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HETEROGENEITY OF PROTEINS IN BIRDS' EGG-WHITES

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ABSTRACT

Little is known about the comparative heterogeneity of protein compounds among a wide variety of birds' egg white, particularly, an analysis of their detailed, in-parallel protein composition. Hence this research is conducted mainly to evaluate the extent of variability among 42 types of birds' egg white. To improve the perception of these biological fluids, the main phenotypic variations of egg whites were evaluated using the discontinuous denaturing polyacrylamide gel electrophoresis (SDS-PAGE), Gradient SDS-PAGE, Native-PAGE, cellulose acetate electrophoresis, and the reverse-phase high-performance liquid chromatography (RP-HPLC). The results showed that the Native-PAGE and SDS-PAGE produced better screening results than other methods in identifying protein. Although Native-PAGE and SDS-PAGE did not show remarkable variability in terms of hydrophobicity, several electrophoretic differences of egg-white proteins were observed. Several unknown proteins in the egg white samples of different bird species were also identified through the electrophoretic experiments. Hence, it might be possible, as in the case of egg white samples, to provide a characterized assessment among birds by using only the available gel electrophoresis techniques. This study also provided a rapid snapshot of the initial identification of several unknown components of egg white proteins. Accordingly, this study constituted the first large-scale comparative proteomics investigation performed among the largely varying types of egg whites from commercial stores and bird keepers in the middle Euphrates areas in Iraq.

Keywords: egg white, gradient-PAGE, HPLC, native-PAGE, SDS-PAGE

INTRODUCTION

One of the main scientific compasses in many egg-related kinds of research is the use of egg white components as a cornerstone in several fields of food and drug industry (Kovacs-Nolan *et al.* 2005; Abu-Ghoush *et al.* 2010; Omana *et al.* 2010). The egg white proteins in birds are rich in essential amino acids and possess, particularly in chickens, valuable nutritional food (Mine 2008). It contains many individual protein components with high potential for several industrial applications, such as ovalbumin, ovotransferrin, ovomucoid, ovomucin, and lysozyme (Abeyrathne *et al.* 2013). Several parameters that affect the egg white were investigated, such as ambient temperature (Akkouche *et al.* 2012), salt content (Kaewmanee *et al.* 2011), pH (Bovskova & Mikova 2011), and storage practices or conditions (Qiu *et al.* 2012). More than forty different egg white proteins were isolated and identified in a previous study (Sunwoo & Gujral 2015).

Most of the known egg white components were usually extracted from chickens (Awade 1996; Raikos *et al.* 2006; Guerin-Dubiard *et al.* 2006; D'Ambrosio *et al.* 2008; Mann 2007; Mann & Mann 2011), yet chickens are not the only birds from which egg whites are highly utilized for the food industry. Other egg-white sources, such as quails and ostriches, or even other related species, are also available for this purpose in several regions around the world. Therefore, the essential importance of chickens as a very abundant and cheap source of egg

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white proteins does not mean neglecting the significant contributions of other species that have remarkable impacts in the food industry. Full reliance on the chicken egg whites as the main and only source of many food and industrial applications may not be sufficient to fulfill all the extended needs of the recent requirements as non-chicken-based protein sources are increasingly extended to suit the multiple expanded needs of society.

Despite the magnitudes of researches conducted on egg whites, there is still a lack of a complete comparative proteomic profile concerning the detailed protein chemical compositions of several egg white varieties. Although several comparative studies on egg white proteomics were performed on many poultry species (Desert *et al.* 2001; Miguel *et al.* 2005; Omana *et al.* 2011; Qiu *et al.* 2012; Wang *et al.* 2012), a complete comparative data to construct a concrete basics on these differences is lacking. Detailed information on the egg whites of other species compared with chicken egg whites, were not abundant enough to build a broader view of the nature and extent of these differences. Thus, this study included other bird species.

A profile of the egg white differences could be a useful tool for researchers in applying the various methods of analyzing the egg white varieties. The egg white protein is one of the best-known bird proteins (Campell *et al.* 2003); hence, the need to start evaluating their differences. The protein profiles of egg whites, the most accessible protein sources, occupy valuable roles in bird protein phenotypic studies. As long as such fluids contain many standardized proteins, many possible variables could be used in the proteomic diagnosis to differentiate among the types of birds. Undoubtedly, knowledge on the protein components of these bird egg whites and their physicochemical properties could enhance its potential applications in the food industry (Nys & Sauveur 2004), and its therapeutic applications (Narat 2003), and could also intensify one's knowledge of various biological processes (Wellman-Labadie *et al.* 2008).

Few large-scale information is available regarding divergence in the whole egg white compositions among the different bird species. Despite previous studies on the egg whites, the number of researches describing the egg whites' variability among birds' genera and species is still very few. Thus, this study on the extent of differences among the egg whites of various bird species.

This task was done by performing a direct screening of the egg white proteins using the simple proteomic separation techniques to identify the molecular categories of birds. Hence, it was not the purpose of this investigation to determine the entire chemical composition of the egg white varieties. Rather, its purpose was to determine whether the protein heterogeneity evidence alone can support the suggested diagnostic approach. Accordingly, this work constituted an initial large-scale study that simplifies in-parallel proteomic investigation as this included a direct comparison among more than forty different types of egg white proteins.

MATERIALS AND METHODS

Sample Collection and Preparation

All the samples were collected from different commercial stores and bird keepers from various regions of the middle Euphrates areas in Iraq (Table 1). For about 120 days, 42 eggs from 42 commercially and locally available types of birds were collected, phenotypically classified, and stored at -20 °C as whole eggs until further processed. In the case of large-sized eggs, the egg white samples were collected from each egg by windowing the sterilized eggshell, while in the small-sized eggs, the egg white samples were obtained by cracking the sterilized eggshells. These egg whites were then centrifuged for 10 min at 3461 xg at room temperature in a clinical centrifuge (EBA 20, Hettich, Germany). Any spoiled egg white was omitted from this study. All the supernatants were kept under -20 °C until further processed.

Table 1 List of the bird species from which egg white samples were collected

No.	Scientific name	Common name	Place of collection
1.	*Columba livia domestica*	Domestic pigeon	Babil
2.	*Columba livia*	Rock dove	Babil
3.	*Streptopelia semitorquata*	Red eye dove	Babil
4.	*Streptopelia tranquebarica*	Red turtle dove	Babil
5.	*Columba palambus*	Common wood pigeon	Kufa
6.	*Streptopelia roseogrisea*	African collared dove	Kufa
7.	*Streptopelia bitorquata*	Island collared dove	Babil
8.	*Streptopelia tranquebarica*	Red turtle dove	Babil
9.	*Streptopelia decaocto*	Eurasian collared dove	Babil
10.	*Meleagris gallopavo*	Domesticated turkey	Babil
11.	*Coturnix adansonii*	African blue quail	Babil
12.	*Agapornis fischeri*	Fischer's fischeri	Babil
13.	*Melopsittacus undulates*	Budgerigar	Babil
14.	*Rollulus rouloul*	Greenwood quail	Kufa
15.	*Ammoperdix griseogularis*	See-see Partridge	Karbala
16.	*Ammoperdix griseogularis*	See-see partridge	Karbala
17.	*Carduelis carduelis*	European goldfinch	Karbala
18.	*Falco peregrinus*	Peregrine falcon	Kufa
19.	*Tadorna tadorna*	Common Shelduck	Babil
20.	*Alopochen aegyptiacus*	Egyptian goose	Babil
21.	*Anser anser rubrirostris*	Iraqi goose	Karbala
22.	*Anas Platyrhynchos*	Domestic duck	Babil
23.	*Coracias garrulous*	European roller	Karbala
24.	*Gallus gallus domesticus*	Chicken	Kufa
25.	*Agapornis personatus*	Yellow-collared lovebird	Karbala
26.	*Agapornis nigrigenis*	Black-cheeked lovebird	Karbala
27.	*Ammoperdix heyi*	Sand partridge	Kufa
28.	*Padda oryzivora*	Java sparrow	Babil
29.	*Streptopelia turtur*	European turtle dove	Karbala
30.	*Agapornis fischeri*	Fischer's lovebird	Karbala
31.	*Gallus domesticus*	Faverolles chicken	Karbala
32.	*Coturnix Coturnix*	Common quail	Karbala
33.	*Glareola pratincola*	Collared pratincole	Kufa
34.	*Agapornis roseicollis*	Rosy-faced lovebird	Karbala
35.	*Alectoris Barbara*	Barbary partridge	Babil
36.	*Charadrius dubius*	Little ringed plover	Karbala
37.	*Galerida crista*	Crested lark	Karbala
38.	*Nymphicus hollandicus*	Cockatiel	Kufa
39.	*Passer domesticus*	House sparrow	Karbala
40.	*Treron phoenicoptera*	Yellow-footed green pigeon	Babil
41.	*Francolinus francolinus*	Black francolin	Babil
42.	*Sturnus vulgari*	Common starling	Babil

Separation of Egg White Samples Using Discontinuous SDS-PAGE

In separating the egg white samples using the discontinuous SDS-PAGE method, the supernatants were diluted (1:1) in the denaturing-loading buffer (0.5M Tris-HCl, pH 6.8; 4% SDS; 20% glycerol; 10% β-mercaptoethanol and 5% bromophenol blue), and then heated for 3 min at 95 °C in a water bath (Memmert, Schwabach, Germany). Each sample was separated by gel electrophoresis at 10% mini vertical gel format, gel size at 10 cm ×10 cm (W×L), and gel thickness at 1 mm (Model OmniPAGE, Cleave Scientific – UK), and for the midi vertical gel format, the gel size was at 12 cm × 14.5 cm (W×L), and gel thickness at 1 mm (Model JY-SCZ9, Junyi-Dongfang Electrophoresis Equipment – China). The discontinuous Laemmli (SDS-PAGE) method was applied (Laemmli 1970) with minor modifications. For the mini gel format, electrophoresis of the egg white proteins was performed using a 10% separating gel buffer [10% of 30:0.8% acrylamide/bisacrylamide, 1.5 M tris-Cl pH8.8, 0.4% (w/v) SDS], and a 6% stacking gel buffer [6% of 30:0.8% acrylamide/ bisacrylamide, 1 M tris-HCl pH 6.8, 0.4% (w/v)

SDS]. For the midi gel format, the concentration of separating gel buffer was changed to 12%. From 9 μg to 15 μg of loaded samples were prepared by mixing 1:1 V/V with sample denaturing-loading buffer (0.5M Tris—HCl, pH 6.8; 4% SDS; 20% glycerol; 10% β-mercaptoethanol and 5% bromophenol blue). The prestained molecular weight standards were also routinely loaded (Bioneer Cat # D-2010). The loaded samples were electrophoresed in 1X of running buffer [25 mM Tris pH 8.3, 250 mM glycine, 0.1% (w/v) SDS] in a vertical electrophoresis tank at 120 V and 30 mA for the mini gel formats, and 200 V and 85 mA for the midi gel formats. Electrophoresis was performed at constant parameters until the tracking dye reached the end of the gel. The gels were stained with Coomassie blue (Candiano *et al.* 2004).

Separation of Egg White Samples Using Gradient SDS-PAGE

In separating the egg white samples by using Gradient SDS-PAGE, the supernatants were diluted (1:1) in the denaturing-loading buffer and then heated at 95 °C in a water bath for 3 min. Each sample was separated by gel electrophoresis at 4 – 10% concentration for the midi vertical gel format. The gradient method of Domingo (1990) was applied, with some modifications. Briefly, two solutions were prepared in the casting of the 4 – 10% gradient gel in midi format gels. The preparation of Solution A (or heavy solution) included 10% acrylamide (2.7mL acrylamide solution, 3.28 mL D.W., 2 mL separating gel buffer, 1.2 g sucrose, 20 μL freshly prepared ammonium persulfate, and 10 μL freshly added TEMED) while Solution B (or light solution) preparation included 4% acrylamide (1.06 mL acrylamide solution, 4.8 mL D.W., 2 mL separating gel buffer, 20 μL freshly prepared ammonium persulfate, and 10 μL freshly added TEMED). The total volume of the light and heavy solution is 15 mL, which is sufficient to prepare a gradient gel in a 50 mL capacity disposable syringe. Then, 5% stacking gel [6% of 30:0.8% acrylamide/bisacrylamide, 1M tris-HCl pH 6.8, 0.4% (w/v) SDS] was applied above the gradient separating gel. From 9 μg to 15 μg of loaded samples were prepared by mixing 1:1 V/V with sample loading buffer. The prestained molecular weight standards were also routinely loaded (Bioneer Cat # D-2010). Loaded samples were electrophoresed in 1X of running buffer in a vertical electrophoresis tank at 180 V and 85 mA, for midi gel formats. Electrophoresis was performed at constant parameters until the tracking dye reached the end of the gel. Gels were stained with Coomassie blue.

Separation of Egg White Samples Using Native-PAGE

The same sample preparation procedure in the SDS-PAGE was followed in the Native-PAGE method. The supernatants were diluted (1:1) in non-denaturing loading buffer (0.5M Tris—HCl, pH 6.8; 4% SDS; 20% glycerol; and 5% bromophenol blue). Each sample was separated by gel electrophoresis on a 10% midi gel format, and the discontinuous Native-PAGE method was applied (Arndt *et al.* 2012). Electrophoresis of egg white proteins was performed using 10% separating gel buffer [10% of 30:0.8% acrylamide/bis acrylamide, 1.5M tris-Cl pH8.8], and 6% stacking gel buffer [6% of 30:0.8% acrylamide/bisacrylamide, 1 M tris-HCl pH 6.8]. From 7 μg to 13 μg of loaded samples were prepared by mixing 1:1 V/V with sample loading buffer. Four molecular weight standard proteins were also routinely loaded (14 kd of lysozyme, 31 kd of carbonic anhydrase, 45 kd of ovalbumin, 66 kd of bovine serum albumin, 97 kd of phosphorylase B). The loaded samples were electrophoresed in 1X of running buffer [25 mM Tris pH 8.3, 250 mM glycine] in a vertical electrophoresis tank at 120V and 30 mA. Electrophoresis was performed at constant parameters until the tracking dye reached the end of the gel. Gels were stained with Coomassie blue.

Separation of Egg White Samples Using Cellulose Acetate

The separation of egg whites using the cellulose acetate electrophoresis was performed according to Keren method (Keren 2003). CellasGEL of 250 μm strips (2.5 cm x 7 cm) were used in these experiments (Cleaver Scientific, Warwickshire, UK). The strips were soaked with agitation for 30 min at room temperature in barbital buffer (Tris Hippurate

0.05 M, pH 8.8, Barbital tris 0.05 M). The strips were briefly blotted and immediately spotted with 2 µL of each egg white sample. Electrophoresis was performed by CSL-CELLAS device (Cleaver scientific, Warwickshire – UK) at 200 volts for 35 min at room temperature in barbital buffer. A standard bovine serum albumin fraction V was used as a size marker (BioLabs, London W1W 6DB, UK). After electrophoresis, the strips were then stained and fixed by immersion in a staining solution [1 g ponceau S, 37.5 g trichloroacetic acid, 37.5 g sulfosalicylic acid in 500 ml water (w/v)] for 10 min. Destaining was then performed by washing the samples several times with gentle agitation in a destaining solution (10% ethanol, 5% glacial acetic acid). The strips were dried at room temperature and imaged by a digital camera (Sony – China). The generated images were analyzed using the CS analyzer software (ATTO, Yushima, Bunkyo-ku, Japan).

Separation of Egg White Samples Using the RP-HPLC

The reverse phase-high performance liquid chromatography (RP-HPLC) separation procedures were performed according to Miguel *et al.* (2005), with some modifications. The egg white proteins were separated by the HPLC system equipped with a UV-Visible detector (Knauer advanced scientific instruments, Berlin, Germany). System control and data acquisition were performed by Clarity chromatography station software (DataApex, Prague, Czech Republic). The analysis was carried out using a Discovery® BIO Wide Pore C18 column, with 4.6 X 250 mm, 5 µm (Supelco, Madrid, USA), at ambient temperature. Two solvents were used in the mobile phase of these experiments: Solvent A at 0.1% (v/v) trifluoroacetic acid (TFA) in HPLC-grade water, and solvent B at 0.1% (v/v) TFA in HPLC-grade acetonitrile. Elution was performed at room temperature with a flow rate of 0.8 ml/min and a linear gradient from 2 to 65% of solvent B for 60 min then to 75% of solvent B at 90 min. Absorbance was monitored at 214 nm. Before the injection, samples were filtered through 0.45-mm filters (Millipore Corporation, Bedford, MA, USA).

RESULTS AND DISCUSSION

To determine their macromolecular components, the egg whites were directly subjected to various electrophoresis techniques; namely, the polyacrylamide gel-based and cellulose acetate-based techniques, and RP-HPLC. Despite the accumulated data on egg-white components in different birds, the direct effect of genera and species of birds on the main components of the egg white was less emphasized. Particularly, the whole egg white proteins of chickens, quails, and ducks have been studied extensively (Mann & Mann 2011; Hu *et al.* 2016; Miguel *et al.* 2005), however, those studies were not performed on a scale large enough to build an initial screening data to identify the nature of these differences. In this study, several routinely used electrophoretic techniques, such as denaturing, non-denaturing, and gradient PAGE were applied to compare between the benefits and limitations of each technique, with accurate judgment, amongst the analyzed egg-white samples. In addition, several routinely used non-electrophoretic experiments were performed to collectively monitor the differences of the whole egg white profile. Therefore, instead of applying the commonly used DNA-based diagnostic tools in birds (Pereira *et al.* 2008), several attempts were carried out to use proteomics identifications as an alternative.

Although the genomic diagnosis is highly accurate, the proteomic diagnosis is characterized with a very high dynamic process since it is directly correlated with the changeable protein expression levels (Corthals *et al.* 2000; Fey & Larsen 2001). Therefore, this study provides an assessment of egg white as a dynamic diagnostic marker using several proteomic routine techniques. The utilization of low-cost and basic analysis techniques may broaden the applications of this diagnosis around the world. SDS-PAGE followed by Coomassie blue detection is one of the routinely available techniques that could be invested at a low cost yet could provide a straightforward identification of the egg white proteins. However, SDS-PAGE alone is limited in terms of its low ability to resolve proteins of similar molecular masses (Cassiday 2007). Thus, it should be aided with another electrophoretic

technique to overcome its shortcomings in detecting several unknown protein bands.

Therefore, in addition to submitting the egg white samples to variable SDS-PAGE conditions, other techniques were applied, such as Native-PAGE and the cellulose acetate techniques. The hydrophobic HPLC was also applied to give a further fingerprint about the whole nature of the samples with regard to proteins function and specificity.

SDS-PAGE

Several denaturing electrophoretic conditions in terms of varying gels and sample concentrations were used to show the most beneficial profile. Several other technical standardizations were made, such as maximizing the sample numbers in each gel format to enhance the chances of the correct in-parallel reading. These were optimized as much as possible to provide a direct and simultaneous comparison among a larger number of samples. The limited dimensions of small mini gel formats did not provide an accurate in-parallel comparison of the egg white bands. Therefore, larger formats and greater well numbers were included to load as many samples as possible in one gel format. Thus, the sizes of gels and the number of wells were approximately duplicated. Moreover, each concentration of separating gel could precisely describe a certain range of proteins and relatively neglect the other proteins of other molecular weights (Rath *et al.* 2009). Therefore, two different concentrations of gel were used in each case. However, since polyacrylamide gel electrophoresis is very sensitive to any tiny changes in protein profile, two different concentrations of egg white proteins were also applied (Fig. 1). Moreover, based on molecular weights (MW), many proteins were identified in many egg white samples (Awade 1996; Cao 2005; Sunwoo & Gujral 2015).

Although silver staining is a very sensitive technique compared to Coomassie (Weiss *et al.* 2009), it was omitted from the staining because of practically several limiting factors. The differences in development time may give a non-real quantitative density of the protein bands as several proteins were obscured because of the dark areas that emerged during development (Gromova & Celis 2006). Some of the protein bands were identified by simple direct comparison with their standards, while other bands were not. This associated difficulty of gel reading interpretations could not be resolved without submitting the same samples to further conditions as several egg white proteins have extremely similar molecular weights (Desert *et al.* 2001).

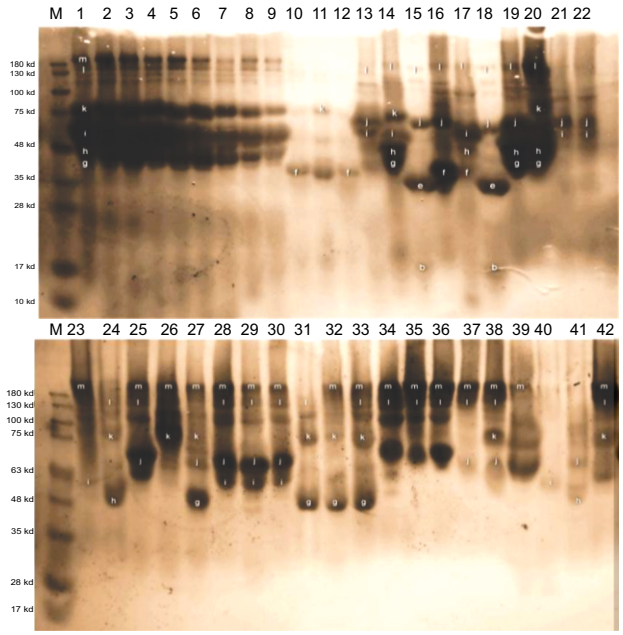

Figure 1 SDS-PAGE analysis of egg white protein samples in 12% midi gel formats
Notes: Lane M = ladder marker. Lanes 1 – 42 = various birds' egg white protein samples.
Letters "a" to "m" = egg white resolved proteins.

In addition to the limited range of proteins to be resolved on the gel, several extremely high and low molecular weight standards were not readily available for comparison (Hu *et al.* 2016). Nonetheless, several simple electrophoretic migrations in this study had provided useful data on the distinct resolving power for many types of egg whites based only on one-dimensional electrophoresis. Despite the high electrophoretic variations among the egg white samples, a particular pattern of distribution of egg white proteins was observed in some phenotypically related samples. This is noticeable in the first nine samples that were very closely related to each other in terms of biological relationships. This highlighted the potential ability of these simple electrophoretic conditions in providing an initial diagnostic marker among the samples. However, remarkable differences exist between the egg white patterns of a species and other distant families. Not only the discrete differences among the isolated and identified egg white proteins identities were known but also the differences in their concentrations (Miguel *et al.* 2005). Thus, this result indicated the potential capability of this simple one-dimensional, low cost, and rapid screening SDS-PAGE method to identify the extent of phenotypic divergence among birds.

In addition to the many repetitive electrophoretic separations, other practical difficulties in separating the egg white samples were encountered as it is relatively hard to simultaneously standardize these variedly viscous specimens in only one gel format. Thus, the specimens were submitted to different concentrations of SDS-PAGE and other electrophoretic environments. Another related factor is the large gap that existed in the protein concentrations of the various egg whites. For instance, ovalbumin, ovotransferrin and ovomucoid represent about 77% of egg white content (Mine 1995), while other components never exceeded 1%, such as avidin and flavoprotein (Desert *et al.* 2001). Therefore, to improve the detection of proteins in such samples, different amounts of proteins were loaded (Fig. 2). Fortunately, several protein bands were unambiguously identified in most of the samples.

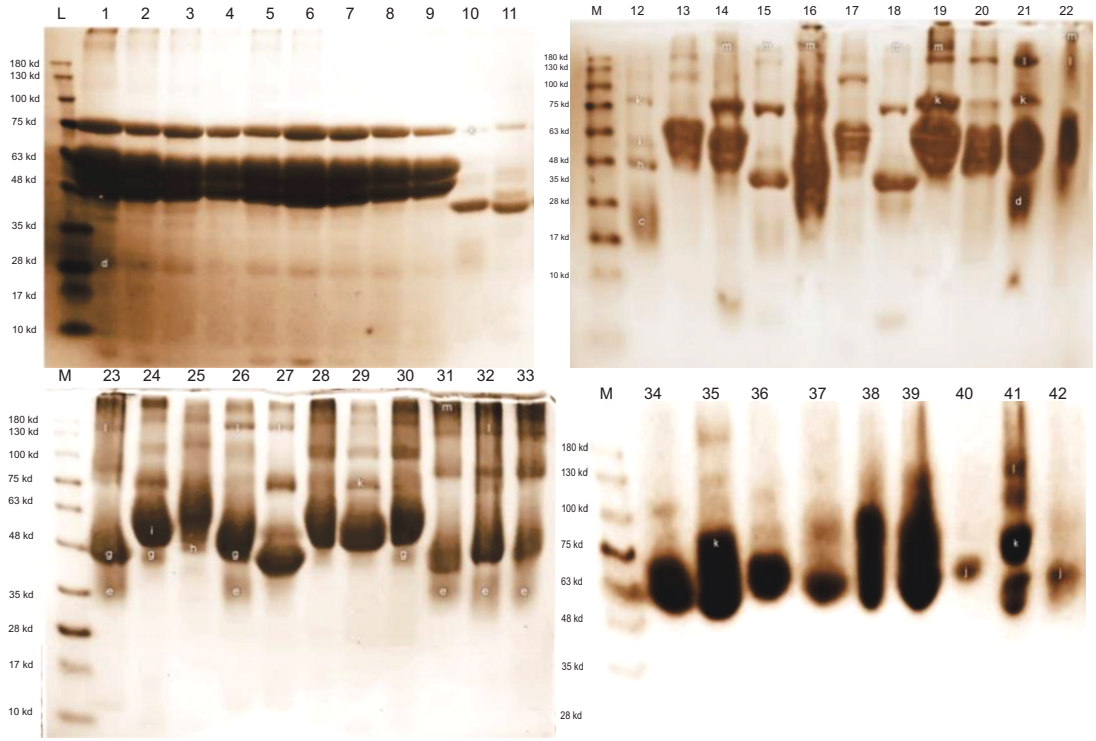

Figure 2 SDS-PAGE analysis of egg white protein samples in 10% mini gel formats
Notes: Lane M = ladder marker. Lanes 1 – 42 = various birds' egg white protein samples.
Letters "a" to "m" = egg white resolved proteins that did not resolve in Fig. 1.

Remarkably, the most abundant proteins in the studied samples were ovomucin proteins (MW 135 – 150 kd, and 220 – 270 kd) (Alleoni 2006) while cystatin, a minor protein with MW 13 kd, was not detected in all samples (Abeyrathne *et al*. 2013) (Table 2).

Table 2 The expected observed bands of the birds' egg white samples and their corresponding proteins according to variable PAGE conditions

	Description of Known Proteins Bands												
	a Cystatin	**b** Lysozyme	**c** Ovoglyco protein	**d** Ovomucoid	**e** Ovoflavo protein	**f** Thiamine binding protein	**g** Ovalbumin	**h** G_3 Ovoglobulin	**i** Ovoinhibitor	**j** Avidin	**k** Ovotransferrin	**l** Ovomucin	**m** Ovomucin
MW (Kd)	**13**	**14**	**24**	**28**	**32-35**	**38**	**45**	**47**	**54**	**67-68**	**76-78**	**135-150**	**220-270**
No. of samples													
1	-	-	-	+	-	+	+	+	+	+	+	+	+
2	-	-	-	+	-	+	+	+	+	+	+	+	+
3	-	-	-	+	-	+	+	+	+	+	+	+	+
4	-	-	-	+	-	+	+	+	+	+	+	+	+
5	-	-	-	+	-	+	+	+	+	+	+	+	+
6	-	-	-	+	-	+	+	+	+	+	+	+	+
7	-	-	-	+	-	+	+	+	+	+	+	+	+
8	-	-	-	+	-	+	+	+	+	+	+	+	+
9	-	-	-	+	-	+	+	+	+	+	+	+	+
10	-	-	-	-	+	+	+	+	-	-	+	-	-
11	-	-	-	-	+	-	+	-	-	+	+	-	-
12	-	-	+	-	+	+	+	+	+	+	+	-	-
13	-	+	-	+	-	-	+	-	+	+	-	+	-
14	-	-	-	-	-	-	+	+	+	-	+	+	+
15	-	+	-	-	+	+	+	-	-	+	-	+	+
16	-	-	+	-	-	+	-	-	-	+	-	+	+
17	-	-	-	-	-	+	-	+	+	-	-	+	-
18	-	+	-	+	+	-	+	-	-	+	-	+	+
19	-	-	-	-	-	-	+	+	-	+	+	+	+
20	-	+	-	-	+	-	+	+	-		+	+	-
21	-	+	-	+	+	-	-	-	+	+	+	+	-
22	-	+	-	-	+	-	-	-	+	+	-	+	+
23	-	-	-	-	+	-	+	-	+	-	-	+	+
24	-	-	-	-	-	-	+	+	+	-	+	+	-
25	-	-	-	-	+	-	-	+	-	+	-	+	+
26	-	-	-	-	+	-	+	-	-	-	+	+	+
27	-	-	-	-	-	-	+	-	+	+	+	+	+
28	-	-	-	-	+	-	-	-	+	+	-	+	+
29	-	-	-	-	+	-	-	-	+	+	+	+	+
30	-	-	-	-	+	-	+	-	+	+	-	+	+
31	-	-	-	-	+	-	+	-	-	-	+	+	+
32	-	-	-	-	+	-	+	-	+	-	+	+	+
33	-	-	-	-	+	-	+	-	-	-	+	+	+
34	-	-	-	-	+	-	-	-	-	-	-	+	+
35	-	-	-	-	-	-	-	-	+	-	+	+	+
36	-	-	-	-	+	-	-	-	-	-	-	+	+
37	-	-	-	-	-	-	-	-	-	+	-	+	+
38	-	-	-	-	-	-	-	-	-	+	+	+	+
39	-	-	-	-	-	-	-	-	-	-	-	-	+
40	-	-	-	-	-	-	-	-	+	+	-	-	-
41	-	-	-	-	-	-	-	+	-	+	+	+	-
42	-	-	-	+	+	-	-	-	-	+	+	+	+

The main electrophoretic limitation for egg white separation was potentially attributed to the ability of each gel concentration to separate certain ranges of protein molecular weights. This resulted in the un-identification of many other MW bands (Table 3).

Table 3 The unknown observed bands of the birds' egg white samples according to variable PAGE conditions

No. of samples	Description of Unknown Proteins Bands							
MW (Kd)	4-6	63	83-85	90-95	100-105	115-117	120-123	125-129
1	+	+	-	-	-	+	-	+
2	+	+	-	-	-	+	-	+
3	+	+	-	-	-	+	-	+
4	+	+	-	-	-	+	-	+
5	+	+	-	-	-	+	-	+
6	+	+	-	-	-	+	-	+
7	+	+	-	-	-	+	-	+
8	+	+	-	-	-	+	-	+
9	+	+	-	-	-	+	-	+
10	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-
13	-	-	-	-	-	+	-	-
14	-	+	-	-	-	+	-	-
15	-	-	-	-	+	+	-	-
16	-	-	-	-	-	+	-	-
17	-	+	-	-	+	-	-	-
18	-	-	-	-	-	-	-	-
19	-	-	-	+	+	+	-	-
20	-	-	-	-	-	-	-	-
21	-	-	-	+	+	-	-	-
22	-	-	-	+	+	-	-	-
23	-	-	+	-	-	-	+	-
24	-	-	-	-	-	+	-	-
25	-	-	-	-	+	-	-	-
26	-	-	-	-	+	-	-	+
27	-	-	-	-	+	-	-	-
28	-	-	-	-	+	-	-	-
29	-	-	-	-	+	-	-	-
30	-	-	-	-	+	-	-	-
31	-	-	-	-	+	-	-	-
32	-	-	-	-	+	-	-	-
33	-	-	-	-	+	-	-	-
34	-	-	-	-	+	-	-	-
35	-	-	-	-	+	-	-	-
36	-	-	-	-	+	-	-	-
37	-	-	-	-	+	-	-	-
38	-	-	-	-	-	-	-	-
39	-	-	-	+	-	-	-	-
40	-	-	-	-	-	-	-	-
41	-	-	-	-	-	-	-	-
42	-	+	-	-	-	+	-	-

This condition, however, was minimized by subjecting the egg white samples to various PAGE conditions. However, even though the various electrophoretic conditions were applied, the procedure still had inevitable limitations due to lack of discrimination among the various forms of proteins because of glycosylation (Jay *et al.* 1990) and phosphorylation (Li *et al.* 2003), or due to the splitting of some proteins into smaller subunits in the reducing conditions (Hoppe 2010). Although these techniques identified many proteins according to their MW differences on the gel, little is known on whether these differences are attributed to the various post-translational modifications that might have been followed by some of these proteins in their three-dimensional structure, their amino acids residues, their backbones, or discrete differences in their amino acid sequences. The majority of the egg white samples, however, are polymorphic (Guérin-Dubiard *et al.* 2006), and this added more complication in their direct comparative visualization. Thus, the presence of certain physical barriers in the egg white samples resisted against the electrophoretic separation of the whole egg white samples. The electrophoretic experiments were repeated several times as it was not easy to directly separate the egg whites because of the steric resistance induced by the carbohydrate moieties (Desert *et al.* 2001). This might be due to the high viscosity resulting from the presence of ovalbumin (Alleoni 2006). Moreover, other difficulties were encountered when glycoproteins migrated randomly during the SDS-electrophoresis because the sugar moieties do not bind SDS (Hames 1998). Hence, if the purpose of this study is to perform an in-depth analysis of these egg white samples, the 2-dimensional electrophoresis and MALTI-TOF analysis are the prerequisites (Hu *et al.* 2016). The gradient gel electrophoresis can allow a greater range of separation if both large and small proteins MW need to be resolved simultaneously in only one gel format (Brunelle & Green 2014). However, several proteins, such as ovalbumin (sample No. 13), ovoflavoprotein (samples No. 25, 28, 29, 30, 36, and 42), and ovomucoid (sample No. 42) were not resolved in discontinuous SDS-PAGE (Fig. 3).

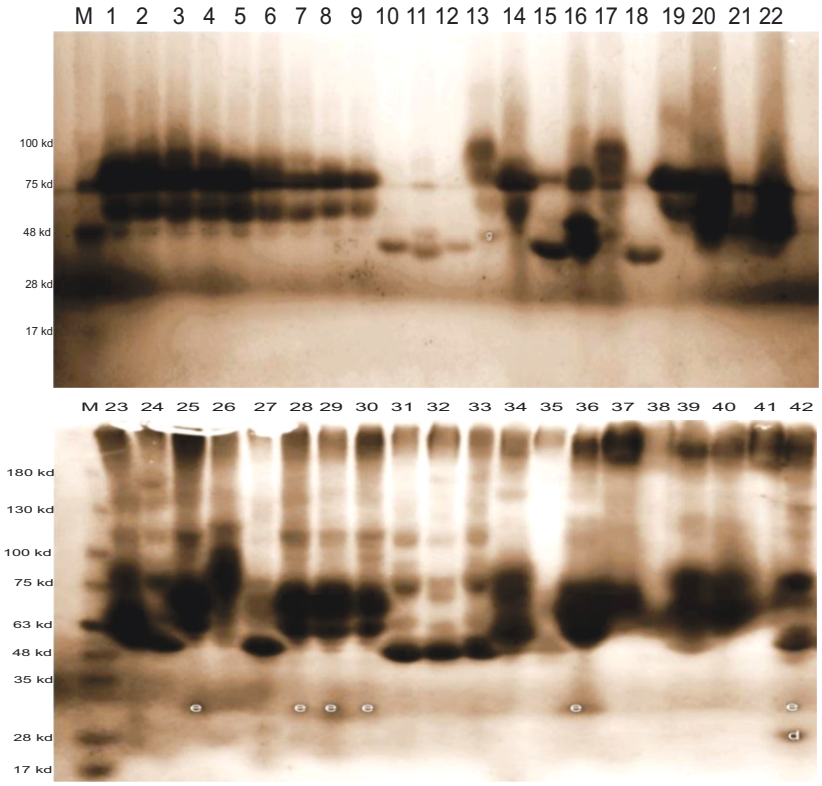

Figure 3 Gradient-PAGE analysis of egg white protein samples
Notes: Lane M = ladder marker. Lanes 1 – 42 = various birds' egg white protein samples.
Letters "a" to "m" = egg white resolved proteins that did not resolve in Fig. 1 and Fig. 2.

Native-PAGE

Although SDS-PAGE is the most popular method due to its availability, reproducibility, and ease of use, the complicated situation of proteins differs in terms of having more reaction sites as seen in these various egg white samples. Hence, SDS-PAGE alone may not offer the best resolution required (Zheng *et al.* 2007).

To achieve a comprehensive understanding of cellular proteins, the limitations of SDS-PAGE was overcome by adding another method, the Native-PAGE. The different egg white samples were immediately subjected to the Native PAGE process as many proteins lose their natural conformations due to the denaturing conditions in the SDS-PAGE. These reducing conditions caused the samples to behave in a manner that does not resemble their natural habit (Nowakowski *et al.* 2014). Although native-PAGE is not commonly applied in the usual diagnosis of many protein samples (Gallagher 1999), it is mandatory to expose these various samples into the non-denaturing conditions to take a snapshot of the many unknown samples that were not easily identified in the SDS-PAGE conditions. As expected, another unique pattern was observed. However, despite this unique resolution, the same pattern of distributions for almost all samples was observed (Fig. 4). Nevertheless, it is still relatively difficult to calculate a lot of proteins MW according to their native separation. The paucity of previous Native-PAGE analyses is the main reason for this difficulty. Hence, monitoring of the natural behavior of many proteins that have relatively close MW may increase the difficulty of this task. However, several proteins, such as ovoglycoprotein (sample No. 16), ovomucoid (samples No. 13 and 18), ovoflavoprotein (samples No. 10, 11, 12, 20, 21, and 22), thiamine binding protein (sample No. 15), ovalbumin (samples No. 10, 11, 12, 15, and 18), G3 ovoglobulin (sample No. 10), ovoinhibitor (samples No. 27, 32, and 53), and avidin (samples No. 10, 11, and 12) that were not resolved in SDS-PAGE were identified using the Native-PAGE.

By using both the denaturing and non-denaturing electrophoretic techniques in this study, the multiple common bands were resolved and identified in most of the samples, such as 32 - 35, 45, 47, 54, 67 - 68, 76 - 78, 135 - 150, and 220 - 270 KDa, which represent ovoflavoprotein, ovalbumin, G_3 ovoglobulin, ovoinhibitor, avidin, ovotransferrin, and ovomucin I and ovomucin II, respectively. Therefore, in the electrophoretic portion of this study, several proteins were localized with certainty, namely; ovoglycoprotein (MW 24kd), ovomucoid (MW 28kd), ovoflavoprotein (MW 32 - 35kd), thiamine binding protein (MW 38kd), ovalbumin (MW 45kd), G3 ovoglobulin (MW 47kd), ovoinhibitor (MW 54kd), avidin (MW 67 - 68kd), ovotransferrin (MW 76 - 78kd), and ovomucins (MW 135 - 150 and 220 - 270kd). However, many bands are still unknown and waiting to be individually recognized. Fortunately, in addition to the collectively high resolving power of these several one-directional electrophoretic techniques in the in-parallel detection of the many protein types, it might be possible for these techniques to give a semi-quantitative indication of the intensity of each particular protein per lane. For instance, in this study, the overall ovalbumin concentration occupied the most noticeable quantity of the separated proteins. This result agrees with that of Stadelman and Cotterill (2001) that ovalbumin constitutes 54% of the total proteins while the overall concentration of ovomucoid bands occupied a very low quantity of the resolved egg white proteins. Ovomucoid is a highly glycosylated protein, so its actual MW is characterized by its changeability in electrophoresis (Kovacs-Nolan *et al.* 2005). Ovomucoid concentration does not exceed 11% of the total egg white proteins (Caubet & Wang 2011).

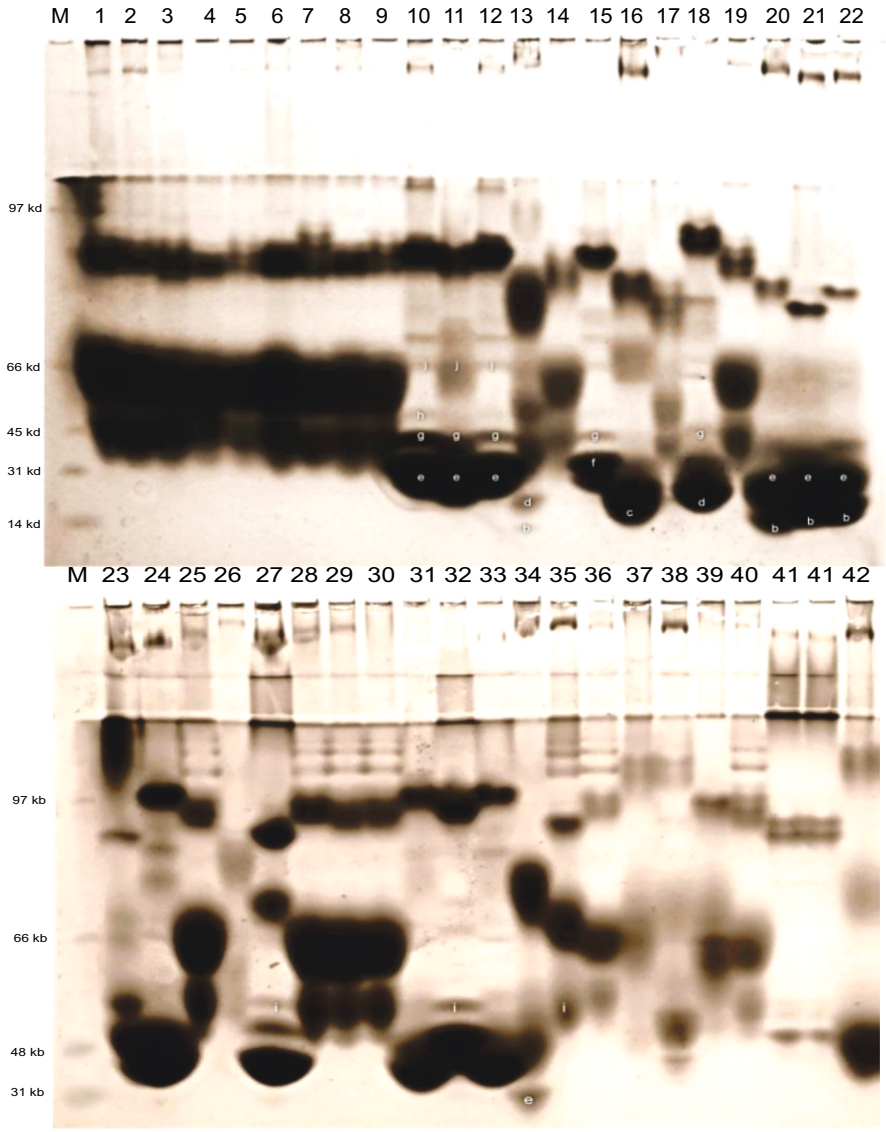

Figure 4 Native Polyacrylamide Gel (Native-PAGE) electrophoresis of high concentrations of egg white protein samples
Notes: Lane M = ladder marker. Lanes 23 – 42 = various birds' egg white protein samples.
Letters "a" to "m" = egg white resolved that did not resolve in Fig. 1, Fig. 2, Fig. 3.

Cellulose Acetate Electrophoresis

To further sustain the screening impression of the natural behavior of the samples, the whole egg whites were subjected to the cellulose acetate electrophoresis technique in a non-biased sequential manner (Fig. 5). Despite the observed low resolution of cellulose acetate method, it provided valuable information about the electrical charges of egg white proteins. Six egg white samples demonstrated one positively charged band (sample No. 9, 11, 18, 24, 27, and 33). In their natural biological fluids, this positively charged proteins or emulsifiers were not abundantly available in food (Decker 1998). In addition to the relatively low resolving power manifested in the reduced number of the observed band (Table 4), these cellulose acetate results did not categorically correspond with the classified phenotypic differences of the electrophoresed egg white samples.

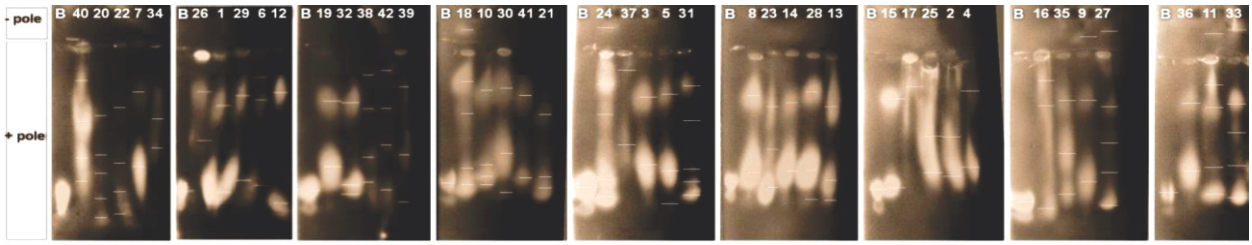

Figure 5 Cellulose acetate gel electrophoresis of egg white proteins samples
Notes: Lane "B" refers to bovine serum albumin fraction V marker.
Lanes 1 – 42 refer to various birds' egg white protein samples. The color is changed to black and white to get a better resolution.

Table 4 Summary of the behavior of each type of egg white in the cellulose acetate electrophoresis

No. of samples	Positive bands	The relative distance of the negative bands concerning bovine albumin				
1	-	0.59	1.00			
2	-	0.72	0.93			
3	-	0.52	0.88			
4	-	0.74	0.88			
5	-	0.50	0.91	1.09		
6	-	0.46	0.57	0.97		
7	-	0.38	0.83			
8	-	0.54	0.95			
9	+	0.51	0.90			
10	-	0.54	0.91	0.97	1.01	
11	+	0.44	0.56	0.87	1.01	
12	-	0.53	1.06			
13	-	0.60	0.99	1.10		
14	-	0.58	0.94			
15	-	0.51	0.99			
16	-	0.41	0.54	1.03		
17	-	0.44				
18	+	0.50	0.66	0.93	1.01	
19	-	0.35	0.83			
20	-	0.52	0.76	0.87	1.02	1.14
21	-	0.66	0.97	1.03		
22	-	0.47	0.81	0.98	1.07	
23	-	0.60	0.83	1.03		
24	+	0.45	0.61	0.96	1.05	
25	-	0.71	0.91			
26	-	0.53	0.76			
27	+	0.54	0.72	0.86	1.02	
28	-	0.52	0.94	1.09		
29	-	0.55	0.95			
30	-	0.53	0.85	1.05		
31	-	0.46	0.64	0.90	0.97	1.04
32	-	0.36	0.96	1.03		
33	+	0.53	0.69	0.86	1.03	
34	-	0.40	0.54	0.71		
35	-	0.51	0.77	0.96		
36	-	0.54	0.89			
37	-	0.38	0.77			
38	-	0.16	0.41	0.76	0.94	
39	-	0.24	0.74	1.09		
40	-	0.31	0.56	0.78	0.91	
41	-	0.57	0.97			
42	-	0.13	0.40	067	1.00	

RP-HPLC

Although the discontinuous and gradient gel electrophoresis method had been prescribed for egg white separation, the use of reverse-phase high-performance liquid chromatography (RP-HPLC) might also help monitor the resolving power, as well as assessing the degree of proteins specificity, particularly in their functions. The relatively similar resolution patterns in almost all samples (Fig. 6) potentially indicated a similar functional specificity that was adopted by most of the egg white proteins within the eggs' environment. However, the typically high resolution of RP-HPLC was significantly reduced in resolving structurally similar components from a complex mixture (Mitulović 2015). In such cases, sufficient time was needed to separate the great number of peaks from each other. This has extended the run time for these 42 samples to more than 60 hours, increasing the runtime to 90 min/sample. Other limitations included the fact that HPLC could not be usually performed for more than one sample at a time.

RP-HPLC is limited in this direct comparative diagnostics scope even when it is being used depending on the size exclusion property. In contrast to the electrophoretic techniques that have given the high diversity of the electrophoresed proteins, RP-HPLC did not provide such high diversity. In this study, the electrophoretic separation had provided remarkable superiority compared to RP-HPLC. Considering variability, RP-HPLC might have failed to give the desired categorical information about the actual heterogeneity of the egg white varieties. Moreover, as in some cases in Fig. 6, the HPLC peaks may be broad and overlapping due to the heterogeneity of the egg white samples. This might be attributed to the complexity of the adsorption mechanism of protein aggregates in hydrophobic interaction chromatography that was not fully understood (Mahn 2012). Nevertheless, by using RP-HPLC, a noticeable conservative nature of almost all of the studied proteins was observed. The predominant characteristic in the egg white could be attributed to the presence of egg white with similar functions, as shown in the similar hydrophobicity peaks (Fig. 6).

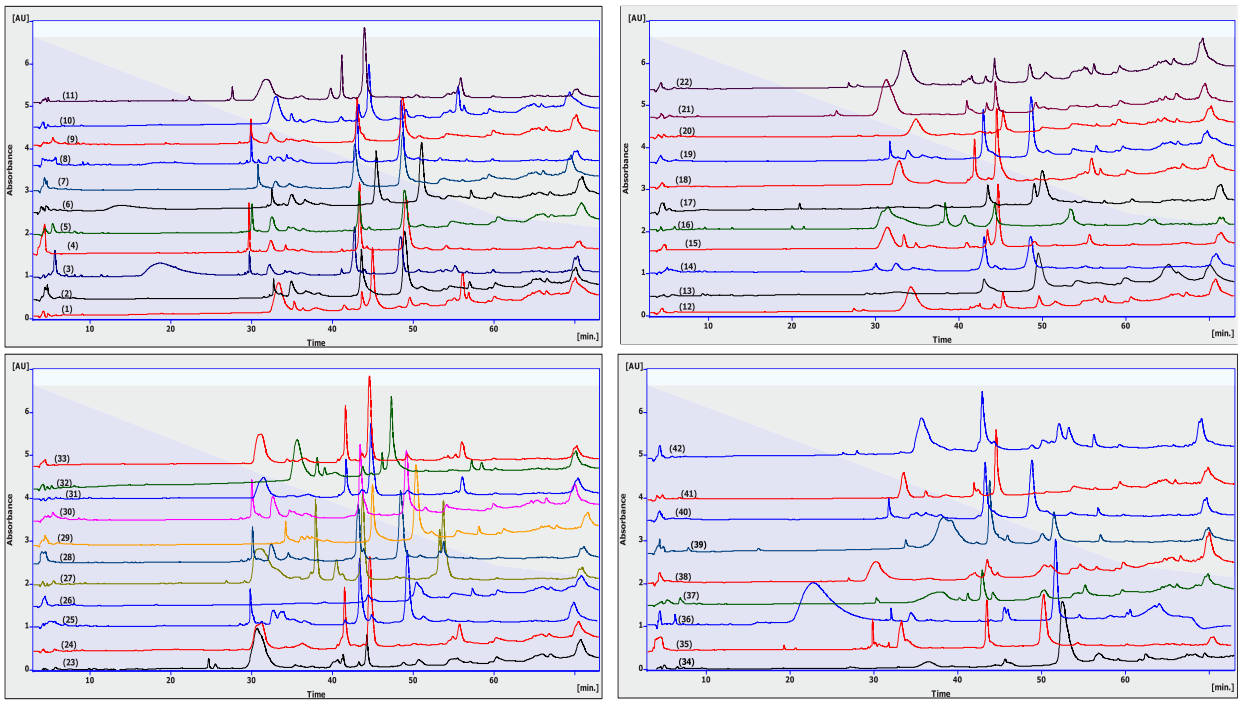

Figure 6 Reverse-phase high-performance liquid chromatography (RP-HPLC) for egg white proteins. The number of each lane is indicated in each chromatogram.

In addition to the longer time run of all of the egg white samples resulting from its inability to provide a simultaneous run of all samples, HPLC has limited ability to verify samples based on their hydrophobicity. Thus, the closely related nature of egg white proteins was elucidated through RP-HPLC. Nonetheless, some samples exerted unique peaks in certain portions of elution, such as sample No. 3 and No. 29. Although RP-HPLC provides an initial clue through the similar peaks despite all the egg whites' high diversity obtained from other techniques, these differences were not attributed to their functions. Instead, other potential factors were involved in the interpretation, such as the classified phenotypic differences. In other words, RP-HPLC results provided an additional indicator for the possibility of using egg whites as initial diagnostic tools or as a basis for the bird phenotypic classification. Therefore, it might be appropriate to describe these differences as "species-related" instead of being "function related".

In contrast to this study, other studies indicated that the differences in the phenotypically diverse eggs are not related to chemical compositions but on the different concentrations of their proteins due to egg whites' species diversity (Wang *et al.* 2012). However, in this study, the egg white-related heterogeneity was obvious; both the qualitative characteristic in protein alterations and the quantitatively discrete variations of egg white proteins were observed.

CONCLUSION

Obvious differences among the egg white proteins of the different bird species were observed electrophoretically. The results indicated that both Native and SDS-PAGE methods produced a better resolution and therefore possessed certain potential applicability as egg white diagnostic tools. These methods might provide an initial diagnostic marker to differentiate bird species through their egg whites. However, without additional data from reverse-phase high-performance liquid chromatography (RP-HPLC), these methods did not give satisfactory reliability to diagnose the bands.

In summary, despite all the differences, the electrophoretic results still provided the way for more rapid screening studies by further optimizing several conditions in the SDS-PAGE, one of which could be done by minimizing the gel-based procedure to an acceptable level to provide a more reproducible diagnostic tool in differentiating the types of various birds' egg whites.

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