STORAGE STABILITY STUDY OF A COMMERCIAL SPRAY-DRIED HEN EGG YOLK POWDER

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Mufan Guo

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Abstract

Dehydration is a good process approach for food preservation. However, dried food products may still suffer from deterioration if store in an abused environment such as high humidity (water activity (a_w) > 0.6) and/or high temperature (> 45°C). These storage conditions can induce undesirable chemical reactions (disulfide bond interactions, Maillard reaction and/or lipid oxidation), resulting in a significant decrease in food quality.

In this study, the storage stability of a commercial spray-dried egg yolk powder was evaluated. The dried egg yolk powder (DEY) was stored at three temperatures (room temperature, 35°C, and 45°C) and at six a_w (0.05, 0.12, 0.37, 0.44, 0.54, 0.66) for at least two months, and several physicochemical changes and extent of protein aggregation were measured.

The overall color change of DEY was that it became slightly darker (decrease of L^* value), more red (increase of a^* value), and less yellow (decrease of b^* value) with increased storage time. The reaction kinetics of the L^* value of DEY was also calculated using a first-order hyperbolic model. Its Q_{10} (rate increase with temperature increase at 10°C) was 2.9, which was more indicative of lipid oxidation, and the E_a (activation energy) was around 83 kJ/mole. The color change was mostly due to the browning pigments that were produced from the Maillard reaction and lipid oxidation. The glucose content went to zero after one-week during storage at 45°C at an a_w of 0.66, confirming the occurrence of the Maillard reaction. The peroxide value of DEY storage at 45°C at a_w of 0.66 was significantly increased compared to the control (vacuum packaged at -20°C),

proving the occurrence of lipid oxidation. In addition, the Maillard reaction products and lipid oxidation products were both detected using the front face fluorescence spectrometer.

After storage at an a_w of 0.66 at 45°C for 8 weeks, protein solubility of DEY in TBS-SDS buffer [Tris-buffered saline (TBS: 20 mM Tris and 500 mM sodium chloride, pH 7.5) containing 1% sodium dodecyl sulfate (SDS, g/ml)] decreased to ~ 78% compared with that of the original DEY. Formations of buffer-soluble and –insoluble protein aggregates were discovered using SDS-PAGE. The protein aggregates were mainly formed through unfolded intermediates and unfolded states as well as direct chemical linkages. The proteins in DEY were all denatured after storage at an a_w of 0.66 at 45°C for 8 weeks, resulting in numerous unfolded intermediates and states that could interact with each other to form aggregates. The spray drying process during the manufacturing of DEY also caused denaturation of protein, which explained the detection of buffer-insoluble protein aggregates in the original sample. Increases of disulfide bond links and protein-lipid interaction during storage were also found using techniques such as Raman spectrometry, fourier transform infrared spectroscopy, and front-face fluorescence spectrometry, indicating that some of the protein aggregates were induced by chemical reactions.

The high molecular weight protein aggregates (HMWPAs) were further evaluated. Results showed that 32 proteins were involved with formation of buffer-soluble and -insoluble HMWPAs. They were products of natural egg yolk proteins and egg white proteins including serum albumin, vitellogenin, apovitellenin, as well as

ovotansferrin, ovalbumin, lysozyme, ovomucoid, and ovastatin. Most of them contain disulfide bonds and some of them contain ligand and fatty acid binding sites, which corresponded with the theory of the direct chemical linkages induced protein aggregates.

Overall, physicochemical changes and protein aggregates were found during the storage of DEY and it is mostly due to three undesirable chemical reactions, i.e., disulfide bond interactions, the Maillard reaction and/or lipid oxidation. Therefore, most effective approaches to reduce and/or inhibit the occurrence of those reactions include adjusting storage temperature and humidity as well as vacuum packaging after drying.

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Chapter 1 Introduction

Food deterioration is a worldwide problem for food products during storage. Deterioration includes not only becoming non-edible, but also the reduction in nutritional quality overtime. In dry and intermediate moisture food systems, the low to moderate water activity (0.2 to 0.85) helps to inhibit the growth of pathogenic microorganisms (Scott, 1957). But in that range, chemical reactions lead to increases by a factor of 2 to 3fold for every 0.1 water activity increase (Labuza, 1970).

Our previous studies showed physicochemical changes and the formation of protein aggregates during storage of pure protein powder such as whey isolates and egg white (Qinchun Rao & Labuza, 2012; Qinchun Rao, Rocca-Smith, Schoenfuss, & Labuza, 2012; Zhou & Labuza, 2007), and protein/buffer systems (Qinchun Rao, Rocca-smith, & Labuza, 2012; Qinchun Rao, Rocca-Smith, & Labuza, 2013; Zhou, Liu, & Labuza, 2008b). Both systems showed an increase in darkening of color and texture hardening as well as a decrease in free amino groups. Disulfide linkage formation between single proteins was the main reason for the formation of protein aggregates in whey protein based systems (Zhou & Labuza, 2007; Zhou et al., 2008b); while in egg protein based systems, the Maillard reaction induced protein aggregation was the more prevalent mechanism (Qinchun Rao & Labuza, 2012; Qinchun Rao, Rocca-Smith, Schoenfuss, & Labuza, 2012; Qinchun Rao, Rocca-Smith, & Labuza, 2013; Qinchun Rao, Rocca-smith, & Labuza, 2012). Besides disulfide linkages and the Maillard reaction, lipid oxidation can also induce the formation of protein aggregates mainly through two pathways: 1) formation of a lipid-protein complex through hydrogen bonding; 2)

formation of protein radicals, which can react with other protein/lipid radicals through covalent bonds (Gardner, 1979).

Dried egg yolk (DEY) is an egg product and has broad application in the food industry. It can be used in infant formula, mayonnaise, salad dressing, and bakery products primarily for emulsification, and also has the potential to be mixed with other protein powders (whey, casein, soy, and egg white) to be applied into high protein nutrition and energy bars. Dried egg yolk contains protein (36%), unsaturated fat (61%), and a small amount of the reducing sugar, glucose (0.12%). Therefore, chemical reactions are highly likely to occur during storage thus resulting in loss of quality. However, few studies have evaluated the storage stability of dried egg yolk. So in this thesis, we want to study the storage induced physicochemical changes and protein aggregation of a commercial spray-dried egg yolk powder with the objective of providing useful suggestions to maximize quality when used by the food industry.

Objectives and Hypothesis

Our long-term goal is to determine the molecular mechanism behind the storage induced physicochemical changes including protein aggregation of a commercial spray-dried egg yolk powder and its relationship with storage environment (humidity and temperature).

Our central null hypothesis is that NO physicochemical changes and formation of protein aggregates of a commercial spray-dried egg yolk powder will be found during storage, and it is not correlated with environmental humidity and storage temperature.

We plan to test our central hypothesis and accomplish our goal through pursuing the following objectives:

1) To elucidate the molecular mechanism of storage induced physicochemical changes of a commercial spray-dried egg yolk powder when stored at several temperatures and different water activities
2) To elucidate the molecular mechanism of storage induced protein aggregation of a commercial spray-dried egg yolk powder when stored at several temperatures and different water activities

Chapter 2 Literature Review

Egg yolk powder

The per capita consumption of shell & processed egg products has remained almost constant (equivalent to around 250 whole eggs) since 1984, but the per capita consumption of egg product has been increasing to an equivalent 37 whole eggs in 1984 to 76 whole eggs in 2014 (American Egg Board: Egg Production and Consumption, 2014), indicating an increasing demand of egg products as ingredients in food products to provide bioactivity and/or functionality. Dried egg yolk powder (DEY) is an egg product, which enjoys several advantages over liquid egg yolk especially in terms of shelf stability (American Egg Board, EGGSolutions - The complete reference for egg products, 2004; American Egg Board, Egg product reference guide, 2006). The low moisture content prevents the growth of microorganisms (Beuchat, 1981; Christian, 1963; Harris, 1981) and reduces chemical reactions (Theodore P Labuza, 1980). DEY has a broad application not only in food but also in medical, pharmaceutical, cosmetic, nutraceutical and biotechnological industries due to its functionality and bioactivity. In the food industry, DEY can be used in many food products, for nutrition like infant formula as well as for physical functionality in mayonnaise, salad dressing, bakery products, and custard. It also has huge potential to be applied in high protein nutrition and energy bars, a current nutrition fed.

Egg yolk powder composition

According to the USDA (United States Department of Agriculture) National Agriculture Library, 100 g of DEY contains approximately 33.7 g of protein, 58.1 g of

lipid, 2.7 g of water, and 2.1 g of carbohydrate. In addition, it contains numerous minerals and vitamins, especially rich in folate, vitamin A and D, as well as calcium (Ca) and iron (Fe) (Table 2.1).

Egg yolk can be separated into plasma (supernatant) and granule (sediment) under certain centrifuge condition (dissolving in 0.16 M NaCl solution, and then centrifuged at 10,000 ×g for 45 min at 10°C) (Anton & Gandemer, 1997; MC Bee & Cotterill, 1979). Plasma represents 77-81% yolk dry matter, while granule represents what is left 19-23%. Plasma is composed of low-density lipoproteins (LDL) (85%) and livetins (15%), accounting for 50% of yolk proteins and 90% of yolk lipids. Granule is mainly composed of high-density lipoprotein (HDL) (70%) and phosvitin (16%), with minor LDL (12%). It accounts for about 50% of yolk proteins and 7% of yolk lipids (Anton, 2013; Burley & Cook, 1961). However, Mann's proteomic study (Mann & Mann, 2008a) of egg yolk found most proteins can be detected in both plasma and granule but with different abundances, indicating that the boundary of plasma and granule is not very strict and proteins are dispersed in the whole yolk.

Table 2.1: Nutrition values of dried egg yolk powder. (USDA Nutrition Database)

Nutrient	Unit	Value per 100 g
Water	g	2.7
Energy	kcal	679
Protein	g	33.7
Total lipid (fat)	g	58.1
Carbohydrate	g	2.1
Total dietary fiber	g	0
Total sugar	g	0.1
Calcium, Ca	mg	307
Iron, Fe	mg	9.5
Magnesium, Mg	mg	26
Phosphorus, P	mg	1040
Potassium, K	mg	264
Sodium, Na	mg	163
Zinc, Zn	mg	7.7
Vitamin C, total ascorbic acid	mg	0
Thiamin	mg	0.4
Riboflavin	mg	1.2
Niacin	mg	0.1
Vitamin B-6	mg	0.8
Folate, DFE	µg	209
Vitamin B-12	µg	6.0
Vitamin A, RAE	µg	785
Vitamin E (alpha-tocopherol)	mg	4.8
Vitamin D (D2 + D3)	µg	11.4
Vitamin K (phylloquinone)	µg	1.5
Fatty acids, total saturated	g	18.1
Fatty acids, total monounsaturated	g	23.7
Fatty acids, total polyunsaturated	g	8.8
Fatty acids, total trans	g	0.2
Cholesterol	mg	2307

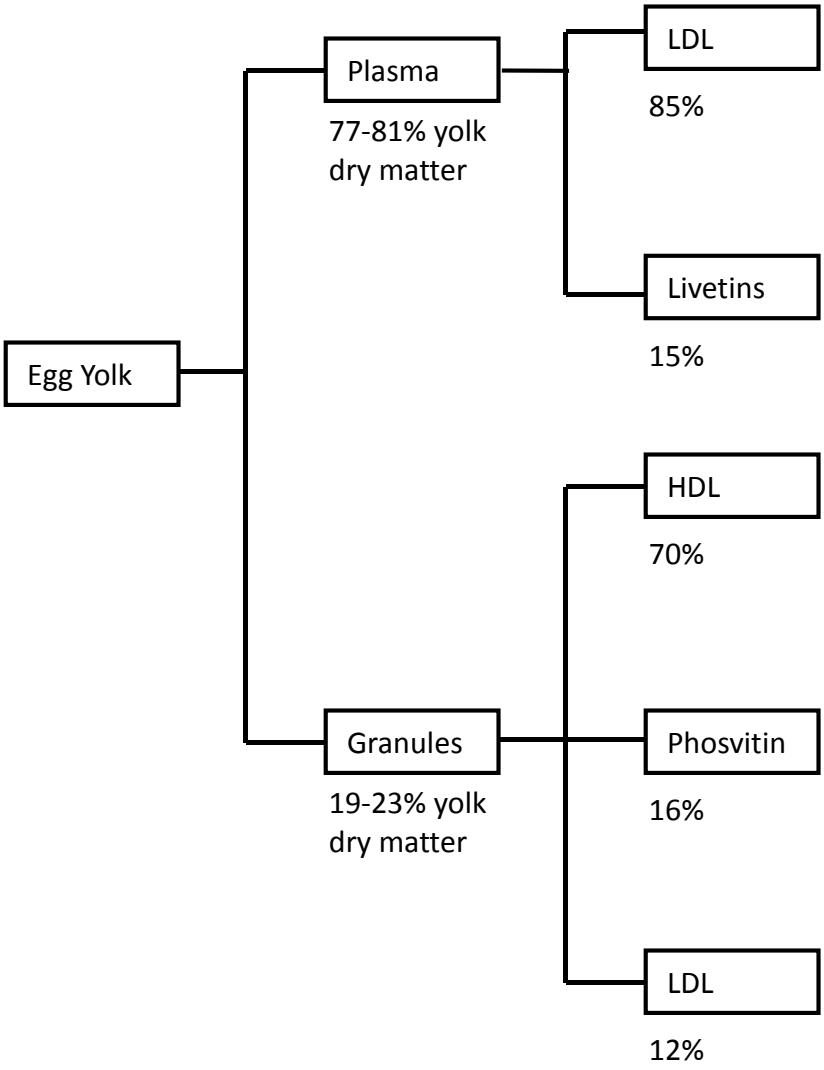


Figure 2.1: The composition of egg yolk. LDL: low-density lipoprotein; HDL: high-density lipoprotein. (Adopted from Anton (2013))

Plasma Structure

Plasma is the clear yellow fluid and is composed of LDL and livetins (Anton, 2013). The structure of LDL is a soluble micelle, with a monofilm of phospholipids and proteins on the outside, and triglycerides and cholesterol esters in a liquid state on the inside (Figure 2.2) (Anton, 2013). Some cholesterol is in the monofilm to increase the rigidity of the monofilm structure (Anton, 2007b). Its diameter is between 17 and 60 nm.

LDL contains only 11-17% protein but 83-89% lipid (74% neutral lipid and 26% phospholipid) (W. H. Cook & Martin, 1969).

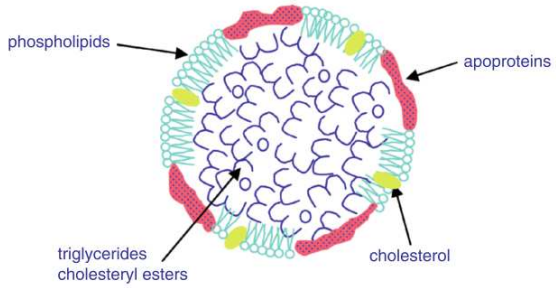


Figure 2.2: The structure of low density lipoproteins. (Adopted from Anton (2007))

LDL is soluble in aqueous solution no matter the ionic strength because of its low density (0.982). The stability of the LDL micelle is mostly dependent on phospholipids, because they provide the hydrophobic interactions, which are the essential association forces of the LDL structure (Anton, 2007b). LDL contains two subgroups: LDL_1 and LDL_2; while LDL_1 accounts for 20% of LDL but the protein content is twice as much as that of LDL_2 (Anton, 2007b; W. Martin, Augustyniak, & Cook, 1964).

Granule Structure

The structure of the granule is HDL-phosvitin circular complexes with diameters ranging from 0.2 to 2 μm under low ionic strength (Bellairs, 1961; C. M. Chang, Powrie, & Fennema, 1977). These complexes are linked through numerous phosphocalcic bridges, leading to a "very compact, poorly hydrated and weakly accessible to enzymes" structure, which effectively protects themselves from denaturation and gelation during heat treatments (Anton, 2013). At high ionic strength (1.71 mol L^{-1} NaCl), the HDL-phosvitin complexes will be disrupted and form soluble micelles with a diameter between

100 and 200 nm instead. Between low ionic strength and high ionic strength, both HDL-phosvitin complexes and micelles are present (Anton, 2013).

HDL has a pseudo-molecular structure (close to globular proteins), which is unlike LDL. Therefore, HDL is not formally a lipoprotein (Anton, 2007a). It is composed of 75-80% proteins and 20 – 25% lipids, leading to a density close to that of proteins (1.120 g/ml). The lipids are composed of 65% phospholipids, 30% triglycerides, and 5% cholesterol (W. H. Cook & Martin, 1969). HDL exists as a dimer of two monomers of around 200 kDa each. The structure of each monomer is a globular protein containing a cavity for phospholipids (Anderson, Levitt, & Banaszak, 1998). Since phospholipids are enclosed within the HDL structure, it is difficult for enzyme access. Phospholipase C will only liberate 6% lipid phosphorus of HDLs whereas it can liberate 95% of that of LDLs (Burley & Cook, 1961). HDL also has two subgroups: α- and β- HDL (Radomski & Cook, 1964). α- HDL accounts for 40% of yolk HDL and is more acidic but less water soluble than β- HDL, because α- HDL contains more sialic acid and phosphorus. They are very similar in terms of chemical composition. They both contain around 30% amino acids that are hydrophobic (Anton, 2007a).

Proteins in egg yolk powder

Egg yolk contains about 36% protein (dry basis), which accounts for about 40% of the protein of a whole egg. In 2008, Mann's lab identified 119 proteins from chicken egg yolk using 1D SDS-PAGE, LC-MS/MS, and MS^3, which is the most in-depth proteomic analysis of hen egg yolk so far (Mann & Mann, 2008b). Among all the proteins, serum albumin (α-livetin), the vitellogenin cleavage products, apovitellenins,

IgY (γ-livetin), ovalbumin, and 12 kDa serum protein with cross-reactivity to β2-microglobulin are the most abundant proteins (Mann & Mann, 2008b). However, the amount of phosvitin was underestimated due to the low detectability of phovitin peptides, which is because of "the dense spacing and high number of phosphorylated amino acids and the correspondingly low number of cleavage sites" (Mann & Mann, 2008b).

The vitellogenin cleavage products

The proteins, apolipovitellins and phosvitin, in HDL are cleaved products from vitellogenin (dimeric protein, 480 kDa) (S. Wang, Smith, & Williams, 1983), which is synthesized in the liver under the regulation of estrogen and transferred from blood to yolk (Anton, 2007a).

Apolipovitellins

As mentioned above, HDL is present as a dimer of two monomers. Each monomer is composed of five main apoproteins. The molecular weights of the five apoproteins range from 35 to 110 kDa (Anton, 2007a). However, in Mann's paper, they only detected two apolipovitellins: apolipovitellins I (125 kDa), and II (35 kDa). They are cleaved products of vitellogenin I and II (Yamamura et al., 1995), and possibly of vitellogenin III, because vitellogenin III has been detected both in yolk plasma and granule and its fragments include partial fragments of apolipovitellins I and II, and phosvitin (Mann & Mann, 2008b).

Phosvitin

Phosvitin is also cleaved from vitellogenin I, II, and III. It is a non-lipid phosphoglycoprotein, which accounts for 60% of phosphoproteins and 90% of

phosphorous in yolk. It is also the most highly phosphorylated protein existing in nature (Mecham & Olcott, 1949), more than 55% of which is composed of serine (Allerton & Perlmann, 1965). The clusters of phosphoserines can effectively bind minerals like calcium and iron, thus improve their bioavailability. In addition, phosvitin contains 6.5% carbohydrate, which includes six hexose, five glucosamine and two sialic acids attached to the N-acetyl derivatives per 40 kDa molecular weight (Samaraweera, Zhang, Lee, & Ahn, 2011; Shainkin & Perlmann, 1971).

Apovitellenins

Apovitellenins are proteins in yolk LDL, which are all constituents of blood derived very-low-density lipoprotein (VLDL). The major blood VLDL, apoprotein B (apo B), which is a 523 kDa protein, very similar to human apoB-100, cleaves into seven fragments, apovitellenins III – VI, during or after transferring from blood to egg yolk by cathepsin D or a cathepsin D-like pepstatin A-sensitive protease (Evans & Burley, 1987). The molecular weights of the seven fragments range from 55 to 190 kDa (Jolivet, Boulard, Beaumal, Chardot, & Anton, 2006). Another major apovetellenin of egg yolk LDL is apovitellenin I, which is not derived from apo B (Anton et al., 2003). Apovitellenin I is a small homodimer linked with disulfide bond. It is present in egg yolk LDL as a monomer (9 kDa) or homodimer (18 kDa) (Jolivet et al., 2006). All apovitellenins have neutral isoelectric point (pI = 6.3 – 7.5) (Kojima & Nakamura, 1985) and contain 40% of hydrophobic amino acids, indicating a highly hydrophobic and flexible structure (Anton, 2007b). The glycosylation sites of apovitellenins are on asparaginyl residues. The composition of carbohydrate in apovitellenins is 1.3% hexose, 0.7% hexosamine, and 0.4% sialic acid (Saari, Powrie, & Fennema, 1964).

Livetins (serum albumin, IgY, α-glycoprotein)

Livetins are water-soluble, non-lipid, globular glycoproteins in yolk. In 1949, Shepard and Hottle discovered that livetins are composed of three fractions (Shepard & Hottle, 1949). Later in 1957, Martin, Vandegaer, and Cook confirmed the three fractions: α-, β-, and γ-livetins (W. G. Martin, Vandegaer, & Cook, 1957). William further identified α-, β-, and γ-livetins as serum albumin, α-glycoprotein, and IgY respectively (Williams, 1962). Serum albumin (α-livetin) is sensitive to high ionic strength and both α- and β-livetin are vulnerable to organic solvents (Juneja & Kim, 1996). Livetins are also the most heat sensitive proteins in yolk due to the globular structure, especially for γ-livetins (Dixon & Cotterill, 1981; Juneja & Kim, 1996; Woodward & Cotterill, 1983).

Other proteins

Yolk also contains other lipid-binding proteins, including apoliporotein A-I and apolipoprotein D. In addition, it contains vitamin- and cofactor-binding proteins, e.g. biotin-binding proteins and vitamin D-binding proteins. In addition, several egg white proteins have been detected in yolk, which are ovalbumin, ovomucoid, ovotransferrin, and ovoinhibitor. This is due to: 1) a possible route with receptor-mediated endocytosis, and 2) the up-to-date breaking technology cannot separate white from yolk completely, which will be considered later in the "Processing of egg yolk powder" section.

Lipids in egg yolk powder

Egg yolk contains 60% of lipids (dry basis), which accounts for all the lipids in a whole egg. The function of lipids is to provide metabolic energy for the embryo. In fresh

egg, lipids are mostly combined with proteins through non-covalent interactions as in lipoproteins. However through dehydration processing, some of the lipids are liberated from the inside core, especially for LDL.

In 1978, Cotterill et al. evaluated the fatty acid composition of commercial dried egg yolk powders and found the fatty acids are composed of 63% unsaturated fatty acids and 37% of saturated fatty acid. Among the unsaturated fatty acids, oleic acid is the majority, followed by linoleic acid and palmitoleic acid. Palmitic acid is the majority in saturated fatty acids, followed by stearic acid (Cotterill, Glauert, & Froning, 1978)(Table 2.2). The fatty acids are present in egg yolk powder, not in their single molecular state, but mostly attached to certain backbones to form neutral lipids (65%) and phospholipids (31%) (Juneja, 1996).

Table 2.2: Fatty acid composition of dried egg yolk powder (100 g).

	Yolk (100 g)	Discrep. [a] (%)
Fatty acids (g)		
Myristic (14:0)	0.25	+ 1.2
Palmitic (16:0)	12.95	+ 3.9
Stearic (18:0)	5.14	- 3.4
Palmitoleic (16:1)	1.49	- 5.9
Oleic (18:1)	23.61	0.0
Linoleic (18:2)	6.58	+ 3.0
Linolenic (18:3)	0.26	- 13.6
Arachidonic (20:4)	0.51	+ 7.5

[a]: Calculation of percent discrepancy – whole egg$^{calc.}$ = 0.28 egg white + 0.72 egg yolk * (whole egg$^{calc.}$ – whole egg$^{obs.}$)/whole egg$^{obs.}$ * 100 = percent discrepancy (Reproduced from Cotterill et al. (1978))

Neutral lipids

The neutral lipids include triacylglycerols, diacylglycerols, monoacylglycerols, fatty acids, carotenoids, and sterols. Triacylglycerols, diacylglycerols, and

monoaceylglycerols are glycerols attached with three, two and one fatty acids respectively (Akoh & Min, 2008a). For triacylglycerols in yolk, palmitic acids mostly occupy the position *sn*-1; oleic and linoleic acids occupy the position *sn*-2; and oleic, palmitic and stearic acids occupy position *sn*-3 (Figure 1.3)(Christie & Moore, 1970).

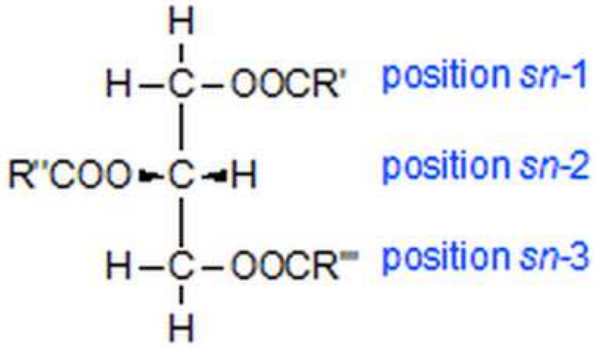


Figure 2.3:Fischer projection of a triacyl-*sn*-glycerol (Adapt from http://lipidlibrary.aocs.org/Lipids/tag1/index.htm)

Phospholipids

Phospholipids are composed of a glycerol-phosphate backbone (phosphate on position *sn*-3), a polar head group (X), and fatty acids. Phospholipids are classified into six major groups according to the X group. Phophatidic acid (PA) is a phospholipid with X group of a hydrogen; phoshatidycholine (PC) is with choline; phosphatidylethanolamine (PE) is with ethanolamine; phosphatidylglycerol (PG) is with glycerol; phosphatidylserine (PS) is with serine; phosphatidylinositol (PI) is with inositol (Akoh & Min, 2008b). The major phospholipids in egg yolk are PC, PE, Lysophophatidylcholine (LPC), and sphingomyelins (SM) (

Table 2.3) (Juneja, 1996).

Table 2.3: Composition of egg yolk phospholipids.

Phospholipids	Concentration %
Phosphatidylcholine	80.8
Phosphatidylethanolamine	11.7
Lysophosphatidylcholine	1.9
Sphingomyelin	1.9
Neutral Lipids + Others	3.7

(Reproduced from Juneja (1996))

Phoshatidycholine (PC)

PC accounts for about 80% of phospholipids in egg yolk (Juneja, 1996). It also has a pattern of attached fatty acids. Studies have shown that saturated fatty acids are mostly present on the *sn*-1 position, while unsaturated fatty acids mostly on the *sn*-2 position. The unsaturated fatty acids on the *sn*-2 position not only refers to oleic and linoleic acid, but also to polyunsaturated fatty acids, such as arachidonic acid (AA), and docosahexaenoic acid (DHA) (Tattrie, 1959).

Phosphatidylethanolamine (PE)

PE accounts for about 11% of phospholipids in egg yolk, which is the second major constituent of egg yolk of phospholipids (Juneja, 1996). The pattern of fatty acids on PE is similar to that of PC: saturated fatty acids on the *sn*-1 position and unsaturated fatty acids on the *sn*-2 position. Saturated fatty acids include palmitic and stearic acid and unsaturated fatty acids are oleic and linoleic acid, AA, and DHA (Juneja, 1996; Tattrie, 1959).

Lysophosphatidylcholine (LPC)

LPC is a PC that is missing a fatty acid (usually on the *sn*-2 position). LPC is a minor component, only accounting for 1.9% of phospholipids in egg yolk (Juneja, 1996).

Sphingomyelins (SM)

SM is composed of a PC polar head group (X), a sphingosine, and a fatty acid (Akoh & Min, 2008a) and accounts for 1.9% of phospholipids in egg yolk (Juneja, 1996). The fatty acids of SM are usually composed of medium chain length fatty acids, e.g. palmitic acid. Therefore, palmitoylsphingosine is the major constituent of SM in egg yolk (Connelly & Kuksis, 1983; Do, Pei, & Minard, 1981).

Cholesterol

Cholesterol is the main sterol in egg yolk. Unlike fatty acids, all cholesterols exist in their free form and are trapped within LDL and HDL. The cholesterol content in yolk varies due to different measurement approaches. As early as 1962, a study evaluated the total cholesterol content in yolk using the Thin Layer Chromatography (TLC) technique. They found that cholesterol accounted for 5.2% (15 mg/g liquid yolk) of yolk lipids (Privett, Blank, & Schmit, 1962). Then another study used high-performance chromatography (HPLC) to measure the total cholesterol and found a lower content (10.97 mg/g liquid yolk) compared with TLC method, which has been considered more accurate (Beyer & Jensen, 1989). Other studies agreed with the lower cholesterol content result since they obtained similar results when using other techniques (on-column capillary gas chromatography and supercritical carbon dioxide extraction) (Dyer-Hurdon & Nnanna, 1993; Fenton & Sim, 1991; R. Sun, Sivik, & Larsson, 1995). However, the

cholesterol content in yolk is dependent on the feed of the chicken. Studies have shown that feeding dietary fiber and probiotics could help reduce the cholesterol content in yolk (Kurtoglu*, Kurtoglu, & Seker, 2004; McNaughton, 1978). But the change is slight.

Carotenoids

Carotenoids are organic pigments, which contribute to the yellow-orange color of egg yolk. Since carotenoids cannot be produced by animals, it is obtained through the pigmented feeding ingredients including yellow corn, alfalfa, and corn gluten meal (Brockman & Volker, 1934; Gillam & Heilbron, 1935; Smith & Perdue, 1966). Therefore, the content of carotenoids is largely dependent on the feed ingredients. Carotenoids have two classes: xanthophylls and carotenes. Xanthophylls are the major constituents of carotenoids in yolk, which can be further divided into lutein, zeaxanthin, and cryptoxanthin. Lutein is the dominant xanthophylls in yolk, followed by zeaxanthin (Stadelman & Cotterill, 1973).

Nutritional value of egg yolk powder

Eggs have been an important and valuable source of food since prehistory. Chicken eggs were consumed as food in Asia and India before 7500 BCE and brought to Sumer, Egypt and other places of the world afterwards (Stadelman, 1995). Nowadays, eggs are still considered to have great nutritional value because they are a rich source of protein, unsaturated fatty acids, iron, phosphorus, trace minerals, and various vitamins (Watkins, 1995).

Proteins

Yolk has a very balanced amino acid composition and contains all the essential amino acids (Table 2.4). In addition, egg protein is known for its high digestibility among major proteins from food and has a Protein Digestibility Corrected Amino Acid Score (PDCAAS) of 1, which is the highest score a protein can get.

Table 2.4: Amino acids composition of dehydrated egg yolk.

Amino acids	Yolk (g per 100g)
Alanine	1.70
Arginine	2.28
Aspartic Acid	3.03
Cystine	0.60
Glutamic acid	4.04
Glycine	1.04
Histidine	0.84
Isoleucine	1.79
Leucicne	2.85
Lysine	2.37
Methionine	0.86
Phenylalanine	1.48
Proline	1.33
Serine	2.74
Threonine	1.72
Trytophan	0.51
Tyrosine	1.48
Valine	2.01

(Reproduced from Watkins, (1995))

Lipids

Yolk is a good source of unsaturated fatty acids. As stated above, 63% of fatty acids in yolk is unsaturated, which is much higher than most animal lipids. In addition, yolk is also a good source of choline due to its high content of PC. Choline is considered

good for brain development, liver function, and cancer prevention (Woodbury & Woodbury, 1993; Zeisel, 1992).

Except for the good, healthy fatty acids, yolk is also rich in cholesterol, which has been considered a not healthy part of the diet. Research evaluating the relationship between dietary cholesterol and several human diseases has been controversial (Hu et al., 2012; McNamara, 2014). For example a great number of animal experiments, epidemiological surveys, and clinical interventions did not find a direct correlational relationship between dietary cholesterol and human disease such as heart disease, cardiovascular disease (Fernandez & Webb, 2008; Fernandez, Wilson, Conde, Vergara-Jimenez, & Nicolosi, 1999; Herron et al., 2002; Kritchevsky & Kritchevsky, 2000; Lee & Griffin, 2006). Some studies also found that dietary fat intake had a more significant affect in increasing the blood cholesterol content, especially dietary saturated fat intake (Moore, 1989). This indicates that eggs are healthier when compared with other animal fats in terms of dietary saturated fat. But researchers do suggest limiting the consumption of eggs for patients at risk of cardiovascular disease (Spence, Jenkins, & Davignon, 2010) and for older people in their 70s (Houston et al., 2011), because the cholesterol content in one fresh egg is 275 mg, which is more than the recommended daily intake value (200 mg/day).

Vitamins

Yolk contains of the vitamins (especially fat soluble vitamins) that are necessary for human being, except vitamin C. Yolk is rich in Thiamin, Riboflavin, Folate, as well as

Vitamin B-6, B-12, A, E, and D (Table 1.5). It has all the nutrients simply because they are all necessary for the development of the embryo.

Table 2.5: Vitamins in egg yolk with Recommended Daily Intake (RDI) based on a 2,000 calorie diet (Stadelman & Cotterill, 1973).

Vitamins	Unit	Value per 100 g	RDI
Vitamin C, total ascorbic acid	mg	0	60
Thiamin	mg	0.4	1.5
Riboflavin	mg	1.2	1.7
Niacin	mg	0.1	20
Vitamin B-6	mg	0.8	1.7
Folate, DFE	µg	209	400
Vitamin B-12	µg	6	6
Vitamin A, RAE	µg	785	900
Vitamin E (alpha-tocopherol)	mg	4.8	15 (Tocopherol)
Vitamin D (D2 + D3)	µg	11.4	10 (D3)
Vitamin K (phylloquinone)	µg	1.5	80

Minerals

Yolk is rich in calcium, iron, phosphorus, calcium, and iron (Table 1.6). However, the bioavailable of iron may be blocked due to the iron binding proteins: phosvitin and ovotransferrin. Studies have shown that when incorporating egg yolk proteins with iron in a diet, the iron absorption decreased probably because phosvitin bound iron was not available during digestion (J. D. Cook & Monsen, 1976; Kim, Lee, & Lee, 1995; Rossander, Hallberg, & Bjorn-Rasussen, 1979) as the iron binding capacity of phosvitin is very significant and not influenced by high temperature and/or pressure (Castellani, Guérin-Dubiard, David-Briand, & Anton, 2004).

Table 2.6: Minerals in egg yolk with Recommended Daily Intake (RDI) based on a 2,000 calorie diet (Stadelman & Cotterill, 1973).

Minerals	Unit	Value per 100 g	RDI
Calcium, Ca	mg	307	1000
Iron, Fe	mg	9.5	18
Magnesium, Mg	mg	26	400
Phosphorus, P	mg	1040	1000
Potassium, K	mg	264	3500
Sodium, Na	mg	163	2400
Zinc, Zn	mg	7.7	15

Bioactivity of egg yolk

Except for the above stated nutritive value, egg yolk is also a source of antibodies, which aim to protect the new offspring from diseases. Hens have been found to have three kinds of Immunoglobulins: IgY, IgA, and IgM. IgY is mainly transferred to egg yolk, while IgA and IgM are mostly found in egg white (U Lösch, Schranner, Wanke, & Jurgens, 1986; Uli Lösch, 1996; Rose, Orlans, & Buttress, 1974; Yamamoto, Watanabe, Sato, & Mikami, 1975).

Discovery of IgY

In 1893, A German scientist Dr. Klemperer first discovered the present of IgY in egg yolk (Klemperer, 1893; Schade et al., 2005). This work was valued as important when Russell & Burch published "The principles of Humane Experimental Techniques" in 1959 in which egg yolk antibodies, due to its non-invasive nature, followed the principles for the consideration of animal welfare (Russell & Burch, 1959). After that,

IgY has been intensively studied during production, extraction and purification as well as clinical needed application into many fields including diagnosis, therapy and prophylaxis (Schade et al., 2005).

Production of Ig

IgY (also known as γ-livetin) is composed of two light and two heavy chains with a molecular weight of about 167 kDa. The isoelectric point (pI) of IgY is between 5.7 and 7.6 (S. Sun, Mo, Ji, & Liu, 2001). It is transferred to yolk through receptor-mediated processes as the receptor is located on the surface of the yolk membrane (Mohammed et al., 1998; Morrison, Mohammed, Wims, Trinh, & Etches, 2002). Therefore, the production of IgY is mainly in the egg yolk. Some studies evaluating the effect of chicken environmental conditions (in cage vs free range) on production of IgY, found that the IgY concentration was higher in eggs laid by chickens kept in cages (Erhard, Ozpinar, & Bilal, 1999).

Specific antibodies can be produced through immunization of the chicken; even though the results are unpredictable due to four main variables: the antigen, the adjuvant, the route of application, and the chicken (Schade et al., 1996, 2005). The concentration of IgY can reach to 15-25 mg/ml yolk, which is 18 times higher than that produced from rabbits (Juneja, 1996).

Extraction and Purification of IgY

IgY is pH and heat sensitive. IgY will lose its activity in acid environment (pH = 3 and 4) (Hatta, Tsuda, Akachi, Kim, & Yamamoto, 1993; Schade et al., 1996; Shimizu et al., 1992) and will be denatured above 70°C (Hatta et al., 1993; Jaradat &

Marquardt, 2000; Shimizu et al., 1992). Therefore, the extraction condition should be mild in case of reduction of destroying the structure of IgY. Many approaches can be applied in extraction and purification of IgY from yolk and can be categorized into three groups: precipitation, chromatographic, and filtration methods (Meulenaer & Huyghebaert, 2001; Schade et al., 2005).

The first step is to separate egg yolk from egg white, which can be done manually or using an egg-breaking machine. However, the yolk can be easily contaminated with 20% egg white when using the machine, which makes the further extraction and purification steps difficult (Fichtali, Charter, Lo, & Nakai, 1992).

Extraction

The crude extraction of IgY from lipoproteins can be mostly achieved through precipitation methods using various solvents. After this, IgY and other soluble proteins are left in the water-soluble fraction.

The simplest method to extract IgY from yolk is the water dilution method. When diluting yolk with water (10-fold), the low ionic strength helps separate IgY from lipids. The results would be better if incorporating pH adjustment (pH 5) and/or a freeze/thaw process. However, the drawback of this method is the loss of IgY in the water-soluble fraction (Jensenius, Andersen, Hau, Crone, & Koch, 1981; Kwan, Li‐Chan, Helbig, & Nakai, 1991).

A more broadly used method for extraction of IgY from yolk is the polyethyleneglycol (PEG) precipitation method. Through adjusting the PEG

concentration in different steps, IgY with concentration of 6 -12 mg/ml is yield (Polson, von Wechmar, & Van Regenmortel, 1980).

Other precipitation substances include natural gums (xanthan and carrageenan) (Hatta, Kim, & Yamamoto, 1990), anionic polysaccharides (H. M. Chang, Lu, Chen, Tu, & Hwang, 2000), acetone (Bade & Stegemann, 1984), and chloroform (Ntakarutimana, Demedts, Van Sande, & Scharpe, 1992).

Purification

Purification methods include precipitation, chromatographic, and filtration methods. Salt precipitation is a traditional method to purify IgY from the water-soluble fraction. The mechanism is to use salt or organic solution to change the environment to precipitate other proteins in the water-soluble fraction and leave IgY in the soluble fraction. The salt and organic solvent used in purification is similar with that used in extraction (Meulenaer & Huyghebaert, 2001).

Chromatographic methods are applied to separate IgY from other proteins based on their different affinity to different kinds of columns. However, the cost of this method is more expensive compared with others, thus may not be applied in large scale in an industrial environment, but more suitable for laboratory environment.

Filtration methods have a potential to be applied in large-scale industrial environment due to the low cost, high recoveries (72 - 89%) and purity (74 - 99%). These methods include funnel filtration, diafiltration, columnfiltration, and ultrafiltration.

General Application of use of IgY

The commercial use of IgY is very broad in the diagnostic and therapeutic field due to its manifold antigen-binding specificity (Schade, 2001). It is able to qualify and quantify certain substances, such as proteins and peptides, when incorporated into diagnostic assays (Kovacs-Nolan, Mine, Immerseel, Nys, & Bain, 2011). Similar to IgG, it can be used for detection of certain proteins and peptides (Schade et al., 2005) and even cancer biomarkers (Xiao & Gao, 2010). Its above-mentioned advantages provide a huge potential to replace IgG in diagnostic area. In addition, it can be also used in immunotherapy to protect human from lung and gastrointestinal infections due to its ability to block and/or inhibit the pathogens, bacterial and virus (Mine & Kovacs-Nolan, 2002). It takes a passive immune approach, which is very effective in reducing bacterial and virus in *vivo* animal studies and clinical trials (Kollberg et al., 2003; Krüger et al., 2003; Sarker et al., 2001). For example, it can neutralize toxins (Pauly et al., 2009) and treat snakebites (Almeida et al., 1998; Araújo, Lobato, Chavez-Olortegui, & Velarde, 2010; de Almeida et al., 2008; Meenatchisundaram, Parameswari, Michael, & Ramalingam, 2008a, 2008b; Paul et al., 2007). IgY is mostly administrated through the oral path, therefore, adding IgY as a function ingredient into food and/or diets is more likely to be a good approach to reduce/prevent the risk of several infectious diseases. But IgY is vulnerable to acid and high temperature environment (as mentioned above); its application in food, particularly processed food products may be limited.

Functionality of egg yolk powder

The main functionalities of egg yolk are emulsion formation and gelation ability, which gain yolk as a broad application in food products including mayonnaise, salad dressing, and bakery products. These functionalities are associated with structures of certain components in yolk, which will be stated in detail in the following two sections.

Emulsion ability

Egg yolk itself is an oil-in-water (o/w) emulsion and it can be used in foods like mayonnaise and salad dressing to perform as an emulsifying agent (S.-C. Yang & Baldwin, 1995). The mechanism of egg yolk as emulsifier is that it can absorb at the o/w interfaces and form a film around the oil phase to prevent oil droplets from coalescence. It stabilizes the emulsion system through reducing the interfacial tension between the two phases.

The major components in egg yolk contributing to the formation of an emulsion have been extensively studied. As early as 1935, scientists found that the lecithoproteins were the key to a good emulsion (Sell, Olsen, & Kremers, 1935). Chapin then found proteins and lipoproteins in yolk were the main components in terms of emulsion formation (Chapin, 1951), especially lipoproteins and phosvitins, because of their amphipholic state which provides for a better ability to absorb at the interface . The main component of emulsion formation is also dependent on the emulsion environment. With different pH, the proteins absorbed at the interfaces are different. Under acidic environment, more plasma proteins, LDL, would be detected at the interface; whereas under neutral pH, the proteins at the interface were composed of 48% plasma protein and 52% granule proteins (Anton, 2013). Another study also showed that after the removal of

livetins, the emulsion is more vulnerable to coalescence, even though the sizes of oil droplets and the emulsion capacity is similar (Navidghasemizad, Temelli, & Wu, 2014). In conclusion, every component of egg yolk works together to provide a stable emulsion. Lipoprotein may be the most important part but other proteins also involve in as a support to enhance the emulsion stability and activity, which is indirectly approved by a recent study finding that even though the egg yolk proteins had not been detected at the interfaces, the emulsion stability was still improved with egg yolk present in emulsion continuous phase, which resulted in the "intensification of interdroplet interaction effects" (Nikiforidis, Biliaderis, & Kiosseoglou, 2012).

Several approaches can be applied to improve egg yolk emulsion stability through modification of the components in egg yolk or adding salts (S. Yang & Cotterill, 1989). For example, enzymatic modification, which converts PC into phosphatidic acid (PA), can enhance the emulsion's thermal stability (Buxmann et al., 2010). However, some manufacturing process can decrease the emulsion capacity such as the drying process, because during drying, the structure of lipoprotein has been disrupted with releasing of triglycerides and cholesterol (Funk & Zabik, 1971). However, the drying process did not prevent dried egg yolk from still being used as a good emulsifier. Moros et al. studied the emulsion formed using spray-dried egg yolk and found it can form a stable emulsion as long as it is at the optimized concentration (Moros, Franco, & Gallegos, 2002).

Gelation ability

Gelation is a process in which heat induced denatured proteins interact with each other and gradually form a cross-linking network with development of a hard and rubbery

structure (A Paraskevopoulou & Kiosseoglou, 1997). Egg yolk gelation, due to its unique structure, can be applied into various food products including sauces, creams, and bakery products (Kiosseoglou, 2003). The main components in terms of gelation of egg yolk are apolipoproteins of LDL, due to their more labile nature and sensitivity to heat treatment (Anton, Le Denmat, Beaumal, & Pilet, 2001; Denmat, Anton, & Gandemer, 1999). The spray drying process, as it is similar to the heat treatment of gel formation, may also be able to denature proteins and lipoproteins in yolk, which then forms a weak gel.

Egg yolk gel stability is also influenced by its lipids since lipids have been found involved in the gel structure formation (Adamantini, KIiosseoglou, Alevisopoulos, & Kasapis, 2000). This is mostly like because the formation of protein-lipid complex, therefore, the gel network is not only with protein-protein cross-linking, but also incorporate protein-lipid polymerization.

Processing of egg yolk powder

The processing of egg yolk powder begins with receiving shell eggs. The shell eggs are then stored under refrigeration temperature (≤ 41°F) before further processing. The next step is to wash and sanitize the shell egg surface. Alkaline detergents (pH = 11) are used to wash away all the adhering materials on shells and chlorinated water is sprayed to sanitize the shell eggs. After that, the shell eggs go through candling, which is a step to look inside the egg without breaking it to determine its quality. If blood spots are observed, the egg is rejected. Then the shell eggs are separated to white, yolk, and shells using breaking machines. However, even the up-to-date breaking machine cannot separate the yolk from white completely, thus the separated yolk liquid still contains up

to 20% residual white liquid (Fichtali et al., 1992; Meulenaer & Huyghebaert, 2001) as mentioned above in the "Extraction and Purification of IgY". After breaking, yolk liquid is de-sugared first before further processing. De-sugaring is conducted through adding a glucose-oxidase-catalase enzyme system. However it cannot remove all the glucose from the yolk liquid. Up to 1.8% of glucose (dry basis) may be present in egg yolk powder. The yolk liquid then goes through a homogenization process to obtain a uniform mixture, which can be effectively pasteurized. Pasteurization conditions for yolk liquid are 61.1°C for 3.5 min or 60.0°C for 6.2 min. This was determined based on the effectiveness of eliminating salmonella (6 log cycle kill) and maintain functional properties. Spray drying is the predominate method for drying egg products (Goresline & Hayes, 1951). For dried egg yolk powder, the spray drying condition is at inlet temperature of 185°C at 21.4 MPa. After spray drying, the dried egg yolk powders are stored in a cool environment (< 21.1°C) (Figure 1.3). The shelf life of unopened egg yolk powder storage at a cool environment is approximately one year.

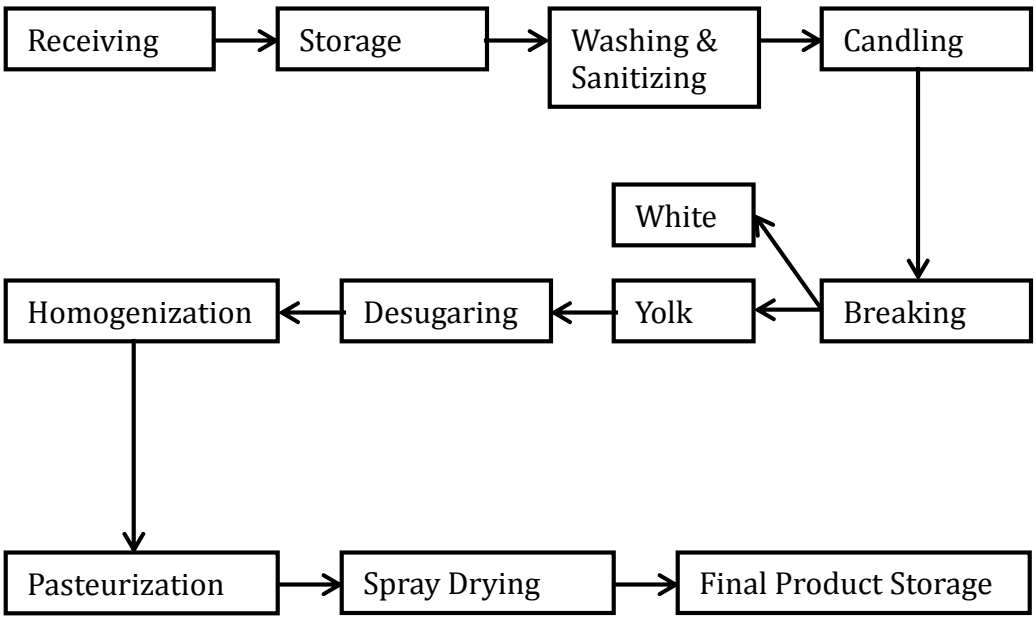


Figure 2.4: The flow chart of processing dried egg yolk powder

Storage stability

Storage of spray-dried egg yolk powder begins right after the production. The time period covers storage at the original manufacture before shipping, during distribution to many other manufactures, storage at those facilities in an ingredients storage room before use, and storage of the processed food products at the facilities, during distribution, on the shelf of grocery stores and supermarkets, and at the consumers' home.

The storage stability of spray-dried egg yolk powder is dependent on several intrinsic (egg yolk composition) and extrinsic (storage environment and processing condition) factors. Since this study evaluates the storage stability of a commercial spray-dried egg yolk powder, it is hard to study alternatives of the intrinsic factors. Therefore, in this thesis, the effects of extrinsic factors, especially the storage environment, on the

storage stability of spray-dried egg yolk have been studied. The storage environment factors include environment water activity and environment temperature.

Water activity

Definition and importance of water activity

Water activity (a_w) is a thermodynamic concept related to free energy, which can be defined as "water activity is, at a given temperature, the ratio of its fugacity, f_w, in a system and the fugacity, f^o_w, of pure liquid water at the same temperature." (Barbosa-Cánovas & Jr, 2008). Fugacity is a term representing the escaping tendency, which can be replaced by the equilibrium vapor pressure upon a good first approximation. Therefore, the definition of a_w becomes "water activity is, at a given temperature, the ratio of its vapor pressure, p_w, in a system and the vapor pressure, p^o_w, of pure liquid water at the same temperature." (Equation 1.1) (Theodore P. Labuza, 1968; Reid, 2008)

$$a_w = \left(\frac{p_w}{p^o_w} \right)_T \qquad \text{(Equation 2.1)}$$

It is also correlates with the percent environment relative humidity (%ERH) at equilibrium (equation 1.2) (Barbosa-Cánovas & Jr, 2008).

$$a_w = \frac{\%ERH}{100} \qquad \text{(Equation 1.2)}$$

Water activity (a_w) is important in food systems because it is more closely related with food products as to their microbial, chemical, and physical properties than the moisture content (Barbosa-Cánovas & Jr, 2008). In other words, a_w is more related with the storage stability because the occurrence of food deterioration is mostly due to microbial, chemical and physical changes during storage. Since the a_w of a food system is at equilibrium with the environment relative humidity, the storage condition is critical in terms of food quality changes during storage.

A moisture sorption isotherm is a curve showing the relationship of moisture content as a function of a_w. In this curve, each moisture content is corresponding to a given a_w value. It can be made using a point by point method, in which each moisture content point and its corresponding a_w will be measured and plotted in a chart with the x axis as water activity and the y axis as moisture content. Since the relationship is not linear, a Guggenheim-Anderson-de Boer (GAB) isotherm model will be applied (Equation 1.3) (Barbosa-Cánovas & Jr, 2008).

$$m = \frac{m_o k_b c_a}{[1-k_b a_w][1-k_b a_w + c_a k_b a_w]} \qquad \text{(Equation 1.3)}$$

where m is the moisture content, m_o is the moisture content at monolayer value, k_b is the multilayer factor, which should be between 0.7 to 1, c_a is the surface heat constant; a higher value means water more tightly bonded. This method is also called the traditional desiccator method (Barbosa-Cánovas & Jr, 2008). Fortunately with the development of

technology, the traditional method has been automated by instruments, which can use both dynamic vapor sorption (static) and the dynamic dewpoint isotherm method.

For each food product, its moisture sorption isotherm is different. In general, there are three types of moisture sorption isotherms: type I, II, and III (Figure 2.5) (Brunauer, 1945) based on the properties of the system constituents. Most of the dehydrated protein powders are of type II.

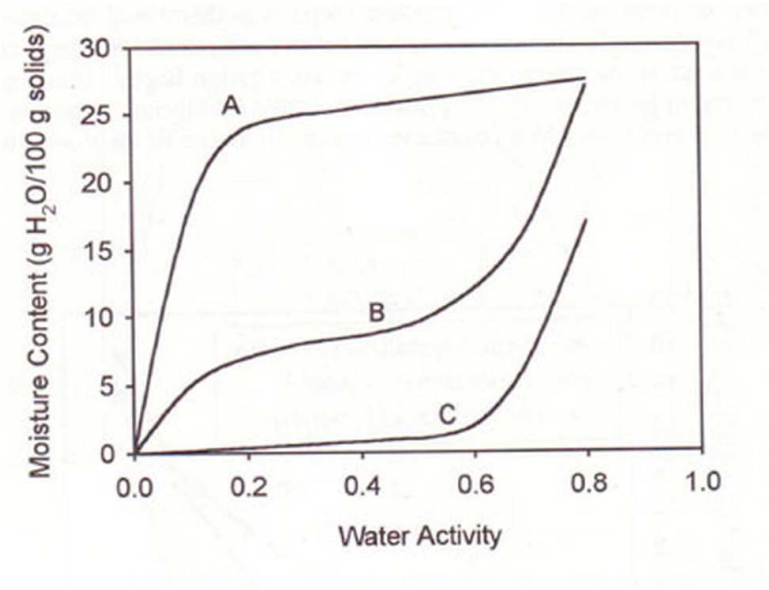


Figure 2.5: Three types of moisture sorption isotherm. Type I: curve A, Type II: curve B, Type III: curve C.

Approaches to decrease water activity (T. P. Labuza, 1975)

Factors of lowering a_w are very important and practical for the food industry in terms of food storage, which needs to be reviewed and emphasized here. The first factor is the solute-water interaction. According to Raoult's Law (Equation 1.4), adding solute,

such as salt and sugar, can reduce the water mole fraction, leading to a lower a_w as shown in Equation 1.4 (T. P. Labuza, 1975).

$$a_w = \gamma \frac{n_{water}}{n_{water} + n_{solute}} \quad \text{(Equation 1.4)}$$

The second factor is the capillary effect. With a decrease of the radius of a capillary, the a_w decreases also according to Kelvin equation (Equation 1.5) (T. P. Labuza, 1975). However, this effect is not significant until the radius capillary size is below 0.01 μm (100 Å), which is not a common size for capillaries in food (10 to 300 μm) but after removal of the majority of water, the water in small capillaries becomes the majority of the total water, which will impact a_w significantly.

$$a_w = \frac{\Delta P \, \nu_m}{RT} \quad \text{(Equation 1.5)}$$

The third one is the interaction of water with solid surface/high molecular weight colloidal material with many hydrophilic groups, e.g., starch or proteins. When water is binding tightly with other polar groups, more energy is required to make them vapor, leading to a decrease of the water vapor pressure, finally resulting in a lower a_w (T. P. Labuza, 1975).

Water activity and glass transition

Water activity (a_w) and glass transition are two different terms, but they are complementary and can provide a better explanation of food deterioration and/or stability during storage. Water activity (a_w) is a thermodynamic property of water in food, while the glass transition is a non-equilibrium property of food solids (amorphous glass vs rubbery transition of food components) (Y. R. Roos, 2002). Glass transition of food indicates the solid rubbery state vs glassy state of food solids, and it is related to the molecular mobility of within a food matrix (Le Meste, Champion, Roudaut, Blond, & Simatos, 2002; Y. H. Roos, 2008; Roudaut, Simatos, Champion, Contreras-Lopez, & Le Meste, 2004; Slade & Levine, 1991, 1995).

Glass transition is a relaxation process where by an amorphous, glassy state product transfers into a rubbery state or vice versa (Y. H. Roos, 2008; Sperling, 1992). The glass transition temperature (T_g) is the temperature of the turning point with the occurrence of such changes. It is moisture content dependent (Levine & Slade, 1986; Y. H. Roos, 1987). A higher moisture content corresponds with a lower T_g of the food matrix.

In many dehydrated food systems, such as protein powders, the mechanism of the physicochemical changes because of a_w is associated with glass transition. Dried food systems generally exist in an amorphous glassy state with little available free volume for mobility (Ferry, 1980). Free volume is defined as "the volume associated with a polymer matrix in which the energy can be distributed without cost." However, with increased moisture content (obtained from the storage environment), they may transition into the rubbery state with a much higher available free volume (Ferry, 1980), a higher diffusion

rate, as well as a much higher chemical reaction rate (Karmas, Pilar Buera, & Karel, 1992). In the rubbery state, molecules gain more mobility, thus they are more likely to react either through covalent or non covalent bonds with other molecules. Therefore, keeping dehydrated food products dry (below the monolayer value) and cool (below T_g) is essential to maintain their quality during storage.

Water activity and food system

The food stability map (Figure 1.6) showed the importance of a_w on chemical reaction (lipid oxidation, nonenzymatic browning, and enzymatic reactions) rates, and microbial (bacteria, yeast, and mold) growth rates (T. Labuza, Tannenbaum, & Karel, 1970). Below is a table summarizing the critical a_w points with its effect (Table 1.8), which shows the critical a_w value associated with microbial, chemical and physical changes. For example, an a_w of 0.6 is a critical point of microbial growth. Therefore, food products with an a_w below 0.6 do not allow microbial growth, although some pathogens may survive and when ingested may rehydrate and cause food poisoning. Despite this, an a_w of 0.6 can set as a hurdle in food preservation. Most reactions requiring a water phase cannot occur below or at the theoretical BET monolayer value, such as nonenzymatic browning and enzymatic reactions. Lipid oxidation shows a different curve as a function of a_w: a U-shape curve with its lowest rate near the monolayer value and the rate increasing on both sides (below and over this value). In conclusion, food systems at their monolayer value obtain the longest shelf life with no microbial growth, and minimal chemical reactions and physical changes (T. Labuza et al., 1970). Therefore, most dehydrated foods are recommended to be dried to their monolayer values. However, the optimum conditions of

preservation are hard to maintain, especially during shipping and long-term storage when the precise control of environment conditions are not feasible (Carpenter, Pikal, Chang, & Randolph, 1997). Therefore, the understanding of the effect of a_w on storage stability of food products is important in terms of a guidance and prediction of food deterioration during storage and shelf life estimation.

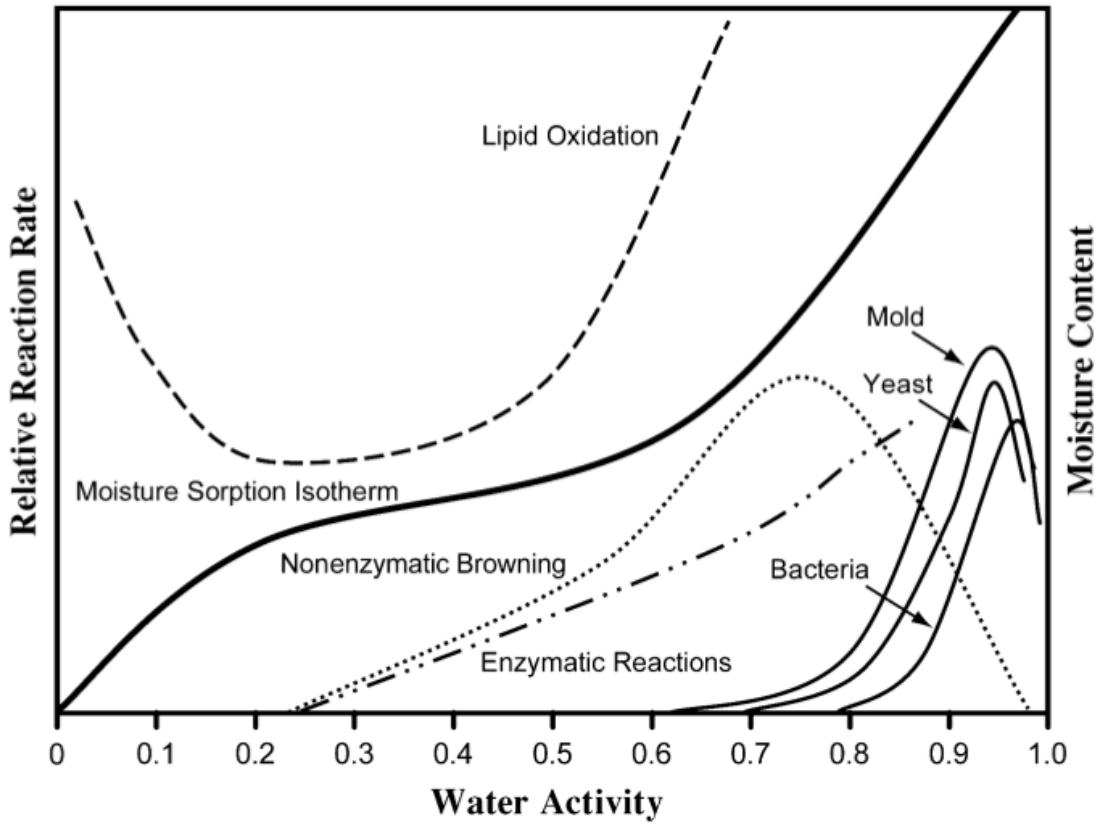


Figure 2.6: Food stability map as a function of water activity (adapted from Labuza (1970)).

Table 2.7: Water activity paradigms.

Water activity range	Effect
0.2 to 0.3	BET monolayer, at and below this value

	reactions requiring water phase do not occur
0.2 to 0.3	Below this rate of lipid oxidation increases
0.35 to 0.4	Onset of stickiness and caking
0.4 to 0.5	Onset of loss of crispness
0.5	Onset of hardening
0.6	Onset of microbial growth
0.65 to 0.85	Maximum reaction rate in amorphous systems
0.85	Onset of growth of bacterial pathogens

(Adapted from lecture slides of Dr. Ted Labuza)

Effect of water activity on storage stability with examples

As shown above based on the food stability map, a_w plays an important role and has a great impact on microbial growth, chemical reaction rate, and physical changes. Those changes result in physicochemical properties changes of food products, such as color, hardness, caking, crispness, and abundant substances declined or produced/increased as the evidences of occurrence of certain chemical reactions (Theodore P Labuza, 1980). The impact of a_w on protein powders and/or protein powders based nutrition bars is tremendous. Since a_w of those are generally below (for protein powder) or around (for nutrition bars) 0.6, microbial growth is not considered a problem. In this thesis, only the chemical and physical changes were evaluated due to the characteristics of the sample.

Dr. Zhou & Labuza found that the glass transition temperature (T_g) decreased with increasing a_w, which resulted in an increased mobility of protein molecules in whey protein powder, and further leading to protein aggregation during storage even at room temperature. These insoluble aggregates resulted mainly from intra- and intermolecular disulfide bond interactions (Zhou & Labuza, 2007). Later, they evaluated the relationship

between whey protein aggregation and moisture content and found the "dependence of aggregation on moisture content was bell-shaped, and the maximal extent of aggregation was achieved at a moisture of 70 - 80% on a dry weight basis" (Zhou et al., 2008b). Dehydrated egg white powders (DEW) found similar results during storage in terms of the impact of a_w. During storage at room temperature (23°C), the higher a_w DEW ($a_w >$ 0.6) was observed with darker color and larger hardness. The physical changes are related with 1) T_g and protein denaturation temperature (T_d) decreases with increasing a_w; 2) the occurrence of the Maillard reaction above a_w of 0.3 (Qinchun Rao & Labuza, 2012). From an accelerated shelf life study (storage at 45°C), DEW was discovered with several physicochemical properties changes: 1) decrease of brightness (L^* value), 2) increase of total color differences (ΔE^* value), 3) increase of florescence intensity (measurement of the Maillard reaction) (Qinchun Rao, Rocca-Smith, et al., 2012).

Temperature

The importance of temperature

Temperature is critical in terms of storage stability because it correlates with the equilibrium constant reaction rate of chemical reactions, including lipid oxidation and the Maillard reaction, according to Gibbs free energy equation (Equation 1.5).

$$\Delta G = \Delta H - T\Delta S = -RT \ln K_{eq} \quad \text{(Equation 1.5)}$$

Where ΔG is the Gibbs free energy, ΔH is enthalpy, ΔS is entropy, R is the gas constant (8.345 J/mole), T is the absolute temperature, and K_{eq} is the equilibrium constant reaction

rate. It can be converted into the Van Hoft relationship (Equation 1.6) (Barbosa-Cánovas & Jr, 2008):

$$\frac{d\ln K_{eq}}{d\ln 1/T} = -\frac{\Delta H}{R} \qquad \text{(Equation 1.6)}$$

This equation suggests that higher temperature leads to an exponentially higher reaction rate, resulting in more deterioration of food products during storage. High temperature also provides energy required for the chemical reactions to start.

During storage of low and intermediate moisture food products, lipid oxidation and the Maillard reaction are the two major reasons leading to food deterioration. Q_{10} is a term commonly used in chemical reaction kinetics. It is defined as the increase of constant reaction rate when the temperature increasing 10°C. Q_{10} of lipid oxidation is around 2 (Theodore P. Labuza & Hurtado, 1972) and of the Maillard reaction is between 4 to 8 (T P Labuza & Saltmarch, 1981). Therefore, the temperature effect of storage stability is tremendous and significant. Temperature is also associated with a_w and glass transition. Higher temperature is corresponding with higher a_w and greater impact from the glass transition temperature, i.e., T - T_g, which affects the increased mobility of food components as a function of $(T - T_g)$.

Examples of the effect of storage temperature on storage stability

To evaluate the storage stability of a whey protein based nutrition bar, our research group designed a bar model with an a_w of 0.6 and stored them at 25, 35, and 45°C for two months. We took samples at designated time intervals and measured their

hardness, progress of the Maillard reaction, and protein solubility. The samples stored at 45°C developed brown color more rapidly and had a higher rate of the Maillard reaction calculated using an apparent first order model compared with samples storage at 25, and 35°C. The calculated Q_{10} from the Arrhenius plot is 6.3. It means that if the bar is shelf stable for 12 months storage at room temperature, it can only be acceptable for 2 months at 35°C, which is 83% of reduction in shelf life. An increased rate of the Maillard reaction, increased hardness and decreased protein solubility were observed as well (Zhou, Guo & Labuza, 2013).

Dr. Rao evaluated the storage stability of egg white protein powder/water dough system, in which he found that samples stored at 45°C had a greater increase in hardness, and a greater decrease of free amino groups and brightness (L^* value) compared with samples stored at room temperature and 35°C (Qinchun Rao, Rocca-Smith, et al., 2013).

Shelf life prediction

Shelf life is defined as "a function of the environmental condition and the amount of quality change that can be allowed over the necessary time to reach the consumers shelf" (T.P. Labuza & Schmidl, 1988). According to shelf life rules, a food can be divided into four categories: 1) Perishable (1-14 days at refrigerated temperature), 2) Extended refrigerated shelf life (60-90 days), 3) Semi perishable (≤ 6 months at room temperature), 4) Shelf stable (≥ 6 months to 3 years). Most of low and intermediate moisture food products are in the shelf stable category due to the hurdles to microorganism growth, which is good in terms of storage stability but not good enough for shelf life testing because it will be very time consuming. Therefore, shelf life

prediction is very important for the food industry. It requires a shorter time but with multiple storage conditions to evaluate the reaction kinetics and build a prediction model. For shelf life estimation, one usually stores the products at three temperatures (room temperature, 35°C, and 45°C) and at least three a_w for each temperature and measure at least 6 different times for each parameter during storage. Then one can plot the 6 points using zero or first order kinetics to calculate the reaction rate constant (k). One then makes a plot of k vs 1/T for the Arrhenius plot to calculate activation energy (E_a) and Q_{10}. Obtaining those factors can help us build the relationship of quality changes as a function of storage time at certain temperature and certain a_w.

Chapter 3 Storage induced physicochemical changes of a commercial spray-dried egg yolk powder

Summary

In dehydrated food products, the physicochemical changes during storage are due to chemical reactions. In this study, a commercial spray-dried egg yolk powder (DEY) was stored at different water activities (a_w), and temperatures and several selected physicochemical changes were evaluated. The moisture sorption isotherm of DEY fitted well to the Guggenheim-Anderson-de Boer (GAB) model and showed a type II curve with monolayer value m_0 of 2.3 g H_2O/100 g DEY (a_w of 0.25). The color of DEY showed a decrease of L^* and a^* value, and an increase of b^* and ΔE^* value during storage. The overall change of color was towards orange-brown in the $L^*a^*b^*$ color system. However, the change of color was very slight as the decline rate constant of L^* value was only 0.0015 day^{-1} after storage at 45°C for two months. To evaluate the progress of the Maillard reaction, glucose content and free amino groups, and the Maillard reaction products were measured. Results showed that the glucose content went to zero after seven days storage at 45°C at an a_w of 0.6. The glucose content of DEY during storage at 23°C

at an a_w of 0.05 only showed a slight decline during 2 month storage. The lipid oxidation progress was also tested and lipid oxidation products were found in all samples, confirming the occurrence of the reaction.

Introduction

Eggs have been an important and valuable source of food since prehistory. Chicken eggs were consumed as food in Asia and India before 7500 BCE and brought to Sumer, Egypt, and other places of the world afterwards (Cotterill, 1995). Nowadays, eggs are still considered to have great nutritional value because they are a rich source of protein, unsaturated fatty acids, iron, phosphorus, and other trace minerals and various vitamins (Stadelman & Cotterill, 1973). In the last decade, the production of hen eggs has increased slightly from 86 to 92 billions of eggs per year in the United States (USDA Layers and Eggs: Production by Year, US, 2014). In the last 25 years, the per capital consumption of eggs has been almost constant, however, the per capital consumption of egg products in 2010 is more than twice as that in 1986 (increased from 36.8 to 75.7 per capital consumption (numbers of shell egg equivalent)) (American Egg Board:Egg Production and Consumption, 2014). It indicates that the market of egg components as ingredients to be applied into food products is growing. The first egg product in the United States was partially dehydrated egg yolk and albumin in 1878 (Stadelman, 1995). After that, with the development of egg-breaking and drying technology, various egg products have been produced including dried egg yolk (DEY). DEY has great advantages over liquid egg yolk (LEY) in terms of preservation and has a broad application in the food industry. It can be used in mayonnaise, salad dressing, and bakery products, and also

has the potential to be mixed with other protein powders (whey, casein, soy, and egg white) to be applied into high protein products such as high protein nutrition and energy bars.

DEY is a shelf stable dry food product given the right packaging and is essentially safe in terms of microorganisms due to its low moisture content if pasteurized before and after drying. It can suffer after drying from physicochemical changes if exposed to an abused environment such as high humidity ($a_w > 0.6$) and/or high temperature (>45°C), which is more likely to happen during transportation and long-term storage when the precise control of conditions is not feasible (Carpenter et al., 1997).

Few studies have evaluated the physicochemical changes in storage of DEY. One study showed that the color changes of DEY is not significant after 90 days storage at room temperature, but the loss of unsaturated fatty acids was significant (Du & Ahn, 2000a). Storage of LEY was found with similar results: no significant color changes during storage at room temperature, but with a significant decrease of protein solubility (Koç et al., 2011). Wahle et al. also found increased peroxidized lipid during storage of whole egg powder while the increase correlated inversely with the Vitamin E content (Wahle, Hoppeb, & Mcintosh, 1993). Other researchers also discovered an increase cholesterol oxidation products (COPs) and early Maillard reaction products during storage of spray-dried whole egg powder at room temperature (Caboni et al., 2005).

Extensive studies have evaluated the physicochemical changes of other important protein powders (whey protein, egg white protein) during storage (Qinchun Rao & Labuza, 2012; Zhou et al., 2013; Zhou & Labuza, 2007). They found a decrease of

brightness (L^* value), free amino groups, protein solubility, glass transition temperature (T_g), and protein denaturation temperature (T_d) with increasing a_w and/or temperature.

Therefore, the objectives of this study were to fill the gap to determine the effect of storage conditions (a_w and temperature) on the physicochemical changes of DEY and provide possible mechanisms for these changes. Possible approaches to reduce/inhibit these changes would be suggested.

Methods and Materials

Materials

The commercial spray-dried egg yolk powder (DEY, H483) was obtained from Deb-EL Food Products, LLC (Elizabeth, NJ, USA). After arrival, a portion of DEY (around 20 g) was taken out for tests of several sample characteristics. The rest of the DEY was vacuum packaged and then stored at -20°C to eliminate any possible chemical reactions before use.

All the other reagents and chemicals used in this study were of analytical grade.

Methods

Characterization of DEY

After receiving the sample, several characteristics were analyzed according to previous work (Qinchun Rao & Labuza, 2012). The initial moisture content of DEY was measured using the Aquatest cma Karl fischer Coulometric Titrator (Photovolt Co., Minneapolis, MN, USA). Water activity (a_w) was measured using the AquaLab Vapor Sorption Analyzer (Decagon Devices, Pullman, WA, USA). The glucose content was measured using the Glucose (HK) Assay Kit (Sigma-Aldrich Co., Saint Louis, MO,

USA). The protein content was measured using TruSpec N (LECO corp., St. Joseph, MI, USA) according to the Dumas combustion method (Padmore, 1990). The specific factor for the conversion of nitrogen content to egg protein content is 6.25 (FAO, 2003).

Moisture sorption isotherm

The moisture sorption isotherm of DEY at room temperature was generated using two methods in the Aqualab Vapor Sorption Analyzer (Decagon Devices, Inc., Pullman, WA, USA): the dynamic vapor sorption (DVS) method for static isotherm and the dynamic dewpoint isotherm (DDI) method for a dynamic isotherm. The GAB isotherm model was used for the prediction of the moisture content and a_w relationship.

DEY storage

DEY samples (pre-weighed) were put into water activity cups (Decagon Devices, Inc., Pullman, WA, USA), glass petri dish, and clear glass bottles without cap based on requirement of selected physicochemical properties' tests. Then each set of samples were put into six plastic desiccators containing either Drierite desiccant or saturated salt slurries (LiCl, $MgCl_2$, K_2CO_3, $Mg(NO_3)_2$, $CoCl_2$) with an a_w range from 0.05 to 0.66 (Greenspan, 1977). Then each set of six desiccators were put into three storage chambers at 23°C, 35°C, and 45°C. Therefore, overall 18 desiccators were used for this storage study and each desiccator was filled with samples of a given amount for future analysis of selected physicochemical properties. The storage time was set based on the presumed Q_{10} value (for lipid oxidation, Q_{10} is around 2). For 45°C storage, the storage time is about two months, which translates to four months and eight months for 35°C and room

temperature storage respectively. Sampling was carried out at designated intervals and put immediately at -30°C until further analysis.

Color measurement of DEY

The color of DEY during storage was measured using a Minolta Chroma Meter CR-221 (Minolta Camera Co., Osaka, Japan). Prior to analysis, the chroma meter was calibrated with a white CR-221 calibration plate (Minolta) and six other color standards (white, pink, green, yellow, blue, and grey) (Hunter Associates laboratory, Inc., Fair fax, VA, USA). The color was measured using the CIT 1976 $L^*a^*b^*$ color space system (International Commission on Illumination 2008). L^* value stands for the brightness with 0 for black and 100 for diffuse white. The a^* value covers from green to red with negative values for green and positive values for red. The b^* value covers from blue to yellow with negative values for blue and positive values for yellow. DEY samples stored at different temperatures (room temperature, 35°C, and 45°C) and a_w (from 0.05 to 0.66) were analyzed. Each sample was scanned for at least four times and the L^*, a^*, b^* values were recorded. The ΔE value was calculated using the equation below (Equation 2.1).

$$\Delta E* = \sqrt{(L*_{\text{sample}} - L*_{\text{original}})^2 + (a*_{\text{sample}} - a*_{\text{original}})^2 + (b*_{\text{sample}} - b*_{\text{original}})^2} \quad \text{(Equation 2.1)}$$

Glucose content

The glucose content of DEY during storage (23°C at a_w of 0.05 and 0.66, 45°C at a_w of 0.05 and 0.66) was analyzed using a Glucose (HK) Assay Kit (Product Code G 3293)(Sigma-Aldrich CO., Saint Louis, Missouri, USA). First, glucose reacts with ATP to generate glucose-6-phosphate (G6P) when catalyzed by hexokinase. Then the

generated G6P reacts with NAD to produce NADH when catalyzed by G6P dehydrogenase. The conversion from NAD to NADH can result in an increase in absorbance at 340 nm, which can be detected using a spectrophotometer. Each sample (0.1 mg) was extracted with 10 ml deionized water and put on a shaker (Thermo Fisher Scientific Inc., Rockford, IL, USA) to shake for at least 18 h to fully extract all the residual glucose. The slurry was then filtered through membrane filters (0.45 μm) for clarification. The glucose (HK) assay reagent was reconstituted with 20 ml of deionized water in the original vial, and after addition of distilled water, the vial was capped and mixed several times by shaking inversely right before the measurement. Then the filtered sample solution (200 μl) was added with/without glucose assay reagent (1 ml) into test tubes to form the sample blank and sample test (Table 3.1).

Table 3.1: Sample blank, reagent blank, and sample test composition chart.

Tube	Glucose Assay Reagent (ml)	Sample Volume (ml)	Volume of Deionized Water (ml)
Sample Blank	---	0.2	1.0
Reagent Blank	1.0	---	0.2
Sample Test	1.0	0.2	---

Each sample has a sample blank and a sample test but shares the same reagent blank. Then the tubes were mixed using a vortex mixer (Henry Troemner LLC, Thorofare, NJ, USA) at 3000 rpm for 30 s before incubating for 15 min at room temperature. After incubation, the absorbance of the solution was measured at 340 nm versus deionized water using a spectrophotometer. The glucose concentration is

calculated based on the direct proportional relationship between the increase in absorbance and the glucose concentration. The equations shown below (Equation 2.2 to 2.5) were used for the calculation.

$$A_{\text{Total blank}} = A_{\text{Sample blank}} + A_{\text{Reagent blank}} \quad \text{(Equation 2.2)}$$

$$\text{mg glucose/ml} = \frac{(\Delta A)(TV)(\text{Glucose Molecular Weight})(F)}{(\varepsilon)(d)(SV)(\text{Conversion Factor for } \mu g \text{ to mg})} \quad \text{(Equation 2.3)}$$

$$\text{mg glucose/ml} = \frac{(\Delta A)(TV)(180.2)(F)}{(6.22)(1)(SV)(1000)} \quad \text{(Equation 2.4)}$$

$$\text{mg glucose/ml} = \frac{(\Delta A)(TV)(180.2)(F)(0.029)}{(SV)} \quad \text{(Equation 2.5)}$$

Where ΔA is $A_{Test} - A_{Total\ blank}$; TV is Total Assay Volume (ml), in this case 1.2 ml; SV is Sample Volume, in this case 0.2 ml; Glucose Molecular Weight is 180.2 g/mole or equivalently 180.2 μg/μmoles; F is Dilution Factor from Sample Preparation; ε is Millimolar Extinction Coeffieient for NADH at 340 nm Millimolar $^{-1}$ cm $^{-1}$ or equivalently (ml/μmoles)(1/cm); d is Light path (cm), in this case 1 cm, Conversion Factor for μg to mg is 1,000.

Solid Fat Index (SFI) of DEY

SFI, the ratio of solid fat content in a fat sample at a given temperature, is generally measured as the change in specific volume with respect to temperature (Shahidi & Wanasundara, 2008). The SFI of DEY was measured using a differential scanning calorimetry (DSC), DSC1 STAR^e system (Mettler-Toledo, LLC, Columbus, OH, USA).

The sample was cycled through a heat-cool-reheat cycle, during which it was heated from -50°C to 80°C at 10°C/min, cooled rapidly back to -50°C and then reheated at 10°C/min back to 80°C. The melting endotherm of the fat during the second heating segment was analyzed to determine the SFI values using STAR^e software (version 11.00a, Mettler-Toledo).

o-Phthalaldehyde (OPA) method

The effect of a_w and temperature on the loss of free amino groups of DEY during storage was measured using the OPA method with minor modifications. The OPA method is based on a reaction involved with free amino groups, which can be detected using a spectrophotometer. Basically, OPA reagent reacts with amino groups on the proteins or free amino acids in an alkaline environment, which gives rise to strongly fluorescing compounds. The OPA reagent is freshly prepared just before the test. Each 100 ml OPA reagent is composed of two parts: 1) 80 mg OPA (dissolved in 1 ml absolute methanol), 2) 200 µl of 2-mercaptoethanol (2-ME). The two parts were diluted to 100 ml volume with CB-SDS buffer (50 nM carbonate buffer (CB) containing 1% SDS (g/ml), pH 9.6). The fresh prepared OPA was protected from direct light and used within 2 h. Each sample (0.1 mg) was diluted with 20 ml CB-SDS and incubated for 2 h on a shaker at 100 rpm. Then 100 µl of sample solution was added into a 96-well clear flat bottom polystyrene microplate (Corning In., Corning, NY, USA) (100 µl/well). The blank was the CB-SDS buffer without DEY (100 µl/well). The OPA reagent (100 µl/well) was then added into each well using a Matrix electronic eight-channel pipette (Thermo Fisher Scientific). Right before measurement, the microplate was shaken briefly. The

absorbance was read using a microplate reader (BioTek Instruments) at 340 nm. In order to obtain the optimal results, the incubation time of each sample after adding the OPA reagent was set to 1.5 min. All the steps were performed at room temperature and each sample was tested at least in duplicate. The remaining free amino groups in each sample were calculated using the following equation (Equation 2.6):

$$\% \text{ Free amino groups} = ((\text{Treated } A_{340} - \text{Blank } A_{340})/(\text{Original } A_{340} - \text{Blank } A_{340}))*100 \quad \text{(Equation 2.6)}$$

Front-face fluorescence

The progress of the Maillard reaction and lipid oxidation were measured using front-face fluorescence at room temperature (LS50 B Perking-Elmer Luminescence Spectrometer, Perkin-Elmer Co., Waltham, MA, USA). The Maillard reaction products emission was monitored from 380 to 580 nm by excitation at 360 nm (Karoui, Schoonheydt, Decuypere, Nicolaï, & De Baerdemaeker, 2007). The slits of excitation and emission are 5.0 and 3.5 nm respectively. The lipid oxidation products emission was monitored from 400 to 640 nm by excitation at 380 nm (Wold & Baardseth, 2002). The slits of excitation and emission are 5.0 and 3.5 nm respectively. DEY samples stored at specific conditions (23°C and 45°C at a_w of 0.66) were tested. Briefly, a certain amount of DEY powder was put into the silica window of the Powder Holder. Then the cap of the Powder Holder was screwed down tightly. The Powder Holder is then put into the Front Surface accessory for scanning. Each sample was scanned at least in triplicate at the speed of 500 nm/min. The data was extracted from the FL WinLab software (Perkin

Elmer) into Excel and undergone principle component analysis (PCA) using R software. The R package for PCA is FactoMineR. The code for PCA is shown in Appendix.

Peroxide value of DEY during storage

The peroxide value of DEY was measured using the ferrous oxidation-xylenol orange (FOX) method (Hermes-lima, Willmore, & Storey, 1995). This method is based on a fluorescence reaction involved with the primary oxidation products of lipid oxidation: hydroperoxides, which can be detected by a spectrophotometer at 560 nm. Basically, the hydroperoxides will convert ferrous ions into ferric ions, which can form a complex with xylenol orange. The complex can be detected at 560 nm. The FOX method is easy, efficient, and sensitive, and has been used for many matrixes including food systems. Briefly, 0.5 g DEY (storage at 45°C and a_w of 0.66) was added into 15 ml cold (-20°C) absolute methanol in a 50 ml centrifuge tube. Then the slurry was homogenized at level 6 (the maximum level) for 1 min. After centrifuging at 1,400 g for 3 min, the supernatant was collected. The supernatant (50 µl) was added into a 2 ml centrifuge tube containing 250 µl 0.25 mM $FeSO_4$, 100 µl 25 mM H_2SO_4, 100 µl 0.1 mM xylenol orange, and 500 µl deionized water. The blank sample is 50 µl of absolute methanol instead of the sample solution. Then the mixture was vortexed for 30 s before incubation in the dark at room temperature for 30 min. After that, the mixture (200 µl/well) was pipetted into a 96-well clear flat bottom polystyrene microplate (Corning In., Corning, NY, USA) and read at an absorbance at 560 nm. The peroxide value was calculated based on a standard curve. The standard curve was performed using 50 µl $FeCl_3$ solutions of different concentration (0.05 – 0.6 mmol) instead of the sample solution. The other steps are the

same with the sample test. The peroxide value was finally expressed as meq active oxygen/g fat.

Results

Characteristics of DEY

DEY was analyzed for its initial moisture content, a_w, glucose content, and protein content. Results showed it contained 36% protein, 0.21% glucose, and 2.1% of moisture (all on a dry basis) as well as being at an a_w of 0.27 (Table 3.2). The fat content (60%, dry basis) and the processing condition of DEY were obtained from Deb-El Food Products, LLC. (Elizabeth, NJ, USA) (Table 3.3).

Table 3.2: Characteristics of a commercial spray-dried egg yolk powder.

	dry basis				
sample	protein	fat	moisture content	glucose content	a_w
DEY	36%	60%	2.10%	0.21%	0.27

Table 3.3: Processing conditions of a commercial spray-dried egg yolk powder (Information obtained from the egg yolk manufacture).

	spray-drying condition		pasteurization	
sample	pressure	inlet Temp.	Temp.	Holding Time
DEY	21.4 Mpa	185°C	62°C	4 min

The moisture sorption isotherm of DEY

Two moisture sorption isotherms of DEY at room temperature, static and dynamic, were generated using the GAB model (Figure 3.1). There was no significant difference ($P > 0.05$) between these two isotherms (Figure 3.1). DEY shows the typical type II isotherm (sigmoidal curve) for proteins (Figure 2.5). The MAPE values of both methods

are small (Figure 3.1), indicating that the GAB model has an excellent fit to the measured data. To maximize its shelf life, the optimal moisture content of DEY should be at its GAB monolayer moisture value (m_o ~ 2.3 g H_2O/100 g solids, a_w of 0.25) (Figure 2.1), which is very close to the initial moisture content of the industrial supplied DEY (Table 3.2). Therefore, if DEY can be maintained at its initial moisture content, it should be able to have a long shelf life. Besides the value of the monolayer value, the GAB model can also provide the value of two parameters: the multilayer factor k (mostly around 1) and surface heat constant C (higher C value means a tighter binding of water). DEY has a k value of around 0.98 and C of around 11.5. When comparing the moisture sorption isotherm of DEY with that of dried egg white powder (DEW) (Figure 3.2), DEY holds less water than DEW at any a_w, which is probably due to the high fat and lipoprotein content of DEY.

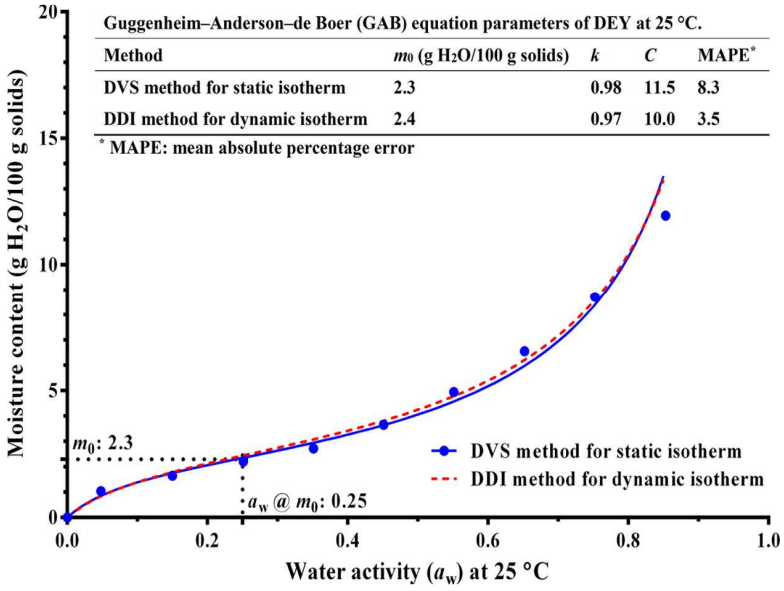


Figure 3.1: Moisture sorption isotherms of a commercial spray-dried egg yolk powder (DEY) generated from the GAB model. The dotted lines indicate the GAB m_o and the relevant a_w of DEY.

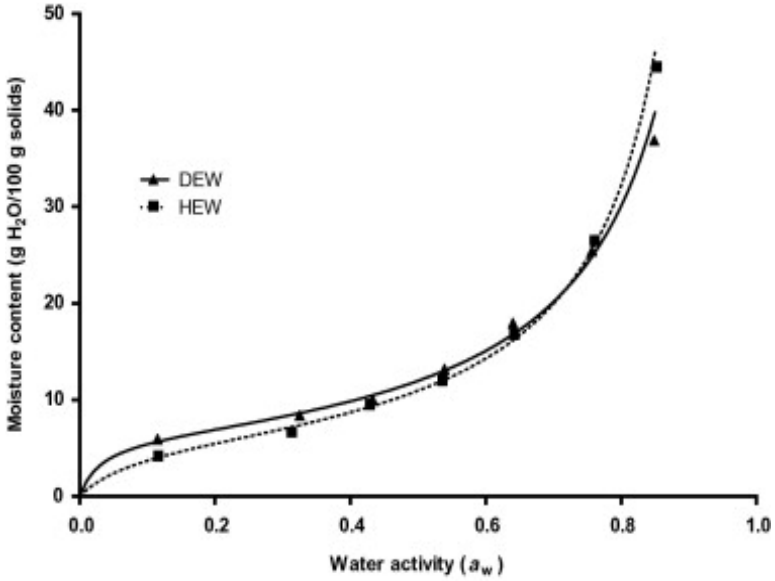


Figure 3.2: Moisture sorption isotherm of dried egg white and hydrolyzed egg white powders. (Rao et al., 2012)

Physicochemical changes of DEY during storage

Color

After two months storage at 45°C, the color change of DEY was significant. For DEY stored at the most extreme condition (45°C at a_w of 0.66), the L^* value decreased with increasing storage time with a rate constant of 1.5×10^{-3} day^{-1} (Figure 3.3(a)); while the total color difference (ΔE) increased with a rate constant of 3.2×10^{-2} day^{-1} (Figure 3.3(b)). The rate constants for both parameters were calculated using an apparent first-order hyperbolic model (T. Labuza & Kamman, 1983) (Equation 2.7(a) and (b)).

L^*: $\Gamma = (Y - Y_{min})/(Y_0 - Y_{min}) = e^{-kt}$ (Equation 2.7(a))

ΔE^*: $\Gamma = (Y - Y_{min})/(Y_0 - Y_{min}) = 1 - e^{-kt}$ (Equation 2.7(b))

For DEY samples stored at 45°C, they all showed a decrease of brightness and increase of total color difference, however, the rate constants were not significant across all a_w, which is different than what one would expect (Figure 3.4, Figure 3.5, Table 3.4, Table 3.5).

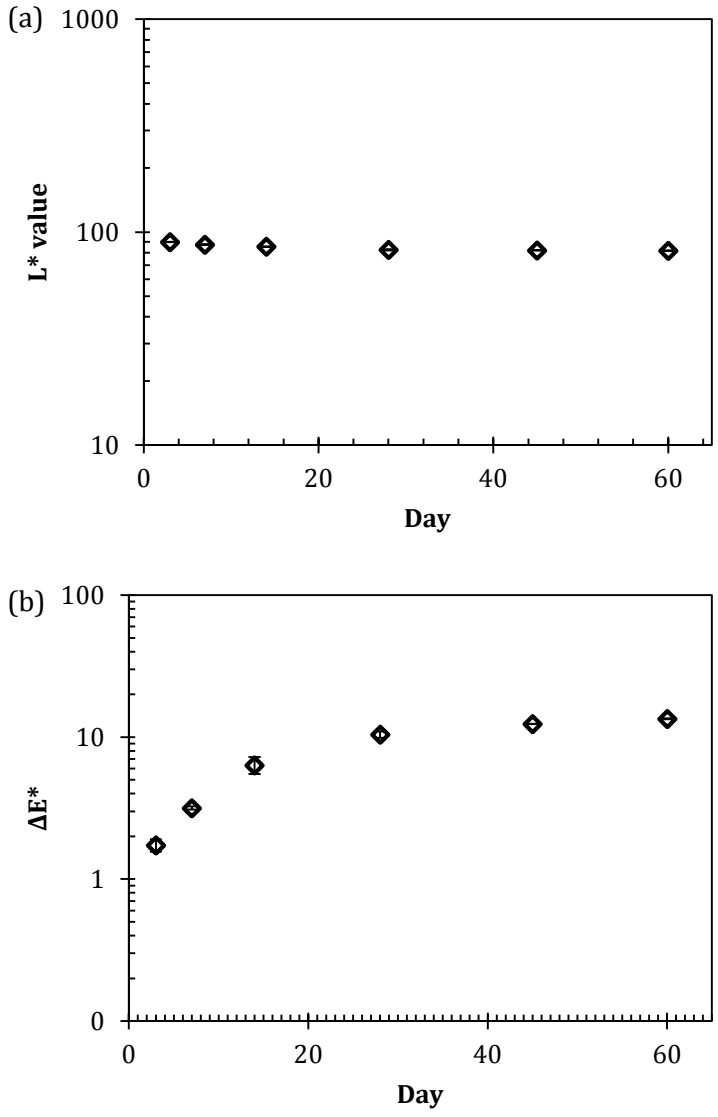


Figure 3.3: (a) L* value and (b) ΔE* value changes of DEY during storage at 45°C at a_w of 0.66 on a semi-log scale.

Our expectation was that with increased a_w, the rate constant of the L* value should increase as found in the previous study that has been done in our lab (Qinchun Rao, Rocca-Smith, et al., 2012). The egg white powder storage study showed significant rate of darkening as a function of a_w (Figure 3.6) (Qinchun Rao & Labuza, 2012),

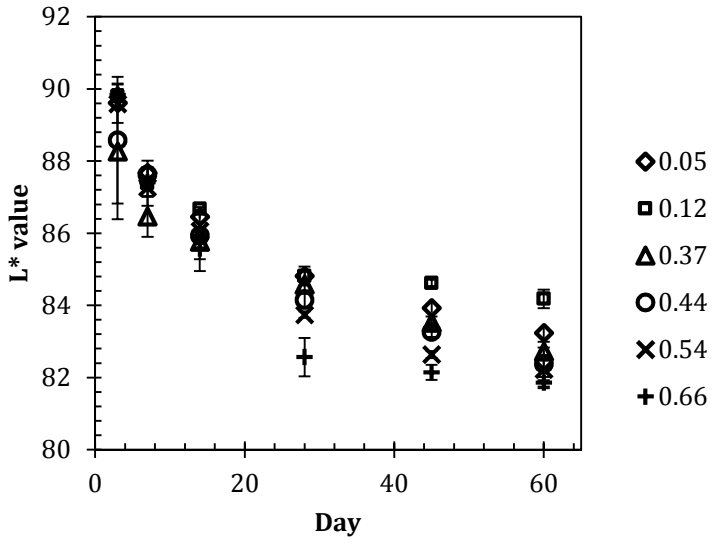


Figure 3.4: Change of L* value as a function of time of DEY during storage at 45°C at different a_w.

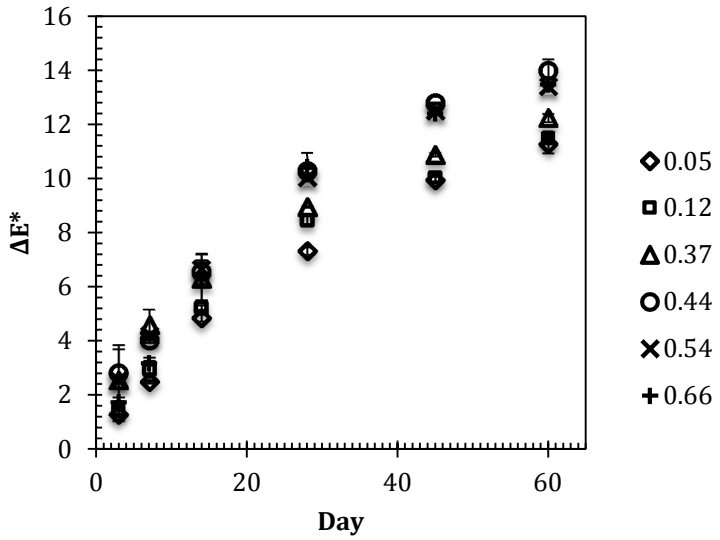


Figure 3.5: Changes of ΔE^* value as a function of time of DEY during storage at 45°C at different a_w.

Table 3.4: Kinetic analysis of apparent first-order hyperbolic model fit for the change in L^* value as a function of water activity (a_w) for DEY stored at 45°C.

Apparent first-order model	a_w					
	0.05	0.1	0.35	0.44	0.5	0.55
Best-fit values						
L^*_{max}	89.6	89.8	88.7	88.8	89.8	89.9
L^*_{min}	83.2	84.2	82.7	82.4	82.2	81.9
$k \times 10^3(day^{-1})$	1.2[a]	0. 9[a]	1.0[a]	1.3[a]	1.4[a]	1.5[a]
95% CL						
L^*_{max}	86.6 - 89.9	85.6 - 90.2	85.4 - 89.0	86.4 - 89.3	85.8 - 90.2	85.4 – 90.6
L^*_{min}	80.7 – 84.6	80.7 - 86.3	80.1 – 84.5	80.1 – 83.6	78.7 - 83.9	77.7 - 83.8
$k \times 10^3(day^{-1})$	0.6 - 1.8	0.1 - 1.8	0.3 - 1.7	0.7 - 1.8	0.6 - 2.2	0.6 - 2.5
Goodness of fit						
R^2	0.878	0.684	0.817	0.912	0.853	0.828

Table 3.5: Kinetic analysis of apparent first-order hyperbolic model fit for the change in ΔE^* value as a function of water activity (a_w) for DEY stored at 45°C.

Apparent first-order model	a_w 0.05	0.1	0.35	0.44	0.5	0.55
Best-fit values						
ΔE^*_{max}	11.3	11.5	12.2	14	13.4	13.5
ΔE^*_{min}	1.3	1.4	2.5	2.8	2.5	1.7
k $*10^2$ (day^{-1})	3.4[a]	3.2[a]	2.4[a]	2.6[a]	2.6[a]	3.2[a]
95% CL						
ΔE^*_{max}	6.1 – 37.6	6.0 – 39.2	7.9 – 27.9	9.6 – 32.6	8.7 – 33.9	7.2 – 48.6
ΔE^*_{min}	1.1 – 4.5	1.2 – 5.2	2.4 – 6.3	2.4 – 6.2	2.2 – 6.5	1.4 – 6.3
k $*10^2$ (day^{-1})	1.1 – 5.7	0.8 – 5.5	0.7 – 4.0	1.1 – 4.2	0.9 – 4.4	0.8 – 5.6
Goodness of fit						
R^2	0.9	0.827	0.811	0.851	0.819	0.774

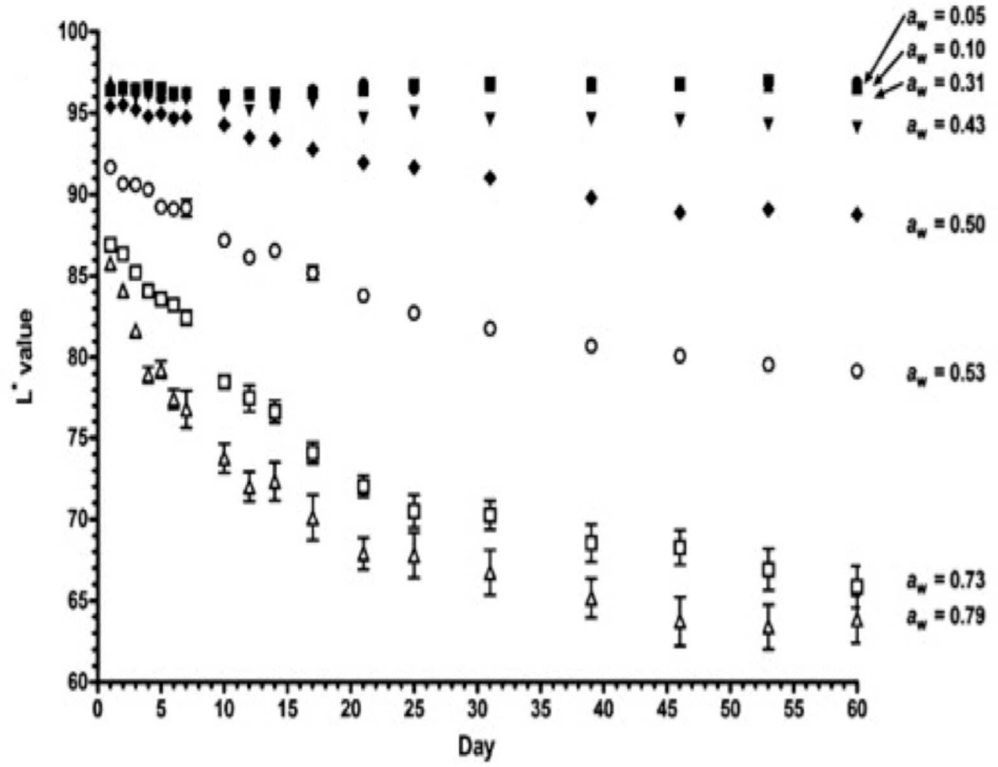


Figure 3.6: Effect of water activity (a_w) on the L* value of hydrolyzed egg white (HEW) during storage at 45°C (Qinchun Rao, Rocca-Smith, et al., 2012).

which is not the case for egg yolk powder. The reason for this differences in the color changes of whey protein and egg white powder is due to the Maillard reaction. The Maillard reaction (refers to nonenzymatic browning, NEB) is a reaction between primary amine groups and reducing sugar, which results in hundreds of products including the brown pigment melanoidin (Hodge, 1953). Its rate constant is a function of a_w as stated in the literature review part: as a_w increases from the dry state, the rate constant of the Maillard reaction increases as well (T. Labuza et al., 1970). However, egg yolk powder may be different most likely due to the composition. For whey protein and egg white protein powder, protein comprises almost 90% of the solid base. But for egg yolk powder, protein is only about 36% of the solid base while fat is at 60%, in which more than half is unsaturated fatty acids (Table 2.2). Therefore lipid oxidation is highly likely. One study assessed the lipid oxidation in freeze-dried egg yolk during storage and found an increase in the 2-thiobarbituric acid (TBA) value after 20 days storage (Hasegawa, Endo, & Fujimoto, 1993). Similarly, another study detected a decline of unsaturated fatty acid content in spray-dried egg yolk powder after 90 days storage (Du & Ahn, 2000b). Lipid oxidation can also result in browning as major products are aldehydes and ketones which can participate in the Maillard reaction. Zamora and Hidalgo claimed that both reactions also share similar pathways and polymerization mechanisms at least from a browning reaction point of view (Zamora & Hidalgo, 2005). According to the food stability map, the relationship of a_w and lipid oxidation rate constant is a U shape curve. As a_w increases from the dry state, the lipid oxidation reaction rate decreases to a minimum just above the m_o, then the rate increases as a_w increases (T. Labuza et al.,

1970). Therefore, it can explain why there is a color changes of DEY even at an a_w of 0.05. The color change of DEY is a result of both the Maillard reaction and lipid oxidation. In the $L^*a^*b^*$ system, the overall color change trend of samples stored at 45°C moved toward the orange/brown region, suggesting that the color change was due to bith the Maillard reaction and Lipid oxidation (Zamora & Hidalgo, 2005).

The samples stored at 35°C had a similar trend as for the samples stored at 45°C with a smaller rate constant as expected (Table 2.4, 2.5, 2.6, 2.7). This indicates that the temperature effect on color change rate constant is significant. The samples storage at room temperature showed only a small decline of L* value going from about 90 to 88. There was an increase of ΔE^* value after 8 month storage going from about 1 to 8 compared to 2 to 11 in Figure 2.5 (Figure 3.7, Figure 3.8). This indicates that DEY is quite stable even at an a_w of 0.66 in terms of color darkening.

Table 3.6: Kinetic analysis of apparent first-order hyperbolic model fit for the change in L^* value as a function of water activity (a_w) for DEY stored at 35°C.

Apparent first-order model	a_w 0.05	0.11	0.36	0.44	0.5	0.6
Best-fit values						
L^*_{max}	89.1	88.2	89.2	89.5	89.3	88.8
L^*_{min}	83.1	85.1	83.2	82.4	79.9	80.2
k $*10^3(day^{-1})$	0.5	0. 3	0.6	0.7	0.9	0.9
95% CL						
L^*_{max}	88.0 – 89.1	87.6 – 88.8	87.2 – 89.3	87.1 – 89.8	87.0 – 89.7	86.9 – 89.4
L^*_{min}	82.2 – 83.7	84.1 – 85.7	81.1 – 83.9	79.8 – 83.4	77.4 – 80.8	77.6 – 80.9
k $*10^3(day^{-1})$	0.4 – 0.6	0.2 – 0.4	0.4 – 0.8	0.4 – 0.9	0.7 - 1.2	0.6 – 1.1
Goodness of fit						
R^2	0.988	0.957	0.958	0.951	0.974	0.974

Table 3.7: Kinetic analysis of apparent first-order hyperbolic model fit for the change in ΔE^* value as a function of water activity (a_w) for DEY stored at 35°C.

Apparent first-order model	a_w					
	0.05	0.11	0.36	0.44	0.5	0.6
Best-fit values						
ΔE^*_{max}	12.4	10.2	13.1	13.5	15.9	14.6
ΔE^*_{min}	1.3	1.3	1.6	1.2	1.2	1
$k * 10^2 (day^{-1})$	2	1.9	1.7	1.9	2	2.4
95% CL						
ΔE^*_{max}	7.2 – 46.0	5.4 – 44.0	8.1 – 45.0	7.3 – 60.0	8.3 – 67.8	8.1 – 75.0
ΔE^*_{min}	0.8 – 2.9	0.7 – 2.9	1.4 – 4.4	1.0 – 4.4	1.1 – 4.6	0.6 – 2.8
$k * 10^2 (day^{-1})$	1.0 – 3.1	0.7 – 3.2	0.7 – 2.7	0.7 – 3.0	0.8 – 3.1	1.2 – 3.7
Goodness of fit						
R^2	0.914	0.886	0.895	0.877	0.884	0.912

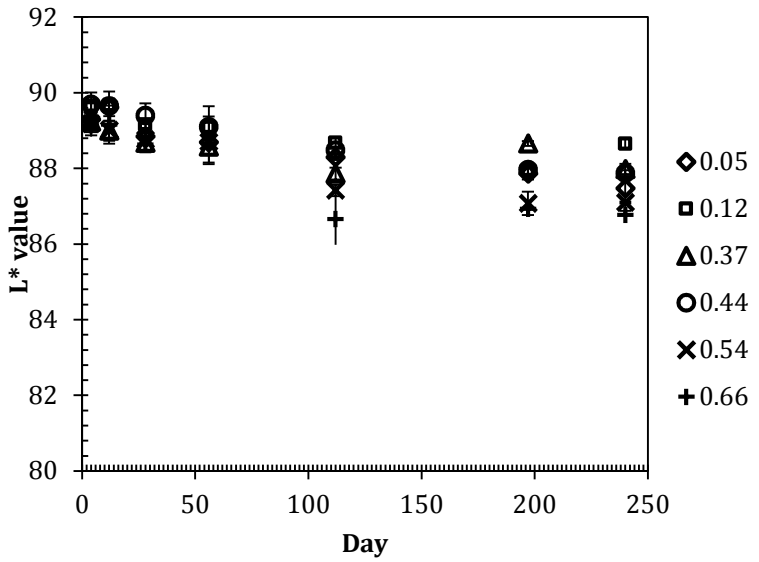


Figure 3.7: Changes of L^* value as a function of time of DEY during storage at room temperature at different a_w.

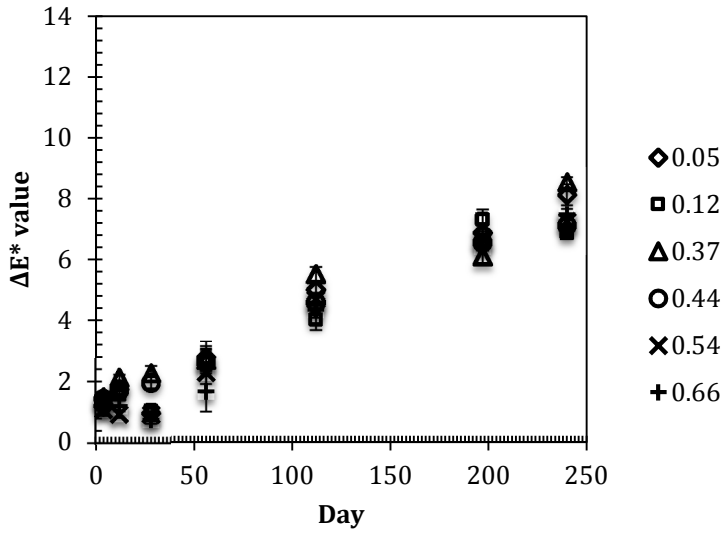


Figure 3.8: Changes of ΔE^* value as a function of time of DEY during storage at room temperature at different a_w.

The decline of L* value of samples during storage at the most extreme condition (with the highest reaction rate of lipid oxidation and the Maillard reaction) is only 9%, which is not a significant change in terms of naked eye observation.

The reaction constants related with L* and ΔE* calculation at three storage temperatures and an a_w of 0.66 were further evaluated in the Arrhenius plots to obtain an activation energy (Ea) of 83.1 and 41.0 kJ/mol respectively (Figure 3.9), which can be transformed to the Q_{10} (rate increases when temperature raises 10°C) of 2.88 and 1.69 respectively. The Q_{10} values were more indicative of lipid oxidation rather than the Maillard reaction(Kaanane & Labuza, 1988; Labuza 1971), which is also consistent with our Q_{10} assumption from the storage experimental design.

Another reason for the color change of DEY is the possible oxidation of carotenoids, which are yellow pigments as stated in the prior "Carotenoids" part. They

are highly unsaturated compounds and considered as antioxidants. Several studies have shown the protection effect of xanthophylls as antioxidants in vitro (Krinsky, 2002; Yen & Chen, 1995). Xanthophylls are capable of reducing the rate of increase of peroxide value of soy oil (lipid oxidation), and are possibly able to protect against macular degradation of the retina (Krinsky, 2002). Therefore, it is highly likely that xanthophylls have been oxidized during storage and resulted in the decrease of the yellow color. So the overall color change is contributed by lipid oxidation and the Maillard reaction and possibly the oxidation of xanthophylls. Lipid oxidation and the Maillard reaction impact on color causing an increase brown color, while the effect of oxidation of xanthophylls is to decrease yellow color. These two trends are contrary to each other, which may be the reason for only a slight decrease of L* value even during storage at the most severe conditions (45°C, a_w of 0.66).

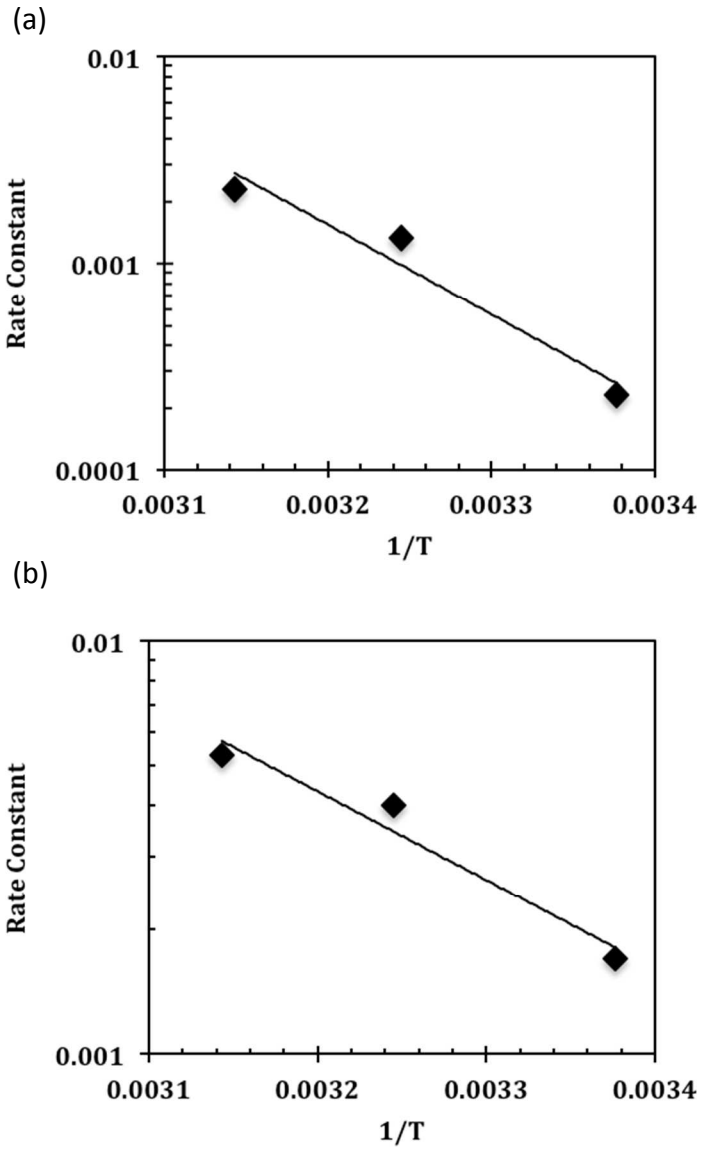
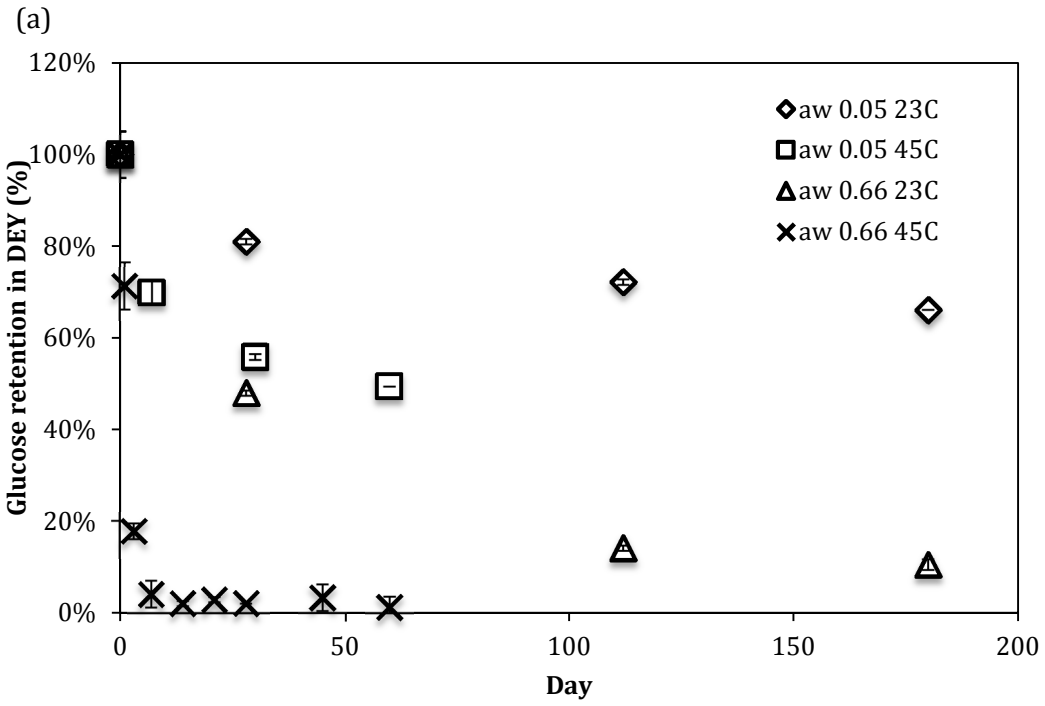


Figure 3.9: Arrhenius plot of (a) L* value and (b) ΔE* value of DEY during storage at 45°C at a_w of 0.66.

Besides the effect of xanthophylls oxidation, the reasons, that the color change of DEY is not as significant as other protein powders, is probably also due to 1) the residual glucose content is small (Table 3.2); 2) the initial color of DEY is yellow, so the decline of L* value from yellow to brown color is not as tremendous as that from white to brown.

Glucose content

To further investigate the progress of the Maillard reaction, the water-extractable glucose content of DEY during storage was determined. The sample stored at 23°C at a_w values of 0.05 and 0.66 and at 45°C at a_w values of 0.05 and 0.66 were both tested. The initial glucose content of DEY is 0.21% (db, Table 3.2). During storage at different a_w values and temperatures, the amount of glucose in DEY decreased as a function of time and followed first-order kinetics (Figure 3.10).



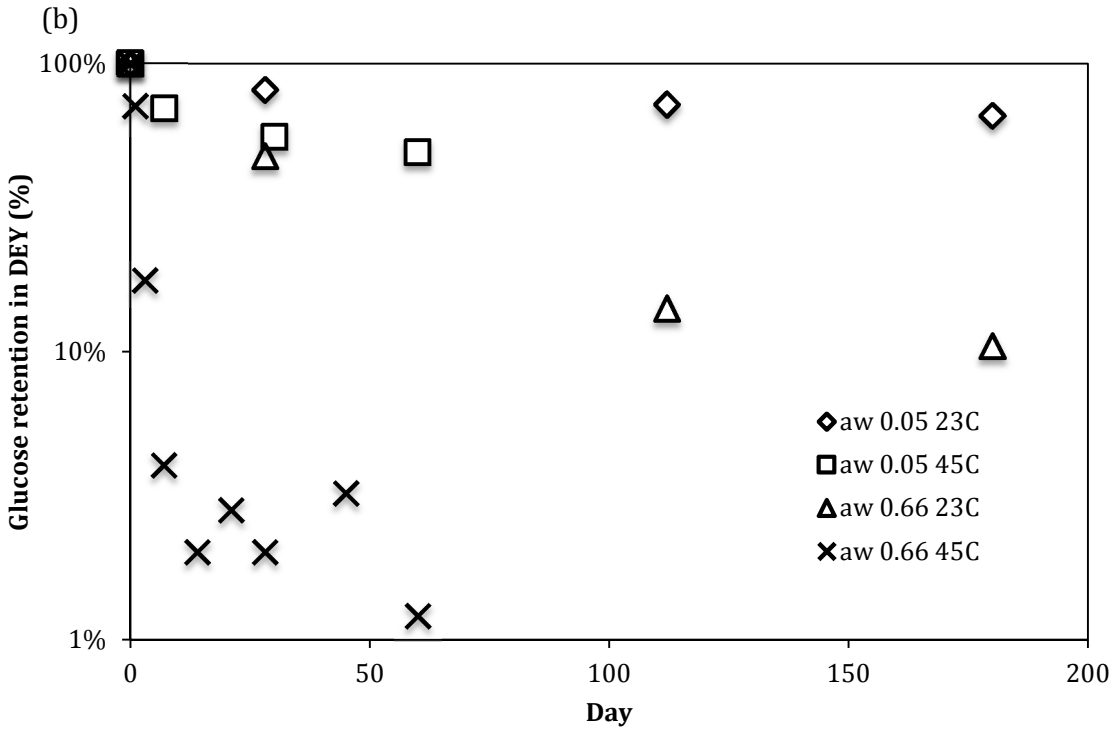


Figure 3.10: Glucose retention in DEY as a function of storage time (a) in a regular scale (b) in a semi log scale

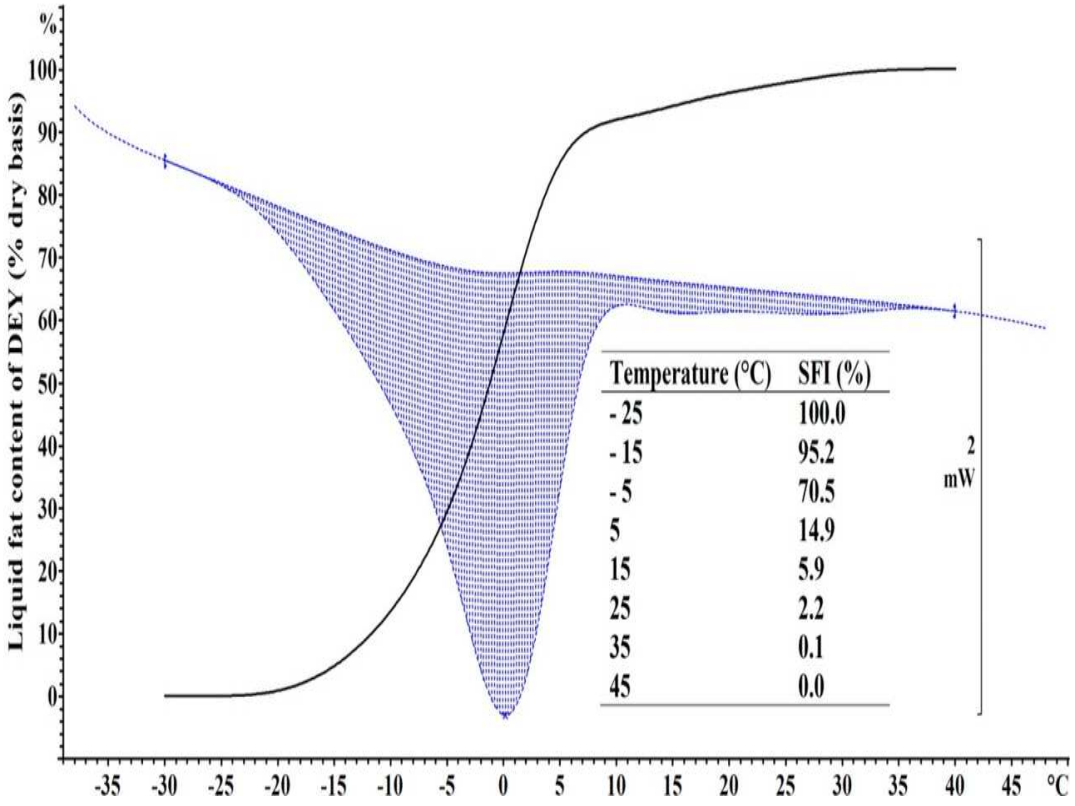


Figure 3.11:Liquid fat content in the initial DEY as a function of temperature. The blue dotted area indicates the total area of fat melting. SFI: solid fat index.

For the sample storage at 45°C at an a_w of 0.66, the glucose content went to zero after 7 days storage (reaction rate of constant (k) = 0.0611), confirming the occurrence of the Maillard reaction. The glucose content of samples stored at 23°C at the same a_w is almost about 92% loss after 6 months storage (k = 0.0045), but with a much smaller rate constant compared with 45°C sample, about 13.5 times slower. This indicates that the temperature effect on the reaction rate of the Maillard reaction is very significant. The Q_{10} for the Maillard reaction generally ranges from 4 to 8, in this case it is around 3.5. The glucose content of samples stored at an a_w of 0.05 showed some loss over time. After 2 months storage at 45°C, the residual glucose declined by 50%. The glucose content

decline of samples stored at 23°C and an a_w of 0.05 showed only 20% loss after 6 months storage. However, even the slight progress of the Maillard reaction can lead to tremendous food quality deterioration (Qinchun Rao & Labuza, 2012). The glucose content decline at very low a_w is very interesting. It should not happen according to the food stability map. When a_w is smaller than that of its m_0, that is, at an a_w of 0.05, the Maillard reaction is stopped due to the lack of moisture as a reaction medium (T. Labuza et al., 1970).

However, the reaction medium is not only restricted to moisture, liquid form lipids can allow for the Maillard reaction (Kamman & Labuza, 1985). As seen in Figure 2.11, the SFI (solid fat index) of DEY samples is 2.2 and 0% at 25 and 45 °C, respectively, indicating that at least 97% of the fats in DEY are in their liquid form during storage at or above room temperature. These liquid form fats can act as a solvent and mobilizing agent for glucose to be able to react with protein at the interface (the Maillard reaction) (Figure 2.10 and 2.11). Overall, when a_w is low; lipids, instead of water, act as the reaction medium for the Maillard reaction. When the a_w is greater than that of its m_0, the moisture is absorbed into the dry matrix and functioning as a solvent for the dissolution of reactants, resulting in a faster reaction rate for the Maillard reaction. Therefore, at an a_w of 0.05 at 23 °C and 45 °C, the decrease of glucose content of DEY was also due to the Maillard reaction while with liquid fats as the reaction medium to provide the mobility and reduce the activation energy (Ea) (Figure 2.10). Similar results have been found in a model system consisting of glucose (10%, g/g), monosodium glutamate (MSG, 11.8%, g/g), and lipids (8%, g/g) during storage (Kamman & Labuza,

1985). Overall, the molecular mobility of food components not only depends on the physical state of the food but also is influenced by water. In addition, water can affect the reaction's sensitivity to temperature. From Figure 2.10, the loss rate of the water-extractable glucose in DEY increased when the storage a_w and/or temperature increased.

The free amino groups

The OPA test at 340 nm did not show significant changes for DEY during storage (Figure 3.12, Figure 3.13, Figure 2.14). Some samples (for example, DEY storage at 45°C at an a_w of 0.66) showed a slight increase rate of free amino groups, which may be due to the protein degradation during storage. Similar results had been found in our lab's previous study for egg white powder storage study (Qinchun Rao, Rocca-Smith, et al., 2012). According to the glucose content study,

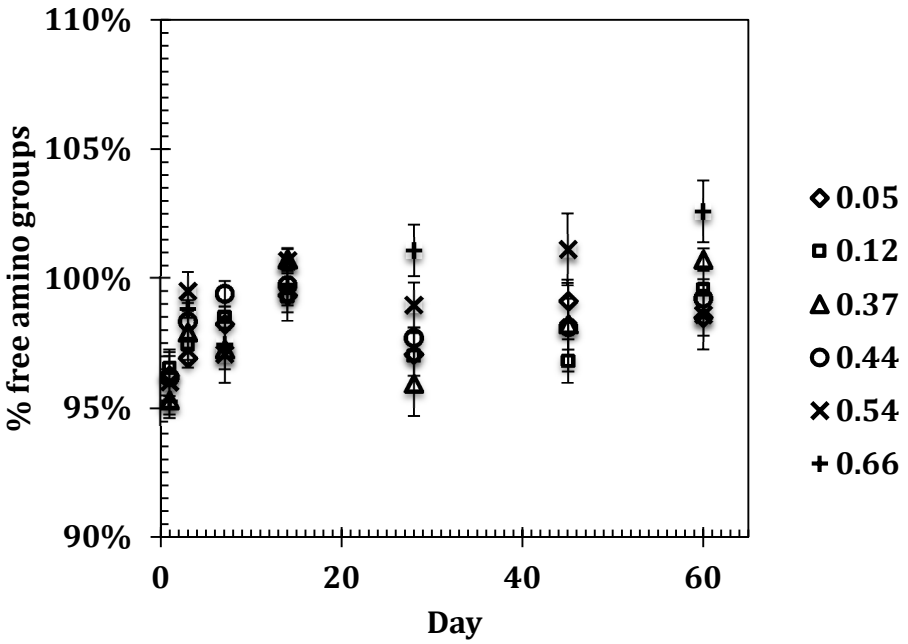


Figure 3.12: Change of % free amino group as a function of water activity (a_w) of DEY during storage at 45°C.

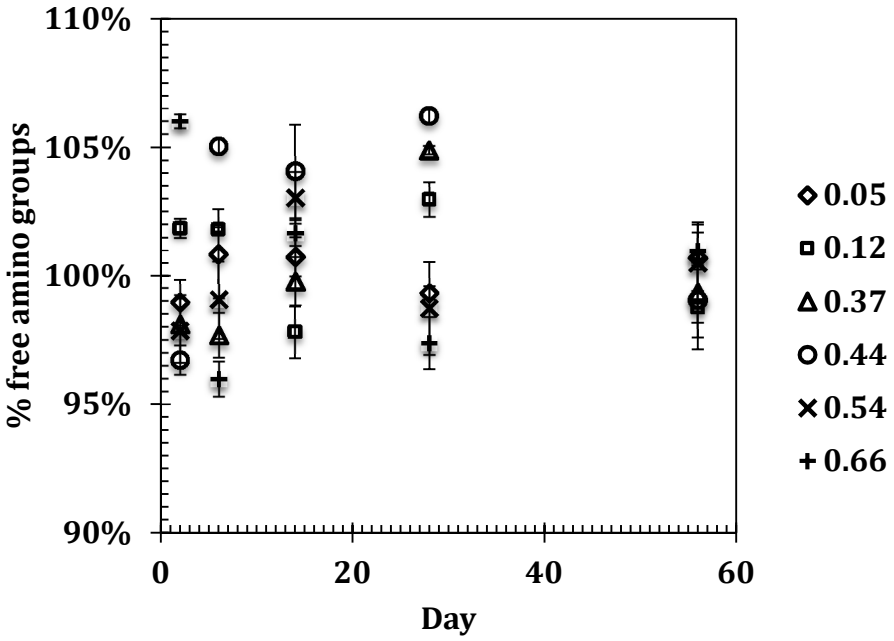


Figure 3.13: Change of % free amino group as a function of water activity (a_w) of DEY during storage at 35°C.

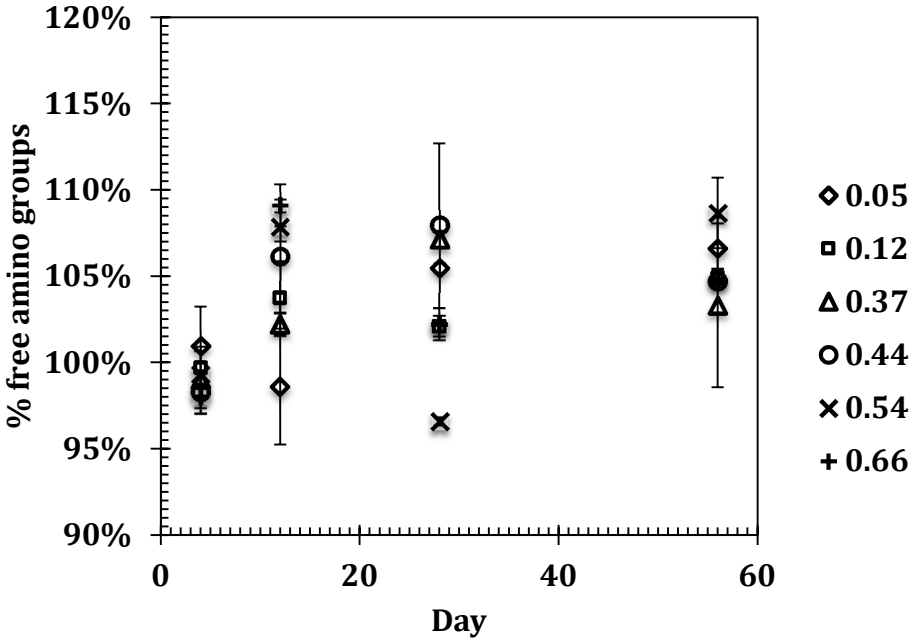


Figure 3.14: Change of % free amino group as a function of water activity (a_w) of DEY during storage at room temperature.

the occurrence of the Maillard reaction is confirmed. Therefore, a decrease of the free amino groups should be observed to correspond with the concurrent loss of glucose. The reason for no decline of free amino groups may be due to the lipid oxidation. Lipid oxidation is an oxidation reaction of unsaturated lipids/fatty acids, producing numerous substances that are fluorescence reactive (Frankel, 2005). Therefore, the reading at 340 nm may be interfered with those lipid oxidation products. Another possible reason may come from the interference of yellow pigments (referred to Carotenoids), as they are hard to wash out during sample preparation. OPA is a reliable chemical method for the measurement of free amino groups, developed by Vigo (Vigo, Malec, Gomez, & Llosa, 1992), if has been found suitable for a protein bar model system, and high carbohydrate samples. However, this method is probably not a good measurement of available amino groups for samples rich in fat/unsaturated fat. As a consequence, for the measurement of the progress of the Maillard reaction in high fat content samples, more direct methodology should be considered, such as for the measurement of glucose, and using mass spectrometry (MS) to detect certain Maillard reaction products.

The Maillard reaction products

The Maillard reaction products were also measured using front-face fluorescence according to Karoui's work (Karoui et al., 2007). Front-face fluorescence is a technology of analysis of fluorescence of a sample. Basically, a beam of light excites the electrons of certain molecules moving from the ground state to an excited state. After that, the excited molecule rapidly relaxes from the higher vibrational state to the lowest vibrational state. During this period, the molecular decay from the excited state to the ground state through

the emission of a photon, can be detected using fluorescence spectroscopy (Strasburg & Ludescher, 1995). Front-face fluorescence has several advantages for the measurements of molecular interactions and reactions: 1) It is much more sensitive than other spectrophometric technologies (Strasburg & Ludescher, 1995)(Karoui et al., 2005); 2) Fluorescence compounds are very sensitive to their environment change, such as tryptophan, the most commonly intrinsic probe used for protein analysis. The fluorescent property of tryptophan buried in the interior core of protein is totally different from that exposed on the surface. This characteristic enables one to identify conformational changes as well as interactions of protein with other compounds in food systems, such as sugar and lipids (Steiner & Weinryb, 1971); 3) It is simple, rapid, and accurate, and only requires very small quantities of a sample (Karoui et al., 2005; Strasburg & Ludescher, 1995).

The results of front-face fluorescence are shown in Figure 3.15. Samples stored at an a_w of 0.66 and either 23°C or 45°C were tested. As we can see, the emission spectra of all samples (vacuum packaged at -20°C) have three peaks, with the highest peak appearing at around 425 nm followed by peak at around 530 nm and 475 nm, which is a little bit different as compared to other Front-face studies (Karoui et al., 2007; A. Kulmyrzaev & Dufour, 2002). The other studies showed either only one peak (at around 425 nm) or two peaks (at around 425 nm and 530 nm) as a result of the Maillard reaction products. The reason for the difference will be stated later in detail (Refer to "The progress of lipid oxidation"). One major Maillard reaction product detected by Front-face fluorescence is furosine. Furosine is one of the hydrolysis products from the Amadori

compounds, which are formed during the Maillard reaction (Hodge, 1953). Research has shown that the intensities of the front-face fluorescence spectra peaks are positively correlated with the furosine content present in the sample (A. Kulmyrzaev & Dufour, 2002). Since all these peaks were detected in all samples, this is an indication that all samples generated the Maillard reaction products during storage, even for the control DEY sample (vacuum packaged at -20°C). Mostly like the peak for the controls shows the effect of the spray drying process as studies have reported the formation of the Maillard reaction products during processing of DEY due to the high temperature and humidity (Caboni et al., 2005; Franke & Kieling, 2002). Our previous study on the storage stability of dried egg white powder also proposed the possibility of the occurrence of the Maillard reaction during manufacturing. The evidence was the browning color generated during storage when the residual reducing sugar content is zero (Qinchun Rao & Labuza, 2012).

The DEY sample stored at 45°C and an a_w of 0.66 for 56 days (8 weeks) has the highest intensity of all peaks, followed by sample stored at 45°C at an a_w of 0.66 for 28 days, at 23°C at an a_w of 0.66 for 56 days, at 23°C at an a_w of 0.66 for 28 days, and the control sample. These results showed the effect of storage temperature and time on the progress of the Maillard reaction. Both have a positive correlation with the Maillard reaction. The peak intensity at 425 nm of sample stored at 45°C and an a_w of 0.66 for 56 days is almost four times as high as that of the control sample, suggesting the progress of the Maillard reaction cannot be ignored even with only 2% reducing sugar left. This

result corresponds with the glucose content study results, confirming again the occurrence of the Maillard reaction.

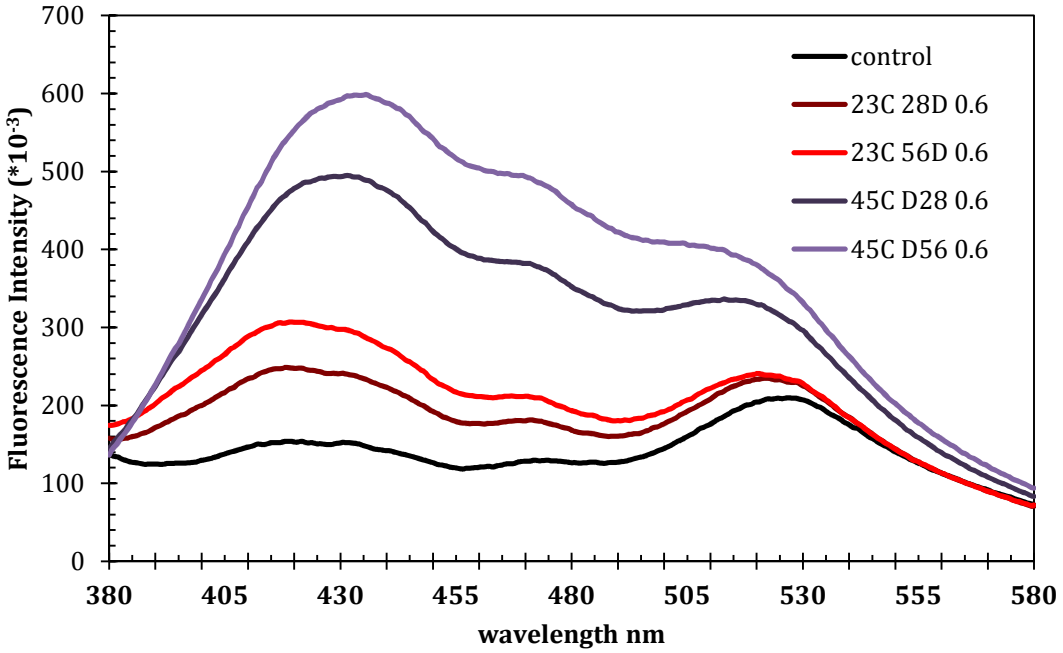


Figure 3.15: Fluorescence spectra of the Maillard reaction products from DEY during storage at 23°C and 45°C and measured at 28 and 56 days at a_w of 0.66.

The PCA (principle component analysis) results are shown in Figure 2.16. PCA is a statistical method that transforms the original data into a new coordinate system using principle components to interpret all the observations. It is a data reduction method used to re-express multivariate data with fewer dimensions. The first principle component (PC1) needs to represent the largest possible variance. The second principle component (PC2) captures most information not captured by PC1 and is also uncorrelated with PC1, and so on. R is free and robust statistical software that can be easily used to conduct the PCA calculation. For the Maillard reaction products spectra data, PC1 captured 92.066% of all the variance, and PC2 captured another 4.702%. Therefore, instead of almost 400

variance, PCA only uses four principle components to capture/interpret the whole data set (all four principle components captured 99.839% of all the variance), Figure 3.16 is the plot of each observation (DEY storage at different conditions) on the new coordinate system.

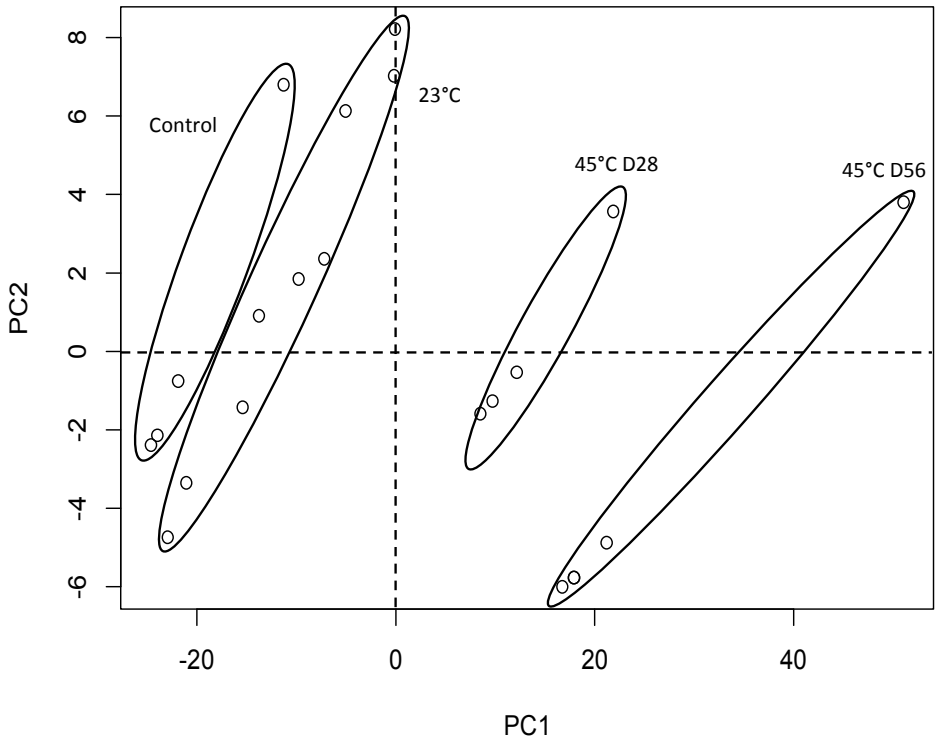


Figure 3.16: Principle component analysis result of the Maillard reaction products spectra of DEY during storage at an a_w of 0.66. Control: the original DEY vacuum packaged storage at -20°C; 23°C: the DEY storage at 23°C for 28 days and 56 days. 45°C D28: DEY storage at 45°C for 28 days; 45°C D56: DEY storage at 45°C for 56 days.

As we can see, the overall trend of the DEY sample moved from the left part to the right part with increasing storage temperature and time, indicating the difference among all the storage conditions. But for the samples stored at 23°C for both 28 and 56 days, the impact of storage time is not significant, as all the observations fell in the same

range. It also suggests the present of outliers in all the samples. Figure 3.17 showed the PCA result after removal of the outliers and it showed clearer the difference in sample at different storage condition. Therefore, the used of front face fluorescence coupled with PCA seems to be a good approach for identification of DEY during storage. In fact, several studies have already been proposed to use this technique to measure the freshness of shell eggs (Karoui et al., 2006, 2007).

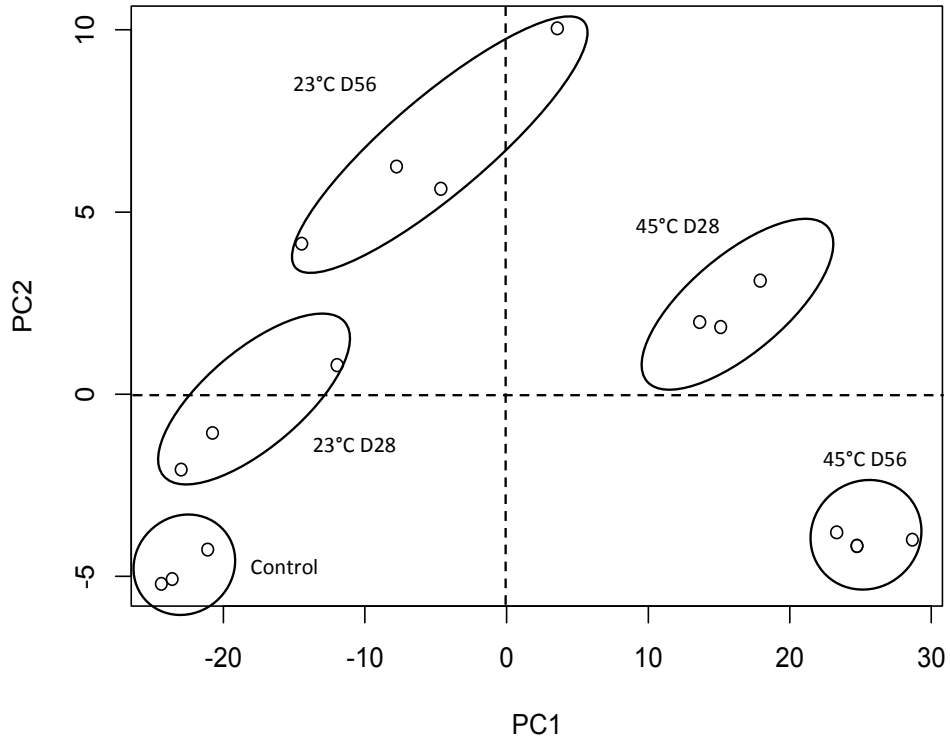


Figure 3.17: Principle component analysis result of the Maillard reaction products spectra of DEY during storage at an a_w of 0.66 after removal of outliers. Control: the original DEY vacuum packaged storage at -20°C; 23C D28: the DEY storage at 23°C for 28 days; 23C D56: the DEY storage at 23°C for 56 days; 45°C D28: DEY storage at 45°C for 28 days; 45°C D56: DEY storage at 45°C for 56 days.

The progress of lipid oxidation

The progress of lipid oxidation can also be measured using Front-face fluorescence (Wold et al., 2002). The result is shown in Figure 3.18. It demonstrated that lipid oxidation products were not increasing for samples stored at 23°C. But with increased temperature, the intensity of each peak was increasing, with almost a three-fold increase. It indicated the significant effect of storage temperature on the progress of lipid oxidation.

The lipid oxidation products spectra are similar to the Maillard reaction products spectra. Both have three peaks around similar emission wavelengths. This explains the difference of the Maillard reaction products spectra from those spectra found in another published papers. It is because the peaks are not only representing the Maillard reaction products, but also lipid oxidation products. It makes sense since two reactions share several pathways, the fluorescence characteristics of their products are similar (Zamora & Hidalgo, 2005).

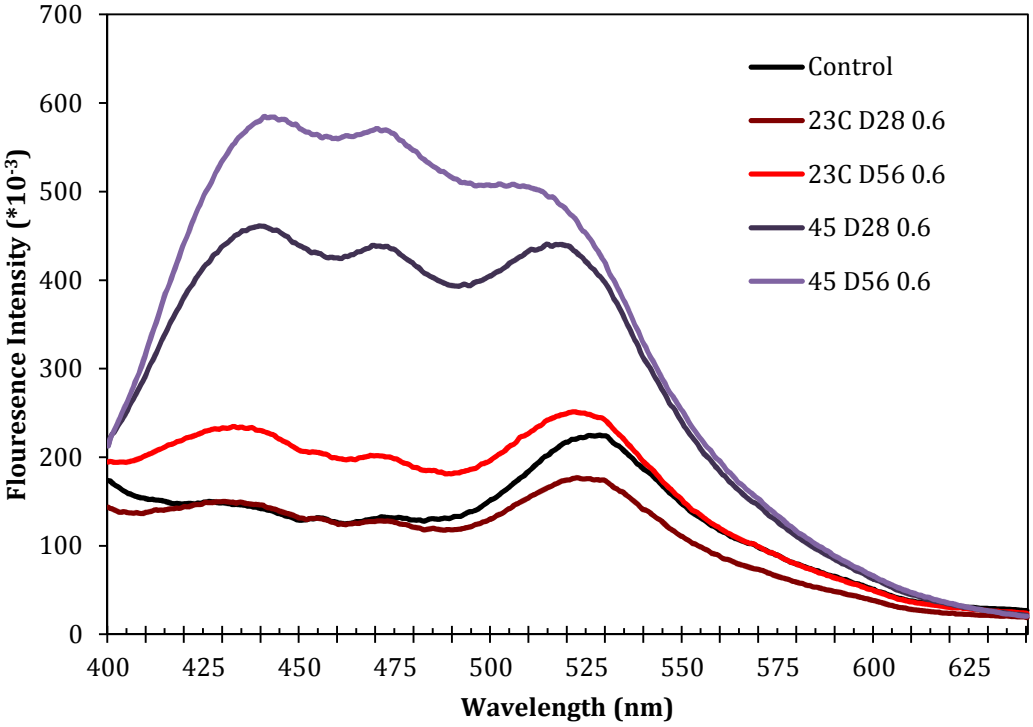


Figure 3.18: Fluorescence spectra of the lipid oxidation products from DEY during storage at 23°C and 45°C and measured at 28 and 56 days at a_w of 0.66.

However, the peak intensities still showed some differences. In the lipid oxidation products spectra, all three peaks increased their intensities almost equally with increased storage temperature and time. Whereas, in the Maillard reaction products spectra, the peak around 425 nm contributed more to the increase of peak intensity compared with the other two peaks. It might indicate that the lipid oxidation products are contributing more to the 475 and 530 nm peaks.

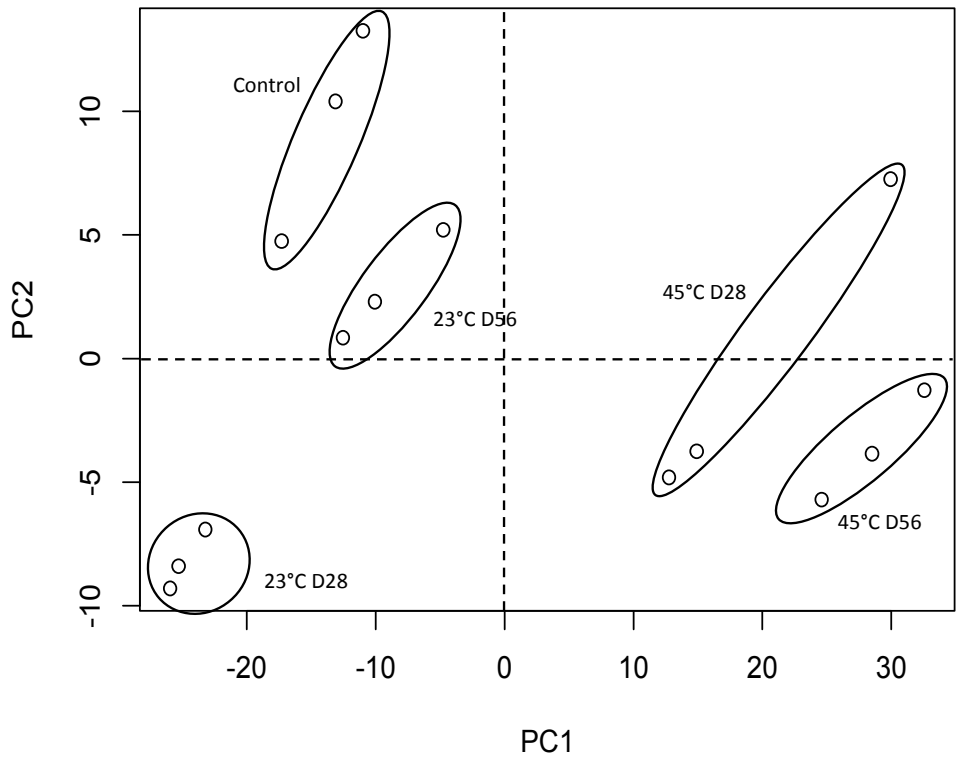


Figure 3.19: Principle component analysis result of the Maillard reaction products spectra of DEY during storage at an a_w of 0.66 after removal of outliers. Control: the original DEY vacuum packaged storage at -20°C; 23C D28: the DEY storage at 23°C for 28 days; 23C D56: the DEY storage at 23°C for 56 days; 45°C D28: DEY storage at 45°C for 28 days; 45°C D56: DEY storage at 45°C for 56 days.

The PCA result (Figure 3.19) showed the distribution of each storage condition on the new coordinate system. The trend was similar with that of the Maillard reaction product PCA result: the spots moved from the left part to the right part with increased storage temperature and time. It also suggested the potential of front-face fluorescence technology to be applied in the area of analysis or identification of the quality of DEY during storage. Since the peak intensity is correlated with the progress of both the Maillard reaction and lipid oxidation, it is hard to differentiate the Maillard reaction products from lipid oxidation products if both reactions occur in the food matrix.

Peroxide value

To further investigate the progress of lipid oxidation, the peroxide value of DEY during storage was determined. Many methods have been developed to measure the peroxide value in food systems, among which the FOX method was applied in this study. The peroxide value was expressed as meq active oxygen/g fat. The meq is the abbreviation term of milliequivalents, where one millequivalent of active oxygen is equal to 0.5 millimole of Fe^{3+} detected in the assay, because 1 meq of oxygen is equal to 0.5 millimole of O_2. The peroxide value of DEY storage at 45°C at an a_w of 0.66 had a bell shape curve as a function of storage time. The increase of peroxide value from day 0 to day 28 confirmed the occurrence of lipid oxidation (Figure 3.20). Even though the increase in terms of peroxide value was slight, it was statistically significant ($P < 0.05$), meaning the peroxide value after 28 days storage was significantly different from that at day 0. Then the peroxide value decreased, which was due to the increased rate of peroxide interaction (Labuza, 1971). They are intermediates produced during the primary oxidation of lipid oxidation and will undergo degradation during the secondary oxidation. Therefore, the decrease of peroxide value does not mean the decrease of lipid oxidation, but indicates the progress of lipid oxidation has moved to the secondary oxidation period.

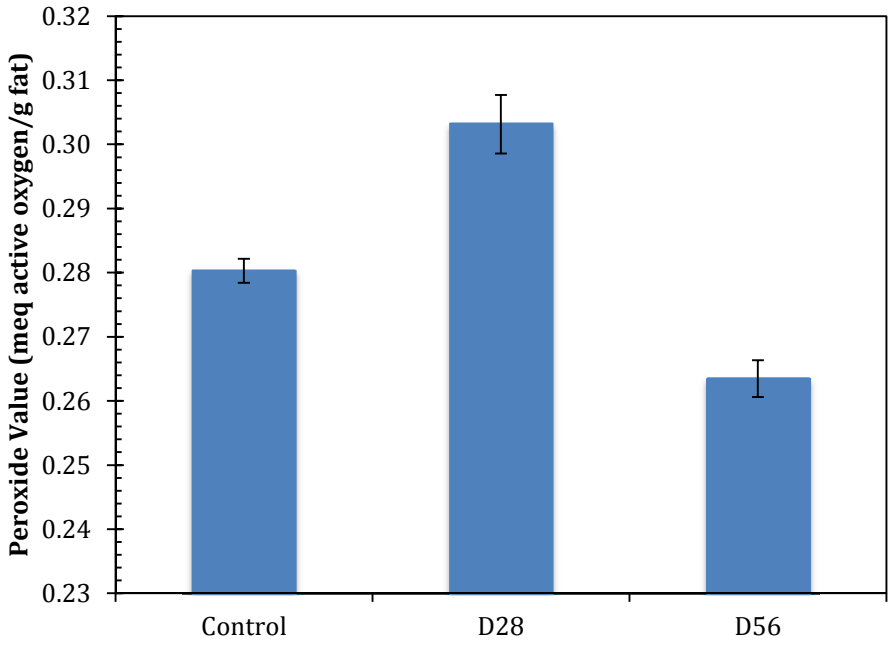


Figure 3.20: The peroxide value of DEY storage at 45°C at an a_w of 0.66. Control: the original DEY vacuum packaged and stored at -20°C; D28: after storage for 28 days; D56: after storage for 56 days.

As noted the control sample also showed present of lipid oxidation with a peroxide value of 0.28 meq active oxygen/g fat. This was probably due to 1) Lipid oxidation that ocurring during processing; 2) Lipid oxidation that occured during distribution and storage after production since the packages had no barrier to oxygen and light; and (3) chemical (antioxidant additives) protection were not in the formula. These results correspond with the front face fluorescence results: 1) peaks of lipid oxidation products have been detected for the control sample; 2) peak intensities increased with increasing storage time.

Discussion

Storage induced changes in physicochemical properties of DEY is caused by two chemical reactions: lipid oxidation and the Maillard reaction, which are also the two major reasons of quality deterioration of intermediate moisture food (IMF) products (Theodore P Labuza, 1980). With the high content of unsaturated fatty acids and 0.21% (Table 3.2) of glucose, lipid oxidation and the Maillard reaction did happen during storage and were confirmed using multiple test procedures.

The effect of a_w is significant on physicochemical properties, as well as the temperature effect. The rate of loss of glucose increased with increasing a_w. Even though the L* value decrease rate constant and ΔE* value increase rate constant did not show a significant difference with different a_w, it still indirectly showed the applicability of the food stability map. When adding up both lipid oxidation and the Maillard reaction rate, because both contributed to the color change, the rate difference is not that much to show a significant difference with a_w. If just looking at the rate of the decline of the L^* value at 45°C (table 2.3), the lowest rate appears at an a_w of 0.12, which is near the a_w value that corresponded to m_o (Figure 2.1), where there is the lowest reaction rate of lipid oxidation and the Maillard reaction. Therefore, storage conditions (temperature and humidity) are crucial in terms of storage stability.

Besides the storage conditions, the processing condition is also important in the storage stability aspect. Our previous paper showed the impact of the drying process on the storage stability (Qinchun Rao & Labuza, 2012). The Maillard reaction occurred during the drying process and had a prolonged effect on the stability of DEW during

storage, as the color of DEW changed from white to yellow with zero glucose content (Qinchun Rao & Labuza, 2012). Other studies have also confirmed the occurrence of the Maillard reaction and lipid oxidation during the drying process of eggs with detection of the Maillard reaction products and cholesterol oxidation products (COPs) (Caboni et al., 2005; Franke & Kieling, 2002). Therefore, possible protection approaches may need to applied to reduce/inhibit those reactions.

Possible approaches include complete removal of reducing sugars (glucose) as well as adding antioxidants. Another approach would be replace spray drying with freeze drying, however, the cost is expensive and may not be practical for the egg ingredient industry. Optimizing spray drying conditions to minimize the heat treatment may be more feasible, factors like inlet and outlet temperature, and spraying time. Therefore, the practical way to limit the damage caused by two chemical reactions is using the way we apply in food preservation, i.e. setting multiple hurdles. As each hurdle can reduce the reactions to some extent, all of them together may be able to inhibit or at least reduce the progress of both reactions.

It may seem that the slight change of color may not be a big problem for the egg ingredient industry. However, the impact of the two chemical reactions is not only on color, but also on protein and lipid quality, which may lead to the loss of functionality and bioactivity, and this part will be presented and discussed in detail in Chapter 3.

Conclusion

Both the Maillard reaction and lipid oxidation are contributing to the physicochemical changes of DEY during storage. The progress of the Maillard reaction

and lipid oxidation are dependent of extrinsic factors: a_w and temperature. Therefore, the physicochemical changes of DEY during storage are related with the storage humidity and temperature. As a consequence, keeping DEY at relatively low humidity and temperature environment helps maintain its quality and maximize its shelf life.

Acknowledgement:

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Chapter 4 Storage induced protein aggregation of a commercial spray-dried egg yolk powder

Summary

Protein aggregation is defined as "any protein species in non-native states and whose sizes are larger than that of the native protein" (W. Wang, Nema, & Teagarden, 2010). The formation of protein aggregation during processing and/or storage is dependent on many factors including intrinsic and extrinsic factors. In this study, we evaluated the extrinsic factors (storage environment) that resulted in protein aggregation during storage of a commercial spray-dried egg yolk powder (DEY). Overall, DEY after 56-day storage at a_w of 0.66 and 45°C (an abuse condition) was studied. We found an increase in both soluble and insoluble protein aggregates as compared to the original DEY. The molecular mechanism of protein aggregate formation was further evaluated. DEY after 56-day storage at 45°C had no native proteins left as observed by an increase in disulfide bonds and lipid-protein interactions. The total of 32 proteins were identified to form soluble and insoluble high molecular weight protein aggregates (HMWPAs of > 200 kDa). They were typical egg yolk proteins and egg white proteins including serum albumin, vitellogenin, apovitellenin, as well as ovotansferrin, ovalbumin, lysozyme, ovomucoid, and ovastatin. Most of them contain disulfide bonds and some of them contain ligand and fatty acids binding sites. The formation of protein aggregates was mainly from disulfide bond formation/exchange or other direct chemical linkages induced

by lipid oxidation and the Maillard reaction. Furthermore, the effective approaches to avoid DEY deterioration during storage are discussed.

Introduction

Chicken egg yolk contains many biologically active substances including phospholipids, long chain polyunsaturated fatty acids, phosvitin, lipoproteins, as well as sialic acid and antibodies (IgY), which provides egg yolk to have a broad application not only in food but also in medical, pharmaceutical, cosmetic, nutraceutical and biotechnological industries (Anton, Nau, & Nys, 2006; Hartmann & Wilhelmson, 2001). Dried egg yolk powder (DEY) is an egg product, which enjoys several advantages over liquid egg yolk especially in terms of preservation stability (American Egg Board Egg product reference guide. 2006)(American Egg Board EGGSolutions - The complete reference for egg products. 2004). However, DEY still suffers from deterioration when stored in an abused environment such as high humidity ($a_w > 0.6$) and/or high temperature (≥45°C). In Chapter 2, we reported a decrease of L^* value (brightness) and glucose content, as well as increase of ΔE^* value and peroxide value during storage of DEY. We proposed that these physicochemical changes were mainly due to the Maillard reaction and lipid oxidation. However, these two reactions can provide more than color changes. They are also related with formation of protein aggregates.

The Maillard reaction was found to correlate with whey protein aggregates as decreasing glucose content/free amino acids correlated with an increase of protein aggregates (Chevalier, Chobert, Dalgalaprondo, & Haertle, 2001; Theodore P Labuza, 1980; Nagaraj, Shipanova, & Faust, 1996; Zhou et al., 2013). However the aggregation

mechanism at the molecular lever has not been completely discovered because of the hundreds or possible thousands of products produced through the Maillard reaction. It is possible that some small molecular compounds such as reducing compounds from lipid oxidation (aldehydes and ketones) and from the reductions produced in intermediate step of the Maillard reaction they may then result in protein cross-linking. Lipid oxidation shares some pathways with the Maillard reaction (Zamora & Hidalgo, 2005), which may be a consequence of protein aggregates. Besides this, the lipid and/or lipid peroxyl radicals can cause damage to proteins and amino acids, leading to further polymerization, which has been shown in lipid-protein/amino acid systems in the 1960s (Desai & Tappel, 1963; Kanner & Karel, 1975; Matsushita, 1975; Roubal, 1971; Rouball & Tappel, 1966; Schaich & Karel, 1975).

Besides these two reactions, another direct chemical linkage that can result in protein aggregates formation is intra and intermolecular disulfide bond formation/exchange. The disulfide bond interaction causing protein aggregation has been studied and reported in several papers (Liu, Zhou, Tran, & Labuza, 2009; Zhou & Labuza, 2007; Zhou, Liu, & Labuza, 2008a; Zhou et al., 2008b; Zhu & Labuza, 2010) including our previous paper (Qinchun Rao, Fisher, Guo, & Labuza, 2013). The thiol group buried in the core of intact proteins may be exposed when unfolding, which can form intra- and intermolecular disulfide bonds with other thiol groups (Jang & Swaisgood, 1990).

Besides direct chemical linkages, additional minor protein aggregates can be formed through protein self-association. However, few studies have conducted an in-

depth study of protein aggregate formation of DEY, so this paper intends to fill the gap through evaluating the protein aggregate formation during storage of DEY and also elucidating the molecular mechanism behind it.

Materials and Method

Materials

The commercial dried egg yolk is the same as in the storage induced physicochemical properties study discussed in Chapter 2. All reagents and chemicals used in this study were of analytical grade.

Storage Study

A certain weight of DEY was put into a sealed desiccator with saturated cobalt chloride slurries at a_w of 0.66 and stored at room temperature (RT) and 45°C for two months. DEY was sampled at designated time intervals for further analysis. The reason we chose a_w of 0.66 is because it generated the most amounts of protein aggregations among DEY storage among all storage conditions and is low enough to limit mold growth.

Denaturation Temperature of DEY

The denaturation temperature of DEY was analyzed using DSC 1 STARe System (Mettler Toledo, Columbus, OH, USA). DEY was hydrated with water (1 to 1 ratio (w:w)) and placed overnight at 4°C for equilibration. About 10 mg of the hydrated DEY was put into the aluminum pan and run under the designated procedure, which includes 5 steps: 1) cool and hold at -40°C for 10 min; 2) heat from -40°C to 100°C at the rate of

10°C/min; 3) cool from 100°C to -40°C at the rate of -10°C/min; 4) hold at -40°C for 10 min; 5) heat from -40°C to 100°C at the rate of 10°C/min. After each run, the curve was analyzed using STARe Excellence Software (Mettler Toledo, Columbus, OH, USA). The endothermic peak above 40°C was recognized as the protein denaturation peak from the second step. The denaturation temperature (T_d) was determined using the 1st derivatives of the curve. T_d was the spot where the 1st derivative value is zero. ΔH was calculated and normalized based on the protein amount in the sample. T_g can be recognized as the inflection point of the slope from the fifth step at which point the slope of the curve changes from a decrease to an increase or vice versa depending on whether the endothermic is up or down.

Secondary structure changes of DEY during storage

Raman spectroscopy (Qinchun Rao & Labuza, 2012)

The secondary structure of DEY powder was analyzed using the DXR Raman microscope (Thermo Fisher Scientific, Madison, WI). Briefly, the DEY powder stored for 8 weeks and the original DEY powder were measured. The instrument is equipped with 780 nm excitation of SERS spetra through a 10 × confocal microscope objective. It can produce an approximately 2.5 μm diameter laser spot, with a 5 cm^{-1} spectral resolution. The laser power is 150 mW with a 25 μm split aperture. Each scan is actually the average of four spots randomly picked by the machine within the selected spot. Each sample was scanned at least 10 times with manually selecting the spot. The spectra of each scan were collected using the OMNIC for Dispersive Raman Software (version 8.2,

Thermo Fisher Scientific). The final spectrum of each sample is thus the average of at least 10 scans.

Front-face fluorescence

The secondary structure was also measured using front-face fluorescence at room temperature (LS50 B Perking-Elmer Luminescence Spectrometer, Perkin-Elmer Co., Waltham, MA, USA). DEY samples storage at certain conditions (RT and 45°C at a_w of 0.66) were tested. Two parameters: tryptophan and Vitamin A, were selected. The tryptophan emission was monitored from 305 to 450 nm by excitation at 290 nm (A. Kulmyrzaev & Dufour, 2002). The slits of excitation and emission are 5.0 and 0 nm respectively. The Vitamin A emission was monitored from 270 to 350 nm by excitation at 410 nm. The slits of excitation and emission are both 5.0 nm. Briefly, a certain amount of DEY powder was put onto the silica window of the Powder Holder. Then the cap of the Powder Holder was screwed down until the sample is held tightly. Then the Powder Holder is put into the Front Surface accessory for scanning. Each sample was scanned at least in triplicate. The spectra data was extracted from FL WinLab (Perkin Elmer) to Excel and the PCA was calculated and plotted using R. The PCA package in R is FactoMineR. The R code for the PCA calculation can be found in the Appendix.

Fourier transform infrared spectroscopy (FTIR)

FTIR Spectrometer Tensor 37 (Bruker Optics Inc., Madison, WI, USA) was used for the FTIR analysis. The instrument was coupled with a horizontal multireflectance ZnSe crystal accessory (sample chamber), a deuterated triglycine sulfate detector, and a

KBr beam splitter. Data were collected in the 600 to 4000 cm^{-1} infrared spectral range at room temperature. Spectral acquisition was conducted at a resolution of 4 cm^{-1}. The background and sample scans were set to 32. Briefly, a background scan was run before the sample scan, which collected the spectra of the empty crystal accessory. Then a measured amount of sample was put into the crystal accessory and fully covered the window part. The final sample spectrum is the spectrum signals of sample test after subtracting the background spectrum signals. Each sample was tested at least in triplicate. The spectra were analyzed using OPUS Software version 6.5 (Bruker). Each spectra was processed through the undergone baseline correction as well as by calculations of the second derivatives, which was used for the estimation of protein secondary structure. The protein secondary structure was estimated according to other literature (Susi & Michael Byler, 1983)(Dong, Huang, & Caughey, 1990)(Dong, Caughey, Caughey, Bhat, & Coe, 1992)(Kalnin, Baikalov, & Venyaminov SYu, 1990)(KONG & YU, 2007). Briefly, the second derivatives of the amide I region (1600 to 1700 cm^{-1}) was used for the secondary structure estimation with the β-sheet assigned for the spectral range of 1600 to 1647 and 1685 to 1701 cm^{-1}, random coil assigned for 1647 to 1654 cm^{-1}, α-helix assigned for 1654 to 1664 cm^{-1}, β-turn assigned for 1664 to 1685 cm^{-1} based on the spectrum of the control sample and the study of Dong et al. (Table 3.1)(Dong et al., 1990).

Table 4.1: Mean absorption frequencies of various secondary structure elements in proteins.

Mean frequencies (cm^{-1})	Secondary structure assignment
1624	β-sheet
1627	β-sheet
1632	β-sheet, extended chain
1638	β-sheet
1642	β-sheet
1650	unordered (random)
1656	α-helix
1666	β-turn
1672	β-turn
1680	β-turn
1688	β-turn
1691	β-sheet
1696	β-sheet

Reproduced from Dong et al. (1990).

Preparation of buffer-soluble and –insoluble protein of DEY during storage

The buffer-soluble and –insoluble protein of DEY during storage at room temperature and 45°C was prepared by adding a certain amount of powder sample (5 mg) into 1 ml TBS-SDS (Tris-buffered saline [TBS: 20 mM Tris and 500 mM sodium chloride, pH 7.5 (Bio-Rad Laboratories, Inc., Hercules, CA)] containing 1% sodium dodecyl sulfate [SDS, g/ml]). The mixture was mixed thoroughly using a vortex mixer (Henry Troemner LLC, Thorofare, NJ, USA) for 1 min at 3000 rpm. The suspension was then shaken on a shaker (Thermo Fisher Scientific Inc., Rockford, IL, USA) at 200 rpm for 2 h. After centrifuging at 20,000*g* for 30 min, the supernatant and precipitate were separated and collected. The supernatant is the buffer-soluble protein.

Simultaneously, the precipitate, i.e. TBS-SDS buffer-insoluble proteins, was washed three times with 1 ml TBS-SDS. For each wash, the mixture was mixed thoroughly using a vortex mixer (Henry Troemner) for 1 min at 3000 rpm. After centrifuging at 15000*g* for 15 min, the liquid portion was removed carefully. After three washes, the precipitate was then mixed with 1 ml TBS-SDS containing 5% 2-mercaptoethanol (ml/ml, TBS-SDS-2-ME). After being mixed thoroughly using a vortex mixer (Henry Troemner) for 1 min at 3000 rpm, the mixture was heated in a water bath (95 °C) for 5 min. After centrifuging at 15,000*g* for 15 min, the supernatant was carefully collected and it is the buffer-insoluble protein. Simultaneously, the control sample (the original DEY powder (vacuum packaged and stored at -20 °C) was prepared using the same procedure. All steps were carried out at room temperature unless otherwise specified.

Protein solubility of DEY during storage

The previous prepared buffer-soluble protein solutions were used for determination of protein solubility. The protein solubility was determined using the Peirce BCA (bicinchoninic acid) Protein Assay Kit (Thermo Fisher Scientific). Bovine serum albumin (BSA) was used as the protein standard (working range 20 – 2000 μg/ml). Briefly, 60 μl buffer-soluble protein solution was added with 480 μl BCA working reagent (ratio 1:8) in 2 ml centrifuge tube. The BCA working reagent was prepared through mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (v:v = 50:1, Reagent A:B; Thermo Fisher Scientific). The reagent is stable for several days in a closed container at room temperature. After mixing the sample with BCA thoroughly using a vortex mixer (Henry Troemner) for 1 min at 3000 rpm, the mixture was incubated at

37°C for 30 min. Then 200 µl of the mixture was pipetted into a 96-well clear flat bottom polystyrene microplate (Corning In., Corning, NY, USA) (200 µl/well) and the absorption was read using a microplate reader (BioTek Instruments) at 562 nm. Each sample was tested in duplicate.

Protein profile of buffer-soluble and –insoluble DEY during storage

Both the buffer-soluble and –insoluble protein solutions were used for the measurement of protein profile. It was analyzed using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (Laemmli, 1970). Before heating at 95 °C for 5 min, one part of the sample was mixed with one part of Laemmli sample buffer (Bio-Rad) with or without 5% 2-ME (ml/ml). The heated sample (20 µl/lane) was then added onto the 4-20% Mini-PROTEAN TGX Precast Gel (Bio-Rad). The running buffer was 25 mM Tris (pH 8.3) containing 192 mM glycine and 0.1% SDS (g/ml). After running at 110 V for a certain time (about 2 h) at 4°C, the gel was stained with the EZBlue Gel Staining Reagent (Sigma-Aldrich Co., Saint Louis, MO, USA). After destained with deionized water, the gel was scanned by the Gel Doc XR system (Bio-Rad). The SDS-PAGE Molecular Weight Standards (broad range, Bio-Rad) were used for accurate molecular weight estimation on the gel.

Identification of HMWPAs of DEY during storage

In-gel trypsin digestion

HMWPAs of DEY were identified using proteomic technologies. HMWPAs were defined as the proteins/protein aggregates with over 200 kDa molecular weight, which were labeled on the SDS-PAGE gel. The HMWPs band was cut using a sterile blade and went through the in-gel trypsin digestion procedure (Shevchenko, Wilm, Vorm, & Mann,

1996). Briefly, the band was cut into ~2×2 mm cubes and place in a 1.5 ml snap-cap microfuge tube. Then 75 µl of 100mM ammonium bicarbonate in 50% acetonitrile was added for the first wash and shake on a vortex (Henry Troemner) at 3000 rpm for 30 s before incubation at room temperature for 15 min. After incubation the solution was removed using a pipette and the gel pieces were pipetted into 75 µl of 100% acetonitrile for the second wash. After the gel pieces shrank and turned white and semi-opaque, the added acetonitrile was removed. The gel pieces were then rehydrated with 75 µl of 10 mM dithiothreitol (DTT) in 10 mM ammonium bicarbonate and then incubated at 56°C in a water bath for 1 h. After that, the tubes were briefly spin down before removal of the DTT solution. Then the gel pieces were added into 75 µl of 55 mM iodoacetamide in 100 mM NH_4HCO_3 and incubated for 30 min at room temperature in dark. Then the first wash and second wash steps as stated above were repeated. The gel pieces after the last wash should be opaque white. They were then rehydrated with digestion buffer (50 mM NH_4HCO_3, 5 mM $CaCl_2$, 12.5 ng/µl trypsin) and incubated at 4°C for 15 min. The digestion buffer was freshly prepared. Then the supernatant was removed and replaced with 70 µl of 50 mM NH_4HCO_3, 5 mM $CaCl_2$ and incubated at 37°C overnight. The supernatant after overnight incubation was collected in a new 1.5 ml tube and was added with 60 µl of 50% acetonitrile and 0.3% formic acid and incubated for 15 min. Then the supernatant in the new 1.5 ml tube was collected and added with 60 µl of 80% acetonitrile and 0.3% formic acid and incubated for 15 min. After that, the supernatant was collected and froze at -80°C for 30 min before speed vacuum dried.

Stop and go extraction (STAGE) tips desalting

Desalting was carried out using C18 stop and go extraction (STAGE)-tips (Rappsilber, Ishihama, & Mann, 2003). Firstly, the stage tip was assembled. Briefly, the Empore disk flat (3M, St. Paul, MN, USA) was placed on a glass microscope slide, and then the 18 gauge blunt ended syringe needle was pressed into the Empore disk to core out a piece of the filter material. The needle was pressed again for a second core into the syringe needle for extra loading capacity. Then the needle was placed into a 200 µl pipette tip and the cored disk pieces were pushed into the pipette tip with fused silica tubing. After that, the material was gently packed into the end of the pipette tip. The pipette tip with Empore disk cores is the stage tip. Secondly, the stage tip/tube was assembled. To begin with, the cap of a 1.5 ml Eppendorf tube was cut and a hole was bored into the center. Then the cap was snapped onto a 2 ml Eppendorf tube. Then the stage tip (pipette tip with Empore disk cores) was placed into the hole in the cap. The tip of the pipette tip should be adjusted to be about 1 cm from the bottom of the tube. The whole tube with stage tip and cap is the stage tip/tube. After the assembly of the stage tip/tube, the desalting can begin. It began with rehydrating the speed vacuum dried sample after trypsin digestion with 60 µl wash solvent (98:2:0.1%, water:acetonitrile:trifluorozcetic acid (TFA)). Then the rehydrated sample was centrifuged at 3000g for 1 min and put at 4°C. The stage tip/tube was first washed by a wetting solvent (80:20:0.1%, water:acetonitrile:TFA) and then centrifuged at 450g for 2 min. After that, the stage tip/tube was washed by wash solvent (98:2:0.1%, water:acetonitrile:TFA) and then centrifuged at the same condition as the first wash. Then the rehydrated sample was loaded (~60 µl) into stage tip and then centrifuged at 450

g for 2 min. The next step is to wash the stage tip/tube with 60 µl wash solvent (98:2:0.1%, water:acetonitrile:TFA) and centrifuged at 450g for 2 min and then repeating the washing step twice. After that, the stage tip with cap was assembled onto a new 1.5 ml Eppendorf tube. The peptides were eluted through pipetting 60 µl elution solvent (60:40:0.1%, water:acetonitrile:TFA) and then centrifuged at 450g for 2 min. The peptide solution was speed vacuum to dryness before mass spectrometry (MS) analysis.

Mass spectrometric analysis and protein identification

A linear ion trap-Orbitrap (LTQ-Orbitrap) Velos instrument (Thermo Fisher Scientific) was used for the MS analysis. The sample after desalting was rehydrated in a load solvent (98:2:0.01%, water:acetonitrile:TFA) and then injected into the instrument for MS analysis. Tandem mass spectra were searched against a NCBI chicken database and common contaminant proteins database using Sequest (Thermo Fisher Scientific, San Jose, CA, USA; version 27, rev. 12). An Iodoacetamide derivative of cysteine was set as the fixed modification. Oxidation of methionine was set as the variable modification. Other parameters included 0.80 Da mass tolerance, two missed cleavages, and partial trypsin specificity. Scaffold (version Scaffold_4.2.1, Proteome Software Inc., Portland, OR) was used to validate Sequest data (Searle, 2010). Peptide identification was accepted if greater than 95% probability (Keller, Nesvizhskii, Kolker, & Aebersold, 2002) and protein identifications were accepted if greater than 99% probability (Nesvizhskii, Keller, Kolker, & Aebersold, 2003) with at least 2 identified peptides.

Results

Protein aggregates generated during DEY storage

Protein solubility changes of DEY during storage

The protein solubility of DEY decreased with increasing storage temperature (Figure 4.1), indicating the formation of buffer-insoluble protein aggregates during storage of DEY. The temperature effect is very significant as the protein solubility of DEY stored at 45°C dropped to 72.5% of the original solubility compared with sample storage at -20°C. But the protein solubility of DEY stored at 23°C had not decreased significantly compared with the -20°C control sample, suggesting the buffer-insoluble protein formation rate speeded up above a certain storage temperature (certainly higher than room temperature). When not reaching it, the activation energy (Ea) required for the reaction cannot be provided, thus, the reaction cannot occur.

Even though the protein solubility of the samples stored at 23°C had not significantly changed, it did not mean none of the buffer-soluble protein aggregates were formed. Since the protein solubility measured both the buffer-soluble protein and buffer-soluble protein aggregates, it was difficult to determine the presence of buffer-soluble protein aggregates. That is why another approach: SDS-PAGE was combined to further understand the formation of protein aggregates, which were discussed below.

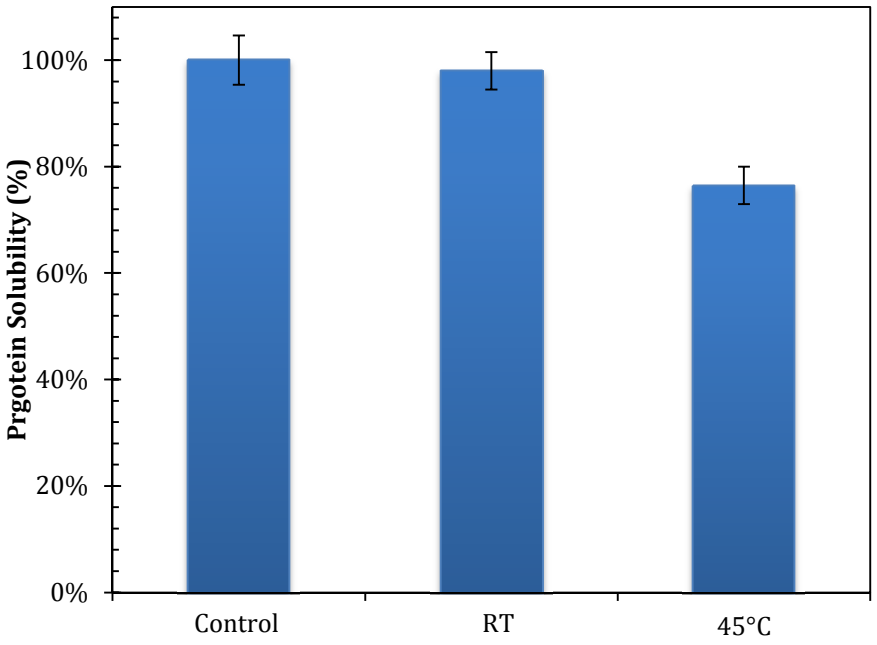

Figure 4.1: Relative protein solubility in TBS-SDS buffer after stored at a_w of 0.66 for 8 weeks at different temperatures. Control: the original sample vacuum package storage at -20°C; RT: room temperature.

Protein profile of DEY during storage

To further study the buffer-soluble and –insoluble protein aggregates of DEY during storage, the profiles of those proteins were analyzed using SDS-PAGE. It must be noted that after treatment with SDS and 2-ME during the sample preparation, both noncovalent (hydrophobic) and disulfide bond interactions can be destroyed. Since

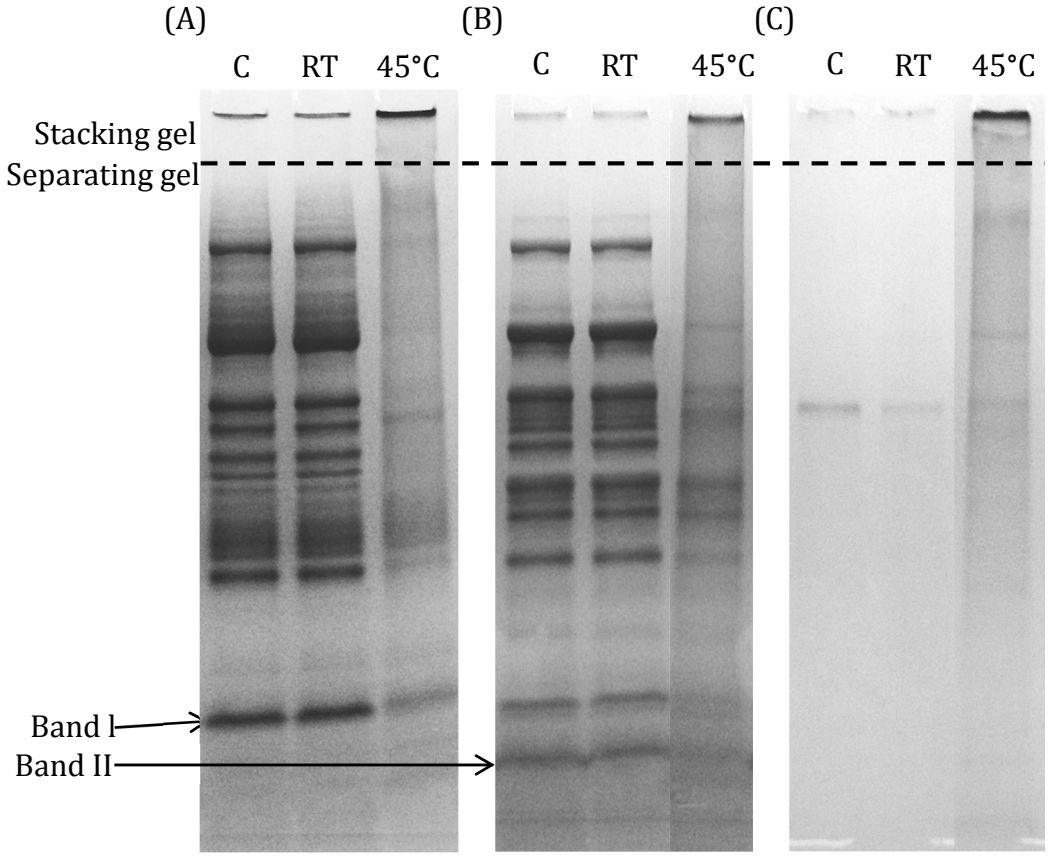

Figure 4.2: SDS-PAGE of DEY stored at an a_w of 0.66 for 8 weeks at different temperatures. (A): Non-reducing gel (without 2-mercaptoethanol) of TBS-SDS buffer soluble protein; (B) Reducing gel (with 2-mercaptoethanol) of TBS-SDS buffer soluble protein; (C): Reducing gel (with 2-mercaptoethanol) of TBS-SDS with 5% 2-mercaptoethanol buffer soluble protein. The dashed line is the boundary of the stacking gel and the separating gel. C: the original sample after vacuum packaged and stored at -20°C; RT: room temperature.

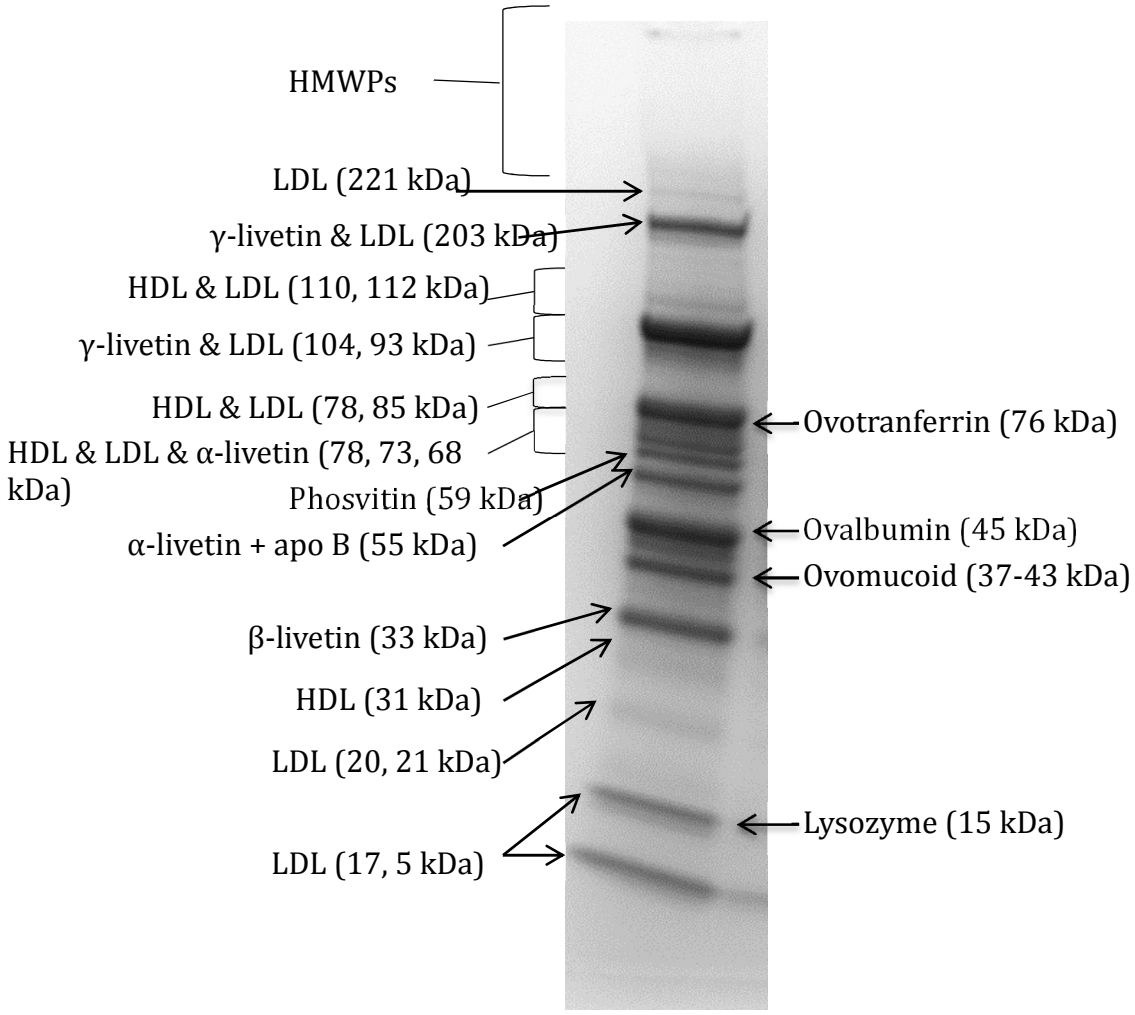

Figure 4.3: DEY (-20°C) protein band labeling on reducing SDS-PAGE.

2-ME is a strong reductant which can cleave the intra and intermolecular disulfide bonds in the aggregates (soluble and insoluble) (Qinchun Rao, Rocca-smith, et al., 2012).

For the control, the original DEY at day 0, its TBS-SDS buffer- soluble protein patterns (Figure 4.2A,B) are similar to those in other studies (Guilmineau, Krause, & Kulozik, 2005; Laca, Paredes, & Díaz, 2010). Compared with that of hen egg white, the protein composition of hen egg yolk is more complex (Guilmineau et al., 2005; Qinchun Rao, Rocca-Smith, et al., 2013). It has been reported that 119 proteins from hen egg yolk

have been identified as noted in the literature review part (Mann & Mann, 2008b). The major egg yolk proteins are labeled in Figure 4.3. For more detailed egg yolk protein information such as protein name and its molecular weight, the readers can refer to several relevant studies (Guilmineau et al., 2005; Laca et al., 2010; Mann & Mann, 2008b). Additionally, no visible TBS-SDS buffer-insoluble precipitate was observed. However, after extraction with the TBS-SDS-ME buffer, a weak protein band (~78 kDa) was found in its TBS-SDS buffer-insoluble precipitate (Figure 4.2C). This protein may be ovotransferrin from egg white, which has 15 disulfide groups (Q Rao, Kamdar, & Labuza, 2013), and/or apovitellin (Guilmineau et al., 2005). This assumption needs to be further confirmed using other methods such as immunoblotting and/or mass spectrometry.

In the gel portion containing the high molecular weight proteins (HMWP, >200 kDa), especially in the stacking gel, the band color intensity of all supernatant samples in the absence of 2-ME (Figure 4.2A) decreased dramatically after the addition of 2-ME to the same sample (Figure 4.2B). Compared with the change in the control, it is clearly shown that TBS-SDS buffer-soluble disulfide bond induced protein aggregates were formed during storage. On the other hand, compared with the pattern of the precipitate from the control in the presence of 2-ME (Figure 4.2B), it is confirmed that many different proteins in DEY were involved in the formation of the TBS-SDS buffer-insoluble disulfide- induced protein aggregates during storage. One typical example is apovitellenin-I (9 kDa, primary accession number P02659), which is one of the low-density lipoproteins (LDL) in hen egg yolk and has a disulfide-linked homodimer (Jolivet

et al., 2006). In the absence of 2-ME, for different storage samples (Figure 4.2A), the color intensity of the band containing the homodimer of apovitellenin- I (Band I) was much greater than that of the band containing its monomer (Band II). However, when its intermolecular disulfide bond was destroyed by 2-ME, the color intensity of Band II (Figure 4.2B) increases dramatically.

As discussed in Chapter 2, when the moisture content of DEY is greater than its m_o, the amino groups can react with glucose to produce the Maillard cross-linked products during storage, which are more likely to contribute to both buffer-soluble and –insoluble protein aggreagtes. Besides disulfide bond interaction and the Maillard reaction, lipid oxidation can also affect the storage stability of DEY due to its high unsaturated fat content (38%, g/g). Two types of lipid oxidation reactions, namely, radical reactions and reactions involving the secondary products, can cause both rancidity and free radical attack on proteins and amino acids (Gardner, 1979). This protein damage caused by oxidized lipids includes (1) formation of various fluorescent chromophores and aggregates (lipid−protein adducts and protein−protein cross-links), (2) protein scission, and (3) amino acid damage (Kikugawa & Beppu, 1987). Additionally, both lipid oxidation and the Maillard reaction pathways (1) can produce common intermediate products such as carbonyl compounds, which results in increased browning, and (2) may have common polymerization mechanisms that lead to hardening (Zamora & Hidalgo, 2005). In summary, direct chemical linkages induced by disulfide bond formation, the Maillard reaction, and lipid oxidation are the primary reasons leading to the formation of buffer-soluble and –insoluble protein aggregates. Due to the co-action of these three

chemical reactions and non-covalent interactions such as hydrophobic interactions, different types and amounts of aggregates (soluble and insoluble) formed in DEY powder under different storage conditions.

Protein aggregates as accessed by tryptophan front-face fluorescence

Tryptophan, a common amino acid present in food proteins, has been well studied for its fluorescence characteristics. Therefore, it is a good probe to study protein structure changes, as tryptophan exhibits different emission fluorescence spectra when present in different environments (Dufour, Genot, & Haertlé, 1994)(Bonomi, Mora, Pagani, & Iametti, 2004)(Kulmyrzaev, Karoui, De Baerdemaeker, & Dufour, 2007)(Huschka, Bonomi, Marengo, Miriani, & Seetharaman, 2012). For example, the tryptophan in a hydrophobic environment has a different emission peak from that in a hydrophilic environment. Besides protein structure, tryptophan is also a good probe for the detection of protein aggregates (Kulmyrzaev, Levieux, & Dufour, 2005).

As noted in "Nutritional value of egg yolk powder" in the literature review, DEY (100 g) contains 0.51 g tryptophan (Table 2.4), which is enough for the front-face fluorescence analysis. Results are shown in Figure 4.4. The spectra was similar to those in published articles (Karoui et al., 2005)(Karoui et al., 2007). The peak shown in each spectrum was tryptophan. The decreases of peak intensity and minor shift of emission peak during storage indicated the formation of protein aggregates and changes of protein structure.

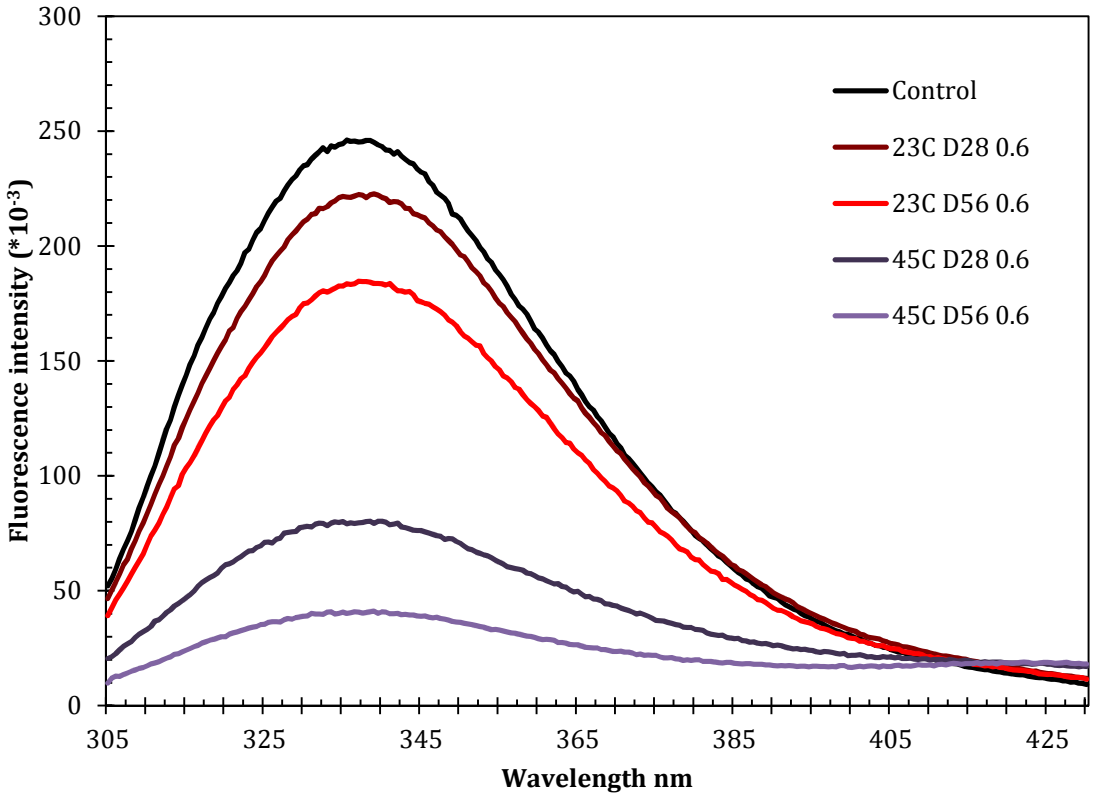

Figure 4.4: Front-face fluorescence spectra of tryptophan from DEY during storage at a_w of 0.66. The D values are days in storage at the given temperature and water activity (0.6 = 0.66).

The decrease of peak intensity suggested less natural tryptophan had been detected with increased storage time and temperature, which indicated the detection available tryptophan had undergone several changes, most probably involved in aggregation, in which protein-protein and/or –other compounds interactions buried tryptophan inside the interior core of the newly formed protein aggregates. As seen at the longest storage time (58 days) and storage at 0.66 a_w at 45°C, 86% of the tryptophan has disappeared (presumably due to aggregates), while at 23°C, after 58 days storage, there was only a loss of 26%. Since the formation of protein aggregates had been confirmed in

the SDS-PAGE study, the decrease of tryptophan fluorescence intensity was correlated with the formation of protein aggregates. A slight shift of emission peak was also observed: the emission peaks of DEY storage at 45°C were slightly left shifted compared with that of DEY storage at 23°C. It indicated more hydrophobic patches were exposed to the outside during the formation of protein aggregates, which has been reported by Andersen et al. (2008). The peak shift towards the lower wavelengths suggests that the tryptophan is exposed to a more hydrophobic environment and vice versa (Andersen & Mortensen, 2008).

The PCA result (Figure 4.5) showed a trend of moving toward the left part with increased storage time and temperature. The control sample and DEY storage at 23°C were not well differentiated from each other, while the DEY storage at 45°C was significantly different on the coordinate system. Therefore, the tryptophan probe may be a less powerful tool to identify and predict quality changes of DEY during storage compared to the Maillard reaction and lipid oxidation products probes. A similar conclusion has been reported in another study, in which the spectra of the Maillard reaction products were more correlated with the freshness of shell eggs (Karoui et al., 2006).

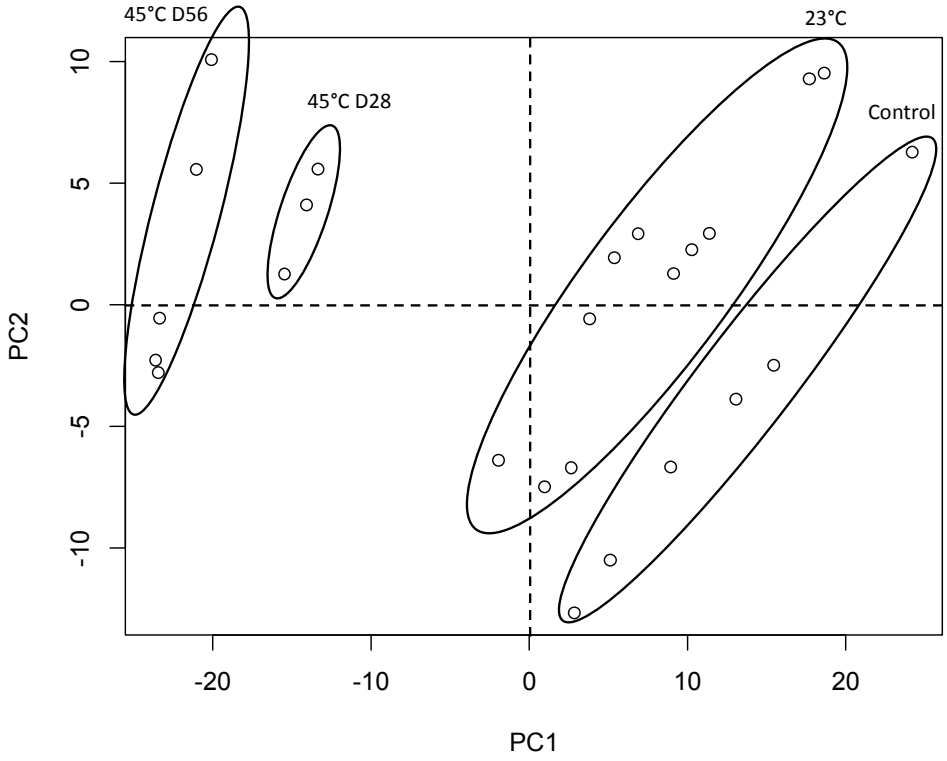

Figure 4.5: Principle component analysis result of the tryptophan spectra of DEY during storage at a_w of 0.66 after removal of outliers. Control: the original DEY vacuum packaged storage at -20°C; 23C: the DEY storage at 23°C for 28 days and 56 days; 45°C D28: DEY storage at 45°C for 28 days; 45°C D56: DEY storage at 45°C for 56 days.

Molecular mechanism of protein aggregates formation during DEY storage

Thermo analysis of DEY during storage

The DSC thermograms of the step 2 scan of liquid egg yolk (LEY), original DEY, and DEY storage after 8 weeks are shown in Figure 4.6. The endothermic peak around 80°C of LEY and original DEY was recognized as the denaturation peak, which disappeared in the step 5 scan (data not shown), indicating the step 2 scan had denatured all native proteins irreversibly. The T_d of LEY was 85.5°C, which is consistent with other

research (Rossi & Schiraldi, 1992). T_d of DEY was 83.5°C. The decline was due to the difference in moisture content. Hydrated DEY contained more moisture leading to a lower T_d. Cotterill et al. (1983) measured T_d of several egg yolk proteins using SDS-PAGE, and found the T_d range is from 62 to 84°C with livetins as the most heat sensitive proteins and phosvitin as the most heat stable proteins (Woodward & Cotterill, 1983). Gandemer et al. also found that granules proteins were more heat stable than plasma proteins (Denmat et al., 1999) due to their compact structure leading to an efficient protection against thermal treatment (refer to "Proteins in egg"; Anton, 2013). They also found that the denaturation of yolk proteins resulted in a decrease of emulsifying activity (Denmat et al., 1999). Furthermore, due to the high oil content, egg proteins are more likely to expose more hydrophobic patches to have more flexibility (W. Wang et al., 2010).

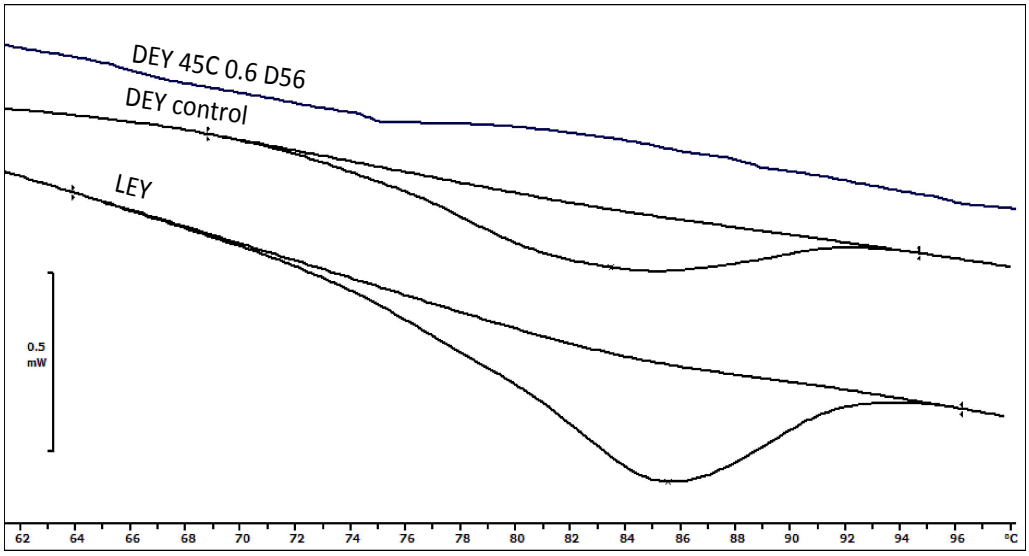

Figure 4.6: DSC thermograms of liquid egg yolk (LEY), dried egg yolk (DEY), and DEY storage for 8 weeks at 45°C at an a_w of 0.66.

The change of enthalpy (ΔH) of LEY (10.9 Jg^{-1}) is larger than that of the original DEY (4.2 Jg^{-1}), demonstrating more proteins present in their native state in LEY because the drying process disrupts intact proteins through removing their hydration layer (Mukherjee, Chowdhury, & Gai, 2009). No peak was detected for DEY stored after 8 weeks at 45°C and a_w of 0.66, indicating that all native proteins in the original DEY had been denatured and changed their conformation during storage. Denaturation is one of many ways that can result in protein aggregation (Figure 4.7). Unfolded intermediates and unfolded states interact with each other to form aggregates whereas unfolded intermediates are precursors of the aggregation process (W. Wang et al., 2010). Therefore, the high temperature and humidity storage condition induced protein denaturation as a main cause of protein aggregation of DEY during storage. So the control of storage temperature and humidity is critical in reducing/inhibiting protein

quality deterioration in DEY and other high protein powders (Qinchun Rao & Labuza, 2012; Zhou & Labuza, 2007).

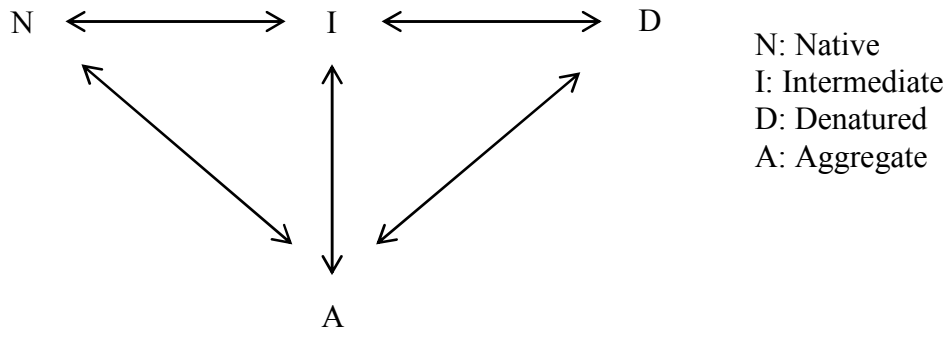

Figure 4.7: Protein aggregations pathways. (Reproduced from Wang et al. (2010))

Secondary structure changes of DEY during storage

To further understand the mechanism of the formation of protein aggregation of DEY during storage, Raman spectrometry, FTIR, and Front-fact fluorescence were used to investigate the changes of secondary structures, namely disulfide bond, protein secondary structure, protein-protein and protein-lipid interaction (Karoui et al., 2005; Li-Chan, Nakai, & Hirotsuka, 1994; Li-chan, 1996; Strasburg & Ludescher, 1995).

Raman spectrometry was used to investigate the changes of disulfide bond, protein secondary structure, and protein-lipid interaction. To normalize the peak, relative peak intensity value (RPIV) was calculated using the equation below

$$RPIV\ (A) = {Peak\ Intensity\ (A)} \Big/ {Peak\ intensity\ of\ phenylalanine}$$

From Figure 4.8, we can see that no peak was observed between 500 and 560 cm^{-1} for LEY and DEY. But it does not mean the disulfide bonds are not present in both samples. It is probably because the disulfide bond peak has been blocked by other compounds with similar Raman detection regions. However the disulfide bond peak was observed in the DEY samples stored for 8 weeks showing a high intensity, indicating that disulfide bond involvement occurred in the protein aggregation of DEY. The β sheet peak was also blocked by other compounds: possibly unsaturated fatty acids, because the peak of –C=C- is around 1665 cm^{-1}. Therefore, from this point of view, it is very hard to illustrate the changes of the protein secondary structure.

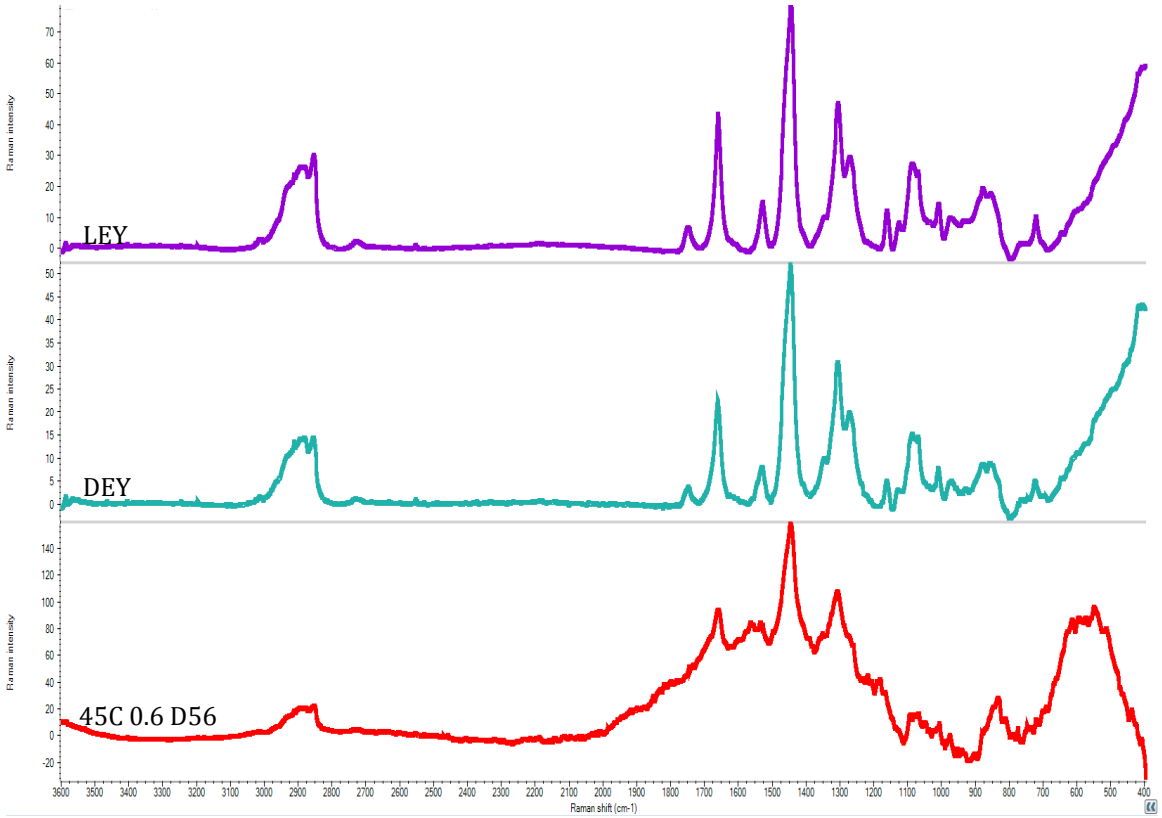

Figure 4.8: Raman spectra of liquid egg yolk (LEY) and dried egg yolk (DEY) as received and after storage at 45°C and 0.66 a_w for 8 weeks.

Table 4.2: Relative intensity values (PRIVs) at selected regions of the Raman spectra of liquid egg yolk, spray dried egg yolk, and spray dried egg yolk storage at a_w of 0.66 after 8 weeks[a].

	-S-S- stretch (wavenumber ±2 cm^{-1})			Lipid-protein interaction (wavenumber ±2 cm^{-1})
	510[b]	545[b]	total	2800-3000[c]
LEY	ND[d]	ND[d]	N/A	221.3
DEY-D0	ND[d]	ND[d]	N/A	231.5
DEY-D56	7.5	8.7	16.2	202.2

[a] Results are expressed using the phenylalanine peak (1006 cm^{-1}) as the internal standard.
[b] PRIVs were calculated using peak height
[c] RPIVs were calculated using peak area
[d] NC: not detected

The lipid-protein interaction peaks are observed between 2800 and 3000 cm^{-1}. The decreasing RPIV of peaks in that area indicated an increase of lipid-protein interaction. DEY after storage at a_w of 0.66 at 45°C had the lowest peak area, indicating the involvement of lipid-protein interactions during storage. The LEY having lower peak area than DEY-D0 may be due to the different moisture contents between the two samples (Carey, 1998). The correlation between lipid-protein interaction and formation of protein aggregates again confirmed the involvement of lipid oxidation in formation of protein aggregates through the pathway of lipid-protein polymerization.

Front-face fluorescence of Vitamin A was used to investigate lipid-protein interaction. The decrease of the Vitamin A peak intensity was detected with increasing storage time and temperature (Figure 4.9), suggesting the amount of detection available Vitamin A was decreasing. Since Vitamin A is only soluble in fat, the decline of available Vitamin A is probably due to: 1) The increase of lipid-protein interaction; 2) Oxidation of Vitamin A. Considering the occurrence of both lipid oxidation and lipid-protein interaction have been confirmed (refer to "The progress of lipid oxidation", "Peroxide value", "Secondary structure changes of DEY during storage"), both reasons are probably contributing to the decline of the Vitamin A peak.

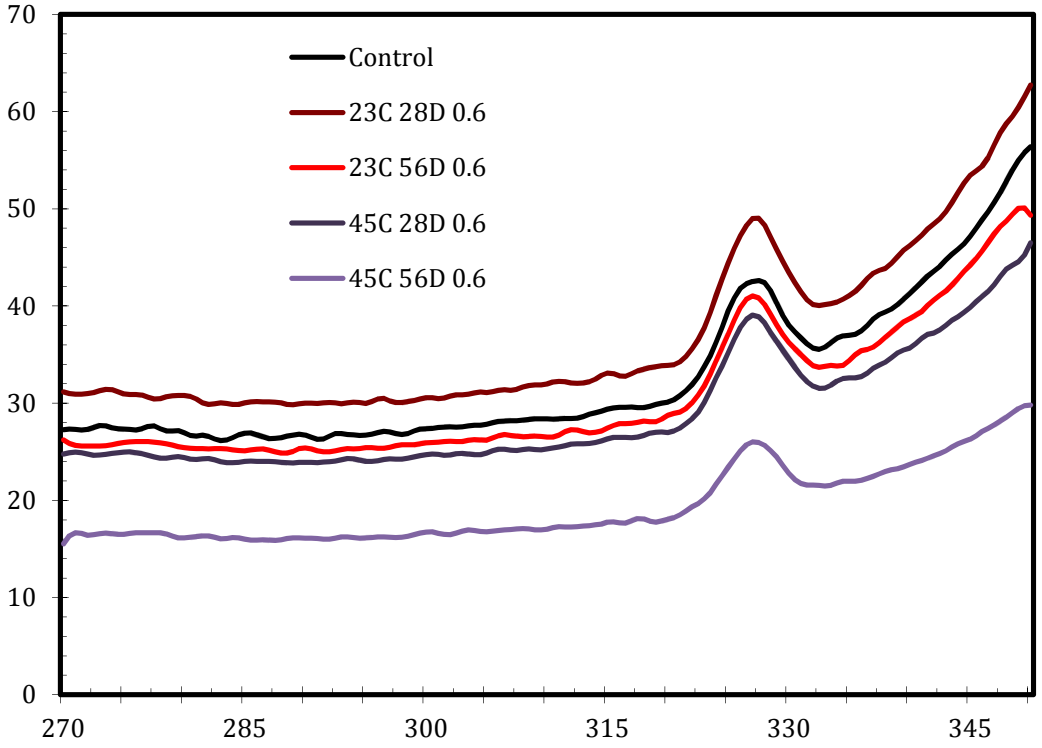

Figure 4.9: Front-face fluorescence spectra of Vitamin A from DEY during storage at a_w of 0.66.

The PCA results showed a trend of decreasing value of PC1 with increased storage temperature and time (Figure 4.10). But similar to the PCA of tryptophan, the control sample and sample storage at 23°C cannot be differentiated from each other. Therefore, it may not be a good tool for identification and prediction of the quality of DEY during storage.

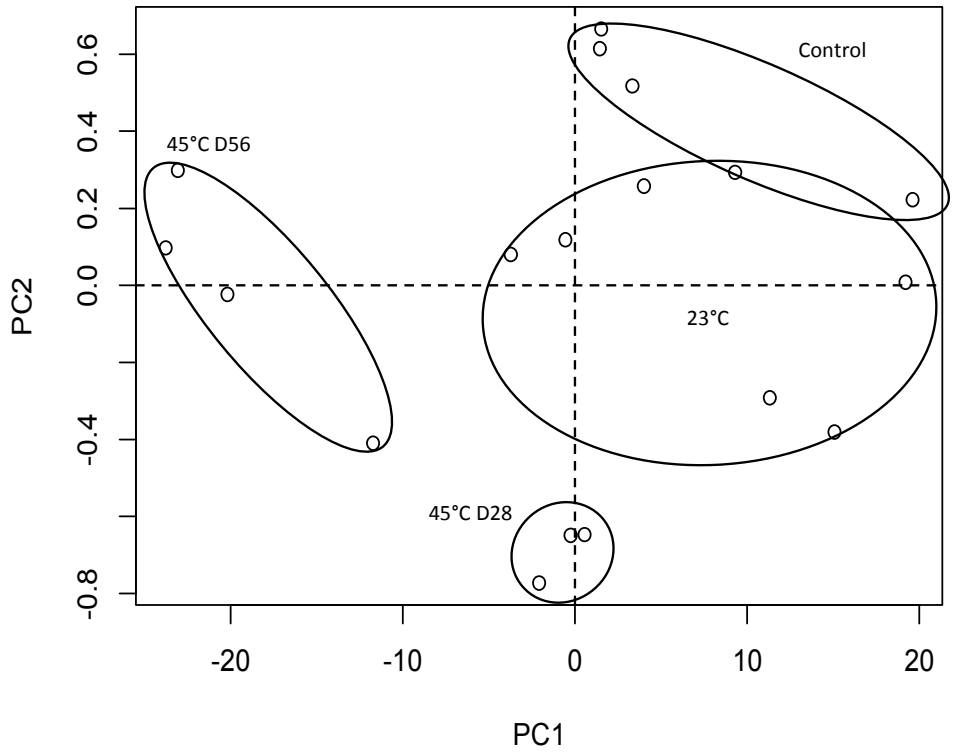

Figure 4.10: Principle component analysis result of the Vitamin A spectra of DEY during storage at a_w of 0.66 after removal of outliers. Control: the original DEY vacuum packaged storage at -20°C; 23C: the DEY storage at 23°C for 28 days and 56 days; 45°C D28: DEY storage at 45°C for 28 days; 45°C D56: DEY storage at 45°C for 56 days.

FTIR is a technique to obtain an infrared spectrum of absorption. It has a broad application including characterization of protein secondary structure (Arrondo, Muga, Castresana, & Goñi, 1993; Byler & Susi, 1986; Michael Jackson, Haris, & Chapman, 1989; Michael Jackson & Mantsch, 1991; Surewicz, Mantsch, & Chapman, 1993), which was first discovered by Elliot and Ambrose (Elliott & Ambrose, 1950). They showed that the FTIR spectra of the amide I and II absorptions were empirically correlated with the predominant secondary structure. For example, a predominantly α-helix structure would

show amide I and II absorptions in the frequency range of 1652 to 1657 and 1545 to 1551 cm^{-1}, respectively (Ambrose & Elliott, 1951; Elliott & Ambrose, 1950). The contribution of amide I adsorption is mostly from the C=O stretching vibration with a minor C-N stretching vibration, whereas, the contribution of amide II adsorption is from both the N-H bending and C-N stretching vibrations (Miyazawa, Shimanouchi, & Mizushima, 1956). Therefore, amide I adsorption has proven to be a better measurement of protein secondary structure than amide II adsorption because it only measures one functional group. The secondary structures can also be quantitatively estimated using curve fitting of the amide I absorption, which is to assign the absorption frequency region to each secondary structure and then calculate the area on a secondary derivative curve.

Figure 4.11 shows the result of the full spectra of DEY during storage for 28 and 56 days storage at 23 and 45°C and the frozen control using FTIR spectroscopy. They looked very similar to each other. The two peaks between 2550 to 3050 cm^{-1} refer to the fat, which does not show any significant difference on the peak height among all samples. The absorption region between 1600 to 1700 cm^{-1} is the amide I absorption, which is used for the estimation of protein secondary structure.

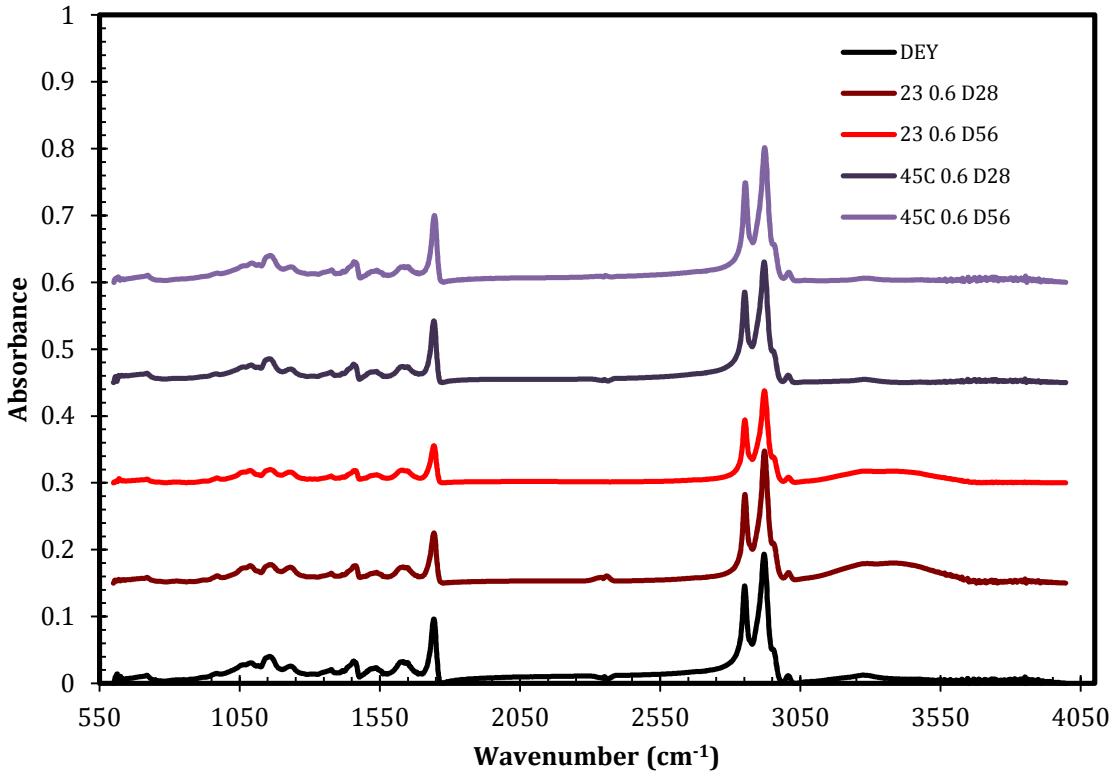

Figure 4.11: FTIR spectra of DEY during storage at a_w of 0.66 at different temperatures.

Figure 4.12 shows the second derivative curve of the control sample with regions labeled for each type of secondary structure. From Figure 4.13, we can see that the second derivative curve shows a similar trend but with intensity differences of several peaks, suggesting a slight change of the distribution of the secondary structure. However, no significant difference of distribution of protein secondary structure was found among the samples stored at different conditions (Table 4.3). But it has a slight trend of the decreasing of α-helix and β-sheet with the increasing of random coil.

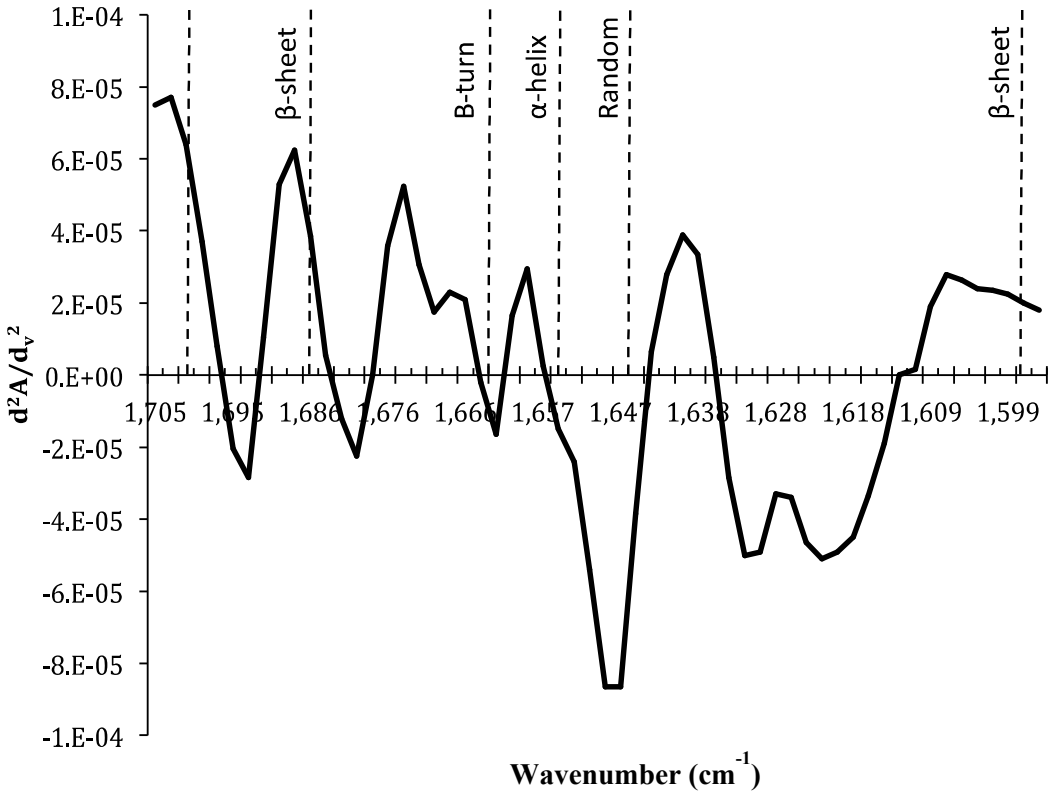

Figure 4.12: Second derivative of the FTIR spectra of the control DEY sample in the amide I region with regions labeled with each secondary structure for the secondary structure estimation.

Meanwhile, the estimation of protein secondary structure using FTIR has been argued about its accuracy since the amide I absorption can be interfered with many factors, such as amino acid side chains (M Jackson & Mantsch, 1995). The frequency region assigned for each secondary structure is also debatable and should be unique for each type of protein/peptides due to their different composition of amino acids and structure. However, most papers set their region according to papers investigating totally different proteins. To this study particularly, the secondary structure region may need to

be set different for each sample since with the formation of protein aggregates, the region for each secondary structure may change as well. Therefore, the information of estimation protein secondary structure changes may not be correct. To get more accurate information, many standards need to be applied, which is not feasible, unfortunately for this study.

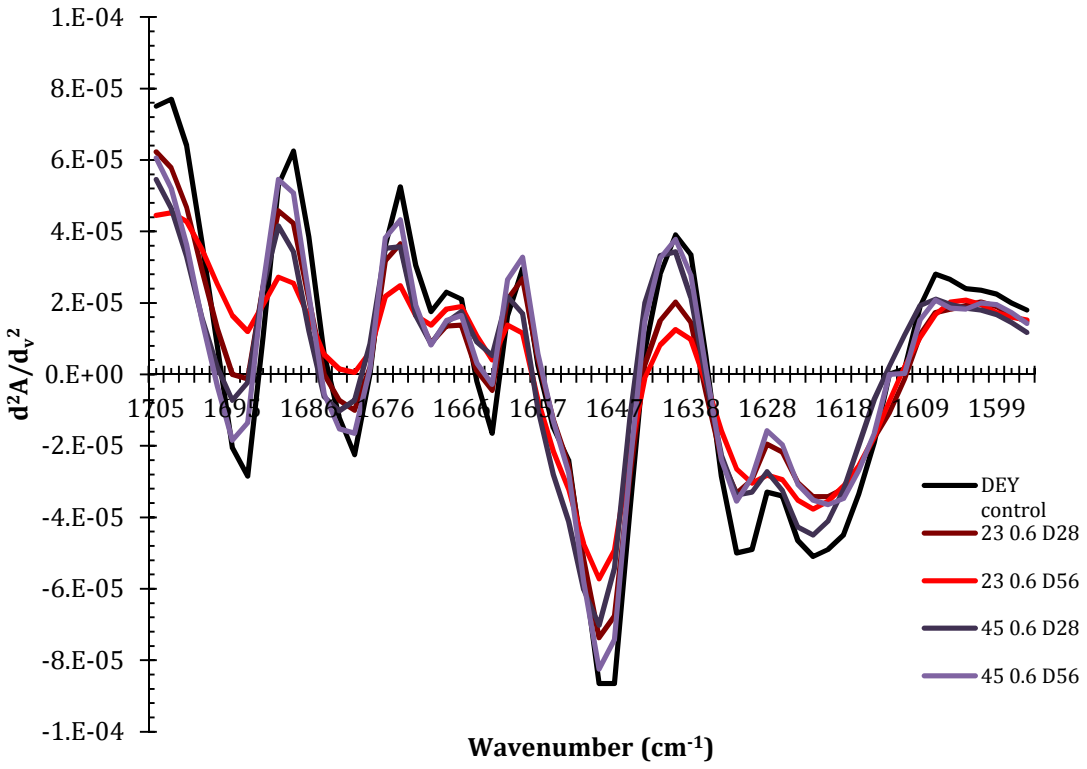

Figure 4.13: Second derivative of FTIR spectra of DEY during storage at a_w of 0.66 at different conditions

Table 4.3: Estimation of protein secondary structure content of DEY during storage

	β-sheet (%)	random (%)	α-helix (%)	β-turn (%)
Control	71.8±2.9	20.6±5.7	2.3±2.3	5.2±0.5
23C D28	68.2±2.6	26.3±5.4	2.0±2.0	3.4±4.1
23C D56	70.3±2.2	27.9±2.2	1.5±0.4	0.4±0.7
45C D28	63.9±2.2	31.0±3.1	1.5±0.8	3.6±2.4
45C D56	65.4±1.2	27.5±6	1.9±1.4	5.2±4.7

HMWPAs formation during storage

Using 1D SDS-PAGE and LC-MS/MS, 33 proteins (including predicted proteins) were identified (Table 4.4), which were involved with HMWPAs formation according to NCBI gallus gallus database. Among them were several typical egg yolk proteins: vitellogenin, apovitellenin, and livetins and egg white proteins: ovotansferrin, ovalbumin, lysozyme, ovomucoid, and ovastatin. Most of the identified proteins contain disulfide bonds. For example, serum albumin (70 kDa), also known as α-livetin, contains approximately 17 disulfide bonds. Another examples is a 12 kDa lipoprotein, apovitellenin-1, which has a subunit of homodimer linked with disulfide bonds. Therefore unfolding induced intra and intermolecular disulfide bond formation/exchange is one pathway to result in the formation of HMWPAs. In addition, many proteins have good binding capacity for metal, fatty acids, and other compounds, indicating lipid oxidation and the Maillard reaction induced direct chemical linkages would be another pathway for the formation of HMWPAs. The similar mechanism of formation of egg white protein aggregates have been found in our lab's previous study (Qinchun Rao &

Labuza, 2012; Qinchun Rao, Rocca-smith, et al., 2012; Qinchun Rao, Rocca-Smith, et al., 2013, 2012).

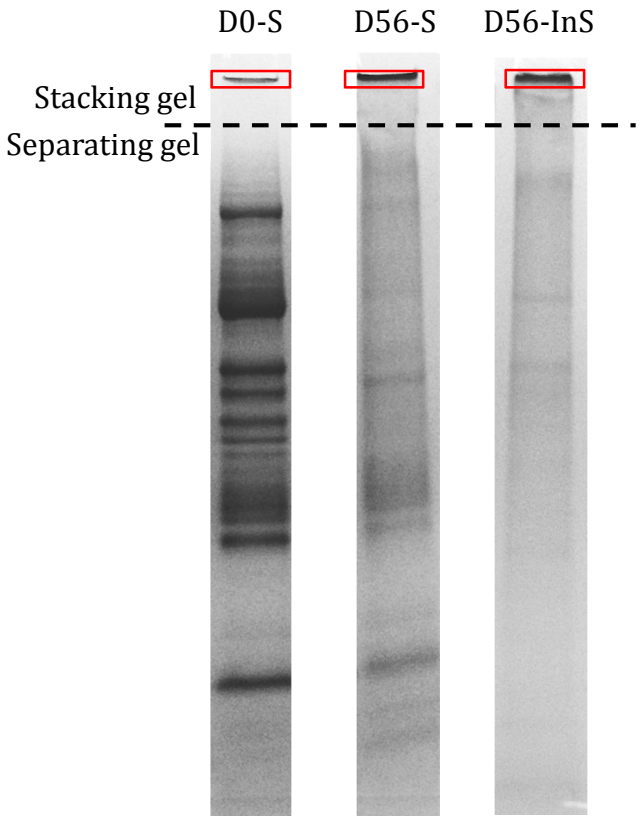

Figure 4.14: SDS-PAGE of D0-S: soluble proteins/protein aggregates in original DEY; D56-S: soluble proteins/protein aggregates in DEY storage 8 weeks at 45°C and 0.66 a_w; D56-InS: insoluble proteins/protein aggregates in DEY storage 8 weeks at 45°C and 0.66 a_w. The HMWPAs band for LC-MS/MS analysis was circled using red rectangles.

HMWPAs are already present in the original DEY due to the spray drying process. During the drying process, proteins suffer from two stresses: 1) elevated temperature, 2) exposure to increased air-water interface because of the reduction to small droplets in the range of 100 to 300 mm. This results in protein destabilization and aggregation (W. Wang et al., 2010). The effect of storage did not induce much HMWPAs formed from different proteins. It caused the same proteins to form more HMWPAs because the

HMWPAs band intensity is much higher for DEY storage after 8 weeks than the original DEY (Figure 4.14).

A rough calculation can be made to obtain an approximate quantification comparison of the same protein in different samples using apolipoprotein B (apo B) as the internal standard. Apo B can be used as the internal standard due to its very large molecular weight (523 kDa). No matter it formed aggregates or not, it would be left on the top of the stacking gel. Since the same amount of DEY was put at the beginning of sample preparation, each sample should contain the same amount of apo B. So the relative protein quantity value (RPQV) of each protein can be calculated using equation below within the same sample (Table 4.5).

$$RPQV(A) = {exclusive\ peptide\ count\ (A)} \Big/ {exclusive\ peptide\ count\ (Apo\ B)}$$

Then we can compare the ratio between samples. As we can see in Table 4.5, if we set the significant difference range to more than two times different, 19 out of 32 proteins were found significantly increasing involvement in the formation of soluble HMWPAs.

Table 4.4: List of proteins involved in the formation of soluble and insoluble HMWPs in original DEY and DEY storage after 8 weeks.

Accession[a]	Protein	MW (kDa)	Exclusive unique peptide count		
			D0-S[b]	D56-S[b]	D56-InS[b]
71896765	vitellogenin-2 precursor	205	165	74	296
113206052	apolipoprotein B precursor	523	444	35	265
52138705	vitellogenin-1 precursor	211	90	11	166
45383974	serum albumin precursor	70	69	36	44

45385813	ovotransferrin precursor	78	51	68	35
129293	ovalbumin	43	47	11	28
45382303	complement C3 precursor	184	8	28	0
513788266	Ig lambda chain V-1 region precursor	24	9	2	0
513225677	PREDICTED: alpha-2-macroglobulin-like protein 1-like	164	4	18	0
45382733	apovitellenin-1 precursor	12	11	0	4
71897377	ovalbumin-related protein Y	44	9	5	2
126608	Lysozyme C precursor	16	3	2	5
485049501	keratin, type I cytoskeletal 19	46	2	1	2
46048814	protein TENP	47	7	1	3
47575885	keratin, type II cytoskeletal 5	62	2	3	2
363729530	PREDICTED: hemopexin	43	5	3	0
363734560	PREDICTED: mucin-5B isoform X1	234	0	1	4
162952006	ovomucoid precursor	22	4	0	2
513226216	PREDICTED: keratin, type I cytoskeletal 12 isoform X2	57	2	3	2
363746526	PREDICTED: uncharacterized protein LOC100858082	25	3	1	1
45382565	ovostatin precursor	164	0	5	0
448824824	ovalbumin-related protein X	44	4	0	1
513206786	PREDICTED: ovoinhibitor	57	5	0	1
513165204	PREDICTED: von Willebrand factor A domain-containing protein 8-like	214	1	2	0
46395491	PIT54 protein precursor	51	3	0	1
118082412	PREDICTED: cathepsin E-A-like	45	0	3	0
118088308	PREDICTED: plasminogen	91	2	2	0
513161014	PREDICTED: alpha-2-macroglobulin-like 1 isoform X3	147	0	1	2
513240096	PREDICTED: uncharacterized protein LOC101750354	32	2	0	1
363736454	PREDICTED: complement factor H	148	2	1	0
487439524	beta-2-glycoprotein 1 precursor	39	2	1	0
50752381	PREDICTED: alpha-2-HS-glycoprotein	37	0	2	0
363729287	PREDICTED: complement C4-A isoform X2	190	0	2	0

[a]: gi number from NCBI database of matched protein

[b]: D0-S: soluble HMWPs in original DEY; D56-S: soluble HMWPs in DEY storage 8 weeks; D56-InS: insoluble HMWPs in DEY storage 8 weeks.

Table 4.5:Relative protein quantity value (RPQV) of proteins involved in formation of soluble and insoluble HMWPs in original DEY and DEY storage after 8 weeks.

Accession[a]	Protein	RPQV		
		D0-S[b]	D56-S[b]	Ratio
71896765	vitellogenin-2 precursor	0.38	2.61	6.80
113206052	apolipoprotein B precursor	1.00	1.00	1.00
52138705	vitellogenin-1 precursor	0.21	0.37	1.72
45383974	serum albumin precursor	0.18	1.34	7.27
45385813	ovotransferrin precursor	0.12	2.37	20.23
129293	ovalbumin	0.11	0.32	2.87
45382303	complement C3 precursor	0.02	0.73	44.41
513788266	Ig lambda chain V-1 region precursor	0.02	0.07	3.42
513225677	PREDICTED: alpha-2-macroglobulin-like protein 1-like	0.01	0.59	59.22
45382733	apovitellenin-1 precursor	0.02	0.00	N/A
71897377	ovalbumin-related protein Y	0.02	0.15	8.08
126608	Lysozyme C precursor	0.01	0.07	11.10
485049501	keratin, type I cytoskeletal 19	0.00	0.05	14.80
46048814	protein TENP	0.01	0.00	N/A
47575885	keratin, type II cytoskeletal 5	0.00	0.10	19.74
363729530	PREDICTED: hemopexin	0.01	0.07	7.40
363734560	PREDICTED: mucin-5B isoform X1	0.00	0.00	N/A
162952006	ovomucoid precursor	0.01	0.00	N/A
513226216	PREDICTED: keratin, type I cytoskeletal 12 isoform X2	0.00	0.07	22.21
363746526	PREDICTED: uncharacterized protein LOC100858082	0.01	0.00	N/A
45382565	ovostatin precursor	0.00	0.15	∞
448824824	ovalbumin-related protein X	0.01	0.00	N/A
513206786	PREDICTED: ovoinhibitor	0.01	0.00	N/A
513165204	PREDICTED: von Willebrand factor A domain-containing protein 8-like	0.00	0.05	∞
46395491	PIT54 protein precursor	0.00	0.00	N/A
118082412	PREDICTED: cathepsin E-A-like	0.00	0.07	∞
118088308	PREDICTED: plasminogen	0.00	0.05	14.80
513161014	PREDICTED: alpha-2-macroglobulin-like 1 isoform X3	0.00	0.00	N/A
513240096	PREDICTED: uncharacterized protein	0.00	0.00	N/A

	LOC101750354			
363736454	PREDICTED: complement factor H	0.00	0.00	N/A
487439524	beta-2-glycoprotein 1 precursor	0.00	0.00	N/A
50752381	PREDICTED: alpha-2-HS-glycoprotein	0.00	0.07	∞
363729287	PREDICTED: complement C4-A isoform X2	0.00	0.05	∞

[a]: gi number from NCBI database of matched protein

[b]: D0-S: soluble HMWPs in original DEY; D56-S: soluble HMWPs in DEY storage 8 weeks; D56-InS: insoluble HMWPs in DEY storage 8 weeks.

The insoluble HMWPAs mostly come from the same group of proteins, indicating that the initial HMWPAs are soluble but gradually become insoluble during storage as they "exceed certain size and solubility limits" (W. Wang et al., 2010). Moreover, the formation of insoluble HMWPAs is not due to disulfide bond interactions but through other direct chemical linkages because when 2-mercaptoethanol was added during the sample preparation, it disrupted all disulfide bonds.

To further differentiate all the proteins, they are divided into four groups: 1) only in original DEY; 2) not in soluble DEY after storage for 8 weeks at 45°C and 0.66 a_w; 3) not in original DEY, 4) in all of them. Group 1 includes ovalbumin-related protein X, PIT54 protein and other 2 predicted proteins. The reason why those proteins did not show up in storage samples may be bacause 1) the proteins degraded during storage, and/or 2) the amounts of those proteins were under the detection limit in the storage sample. Apovitellenin-1 and ovomucoid are in group 2. It indicates that both proteins ended up with insoluble HMWPAs and not left as soluble HMWPAs. Group 3 includes ovastatin and five predicted proteins. Ovastatin is a large protein with a MW of 164 kDa and its subunit is linked through a disulfide bond. It passed through the spray drying process

without forming HMWPAs, indicating that the unfolding induced direct chemical linkages may not be the main reason. The main reason would be direct chemical linkages induced aggregates due to Maillard reaction and Lipid oxidation.

Discussion

The mechanism of protein aggregate formation of DEY proteins during storage is dependent on two extrinsic factors, mainly storage temperature and humidity. High temperature and humidity will lead to the occurrence of several chemical reactions resulting in protein aggregates. The spray drying process could cause protein aggregation to a different degree due to its high temperature. During the process, proteins will be partially denatured and expose its potential binding sites (thiol group, ligand and fatty acid binding sites), which can interact with other denatured proteins to form protein aggregates. Storage at high temperature and humidity can promote further denaturation of proteins, leading to more unfolded protein interactions, which ends up with formation of protein aggregates. It also promotes lipid oxidation and the Maillard reaction, which provides non-disulfide cross-linking pathways leading to protein aggregation.

The formation of protein aggregates may have an impact on the nutrition value of DEY as it involves lipid and protein interactions. Lipid oxidation can cause damage to the unsaturated fatty acid and can produce multiple substances that may have adverse effects on human health (Frankel, 2005). The formation of protein aggregates may have adverse effect on the digestion in the gastrointestinal tract, which may block the bioactivity of certain proteins, such as IgY.

The formation of protein aggregates may also have adverse effects on the functionality of DEY as well. As stated in the "Functionality of egg yolk powder" part in the literature review, egg yolk has the abilities to form emulsion and gels. The functionality of DEY is closely related with its protein structure. Therefore, the changes of protein structure resulting from the formation of protein aggregates probably alters the emulsion and gelation ability, but was measured in this study. Research has shown that the denaturation of proteins during spray-drying process may improve the emulsion ability of DEY (Caboni et al., 2005), which is due to the partially unfolded protein exposing several hydrophobic patches outside, leading to a better balance of hydrophobic/hydrophilic ratio, which can help the stabilization of the fat in water. However, the damage of protein from the spray-dried process is minor compared to that caused during the storage. With completely denatured proteins and rearrangement of protein structure, the decline of functionalities of DEY is possible. But since the functionality changes of DEY during storage has not been measured in the study, it is hard to make any assumption on that.

Besides the nutritional value and functionality, lipid oxidation also produces off-flavor and rancidity, which is definitely not welcomed for any DEY made products. Therefore, effective approaches need to be applied to prevent the deterioration of DEY during storage. The main stress to protein during spray drying is the exposure to the air-water interface; therefore adding surfactants is an effective approach avoiding protein aggregation. Controlling the storage condition of DEY is very important as stated in Chapter 2. According to the food stability map (T. Labuza et al., 1970), the Maillard

reaction will not occur at very low a_w (usually below 0.3) due to the lack of water as the reaction medium, however, it did occur in DEY system due to the high liquid fat content at 23°C. But the Maillard reaction rate at low water activity is much lower than the high water activity. The relationship of lipid oxidation and a_w is a U shape curve with the lowest oxidation rate at its monolayer moisture value (2.3 g H_2O for 100 g solids for DEY, Figure 3.1). Therefore, storing DEY at its monolayer moisture value can reduce both Maillard reaction and lipid oxidation. If reducing both reactions is not enough for protecting deterioration of DEY, completely remove glucose and better packaging techniques such as vacuum package can be applied to obtain a longer shelf life as long as the water of the film is also low so as to not allow water vapor transmission into the package.

Conclusion

The mechanism of formation of protein aggregation of dried egg yolk (DEY) at different temperature and a_w was investigated in this paper and effective approaches to avoid DEY deterioration during storage were discussed. Overall, the denatured proteins are precursors of the aggregation process. Unfolded intermediates and states interact with each other through disulfide bonds or other chemical linkages to form protein aggregates. With further denaturation of proteins and involvement of chemical reactions, lipid oxidation and the Maillard reaction, more disulfide bonds and direct chemical linkages are involved to build a protein-protein cross linking network, leading to the formation of high molecular weight protein aggregates (HMWPAs). Therefore the best way to avoid the formation of protein aggregation is to maintain proteins in their native states and

inhibit potential chemical reactions by control of storage condition, i.e., humidity and temperature.

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Thesis Conclusion:

From the storage study of a commercial spray-dried egg yolk powder (DEY), we showed results that give evidence negating our central null hypothesis as we showed physicochemical changes and the formation of protein aggregates of DEY during storage were closely related with storage environment (a_w and temperature).

The DEY samples stored at 45°C showed significant changes in physical and chemical properties such as protein aggregation. The color change (a decrease of L^* value and an increase of ΔE^* value) was mostly due to the Maillard reaction and lipid oxidation as the products of both reactions have been found in all DEY samples stored at 45°C (at a_w of 0.05, 0.10, 0.37, 0.44, 0.54, 0. 66) using multiple techniques and their quantities increased with increasing storage time. Both soluble and insoluble protein aggregates were also found. The protein compositions of both soluble and insoluble high molecular weight protein aggregates were similar, including major egg yolk and white proteins, indicating that certain proteins are more suspectable than others to form aggregates when placed in an abused environment, probably due to their intrinsic structures.

The molecular mechanism of the formation of protein aggregates is the result of three mechanisms. The first is through the unfolding of intermediates and unfolded states; The second is through direct chemical linkages, i.e., disulfide bonding, while the third one is through self-association. The first two are the major ones as we have found protein denaturation during storage, and again the occurrence of the Maillard reaction, lipid oxidation and disulfide bond interactions, while the third one is a minor one. Therefore, the effective approach to limit the formation of protein aggregates is to control the

temperature during manufacturing and storage (to avoid protein denaturation), as well as to reduce/inhibit the Maillard reaction, lipid oxidation, and disulfide bond interaction through adjusting environmental humidity.

The DEY samples stored at room temperature were very stable in terms of physicochemical changes and the formation of protein aggregation even at an a_w of 0.66. The color had almost no decline in the brightness (L^* value) and there were no visible soluble or insoluble aggregates on a SDS-PAGE gel. Therefore, as long as DEY is stored at a temperature below 25°C and at a_w of 0.05 to 0.66, the powder should have at least a year long shelf life with minimal and definitely acceptable quality changes. We have not studied the storage stability of DEY with an a_w higher than 0.66, because DEY gets moldy easily, which confounds the results. We also did not add anti-microbial chemicals, such as sodium azide, into our samples, because we did not want to introduce any interference. However from other studies, we do see dramatic changes when the a_w is higher than 0.66 as shown on the food stability map. However, high a_w (above 0.80) may not be practical for a dehydrated products to achieve desired shelf life. Therefore, it may not be a concern for the food industry. For example, the commercial egg yolk powder that we received for our project has a moisture content of 2.3% of moisture (db) and was contained in an opaque plastic package.

Even though little changes were found for color and protein aggregation of DEY storage at room temperature, the occurrence of both the Maillard reaction and lipid oxidation were confirmed with detection of decreased glucose content, increased peroxide value, as well as increased other Maillard reaction and lipid oxidation products.

It must be noted that some of the reaction products are generated during the manufacturing process, which include liquid pasteurization followed by spray drying. The progress of both the Maillard reaction and lipid oxidation is mild and does not result in significant changes in terms of physicochemical properties.

The impact of the a_w is significant. It basically corresponds with the food stability map. However due to the complexity of the sample system, with the Maillard reaction and lipid oxidation occurring at the same time, several parameters do not show significant difference as a function of a_w. This is because when evaluating the reaction rates of both reactions, the differences are not large enough to result statistical significant.

The storage temperature also has a huge impact on the storage stability of DEY. If the Q_{10} of color change is adapted, which is around two, the shelf-life of DEY storage at 45°C would only be six months assuming that the shelf-life of DEY stored at room temperature is about two years. The shelf-life decline can be bigger if the Q_{10} of protein aggregation is adapted considering the tremendous difference between DEY stored at room temperature and at 45°C in terms of formation of protein aggregates.

Therefore, both storage humidity and temperature are key factors in terms of DEY storage stability. An appropriate storage environment can help maintain the quality of DEY and probably extend the shelf life as well. Through adjusting storage environment, the Maillard reaction, lipid oxidation, and disulfide linkages can be reduced, which further decreases or eliminates the physicochemical changes and protein aggregates formation during storage.

Besides storage environment, the manufacturing process can be adjusted to obtain a better quality DEY as well through avoiding high temperature, humidity, and/or pressure treatment. However, those technologies such as freeze drying may be too expensive for the food industry to afford. While, from our storage study, the manufacturing treatment, including spray drying and homogenization, does not affect the overall quality of DEY as long as it is stored at $\leq 23°C$ and $a_w \leq 0.66$.

In conclusion, commercial spray-dried egg yolk powder is a shelf-stable product if kept in an appropriate storage condition i.e., $\leq 23°C$ and $a_w \leq 0.66$, which is not hard to maintain during distribution and storage. However, since DEY is not the final product, it is mostly applied as an ingredient to put into food products such as mayonnaises, salad dressing, baking products, and infant formula. Therefore, further studies of evaluating the storage stability of those food products are necessary, as minor physicochemical changes of DEY may result in significant quality changes of the final food products. Further studies of analyzing all the protein aggregates (instead of just the high molecular weight protein aggregates) may be necessary as well, because knowing the basic principle is important in terms of problem solving and trouble shooting in the future.

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Appendix

Appendix 1: Selected raw data of the color measurement

Table 1: L^*, a^*, and b^* values of DEY during storage at 45°C at a_w of 0.66

Day	L^*	a^*	b^*
0	88.52	-2.98	54.67
	88.33	-3.17	55.13
	88.40	-2.91	55.92
	88.42	-3.02	55.24
3	89.65	-2.26	54.20
	89.85	-2.51	55.00
	89.99	-2.38	54.44
	89.93	-2.32	54.92
7	87.79	-1.54	52.44
	87.46	-1.64	52.67
	87.00	-1.70	52.68
	87.11	-1.63	52.61
14	85.70	-0.54	50.69
	85.83	-0.37	50.57
	84.88	0.50	49.78
	84.91	0.49	50.07
28	83.16	0.94	47.85
	83.55	0.87	47.85
	83.25	0.78	47.52
	83.30	0.60	47.86
	84.06	1.53	47.54
	83.94	1.42	47.69
45	82.38	1.76	45.55
	83.42	1.60	46.48
	83.10	2.01	46.16
60	81.69	2.03	44.71
	81.88	2.01	44.48
	82.00	1.92	44.59

Table 2: L^*, a^*, and b^* values of DEY during storage at 35°C at a_w of 0.66

Day	L^*	a^*	b^*
0	88.52	-2.98	54.67
	88.33	-3.17	55.13
	88.40	-2.91	55.92
	88.42	-3.02	55.24
2	87.22	-3.59	54.19
	89.36	-3.38	55.45
	88.64	-3.42	55.58
	88.45	-3.45	55.33
	89.27	-3.56	55.66
	89.69	-3.37	55.69
6	87.55	-2.88	55.07
	88.35	-2.65	55.43
	88.62	-2.80	55.89
	88.01	-2.55	56.13
	88.82	-2.70	55.83
	88.92	-2.60	55.54
	88.89	-2.81	55.61
	88.39	-2.58	55.44
	88.62	-2.76	56.09
14	87.51	-1.97	53.80
	86.69	-1.71	54.95
	87.59	-2.05	54.68
	87.49	-1.87	55.17
28	85.80	-0.57	52.88
	85.36	-0.35	53.90
	85.34	-0.67	52.93
56	83.48	0.90	48.13
	83.19	1.37	49.52
	82.32	1.32	49.79
	82.33	1.33	49.30
90	80.71	2.77	46.65
	81.22	2.29	45.20
	80.73	2.63	46.25
123	80.84	2.74	44.22
	79.58	3.43	45.17
	80.31	3.03	44.83
	79.94	3.26	44.85
	80.88	2.79	44.28

Table 3: L^*, a^*, and b^* values of DEY during storage at room temperature at a_w of 0.66

Day	L^*	a^*	b^*
0	88.52	-2.98	54.67
	88.33	-3.17	55.13
	88.40	-2.91	55.92
	88.42	-3.02	55.24
4	88.79	-3.80	56.05
	89.47	-3.60	55.70
	89.41	-3.87	56.06
	88.77	-3.56	55.62
	89.39	-3.86	56.35
	89.15	-3.82	56.07
	89.01	-3.55	55.42
12	89.26	-3.60	56.12
	89.17	-3.70	55.31
	89.05	-3.72	56.06
28	88.78	-3.41	55.54
	88.40	-3.83	55.37
	88.72	-3.77	55.38
56	88.85	-2.86	52.64
	88.51	-3.04	53.99
	88.59	-3.34	53.93
	88.73	-3.31	53.91
112	86.09	-2.30	51.48
	87.41	-2.37	51.36
	86.48	-2.68	51.15
197	87.04	-1.35	49.23
	86.89	-1.29	49.35
	86.86	-1.28	49.09
240	86.85	-1.08	48.21
	86.72	-1.00	48.42
	86.74	-0.95	48.04

Appendix 2: Selected raw data of the glucose content

Table 4: Absorption at 340 nm of DEY during storage at 45°C at a_w of 0.66

Water		Day	Sample blank		Test	
0.000	0.001	1	0.000	0.005	0.179	0.188
Reagent blank		3	0.001	0.003	0.115	0.118
0.091	0.094	7	0.006	0.002	0.099	0.104
		14	0.005	0.004	0.100	0.099
		21	0.007	0.004	0.102	0.101
		28	0.008	0.014	0.106	0.106
		45	0.007	0.007	0.101	0.106
		60	0.013	0.011	0.104	0.108

Appendix 3: Selected raw data of the peroxide value

Table 5: Absorption at 560 nm of DEY during storage at 45°C at a_w of 0.66

Day	Absorption at 560 nm	
0	0.199	0.201
28	0.215	0.22
56	0.186	0.189

Appendix 4: Selected raw data of the protein solubility

Table 6: Absorption at 562 nm of DEY during storage at 45°C at a_w of 0.66

Day	Absorption at 562 nm	
0	1.551	1.646
28	1.533	1.605
56	1.222	1.294

Appendix 5: Selected raw data of the free amino group measurement

Table 7: Absorption at 340 nm of DEY during storage at 45°C at a_w of 0.66

Day	Absorption at 340 nm

1	2.509	2.506	2.516
3	2.602	2.603	2.588
7	2.604	2.534	2.554
	2.574	2.538	2.570
14	2.609	2.643	2.611
28	2.682	2.641	2.638
45	2.694	2.632	2.638
60	2.725	2.671	2.677

Appendix 6: Selected spreadsheet of kinetic analysis and Arrhenius plot of L^* and ΔE^* value

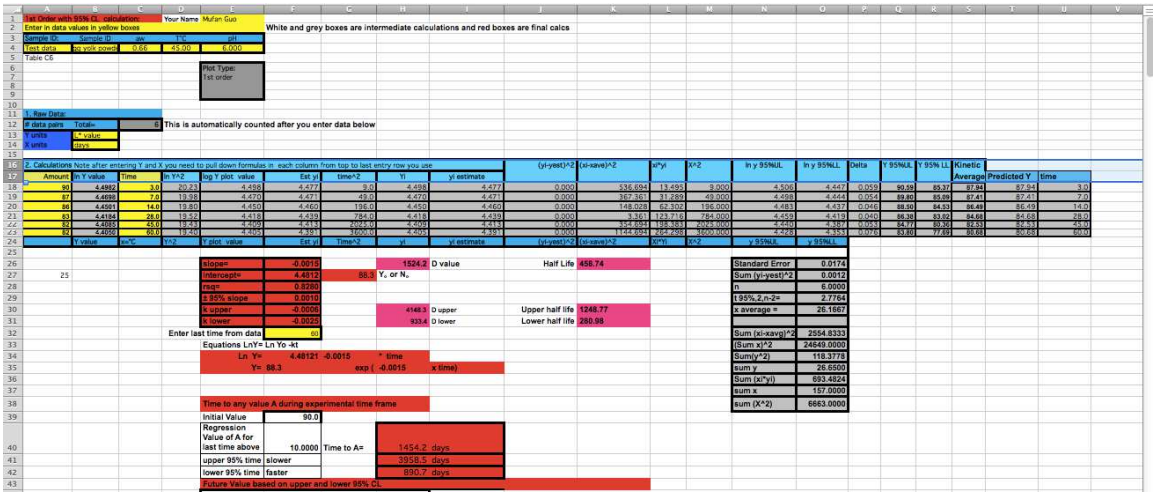


Figure 1: The spreadsheet of the kinetic analysis for the change of L^* value of DEY during storage at 45°C at a_w of 0.66

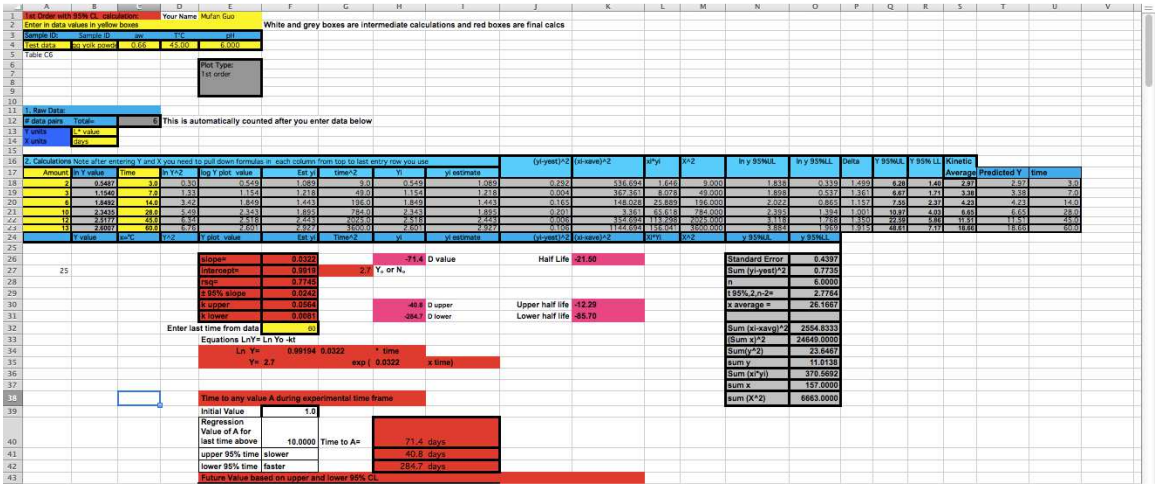


Figure 2: The spreadsheet of the kinetic analysis for the change of ΔE^* value of DEY during storage at 45°C at a_w of 0.66

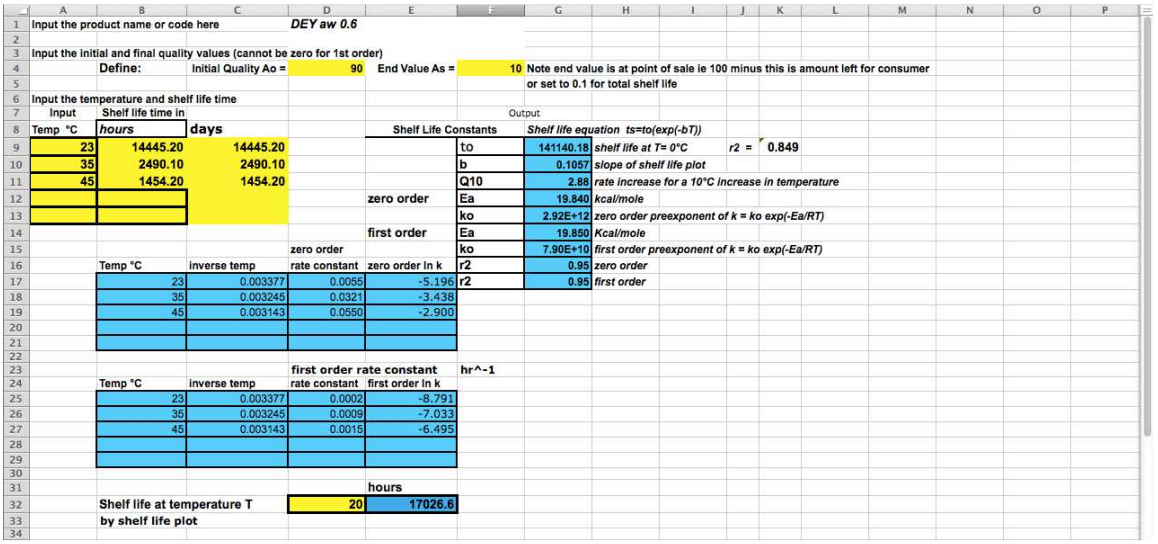
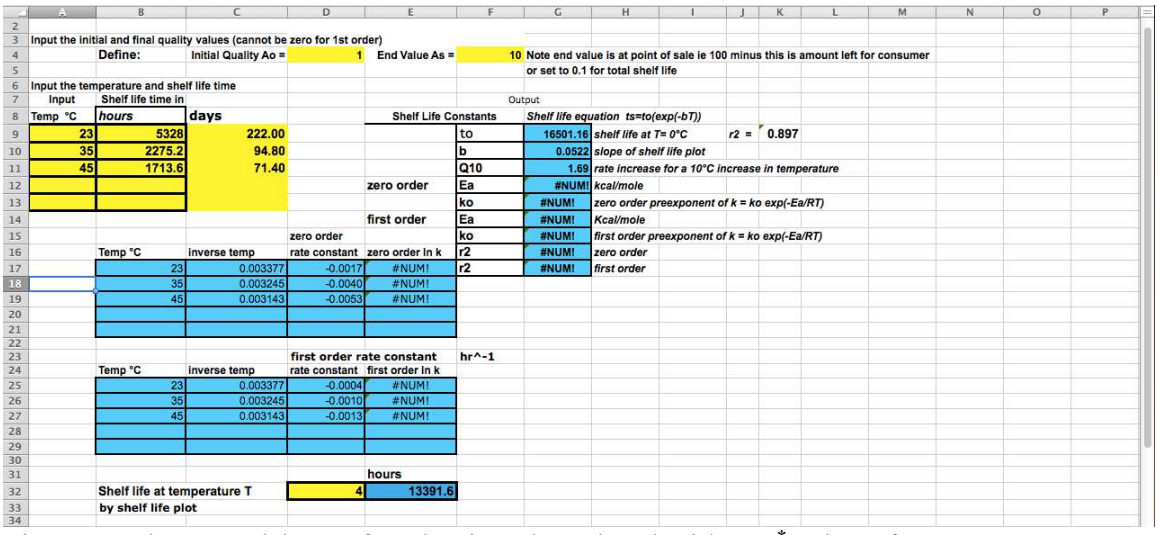


Figure 3: The spreadsheet of Arrhenius plot related with L^* value of DEY



Input the initial and final quality values (cannot be zero for 1st order)

Define: Initial Quality Ao = 1 End Value As = 10 Note end value is at point of sale ie 100 minus this is amount left for consumer or set to 0.1 for total shelf life

Input the temperature and shelf life time

Input Temp °C	Shelf life time in hours	days
23	5328	222.00
35	2275.2	94.80
45	1713.6	71.40

Output

Shelf Life Constants

	to	16501.16	shelf life at T= 0°C
	b	0.0522	slope of shelf life plot
	Q10	1.69	rate increase for a 10°C increase in temperature
zero order	Ea	#NUM!	kcal/mole
	ko	#NUM!	zero order preexponent of k = ko exp(-Ea/RT)
first order	Ea	#NUM!	Kcal/mole
	ko	#NUM!	first order preexponent of k = ko exp(-Ea/RT)
	r2	#NUM!	zero order
	r2	#NUM!	first order

Shelf life equation ts=to(exp(-bT)) r2 = 0.897

Temp °C	inverse temp	zero order rate constant	zero order ln k
23	0.003377	-0.0017	#NUM!
35	0.003245	-0.0040	#NUM!
45	0.003143	-0.0053	#NUM!

first order rate constant hr^-1

Temp °C	inverse temp	first order rate constant	first order ln k
23	0.003377	-0.0004	#NUM!
35	0.003245	-0.0010	#NUM!
45	0.003143	-0.0013	#NUM!

		hours
Shelf life at temperature T	4	13391.6
by shelf life plot		

Figure 4: The spreadsheet of Arrhenius plot related with ΔE^* value of DEY

Appendix 7: R code for the PCA analysis

For Figure 2.15

```
#read data
> mrps <- read.csv("/Users/Mufan/Dropbox/research work related/PHD thesis/data/Mufan LS55/PCA new/mrps.csv")
#invert x axis and y axis
> Tmrps<-t(mrps)
#PCA calculation
> mrpspca <- PCA(Tmrps)
> summary(mrpspca)

Call:
PCA(Tmrps)


Eigenvalues
                       Dim.1   Dim.2   Dim.3   Dim.4   Dim.5   Dim.6   Dim.7
Variance             369.186  18.855  12.312   0.404   0.115   0.023   0.021
% of var.             92.066   4.702   3.070   0.101   0.029   0.006   0.005
Cumulative % of var.  92.066  96.768  99.839  99.940  99.968  99.974  99.979
                       Dim.8   Dim.9  Dim.10  Dim.11  Dim.12  Dim.13  Dim.14
Variance               0.014   0.012   0.009   0.008   0.007   0.007   0.005
% of var.              0.004   0.003   0.002   0.002   0.002   0.002   0.001
Cumulative % of var.  99.983  99.986  99.988  99.990  99.992  99.994  99.995
                      Dim.15  Dim.16  Dim.17  Dim.18  Dim.19  Dim.20  Dim.21
Variance               0.005   0.004   0.004   0.003   0.002   0.002   0.000
```

```
% of var.              0.001   0.001   0.001   0.001   0.001   0.001   0.000
Cumulative % of var.  99.996  99.997  99.998  99.999  99.999 100.000 100.000

Individuals (the 10 first)
                Dist    Dim.1    ctr   cos2    Dim.2    ctr   cos2    Dim.3
X45C.D56.0.6   | 17.796 | 16.738  3.449  0.885 | -6.002  8.684  0.114 | -0.417
X45C.D56.0.6.1 | 21.830 | 21.194  5.531  0.943 | -4.876  5.732  0.050 | -1.837
X45C.D56.0.6.2 | 51.370 | 51.045 32.080  0.987 |  3.798  3.477  0.005 | -4.251
X45C.D56.0.6.3 | 18.862 | 17.913  3.951  0.902 | -5.771  8.028  0.094 | -1.114
X45C.D28.0.6   | 12.586 | 12.168  1.823  0.935 | -0.536  0.069  0.002 |  2.943
X45C.D28.0.6.1 | 22.247 | 21.868  5.888  0.966 |  3.565  3.063  0.026 |  1.616
X45C.D28.0.6.2 |  9.097 |  8.506  0.891  0.874 | -1.593  0.612  0.031 |  2.661
X45C.D28.0.6.3 | 18.862 | 17.913  3.951  0.902 | -5.771  8.028  0.094 | -1.114
X45C.D28.0.6.4 | 10.118 |  9.730  1.166  0.925 | -1.270  0.389  0.016 |  2.173
X23C.28D.0.6   |  8.295 | -0.077  0.000  0.000 |  8.213 16.260  0.980 | -0.199
                ctr   cos2
X45C.D56.0.6    0.064  0.001 |
X45C.D56.0.6.1  1.245  0.007 |
X45C.D56.0.6.2  6.671  0.007 |
X45C.D56.0.6.3  0.458  0.003 |
X45C.D28.0.6    3.198  0.055 |
X45C.D28.0.6.1  0.964  0.005 |
X45C.D28.0.6.2  2.615  0.086 |
X45C.D28.0.6.3  0.458  0.003 |
X45C.D28.0.6.4  1.743  0.046 |
X23C.28D.0.6    0.015  0.001 |

Variables (the 10 first)
              Dim.1   ctr  cos2   Dim.2   ctr  cos2   Dim.3   ctr  cos2
V1           | 0.124 0.004 0.015 | 0.933 4.619 0.871 | 0.281 0.643 0.079 |
V2           | 0.233 0.015 0.054 | 0.917 4.457 0.840 | 0.283 0.650 0.080 |
V3           | 0.314 0.027 0.099 | 0.891 4.211 0.794 | 0.296 0.710 0.087 |
V4           | 0.374 0.038 0.140 | 0.867 3.990 0.752 | 0.311 0.787 0.097 |
V5           | 0.438 0.052 0.192 | 0.834 3.691 0.696 | 0.322 0.842 0.104 |
V6           | 0.499 0.068 0.249 | 0.790 3.313 0.625 | 0.340 0.939 0.116 |
V7           | 0.550 0.082 0.302 | 0.750 2.981 0.562 | 0.354 1.018 0.125 |
V8           | 0.601 0.098 0.361 | 0.710 2.675 0.504 | 0.357 1.037 0.128 |
V9           | 0.648 0.114 0.420 | 0.666 2.355 0.444 | 0.364 1.073 0.132 |
V10          | 0.685 0.127 0.469 | 0.627 2.082 0.393 | 0.369 1.108 0.136 |

> plot(mrpspca$ind$coord[,1],mrpspca$ind$coord[,2],xlab="PC1",ylab="PC2")
```

For Figure 2.16

```
> mrpswo <- read.csv("/Users/Mufan/Dropbox/research work related/PHD
thesis/data/Mufan LS55/PCA new/mrpswo.csv")
> Tmrpswo<-t(mrpswo)
> mrpswopca <- PCA(Tmrpswo)
> plot(mrpswopca$ind$coord[,1],mrpswopca$ind$coord[,2],xlab="PC1",ylab="PC2")
> summary(mrpswopca)

Call:
PCA(Tmrpswo)


Eigenvalues
                       Dim.1   Dim.2   Dim.3   Dim.4   Dim.5   Dim.6   Dim.7
Variance             372.177  20.463   7.571   0.561   0.072   0.041   0.027
% of var.             92.812   5.103   1.888   0.140   0.018   0.010   0.007
Cumulative % of var.  92.812  97.915  99.803  99.943  99.961  99.971  99.978
                       Dim.8   Dim.9  Dim.10  Dim.11  Dim.12  Dim.13  Dim.14
Variance               0.020   0.015   0.014   0.011   0.010   0.009   0.005
% of var.              0.005   0.004   0.003   0.003   0.003   0.002   0.001
Cumulative % of var.  99.983  99.987  99.990  99.993  99.995  99.997  99.999
                      Dim.15  Dim.16
Variance               0.005   0.000
% of var.              0.001   0.000
Cumulative % of var. 100.000 100.000

Individuals (the 10 first)
                  Dist    Dim.1     ctr    cos2    Dim.2     ctr    cos2
X45C.D56.0.6   | 23.678 | 23.312   8.589   0.969 | -3.794   4.138   0.026 |
X45C.D56.0.6.1 | 28.955 | 28.652  12.975   0.979 | -3.995   4.589   0.019 |
X45C.D56.0.6.2 | 25.103 | 24.737   9.671   0.971 | -4.164   4.984   0.028 |
X45C.D28.0.6   | 18.232 | 17.908   5.069   0.965 |  3.114   2.788   0.029 |
X45C.D28.0.6.1 | 13.843 | 13.633   2.937   0.970 |  1.977   1.124   0.020 |
X45C.D28.0.6.2 | 25.103 | 24.737   9.671   0.971 | -4.164   4.984   0.028 |
X45C.D28.0.6.3 | 15.289 | 15.116   3.612   0.978 |  1.843   0.976   0.015 |
X23C.28D.0.6   | 23.638 |-22.981   8.347   0.945 | -2.068   1.230   0.008 |
X23C.28D.0.6.1 | 12.274 |-11.961   2.261   0.950 |  0.800   0.184   0.004 |
X23C.28D.0.6.2 | 21.084 |-20.763   6.813   0.970 | -1.063   0.325   0.003 |
                  Dim.3     ctr    cos2
X45C.D56.0.6    -1.481   1.705   0.004 |
X45C.D56.0.6.1   1.064   0.880   0.001 |
X45C.D56.0.6.2  -0.607   0.286   0.001 |
X45C.D28.0.6     0.102   0.008   0.000 |
X45C.D28.0.6.1  -0.841   0.550   0.004 |
X45C.D28.0.6.2  -0.607   0.286   0.001 |
```

```
X45C.D28.0.6.3  -0.012   0.000   0.000 |
X23C.28D.0.6    -5.052  19.833   0.046 |
X23C.28D.0.6.1   2.572   5.139   0.044 |
X23C.28D.0.6.2  -3.486   9.444   0.027 |

Variables (the 10 first)
            Dim.1    ctr   cos2    Dim.2    ctr   cos2    Dim.3    ctr
V1        | -0.069  0.001  0.005 |  0.954  4.446  0.910 |  0.189  0.470
V2        |  0.030  0.000  0.001 |  0.962  4.523  0.926 |  0.208  0.572
V3        |  0.117  0.004  0.014 |  0.962  4.522  0.925 |  0.197  0.511
V4        |  0.192  0.010  0.037 |  0.957  4.479  0.916 |  0.185  0.452
V5        |  0.270  0.020  0.073 |  0.943  4.346  0.889 |  0.168  0.371
V6        |  0.342  0.031  0.117 |  0.924  4.176  0.855 |  0.135  0.239
V7        |  0.404  0.044  0.164 |  0.903  3.985  0.815 |  0.109  0.156
V8        |  0.470  0.059  0.221 |  0.874  3.733  0.764 |  0.092  0.113
V9        |  0.529  0.075  0.280 |  0.843  3.471  0.710 |  0.080  0.085
V10       |  0.577  0.089  0.333 |  0.812  3.225  0.660 |  0.074  0.072
           cos2
V1         0.036 |
V2         0.043 |
V3         0.039 |
V4         0.034 |
V5         0.028 |
V6         0.018 |
V7         0.012 |
V8         0.009 |
V9         0.006 |
V10        0.005 |
```

For Figure 2.18

```
> los <- read.csv("/Users/Mufan/Dropbox/research work related/PHD thesis/data/Mufan
LS55/PCA new/los.csv")
> Tlos<-t(los)
> lospca <- PCA(Tlos)
> plot(lospca$ind$coord[,1],lospca$ind$coord[,2],xlab="PC1",ylab="PC2")
> summary(lospca)

Call:
PCA(Tlos)


Eigenvalues
                Dim.1   Dim.2   Dim.3   Dim.4   Dim.5   Dim.6   Dim.7
```

```
Variance            432.205  45.603   1.472   1.277   0.094   0.061   0.054
% of var.            89.855   9.481   0.306   0.266   0.020   0.013   0.011
Cumulative % of var.  89.855  99.336  99.642  99.908  99.928  99.940  99.952
                       Dim.8   Dim.9  Dim.10  Dim.11  Dim.12  Dim.13  Dim.14
Variance             0.048   0.045   0.038   0.033   0.026   0.023   0.019
% of var.            0.010   0.009   0.008   0.007   0.005   0.005   0.004
Cumulative % of var.  99.962  99.971  99.979  99.986  99.991  99.996 100.000

Individuals (the 10 first)
                Dist    Dim.1     ctr    cos2    Dim.2     ctr    cos2
Control       | 16.801 | -13.119   2.655   0.610 |  10.408  15.837   0.384 |
Control.1     | 18.029 | -17.300   4.617   0.921 |   4.751   3.299   0.069 |
Control.2     | 17.275 | -10.984   1.861   0.404 |  13.264  25.720   0.590 |
X23C.D28.0.6  | 24.237 | -23.215   8.313   0.917 |  -6.916   6.992   0.081 |
X23C.D28.0.6.1| 26.661 | -25.293   9.868   0.900 |  -8.405  10.327   0.099 |
X23C.D28.0.6.2| 27.577 | -25.949  10.387   0.885 |  -9.305  12.658   0.114 |
X23C.D56.0.6  |  7.528 |  -4.764   0.350   0.400 |   5.207   3.963   0.478 |
X23C.D56.0.6.1| 12.726 | -12.539   2.425   0.971 |   0.840   0.103   0.004 |
X23C.D56.0.6.2| 10.479 | -10.069   1.564   0.923 |   2.299   0.773   0.048 |
X45.D28.0.6   | 15.527 |  14.900   3.424   0.921 |  -3.752   2.059   0.058 |
               Dim.3     ctr    cos2
Control         0.466   0.985   0.001 |
Control.1      -0.015   0.001   0.000 |
Control.2       0.587   1.559   0.001 |
X23C.D28.0.6   -0.053   0.013   0.000 |
X23C.D28.0.6.1  0.172   0.134   0.000 |
X23C.D28.0.6.2  0.007   0.000   0.000 |
X23C.D56.0.6   -0.198   0.178   0.001 |
X23C.D56.0.6.1  0.382   0.663   0.001 |
X23C.D56.0.6.2  0.174   0.138   0.000 |
X45.D28.0.6    -2.139  20.731   0.019 |

Variables (the 10 first)
             Dim.1    ctr   cos2    Dim.2    ctr   cos2    Dim.3    ctr
V1         | 0.893  0.185  0.797 |  0.327  0.235  0.107 | -0.081  0.451
V2         | 0.920  0.196  0.847 |  0.280  0.172  0.079 | -0.103  0.726
V3         | 0.936  0.203  0.876 |  0.235  0.121  0.055 | -0.100  0.677
V4         | 0.949  0.208  0.901 |  0.198  0.086  0.039 | -0.089  0.535
V5         | 0.958  0.212  0.918 |  0.169  0.063  0.029 | -0.087  0.512
V6         | 0.964  0.215  0.930 |  0.146  0.047  0.021 | -0.089  0.534
V7         | 0.970  0.218  0.940 |  0.123  0.033  0.015 | -0.080  0.436
V8         | 0.975  0.220  0.950 |  0.095  0.020  0.009 | -0.069  0.327
V9         | 0.979  0.222  0.958 |  0.066  0.010  0.004 | -0.069  0.328
V10        | 0.982  0.223  0.963 |  0.044  0.004  0.002 | -0.073  0.360
```

```
            cos2
V1          0.007 |
V2          0.011 |
V3          0.010 |
V4          0.008 |
V5          0.008 |
V6          0.008 |
V7          0.006 |
V8          0.005 |
V9          0.005 |
V10         0.005 |
```

Vitawo

```
> vitawo <- read.csv("/Users/Mufan/Dropbox/research work related/PHD
thesis/data/Mufan LS55/PCA new/vitawo.csv")
> Tvitawo <- t(vitawo)
> vitawopca <- PCA(Tvitawo)
> plot(vitawopca$ind$coord[,1],vitawopca$ind$coord[,2],xlab="PC1",ylab="PC2")
> summary(vitawopca)

Call:
PCA(Tvitawo)


Eigenvalues
                       Dim.1   Dim.2   Dim.3   Dim.4   Dim.5   Dim.6   Dim.7
Variance             160.384   0.215   0.080   0.060   0.044   0.039   0.034
% of var.             99.617   0.134   0.050   0.037   0.028   0.024   0.021
Cumulative % of var.  99.617  99.751  99.801  99.838  99.865  99.889  99.911
                       Dim.8   Dim.9  Dim.10  Dim.11  Dim.12  Dim.13  Dim.14
Variance               0.029   0.027   0.022   0.021   0.018   0.015   0.012
% of var.              0.018   0.017   0.014   0.013   0.011   0.009   0.007
Cumulative % of var.  99.929  99.946  99.959  99.972  99.983  99.993 100.000

Individuals (the 10 first)
                  Dist    Dim.1     ctr    cos2     Dim.2     ctr    cos2
X23C.56D.0.6   |  2.672 | -2.554   0.271   0.913 |   0.053   0.088   0.000 |
X23C.56D.0.6.1 |  5.566 |  5.513   1.263   0.981 |   0.259   2.087   0.002 |
X23C.56D.0.6.2 |  0.976 |  0.757   0.024   0.602 |   0.088   0.240   0.008 |
Control        |  4.887 |  4.816   0.964   0.971 |   0.523   8.461   0.011 |
Control.1      |  3.000 |  2.839   0.335   0.896 |   0.618  11.843   0.042 |
Control.2      |  3.049 |  2.926   0.356   0.921 |   0.673  14.038   0.049 |
X45C.28D.0.6   |  2.145 |  1.913   0.152   0.795 |  -0.726  16.345   0.115 |
```

```
X45C.28D.0.6.1 |   1.331 | -0.842   0.029   0.400 | -0.829  21.293   0.388 |
X45C.28D.0.6.2 |   1.448 |   1.073   0.048   0.549 | -0.718  15.988   0.246 |
X45C.56D.0.6   |  23.450 | -23.446  22.850   1.000 |   0.015   0.007   0.000 |
              Dim.3     ctr    cos2
X23C.56D.0.6     0.494  20.390   0.034 |
X23C.56D.0.6.1   0.267   5.924   0.002 |
X23C.56D.0.6.2   0.029   0.070   0.001 |
Control          0.211   3.727   0.002 |
Control.1       -0.141   1.665   0.002 |
Control.2       -0.102   0.872   0.001 |
X45C.28D.0.6     0.303   7.651   0.020 |
X45C.28D.0.6.1  -0.060   0.304   0.002 |
X45C.28D.0.6.2  -0.455  17.259   0.099 |
X45C.56D.0.6     0.025   0.054   0.000 |
```

Variables (the 10 first)

```
              Dim.1    ctr   cos2    Dim.2    ctr   cos2    Dim.3    ctr
V1          | 0.977  0.595  0.955 | -0.087  3.488  0.008 |  0.177 39.062
V2          | 0.996  0.619  0.993 | -0.061  1.738  0.004 |  0.036  1.635
V3          | 0.996  0.619  0.993 | -0.051  1.201  0.003 | -0.037  1.732
V4          | 0.997  0.620  0.995 | -0.048  1.068  0.002 | -0.033  1.385
V5          | 0.998  0.622  0.997 | -0.034  0.528  0.001 | -0.016  0.305
V6          | 0.999  0.622  0.998 | -0.011  0.053  0.000 | -0.022  0.603
V7          | 0.998  0.622  0.997 | -0.005  0.011  0.000 | -0.038  1.793
V8          | 0.998  0.621  0.996 | -0.014  0.095  0.000 | -0.045  2.544
V9          | 0.998  0.622  0.997 | -0.031  0.449  0.001 | -0.036  1.610
V10         | 0.999  0.622  0.997 | -0.040  0.727  0.002 | -0.013  0.197
             cos2
V1           0.031 |
V2           0.001 |
V3           0.001 |
V4           0.001 |
V5           0.000 |
V6           0.000 |
V7           0.001 |
V8           0.002 |
V9           0.001 |
V10          0.000 |
```

For Figure 3.5

```
> trypwo <- read.csv("/Users/Mufan/Dropbox/research work related/PHD
thesis/data/Mufan LS55/PCA new/trypwo.csv")
```

```
> Ttrypwo <- t(trypwo)
> trypwopca <- PCA(Ttrypwo)
> plot(trypwopca$ind$coord[,1],trypwopca$ind$coord[,2],xlab="PC1",ylab="PC2")
>
text(x=trypwopca$ind$coord[,1],y=trypwopca$ind$coord[,2],labels=rownames(trypwopc
a$ind$coord))
> summary(trypwopca)

Call:
PCA(Ttrypwo)


Eigenvalues
                       Dim.1   Dim.2   Dim.3   Dim.4   Dim.5   Dim.6   Dim.7
Variance             212.314  37.051   0.621   0.170   0.120   0.105   0.092
% of var.             84.587  14.761   0.247   0.068   0.048   0.042   0.037
Cumulative % of var.  84.587  99.348  99.596  99.663  99.711  99.753  99.789
                       Dim.8   Dim.9  Dim.10  Dim.11  Dim.12  Dim.13  Dim.14
Variance               0.070   0.066   0.056   0.052   0.051   0.046   0.043
% of var.              0.028   0.026   0.022   0.021   0.020   0.018   0.017
Cumulative % of var.  99.817  99.844  99.866  99.887  99.907  99.925  99.942
                      Dim.15  Dim.16  Dim.17  Dim.18  Dim.19
Variance               0.040   0.035   0.027   0.025   0.017
% of var.              0.016   0.014   0.011   0.010   0.007
Cumulative % of var.  99.958  99.972  99.983  99.993 100.000

Individuals (the 10 first)
                Dist    Dim.1    ctr   cos2    Dim.2    ctr   cos2
Control       | 13.475 |  8.856   1.847   0.432 | -10.040  13.604   0.555 |
Control.1     | 14.012 |  6.388   0.961   0.208 | -12.415  20.801   0.785 |
Control.2     | 14.314 | 12.990   3.974   0.823 |  -5.811   4.557   0.165 |
X23C.D28.0.6  |  8.509 |  5.961   0.837   0.491 |  -5.907   4.708   0.482 |
X23C.D28.0.6.1 | 25.105 | 22.211  11.618   0.783 |  11.649  18.314   0.215 |
X23C.D28.0.6.2 | 16.114 | 15.390   5.578   0.912 |   4.637   2.901   0.083 |
X23C.D28.0.6.3 | 13.271 | 12.917   3.929   0.947 |   2.819   1.072   0.045 |
X23C.D28.0.6.4 |  8.075 |  4.112   0.398   0.259 |  -6.795   6.231   0.708 |
X23C.D28.0.6.5 | 14.747 | 14.168   4.727   0.923 |   3.875   2.026   0.069 |
X23C.D56.0.6  |  7.203 |  7.064   1.175   0.962 |   0.673   0.061   0.009 |
               Dim.3    ctr   cos2
Control        1.299  13.588   0.009 |
Control.1      0.828   5.516   0.003 |
Control.2      1.291  13.415   0.008 |
X23C.D28.0.6  -1.019   8.363   0.014 |
X23C.D28.0.6.1  0.690   3.834   0.001 |
```

X23C.D28.0.6.2 0.385 1.196 0.001 |
X23C.D28.0.6.3 -0.215 0.372 0.000 |
X23C.D28.0.6.4 -1.210 11.787 0.022 |
X23C.D28.0.6.5 0.289 0.673 0.000 |
X23C.D56.0.6 -0.671 3.621 0.009 |

Variables (the 10 first)

	Dim.1	ctr	cos2	Dim.2	ctr	cos2	Dim.3	ctr	cos2
V1	0.990	0.461	0.979	-0.045	0.006	0.002	0.018	0.052	0.000
V2	0.997	0.468	0.994	-0.053	0.008	0.003	0.036	0.205	0.001
V3	0.997	0.468	0.993	-0.052	0.007	0.003	0.025	0.103	0.001
V4	0.996	0.468	0.993	-0.056	0.008	0.003	0.031	0.157	0.001
V5	0.997	0.468	0.995	-0.047	0.006	0.002	0.034	0.187	0.001
V6	0.996	0.467	0.992	-0.056	0.009	0.003	0.044	0.307	0.002
V7	0.997	0.468	0.994	-0.049	0.006	0.002	0.044	0.313	0.002
V8	0.996	0.467	0.992	-0.067	0.012	0.005	0.043	0.293	0.002
V9	0.995	0.466	0.990	-0.074	0.015	0.005	0.058	0.535	0.003
V10	0.994	0.466	0.989	-0.074	0.015	0.006	0.063	0.648	0.004