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Determination of intramuscular phospholipid classes and molecular species in Gaoyou duck

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ABSTRACT

In this study, intramuscular phospholipid classes and molecular species in Gaoyou duck meat were determined. Classes of phospholipids were identified and quantified by normal phase HPLC combined with UV and evaporative light scattering detectors (ELSD). The main phospholipid classes (phosphatidylcholine and phosphatidylethanolamine) were prepared on a semi-preparative silica gel column by HPLC. Reverse phase HPLC was coupled in parallel with both an ELSD and a mass spectrometry in order to characterise molecular species of phosphatidylcholine (PC) and phosphatidylethanolamine (PE). The results showed that Gaoyou duck meat had high quantities of PC and PE (64.66% and 28.10% of total phospholipids, respectively). Arachidonic acid was mainly present in PE and formed molecular species containing a saturated fatty acid, such as stearic or myristic acid; however, oleic acid together with palmitic or stearic acid formed the main molecular species in PC. The content of the molecular species with polyunsaturated fatty acids in PE accounted for 98.33%, while that in PC only 46.20%.

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1. Introduction

Cooked and dry-cured duck products are well accepted by consumers in China and Southeast Asia due to its delicate flavour and texture (Xu, Xu, & Zhou, 2008). In Nanjing city alone, about thirty million ducks are consumed annually (Liu, Xu, & Zhou, 2006). Gaoyou duck is widely used to produce traditional Chinese duck meat products, including the famous Nanjing cooked duck or Nanjing dry-cured duck.

The amount of intramuscular phospholipids in the meat is an important factor (Gray, Gomaa, & Buckley, 1996) for the flavour and nutritive quality of fresh cocked and dry-cured meat products such as ham, sausage, and salami. Phospholipids consist of long-chain fatty acids attached to a phosphoryl group. Because the fatty acid chains can vary in length and degree of saturation, each phospholipid class has numerous molecular species with different chemical and biological properties (Marco, Fabio, & Emanuele, 2004). Several workers have found that fatty acid profiles of phospholipids are correlated with differences in flavour characteristics of meat (Larick & Turner, 1990). There are two major classes of phospholipids, the phosphatidylethanolamines (PE) and the phosphatidylcholines (PC). In our previous study, we found that PE and PC decreased by 50% and 30%, respectively during the Nanjing dry-cured duck processing (Xu et al., 2008). Similar trend was also observed while cooking chicken. To explain the mechanism of this phenomenon, an analysis of the molecular structures of PE and PC in the meat is necessary. Usually, the analysis of phospholipids involves either the determination of total fatty acids by gas chromatography after purification of phospholipids, or the determination of the phospholipid classes with thin-layer chromatography (TLC) or preparative high performance liquid chromatography (HPLC) (Peterson & Cummings, 2005). However, practically no literature data are available of phospholipid molecular species in duck meat which play a pivotal role in the study of nutritional value and flavour of meat. The objective of this study was to determine intramuscular phospholipid classes and molecular species in Gaoyou duck. After purification of the phospholipids by solid phase extraction (SPE), classes of phospholipids were identified and quantified by normal phase HPLC combined with UV and evaporative light scattering detectors, molecular species of the main phospholipid classes (PC and PE) were characterised by reverse phase HPLC which was coupled in parallel with both an electrospray ionisation mass spectrometer and an evaporative light scattering detector.

2. Materials and methods

2.1. Materials

Gaoyou ducks (fed for 10 mouths) from Jiangsu Waterfowl Research and Development Center were slaughtered humanely in a

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commercial meat processing company (Jiangsu Yurun Food Ltd.). After chilling (2 h), six ducks were selected for analyses of phospholipid classes, and four of molecular species. One biceps femoris muscle was removed from each duck.

Phospholipid standards (purity higher than 99%), including phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI) phosphatidylserine (PS), sphingomyelin (SPH) and lysophosphatidylcholine (LPC) standards were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA); methanol, *n*-hexane, 2-propanol, acetamide, chloroform and acetonitrile were chromatographic pure grade. Acetic acid, diethyl ether, NH_4Ac, NaCl and $CaCl_2$ were analytic pure grade.

2.2. Extraction and purification of phospholipids

2.2.1. Intramuscular lipid extraction

Biceps femoris muscles were removed from duck carcasses and trimmed of all visible subcutaneous fat and connective tissue. Lipids were extracted from muscle samples according to the method of Folch, Lees, and Stanley, (1957) with small modifications. Briefly, 3.0 g of muscle sample was homogenised with 60 ml of chloroform/methanol (2/1, V/V) solution at 1500 rpm using an Ultra Turrax (T25, IKA, Germany). The homogenate was allowed to stand for 1 h and then pass through a layer of filter. 0.2-fold the filtrate's volume of a solution containing 7.3 g/L NaCl, and 0.5 g/L $CaCl_2$ was added to filtrate. The mixture was centrifuged for 15 min at 3000 rpm (Allegra 64R, Beckman, USA) and the lower phase was dried under vacuum on a rotary evaporator (RE-85C, Yarong, China) in a 44 °C water bath and then stored at −20 °C.

2.2.2. Phospholipid purification

Phospholipids were separated from intramuscular lipids according to the procedure of García, Gibert, and Díaz (1994). Briefly, 20.0 mg of total lipid extract was dissolved in 1.0 ml of chloroform, and 0.5 ml of the solution was transferred into an aminopropyl-silica minicolumn (100MG, VARIAN, USA) that was activated with 1.0 ml of chloroform before transfer. The minicolumn was washed with 2.0 ml of chloroform/2-propanol (2/1, V/V) to remove hydrocarbons, cholesterol esters and triacylglycerols, and then free fatty acids were eluted with 3.0 ml of 2% acetic acid in diethyl ether (W/W). Finally, phospholipids were eluted with 3.0 ml of methanol. The solvent was removed by rotary evaporation and the residue was dissolved in 0.3 ml of mobile phase C solution (hexane/2-propanol/water, 120/80/11, V/V/V) for HPLC analysis.

2.3. Separation and identification of phospholipid classes

The sample was analyzed in an Agilent 1100 HPLC system (equipped with an autoinjector, HPLC workstation, UV detector, Palo Alto, CA, USA) using a Lichrosorb SI 60-5 silica gel column (5 μm, 250 mm × 4.0 mm i.d.) operating at 30 °C. A gradient elution was carried out at a flow rate of 1.0 mL/min using different ratios of solutions A (*n*-hexane/2-propanol, 3/2, V/V), B (*n*-hexane/2-propanol/25 mmol L^{-1} NH_4Ac, 120/80/11, V/V/V), and C (*n*-hexane/2-propanol/H_2O, 120/80/11, V/V/V). The best separation was obtained using the following gradient: from 0 to 5 min, B was increased from 0% to 50%; from 5 to 30 min, B was increased from 50% to 100%; from 30 to 45 min, B was kept constant at 100%; from 45 to 50 min, C was increased from 0% to 100%; from 50 to 60 min, C was kept constant at 100%; from 60 to 62 min, A was increased from 0% to 100%; from 62 to 70 min, solution A was kept constant at 100%. Chromatographic peaks were detected with a UV detector and an ELSD, which were installed in series; the UV absorbance was measured at 205 nm, and the ELSD was run at 70 °C with N_2 at 1.8 L/min. Peaks were identified by comparison with known standards.

For the quantitative analysis of phospholipids, a calibration curve for each phospholipid classes was obtained by injecting standard solutions of PC, PE, PC, PI, PS, SPH and LPC at five different concentrations, calibration curves were reported in Table 1. Recoveries, estimated on the basis of determinations after spiking samples with known amounts of standards. The intra-day precision, expressed as the relative standard deviation (RSD, %) of peak area measurements ($n = 5$), was evaluated through the results obtained from the method operating over one day under the same conditions, concentration levels of PE and PC were 2.5 mg/mL, PI, PS, SPH, LPC were 0.05 mg/mL. The inter-day precision was determined at the same concentration levels, and the analyses were performed for 5 days.

2.4. Determination of PC and PE molecular species

2.4.1. Preparation of PC and PE

The sample of phospholipids was prepared by Agilent 1100 HPLC system using a μ-Porasil semi-preparative silica gel column (10 μm, 300 mm × 10 mm i.d.) operating at 30 °C, with an injection volume of 200 μL. Chromatographic peaks were identified using UV absorbance at 205 nm. The programme of gradient elution was identical to that described in 2.3, but carried out at a flow rate of 3.0 mL/min. The total separation time was 70 min. The identification of PC and PE was performed by comparing their retention times with standard samples. PC and PE were collected five times from the column outlet and were dried under vacuum with a rotary evaporator at 40 °C. Two portions of 0.5 mL *n*-hexane were used to transfer the residues into a glass conical tube, then the *n*-hexane was evaporated under N_2 and 0.5 mL chloroform/methanol (1/17.5, V/V) was added. Finally, the samples of PC and PE were kept at −20 °C for further analysis.

2.4.2. Quantitative determination of PC and PE molecular species by high performance liquid chromatography–evaporative light scattering–electrospray ionisation mass spectrometry (HPLC–ELSD–MS)

A reconstructed HPLC–ELSD–MS (HPLC, Waters 2690; ELSD, Alltech 2000; MS, Waters Platform ZMD 4000) system was used to analyze PC and PE molecular species. The separation was performed on the HPLC using a Symmetry C_{18} RP column (5 μm, 250 × 4.6 mm i.d.) operating at 25 °C. A gradient elution was carried out using various ratios of solvent A (chloroform/methanol, 1/17.5, V/V) and solvent B (acetonitrile/water: 1/1, V/V). The elution was begun with solvent A, which was maintained at 80% for 5 min, then increased to 100% in 5 min, and maintained at this level for 45 min. The flow rate was 1.0 mL/min and the injection volume was 5 μL. The HPLC system was coupled in parallel to both an electrospray ionisation mass spectrometer and an evaporative light scattering detector. The HPLC effluent was splitted: 0.25 ml/min entered the MS detector and 0.75 mL/min were delivered to the ELSD.

The ELSD was run at 70 °C with N_2 at 2.0 L/min. The mass detector was operated in positive ion electrospray ionisation mode. The nebulizer gas and desolvation gas were nitrogen. The velocity of the solution entering the MS was 10 μL/min. Typical operating parameters were as follows: capillary voltage 3.3 kV, cone voltage 30 V, source temperature 100 °C, desolvation temperature 400 °C, gas flow 4.0 L/h, m/z range 200–1000, and multiplier voltage of 700 V.

2.5. Statistical analyses

Results were presented as mean ± SD, percentage of phospholipid classes were from six different samples, molecular species from four, the value for each sample was the average of two

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