody against different resin acids and the lower and upper detection limit**

| Resin acids | IC50 (ng/mL) | CR (%) | IC20 (ng/mL) | IC80 (ng/mL) |
|-------------|--------------|--------|--------------|--------------|
| AA          | 12.274       | 2.31   | 150.8        | 9.8×10⁴      |
| PaA         | 10.819       | 2.62   | 49.0         | 3.4×10⁵      |
| PiA         | 13.112       | 2.17   | 29.8         | 5.8×10⁵      |
| iPiA        | 49.808       | 0.56   | 30.8         | 1.2×10⁸      |

Abbreviations: AA, abietic acid; DHAA, dehydroabietic acid; IC, inhibitory concentration; iPiA, isopimaric acid; PaA, Palustric acid; PiA, pimaric acid; SD, standard deviation.

1 All the IC50, IC20 and IC80 were expressed as mean ± SD (n = 3).
results were summarized in Table 2. The intraday and interday precision ranged from 5.9 to 8.7% and 5.5 to 10.2%, respectively. The accuracy of indirect competitive ELISA was verified by analyzing the representative samples spiked with DHAA at levels ranging from 100 to 5,000 ng/g. The average percentage recoveries ranged from 78.2 to 97.2%. These results indicated that the indirect competitive ELISA developed in the present study had a good precision and accuracy for the determination of DHAA in duck skin.

**Application of Indirect Competitive ELISA and Comparison With HPLC-FLD**

Contents of DHAA in 32 duck samples were determined by the indirect competitive ELISA developed in the present study and HPLC-FLD method, and the results were listed in Table 3. Twenty of 32 duck samples were found contaminated with DHAA by both methods, with ranges of 118.6 to 1,199.2 ng/g and 102.8 to 1,235.6 ng/g, respectively. The positive rate and the range of DHAA content in positive samples were in good agreement with the earlier studies (Zhu et al., 2014a). Because the limits of detection of the 2 methods (ie, 16.4 ng/g and 5 ng/g, respectively) were much lower than the DHAA contents in positive samples, it was reasonable that same positive rate was yielded by both methods.

For comparison, a Pearson’s correlation between DHAA concentrations generated by the indirect competitive ELISA and HPLC-FLD method was conducted for the 20 positive samples contaminated with DHAA. A significantly positive correlation was found between results from the 2 methods ($r = 0.976, P < 0.001$), which suggested the suitability of indirect competitive ELISA developed in the present study for DHAA determination in duck skins. To further evaluate the difference between the indirect competitive ELISA and HPLC-FLD method, the values of DHAA from the 2 methods were compared with independent t test for every single positive sample. No significant difference was found between DHAA concentrations from the indirect competitive ELISA and HPLC-FLD method for all samples, which indicated the indirect competitive ELISA established in this study could be regarded to be of the same accuracy as the HPLC-FLD method, and these 2 methods could be used interchangeably in determination of DHAA residue in duck skins.

In addition to the same accuracy, the indirect competitive ELISA established in this study exhibited higher effectiveness than the HPLC-FLD method and was

| Sample | ELISA | HPLC-FLD |
|--------|-------|----------|
| 1      | 119.2 | 102.6    |
| 2      | 74.5  | 63.9     |
| 3      | 207.7 | 222.2    |
| 4      | ND    | ND       |
| 5      | 308.2 | 298.7    |
| 6      | ND    | ND       |
| 7      | 467.6 | 500.6    |
| 8      | ND    | ND       |
| 9      | ND    | ND       |
| 10     | 507.1 | 512.4    |
| 11     | 414.3 | 406.2    |
| 12     | ND    | ND       |
| 13     | 395.3 | 362.8    |
| 14     | ND    | ND       |
| 15     | 168.4 | 172.8    |
| 16     | 193.2 | 192.8    |
| 17     | 190.3 | 163.2    |
| 18     | 540.8 | 542.9    |
| 19     | 534.0 | 528.7    |
| 20     | ND    | ND       |
| 21     | 118.6 | 102.8    |
| 22     | ND    | ND       |
| 23     | 202.0 | 224.3    |
| 24     | 496.5 | 508.3    |
| 25     | ND    | ND       |
| 26     | ND    | ND       |
| 27     | 708.3 | 692.8    |
| 28     | 615.8 | 659.2    |
| 29     | 170.7 | 154.4    |
| 30     | ND    | ND       |
| 31     | 1,178.4 | 1,235.6 |
| 32     | ND    | ND       |

Abbreviation: DHAA, dehydroabietic acid.

1All samples were just marked with different numbers for commercial reason.

2ND stood for not detected.

| Precision/accuracy | Sample-13 | Sample-28 | Sample-31 |
|--------------------|-----------|-----------|-----------|
| Intra-day precision |           |           |           |
| Average DHAA content (ng/g) | 395.3 | 615.8 | 1,178.4 |
| SD (ng/g) | 34.5 | 36.2 | 77.8 |
| RSD (%) | 8.7 | 5.9 | 6.6 |
| Inter-day precision |           |           |           |
| Average DHAA content (ng/g) | 379.3 | 624.6 | 1,170.6 |
| SD (ng/g) | 38.6 | 44.1 | 64.9 |
| RSD (%) | 10.2 | 7.1 | 5.5 |

Abbreviation: DHAA, dehydroabietic acid.

1Intra-day precision was determined by assaying 6 replicates in the same day.

2Inter-day precision was determined by assaying triplicate in 3 D.

Table 2. Precision and accuracy of the method for determination of DHAA in 3 samples.

| Recoveries (%) of DHAA spiked at different levels (ng/g, mean ± SD, n = 3) |
|------------------|------------------|------------------|
| Sample-13         | Sample-28         | Sample-31         |
| 100               | 78.2 ± 7.1       | 97.2 ± 8.7       | 93.7 ± 6.6       |
| 400               | 85.8 ± 5.9       | 89.9 ± 5.6       | 83.4 ± 5.5       |
| 2,000             | 85.2 ± 7.1       | 95.6 ± 5.9       | 86.0 ± 5.5       |
| 5,000             | 83.9 ± 7.1       | 81.5 ± 5.9       | 79.1 ± 5.5       |

Abbreviation: DHAA, dehydroabietic acid.

Table 3. Concentrations of DHAA determined in duck skins by ELISA assay and HPLC-FLD (ng/g, mean ± SD, n = 3).
more suitable for the determination of rosin residue in a large number of duck carcasses in the market.

CONCLUSION

The present study developed an indirect competitive ELISA for determination of DHAA in duck skin. To our knowledge, this is the first indirect competitive ELISA dedicated to analysis of DHAA in ducks defeathered by rosin. The indirect competitive ELISA was demonstrated to be excellently specific against DHAA. The LOD and the working concentration range of DHAA in duck skin were 16.4 ng/g and from 40 to 8,060 ng/g, respectively. The application in determination of DHAA contents in commercial duck samples and comparison with the HPLC-FLD method indicated the indirect competitive ELISA established in this study was of the same accuracy as the HPLC-FLD method with higher effectiveness. The indirect competitive ELISA was simple, rapid, and reliable and could be used in the determination of rosin residue in a large number of samples and identify the duck carcasses defeathered with rosin in the market.

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