Comparative study of herbal and non-herbal egg protein profile using High Performance Liquid Chromatography (HPLC)

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Abstract. The purpose of this research is to compare the protein profile of herbal and non-herbal egg using High Performance Liquid Chromatography (HPLC). This research used HPLC Shimadzu 6.1 with column C18, flow rate mobile phase 1 ml/min, wavelength detector UV Vis 220 nm and temperature 50°C. Mobile phase used in this research was 10 and 60% acetonitril (CH₃CN) in water containing 0.05% trifluoroacetic acid. Supplementation of herb containing bioactive antioxidant compounds affects the formation of egg yolk protein containing immunoglobulin. In this study, herbal eggs and non-herbal eggs were seen from their protein profile using the high performance liquid chromatography (HPLC) method and then analyzed using descriptive qualitative analysis. The result show that herbal egg yolk sample has a dominant protein with a molecular weight of 50.41 kDa. Herbal egg yolk protein appears at a retention time (RT) of 56.87 minutes, an area of 3303488 units of area, and the peak height of the graph / peak at 50,974 µAU. Meanwhile, non-herbal egg yolk sample has a dominant protein with a molecular weight of 49.94 kDa. This protein appears at a retention time of 1.307 minutes, an area of 149445550 units of area, and the peak height of the graph / peak at 402.6026 µAU. The results showed that the the peak of HPLC indicated an antioxidants were bound to the bioactive protein fractions of egg yolk. It could be concluded that bioactive herbal bound to egg yolk IgY, but the bioactive compounds have not been identified yet.

Keywords: Albumen, Egg Yolk, HPLC, Herbs

1. Introduction
Regulation Minister of Agriculture Republic Indonesia Number 14 / PERMENTAN / PK.350 / 5/2017 concerning the rules for the use of feed additives in the form of antibiotics in livestock, has an impact on the layer hens industry. The use of antibiotics as a growth promoter in raising hens was prohibited in order to protect consumers against the dangers of antibiotic resistance. The results of a study showed that the ration of laying hens containing the antibiotic tetracycline 200 mg per kg of feed (= 200 ppm) a week after feeding, residue of tetracycline antibiotics also found 0.017 μg / g in eggs (= 0.017 ppm). This antibiotic residue is above the maximum residue limit allowed by The Codex's recommended maximum residue level (MRL) for tetracycline antibiotics in eggs [1]. In another study it was also reported that giving oxytetracycline antibiotics in a mixture of drinking water at a dose of 2 g / l for 7 consecutive days left oxytetracycline antibiotic residues on the shell and egg white on day 2. The oxytetracycline antibiotic residue was still found in egg yolks on day 4 and a residue of 0.01 ppm was still found in egg white and egg yolk after 7 days of stopping oxytetracycline antibiotics in drinking water [2].
The use of growth promoters antibiotics, which can trigger the danger of antimicrobial resistance in consumers, has prompted the laying hen industry to start looking for alternative additives that are safer and do not leave antibiotic residues on egg products. Case studies in the layer farming industry show that some layer farms had started to apply antibiotic-free growth promoters to produce eggs that are free of antibiotic residues. One of the alternatives chosen to replace the role of antibiotics was herbal additives. Herbals are medicinal plants that contain certain bioactive compounds and can be used in the livestock industry as feed additives [3]. Herbals can be used as natural growth promoters (NGPs) which have been identified as capable natural antibiotics. Natural growth promoters (NGPs) that develop as additives in feed are expected to provide more value as organic eggs.

Previous research has shown that herbal provides benefits in raising chickens. One of the herbs that contains androgofolid bioactive compounds (sambiloto leaf) was reported could be able to optimize egg production when added with a level of 0.4% in the ration of laying hens [4]. The herbal extract of noni fruit was reported could increase the amount of specific antibody production, namely Immunoglobulin Yolk (IgY anti AI) [5]. Based on this, a more in-depth study was conducted to prove the differences in the protein profile of herbal eggs and non-herbal eggs using the HPLC method. HPLC is a separation technique based on the partition of the sample between a mobile phase which can be a gas or a liquid mixture and a stationary phase which can be a liquid or a solid. In principle, the sample is carried by the mobile phase through a column containing a stationary phase to separate the components in the sample being tested. The purpose of this study was to determine the differences in the protein profile of herbal eggs and non-herbal eggs based on bioactive peptide compounds in egg yolks and determine the antioxidant interaction of herbs with bioactive peptide compounds. The expected benefit from this research is that the laying hen farming industry will receive scientific information. This information was expected could used as a scientific reference for evaluating the effectiveness of using herbs as feed additives.

2. Materials and methods

2.1. Material

Material that used in this research was HPLC Shimadzu 6.1 with column C18, flow rate mobile phase 1 ml/min, wavelength detector UV Vis 220 nm and temperature 50°C. The chemicals used include ultrapure water, 10 and 60% acetonitrile, sterile aquabidest, and 0.05% triflouro acetic acid (TFA). Supporting equipment includes camry digital scale, micropipette, eppendorf, vortex, object glass, beaker glass, and egg separator. This study used 54 eggs produced in the last three days in a 28-day laying cycle of all eggs. Herbal eggs are eggs from chickens that are given herbal supplementation in the feed, while non-herbal eggs are eggs from chickens that are not given herbal supplementation. The feed given was a basal feed of laying hens containing 17-19% crude protein and 2800 kcal metabolism energy / kg. The basal feed given an additional 36 ml of herbal mixtures for 10 kg mixture of feed. Herbs are given in the form of a mixture of several herbs consisting of turmeric, ginger, sambiloto, noni, red ginger, soursop leaves, bangle, and lemongrass.

2.2. Methods

A number of eggs were separated between the yolk and albumen, then 1 ml of yolk was taken and homogenized with an additional 9 ml of ultrapure water. The insoluble material was removed by filtering using 0.45 micron milliphore. The mobile phase used in running consisted of 10 and 60% acetonitrile (CH$_3$CN) in water containing 0.05% trifluoroacetic acid. Linear elution of the CH$_3$CN gradient from 10% to 60% was programmed for 60 minutes (1 ml/min) at 50°C. Detection was completed at 220 nm. The peak chromatogram was identified by comparing the retention time with the reference protein. The protein fraction component is seen from the peak area.
2.3. Data analysis
Egg protein profile data were analyzed using descriptive analysis to see the protein component of the peak area that appeared after running.

3. Result and discussion
In this study, egg samples from chickens fed with herbal and non-herbal feeds were evaluated to determine the protein profiles based on differences in peaks on the HPLC chart, then compared with the standard protein. The peak that appears shows the dominant protein fraction according to molecular weight [6]. In this study, the results of the analysis of herbal and non-herbal egg yolk protein can be seen in Figure 1-3.

![Figure 1. HPLC standar protein (40,13;46,39;50,40 kDa)](image1.png)

![Figure 2. Herbal egg yolk protein](image2.png)

![Figure 3. non-herbal egg yolk protein](image3.png)

In this research a small pore-size stationary (C4 guard coloum) was used. Peak were identified by the addition of protein reference to the sample prior HPLC. Based on the graphic, it is known that the herbal egg yolk sample has a dominant protein with a molecular weight of 50.41 kDa. Herbal egg yolk protein appears at a retention time (RT) of 56.87 minutes, an area of 3303488 units of area, and the peak height of the graph / peak at 50,974 µAU. Meanwhile, non-herbal egg yolk sample has a dominant protein with a molecular weight of 49.94 kDa. This protein appears at a retention time of 1.307 minutes, an area of 149445550 units of area, and the peak height of the graph / peak at 402.6026 µAU. The standard protein that used in this research was an apolipoprotein protein component with a molecular weight of 40.13; 46; 39; 50.40 kDa. In this study, HPLC method involves the separation of protein molecules on the basis of hidrophobicity. The separation depend on the hydrophobic binding of protein from mobile phase to immobilized hydrophobic protein that attached to stationary phase. Sample of herbal egg yolk applied to the stationary phase in the presence of aqueous buffer then the sample are eluted by the addition of organic solvent to mobile phase. Elution can proceed either under isocratic conditions where the concentration of organic solvent remains constant, or by gradient elution whereby the amount of organic solvent is gradually increased over a period of time resulting in elution of solutes in the order of increasing hydrophobicity. All peptides and proteins carry a mix of hydrophilic and hydrophobic amino acids, but those with highnet hydrophobicity will be able to participate in hydrophobic interactionswith the stationary phase. As mixtures of proteins are applied to the column, polar proteins will elute first whilenon-polar proteins will bind to the column. Previous research about
egg protein separation using other methods [7], reported that herbal and non-herbal egg has a molecular weight ranging from 31-97 kDa with details as in Table 1.

**Table 1.** Molecular weight, and estimates of bioactive protein compounds from herbals and non-herbals eggs.

| Egg Type               | Fraction Number | Rf (cm) | Molecular Weight (kDa) | Estimated Bioactive Protein Compounds |
|------------------------|-----------------|---------|------------------------|--------------------------------------|
| Non Herbal Egg Yolk    | 1               | 0.06    | 96.38                  | Livetin (α-albumin)                  |
|                        | 2               | 0.08    | 89.53                  | LDL-1 apoprotein                     |
|                        | 3               | 0.14    | 71.77                  | LDL-2 apoprotein                     |
|                        | 4               | 0.20    | 57.54                  | LDL-3 apoprotein                     |
|                        | 5               | 0.26    | 46.13                  | β-α2-glycoprotein-1                  |
|                        | 6               | 0.28    | 42.85                  | β-α2-glycoprotein-2                  |
|                        | 7               | 0.30    | 39.81                  | Apolipoprotein                       |
|                        | 8               | 0.52    | 31.91                  | Apolipoprotein                       |
| Herbal Egg Yolk        | 1               | 0.06    | 96.38                  | Livetin (α-albumin)                  |
|                        | 2               | 0.08    | 89.53                  | LDL-1 + antioxidant apoprotein       |
|                        | 3               | 0.10    | 83.17                  | LDL-2 + antioxidant apoprotein       |
|                        | 4               | 0.16    | 66.06                  | LDL-3 + antioxidant apoprotein       |
|                        | 5               | 0.20    | 57.54                  | Apoprotein LDL-3 + antioxidant       |
|                        | 6               | 0.24    | 49.65                  | β-α2-Glycoprotein-2                  |
|                        | 7               | 0.30    | 39.81                  | Apolipoprotein + antioxidant         |
|                        | 8               | 0.36    | 36.98                  | Apolipoprotein                       |
|                        | 9               | 0.52    | 31.91                  | Apolipoprotein                       |

Herbal mix that given in chicken feed contains several kinds of herbal include turmeric, ginger, sambiloto, noni, soursop leaves, bangle, and lemongrass. Each of these bioactive compounds dominated by flavonoids [8] [9] [10]. Flavonoid can act as antioxidants by inhibiting the oxidation process of the substrate on one side of the free chain that does not have an electron pair so that free radicals become stable. Bioactive flavonoid compounds are strong inhibitors of lipid peroxidation which can capture oxygen or nitrogen compounds (ROS or RNS) [11]. The presence of hydroxylation and the relative position of the OH group are important factors that determine the bioactive ability of flavonoids as antioxidants. Flavonoids can bind superoxides, hydroxyl and peroxyl radicals. Flavonoid also affect arachidonic flow through cyclooxygenase-2 or lipoxygenase in the lipid oxidation process [9] [12] [13]. The activity of bioactive flavonoid compounds from herbals affect the protein fraction that appears at the peak. Flavonoid bioactive compounds affect the process of egg protein biosynthesis and causes the protein molecular weight of herbal egg yolks was higher than non-herbal eggs. The difference in molecular weight tought as antioxidant bound to IgY. These antioxidants are likely to bind to the egg protein complex so that the herbal egg yolk protein fraction that appears has antioxidant characteristics (apoprotein and apoproproteinc). Those protein reported be able to show antioxidant activity by inhibiting lipid oxidation. The ability of appoprotein as a natural antioxidant from egg can be increased by activating the sulfihydrile groups in the molecule during the conjugation process with polysaccharides such as galactomannan.

4. Conclusion
From the result it can be concluded that herbalss affect the characteristics of egg protein fractions. Based on HPLC peak, the alleged antioxidant were bound to IgY, but the bioactive compounds that play a role can not be determined (not detected). The findings in this study need further elaboration through other analytical methods including quantitative analysis of antioxidants and qualitative analysis that can show the types of antioxidants that are bound to bioactive peptides in eggs.

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