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UHPLC–HRMS-based tissue untargeted metabolomics study of naringin and hesperidin after dietary supplementation in chickens

Eirini Baira[a], Ioanna Dagla[a], Eleni Siapi[b], Panagiotis Zoumpoulakis[b], Panagiotis Simitzis[c], Michael Goliomytis[c], Stelios G. Deligeorgis[c], Alexios-Leandros Skaltsounis[d], Evagelos Gikas[a,*]

[a] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, School of Health Sciences, National and Kapodistrian University of Athens, Panepistimiopolis, 15771 Athens, Greece
[b] Institute of Biology, Medicinal Chemistry and Biotechnology, National Hellenic Research Foundation, 48 Vassileos Constantinou, 11635 Athens, Greece
[c] Department of Animal Breeding and Husbandry, Faculty of Animal Science and Aquaculture, Agricultural University of Athens, 75 Iera Odos, 11855 Athens, Greece
[d] Department of Pharmacognosy and Natural Products Chemistry, Faculty of Pharmacy, School of Health Sciences, National and Kapodistrian University of Athens, Panepistimiopolis, 15771 Athens, Greece

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ABSTRACT

To date numerous metabolomic studies have been performed in order to characterize nutritional intervention studies. The aim of the current study was to present a comprehensive pipeline for characterizing the metabolic changes that occur in chickens tissues in response to naringin and hesperidin dietary supplementation. Forty-nine chickens were randomly divided into 3 groups: the first one fed with diet supplemented with naringin, the second with hesperidin whereas the control group was fed by commercial basal diet. After 30 days of administration chicken muscle samples were analyzed by UHPLC–HRMS whereas data were analyzed by the proposed pipeline. Three significant variables were detected to discriminate the control from the group after naringin administration and thirteen variables after hesperidin supplementation. Furthermore, a more detailed pipeline (encompassing multiple internal standards, internal validation of the clustering, extended statistical significance scores and multiple identification procedures) has been proposed aiming towards a more accurate untargeted analysis.

1. Introduction

Metabolomics is a widespread “omics” strategy that offers quantitative and qualitative analysis of endogenous low-molecular-weight compounds (e.g., < 1 kDa) in a biological system. Over the past decade, metabolomics has been widely used to study both pathological and physiological states, mainly aiming towards biomarkers identification in biological fluids and tissues, whereas it has been found extensive acceptance in the clinical and nutritional field in exploring underlying biochemical mechanisms of action. In metabolomics, biomarkers can be measured in any biological sample, e.g., blood, tissue or urine (Hanhineva et al., 2015; Liesenfeld et al., 2015). The use of metabolomics in the identification of biomarkers is based on the hypothesis that abnormalities like disease, environmental alterations or changes on dietary pattern, cause changes on the biochemical pathways in response, leading to a metabolic profile characteristic of the disease. To date, a significant number of potential metabolite biomarkers have been discovered by profiling the human metabolome (Gika, Theodoridis, Plumb, & Wilson, 2014). Metabolomics covers a wide range of applications on diverse research areas such as drug discovery (Kell & Goodacre, 2014), nutrition (Gibbons, O’Gorman, & Brennan, 2015) and the study of human diseases (James & Parkinson, 2015). Additionally, has been used as a promising tool to discriminate different dietary patterns (O’Sullivan, Gibney, & Brennan, 2011) and also to identify dietary biomarkers (O’Gorman, Gibbons, & Brennan, 2013).

Predominantly, two analytic platforms are used for data acquisition in metabolomics i.e. mass spectrometry (MS) coupled to a separation technique e.g. LC–MS and nuclear magnetic resonance (NMR) spectrometry. These platforms are considered as the most effective techniques in quantitative and qualitative metabolomics. Both techniques have the ultimate goal of identifying small molecules that are responsible for a particular activity or phenotype (Nicholson, Connelly, Lindon, & Holmes, 2002). The choice of either one of these technologies depends on the sensitivity and selectivity requirements of the study.

Nowadays, a variety of software tools have been developed for the primary processing procedure after the data analysis employing MS

Abbreviations: UHPLC–HRMS, ultra-high performance liquid chromatography–high resolution mass spectrometry

* Corresponding author.
E-mail address: vgikas@pharm.uoa.gr (E. Gikas).

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such as apLCMS, Mzmine, XCMS, MAIT, XCMS online, MetAlign etc. (Fernández-Albert, Llorach, Andrés-Lacueva, & Perera, 2014; Lommen & Kools, 2012; Pluskal, Castillo, Villar-Briones, & Orešič, 2010; Smith, Want, O'Maille, Abagyan, & Siuzdak, 2006; Tautenhahn, Patti, Rinehart, & Siuzdak, 2012; Yu, Park, Johnson, & Jones, 2009). Following data processing, various statistical tools are typically used to identify significant metabolic differences between distinct groups. Finally, the metabolites represented by the selected peaks (accurate mass_retention time) are verified and identified by searching on online databases.

In this work, an analytical and computational method along with a data processing pipeline is suggested in order to characterize the metabolic changes that occur in tissues in response to the administration of naringin and hesperidin as additives in the nutrition of chickens. Naringin is the major flavanone glycoside in grapefruit, citrus fruits and exerts a variety of pharmacological effects such as antioxidant activity, anti-inflammatory, anti-mutagenic and analgesic (Pang et al., 2010). Additionally, hesperidin belongs to the flavanone class of flavonoids that is found abundantly in citrus fruits (Nielsen, Freese, Kleemola, & Mutanen, 2002) and has been reported to possess antioxidant and anti-inflammatory activity (Hirata, Murakami, Shoji, Kadoma, & Fujisawa, n.d.; Zhang et al., 2006). As it has been described, naringin and hesperidin affect positively meat's antioxidative properties in chickens after dietary administration without effecting their growth performance and meat quality characteristics (Goliomytis et al., 2015).

To our concern, this is the first metabolomic study employing UHPLC–HRMS in tissue samples after dietary supplementation of hesperidin and naringin in chickens. A second equally important goal of the current work is to propose a detailed workflow for the data processing of metabolomics experiments, incorporating a number of steps that would reduce the bias, inevitably introduced in such complex experiments.

2. Materials and methods

2.1. Chemicals and reagents

All solvents were of LC–MS grade. Acetonitrile was purchased from Fluka/Riedel-de Haën (Seelze, Germany). Acetone, chloroform and formic acid were purchased from Sigma-Aldrich (Steinheim, Germany). Naringin was purchased from Alfa Aesar GmbH & Co KG (Karlsruhe, Germany) and hesperidin from TSI Europe NV (Belgium). High purity water was prepared by using Millipore Milli-Q Plus water purification system (Millipore, Milford, MA, USA). Reserpine, yohimbine and 2-aminophenol were purchased from Sigma-Aldrich (Steinheim, Germany).

2.2. Animal and samples treatment

Forty nine 1-day-old Ross 308 broiler chickens were randomly divided into 3 groups. The chickens were obtained from a commercial hatchery and were reared in pens, of a surface area of 2 m^2 in a controlled environment. The lighting program consisted of 23L:1D on arrival, and was decreased to 18L:6D at day 7, remained constant until day 35, and thereafter gradually increased to 23L:1D at slaughter, with access to feed, in mash form, and water ad libitum. The treatment groups were: 17 chickens, after dietary supplementation with naringin (1.5 g/kg of feed), 15 chickens after dietary supplementation with hesperidin (1.5 g/kg of feed) and 17 chickens that were given commercial basal diets (control chickens). The administration of naringin and hesperidin was started from the 11th day of age until sacrificed at the age of 42 days. Tissue samples, from pectoralis major and biceps femoris muscles, were collected, dried by lyophilization and subsequently stored at −80 °C until the analysis.

2.3. Sample preparation

For the metabolomic analysis 0.020 g of lyophilized tissue were used. Briefly, a solution of 0.1 M acetic acid-ammonium acetate (pH = 5 adjusted by aq. acetic acid), was added to reach a final ratio of 1:10 tissue/solution (v/v) for the tissue homogenization. The samples were then homogenized for at least 5 min. 1 mL of cold chloroform was added to the homogenizer and the samples were kept at −20 °C for 10 min, in order to achieve total lipid extraction. After the removal of the lipid phase, 1 mL of cold acetone was added for the protein precipitation. Extraction was conducted by vortexing for 1 min. The extracts were centrifuged at 13,500 rpm at 4 °C for 10 min. After phase separation, the aqueous layer (aq. acetone) was collected and additional of 0.2 mL of cold acetone was added to the tissue pellet for extracting any additional metabolites captured to the solid and the supernatants were mixed and evaporated to dryness under vacuum. The residue was reconstituted with 0.2 mL of the three internal standard mixture (each added at a final concentration of 1 μg/mL). The internal standard mixture, comprised from the compounds reserpine, 2-aminophenol and yohimbine in methanol/water (50/50, v/v).

A pooled quality control (QC) sample was prepared in order to monitor LC and MS performance across sample runs by combining equal small aliquots from all the samples of the experimental set. The QC sample was prepared by mixing the supernatants of all the samples before the evaporation. Subsequently the mixture was evaporated to dryness and processed as described before throughout the experiments as a process control.

2.4. Instrumentation

The metabolomic analysis of tissue samples was performed employing an ESI-LTQ-Orbitrap Discovery XL mass spectrometer (Thermo Scientific, Germany) connected to an Accela UHPLC system (Thermo Scientific, Germany). The UHPLC system was equipped with an autosampler, a vacuum degasser, a binary pump and a temperature-controlled column. An ACQUITY UPLC BEH C18 (2.1 × 100 mm, 1.7 μm) reversed phase column (Waters Corp. Milford, MSA, USA) was used for the analysis.

The system was run in a binary gradient solvent mode consisting of 0.1% (v/v) formic acid/water (solvent A) and acetonitrile (solvent B). Sample analysis was carried out in both positive (ESI+) and negative (ESI−) ion modes. The flow rate was 0.4 mL/min. A gradient method of 32 min was used as follows: 0 to 24 min: 95% A: 5% B, 24 to 28 min: 5% A: 95% B, 28 to 32 min: 95% A: 5% B. The column temperature was maintained at 40 °C while the autosampler tray temperature was kept at 8 °C. The injection volume was 5 μL.

For the positive ion mode, the capillary temperature and voltage were set at 350 °C and 60 V, respectively. The sheath gas flow was set to 30 and the aux gas flow to 10 arb. units. The spray voltage was set to 3.5 kV and the tube lens voltage to 110 V. For the negative ion mode, the capillary temperature and voltage was set at 350 °C and −40 V, respectively. The sheath gas flow was set to 30 and the aux gas flow to 10 arb. units. The spray voltage was set to 3.1 kV and tube lens voltage to −50 V. In both positive and negative ion modes, analysis was performed using the Fourier transform mass spectrometry (FTMS) full scan ion mode, applying a mass scan range of m/z 100–1000 and a resolution of 30,000 FWHM while spectra were acquired in centroid mode.

Centrifugation of the samples was performed by a Mikro 200R centrifuge (Hettich Lab Technology, Germany). Evaporation of the samples was performed by GeneVac HT-4X EZ-2 series evaporator Lyospeed ENABLED (Genevac Ltd, UK). Tissue homogenization was performed by a Kinematica Polytron PT 1200C homogenizer (Brinkmann, Westbury, NY).