QUANTIFICATION OF SOY ISOFLAVONES IN COMMERCIAL EGGS AND
THEIR TRANSFER FROM POULTRY FEED INTO EGGS AND TISSUES

THESIS

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ABSTRACT

Isoflavones are potent phytoestrogens found in soybeans. Soybean meal constitutes a main ingredient of poultry feed and isoflavones may transfer into eggs and tissues. Our objective was to determine the transfer and accumulation of isoflavones from the feed into hen eggs and tissues, making them isoflavone sources in the human diet. Isoflavone content of commercial eggs with different claims were analyzed by HPLC-MS after hydrolysis. All commercial samples contained soy isoflavones and the metabolite equol. Then, 48 laying hens were fed soy-free, regular (25% soybean meal) or isoflavone-rich diet. Isoflavones were found in experimental eggs and tissues. Enhancement of the diet with 500 mg isoflavones/100g feed resulted on egg yolks containing 1000μg isoflavones/100g while livers, kidneys, hearts and muscles contained 7162 μg/100g , 3355 μg/100g , 272 μg/100g and 97 μg/100g , respectively. The results showed that diet can be altered to modulate isoflavone content in hen eggs and tissues.

Keywords: transfer, isoflavones, tissues, hens.

DEDICATION

This thesis is dedicated to the two loves in my life, my daughter Ariana Vargas and my

wife Claudia Collantes.

ACKNOWLEDGEMENT

sacrifice and love to me. I also would like to thank my daughter, Ariana who gave me a smile every morning that helped me get through the hardest times.

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LIST OF ABBREVIATIONS

ER   Estrogen receptor

GI   Gastrointestinal tract

ODMA   O-desmethyllangolensin

LDL   Low density lipoprotein

HDL   High density lipoprotein

HPLC   High pressure liquid chromatography

MS   Mass spectrometry

PDA   Photo diode array detector

CRBD   Complete random block design

UV   Ultraviolet light

CHAPTER 1

INTRODUCTION

Isoflavones are phytochemicals from soybeans widely studied because of their potent phytoestrogenic and antioxidant activities. They have been related mainly with the prevention of chronic diseases and in women's health. Isoflavones and their preventive effect in diseases such as cardiovascular disease, bone mass loss (osteoporosis), breast and colon cancers have been extensively reported. Isoflavone consumption by humans has been mainly attributed to soy foods and foods with soy additives; however, soy is used for animal feed and limited information is available regarding foods from animal sources.

The first part of the project (Chapter 3) was dedicated to the analysis of commercial eggs and their potential as unexpected sources of isoflavones in the human diet. Previous research had proven eggs are used extensively as nutrient carriers. Other studies have shown that there is isoflavone transfer from the feed into poultry egg yolks. Isoflavones found in these yolks, not only include the soy native daidzein, glycitein and genistein, but also, equol, a daidzein metabolite created in the intestine by micro flora. Equol is a more estrogenic form of isoflavone, and we considered it to be important to

find out if regular commercial eggs had significant amounts of equol. Also, isoflavone content was compared to different claims on egg packaging. Overall, the objectives of the first part were to quantify isoflavone concentrations in commercial eggs and to determine if there existed a significant difference between eggs that were reported to have specific claims.

The second part of the research project (Chapter 4) was based on a 28 day feeding trial. During this trial, three experimental feeds, with different isoflavone levels were provided to laying hens. One diet was soy free, a second diet was a regular 25% soybean meal diet and finally one was a soy isoflavone enhanced diet. The transfer of isoflavones into the egg yolks was monitored. At the end of the trial period, tissues were collected and isoflavone concentrations were also measured. Isoflavone effects of animals have been studied for many years. Most researchers have focused on health effects that isoflavones may have in animals. However, they have not considered animal tissues as potential sources of isoflavones in the human diet. Therefore, the objectives for the second part of the thesis were to monitor changes of isoflavone concentrations in egg yolks over a 4 week period and quantify the transfer and concentrations into tissues after the feeding trial.

CHAPTER 2

LITERATURE REVIEW

2.1. Isoflavones

Isoflavones are flavonoids which structure is characterized by the presence of two phenol rings linked together by a three carbon bridge. The structure is simplified as C6-C3-C6 (1). Isoflavones are phytoestrogens and behave as non-esteroidal estrogen mimics; this refers to isoflavones as being capable of having hormone-like activity and having the ability to attach to estrogen receptors (ER) (2). Other well known phytoestrogens are lignans such as secoisolariciresinol, coumestans such as coumestrol and prenyl flavonoids such as 8-prenylnaringenin. All these have been extensively investigated (3) due to their potential as protectors of human health.

2.1.1. Occurrence & Chemistry

Flavonoids, are widely distributed in terrestrial plants, but isoflavonoids are restricted primarily to leguminous plants. They have been widely used as taxonomic markers within the leguminoseae (4). Isoflavones are the most abundant isoflavonoids (4). Isoflavones and coumestants have been identified as the most common estrogenic

compounds in these plants (5). Overall, it seems that nearly every plant is able to synthesize phytoestrogens, but their concentrations in most plants are negligible (6). Soybeans are the largest source of isoflavones with concentrations ranging from 118 mg/100g to 306 mg/100g; therefore, soy foods and some foods with soybean additives have the largest concentration of these estrogenic compounds (7-9). Isoflavones are found mainly in their glycoside form and this has an effect on their absorption and retention within the human body. In soybeans there are 12 different isoflavone isomers (**Figure 2.1**): three aglycones (free isoflavones), three β-glucoside derivatives, three acetyl-glucoside derivatives and three malonyl-glucoside derivatives (10).

Isoflavones are flavonoids because of their C6-C3-C6 structure; however, they differ from flavones in that the B ring is attached to position 3 of the C ring instead of position 2 (see **Figure 2.2**) It is considered that the phenol ring is a key structural element to be able to attach to the estrogen receptors (11) and the flavonoid isomeric configuration increases their similarity to human estrogens (aligned OH groups) which seems to be the foundation of their phytoestrogenic activity(12). If superimposed, genistein (as well as other isoflavones) and estradiol structures are very similar (**Figure 2.3**).

Aglycones
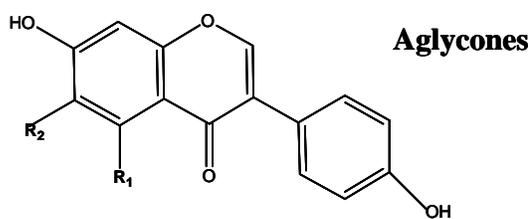
Compound	R_1	R_2
Daidzein	H	H
Genistein	OH	H
Glycitein	H	OCH_3

Glucosides
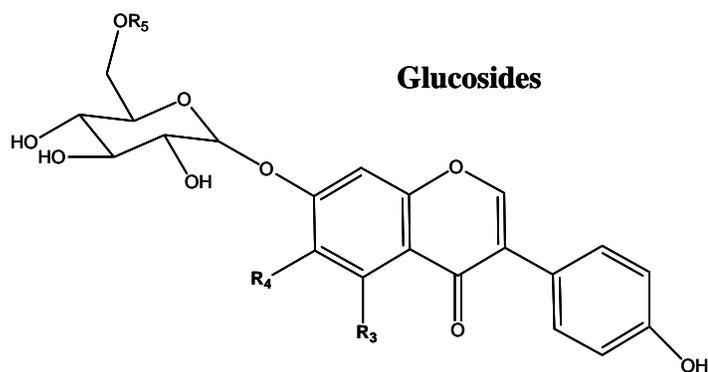
Compound	R_3	R_4	R_5
Daidzin	H	H	H
Genistin	OH	H	H
Glycitin	H	OCH_3	H
6"-O-Acetyldaidzin	H	H	$COCH_3$
6"-O-Acetylgenistin	OH	H	$COCH_3$
6"-O-Acetylglycitin	H	OCH_3	$COCH_3$
6"-O-Malonyldaidzin	H	H	$COCH_2COOH$
6"-O-Malonylgenistin	OH	H	$COCH_2COOH$
6"-O-Malonylglycitin	H	OCH_3	$COCH_2COOH$

Figure 2.1: Soybean isoflavones and derivatives. (61)
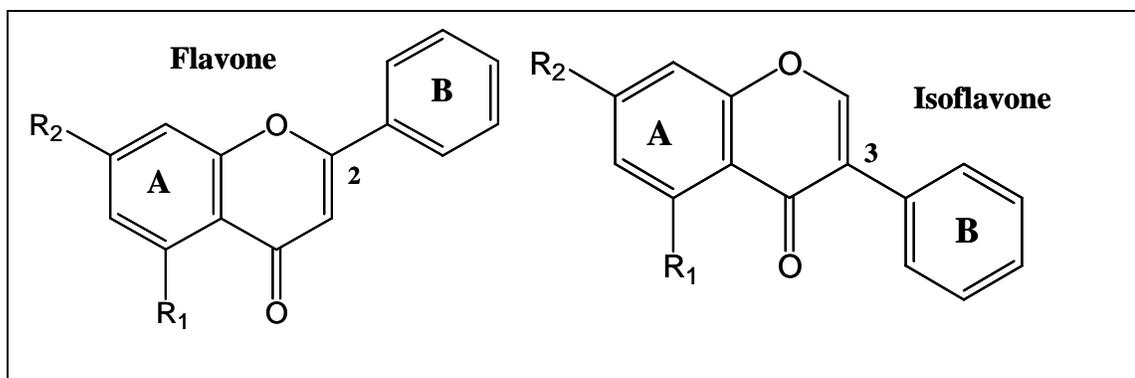
Figure 2.2: Structure comparison of a flavone and an isoflavone
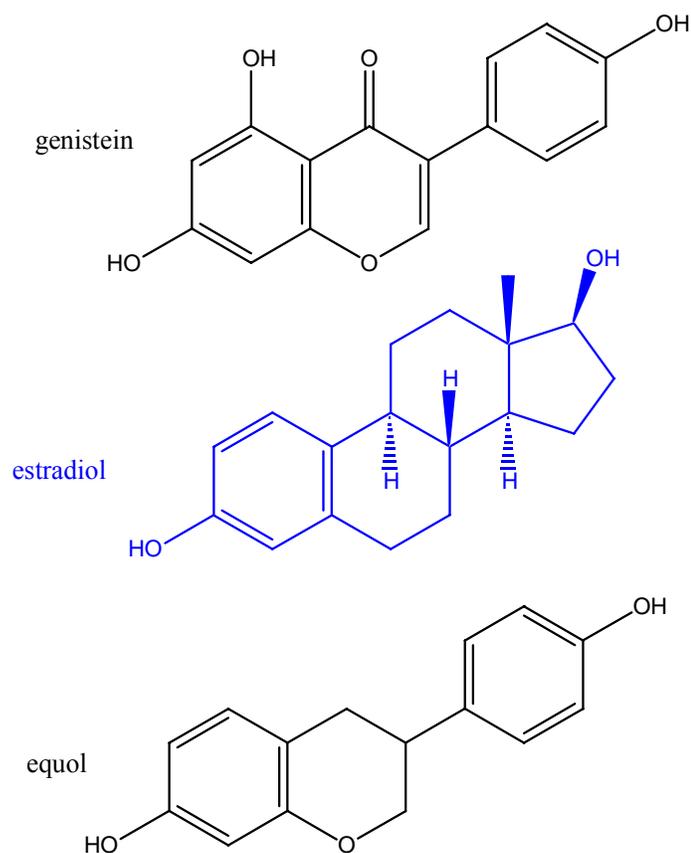
Figure 2.3: Structural comparison between genistein, estradiol and equol

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2.1.2. Isoflavone Bioavailability

For isoflavones to be able to protect against different chronic diseases, the main requirements to consider are their effective absorption, metabolism, distribution and excretion, in other words, their bioavailability (13). Isoflavones are consumed with food, absorbed in the gastrointestinal (GI) tract, and finally, excreted in the urine. They will be subjected to different chemical and physical environments throughout the GI tract. Each of these environments may have specific flora that can be relevant for isoflavone digestion and absorption (14).

The isoflavones found in soybeans are mainly glycosides; very small concentrations are found as aglycones (15,16). These isoflavone glycosides will pass through the GI tract and be hydrolyzed by intestinal β-glucosidases. This hydrolysis will produce aglycones in the small intestine (17, 18). Isoflavone digestion will depend on the individual; it has been shown that not all women can turn isoflavone glycosides into their respective aglycones, due to differences in their micro flora. This is crucial in the absorption process since glycosides cannot be absorbed by enterocytes (6, 19).

After absorption, isoflavones pass through the liver where they can be glucuronidated or sulfated or left as aglycones. This can also significantly affect their bioactivity since glucuronides are believed to be pharmacologically inactive (20). Isoflavones may be further metabolized by the large intestine's microflora into other metabolites such as equol (**Figure 2.3**) and O-desmethylangolensin (O-DMA) (21).

Several studies (22, 23) focus on bioavailability based on the urinary excretion and plasma levels of isoflavones. It has been noted that after absorption most isoflavones are found in urine and plasma as glucuronides, aglycones and sulfates. Isoflavones glucuronides represent 60% and 70% of total isoflavones in urine and plasma, respectively of women that consistently consume soy milk (23). Aglycones only accounted for 5% in urine and 20% in plasma of the total isoflavones. Isoflavones seem to be glucoronidated rapidly in the intestinal mucosa while further glucuronidation occurs in the liver. The major end point of isoflavones seems to be biliary excretion where close to 70% of an isoflavone dose was found (24).

To a lesser extent there are other aspects that can also influence isoflavone bioavailability, gender (25), food matrix and processing conditions (18) among them.

2.1.3. Isoflavones as Phytoestrogens

Phytoestrogens have been noted to have an important role in hormone dependent conditions (26). They have been found to have potential health benefits in age-related and hormone dependent diseases; these include cancer, menopausal symptoms, cardiovascular diseases, osteoporosis, cognitive function, among others (8, 27, 28). Isoflavones behave similarly to hormone treatment estrogens but their estrogenic strength is weaker (6, 29, 30). However, despite the relative low strength of isoflavones in comparison to estrogenic hormones, they may still exert physiological effects because isoflavone levels found in serum of soy consuming individuals can be as much as 1000 fold higher than endogenous estrogens (1).

The main estrogen which isoflavones mimic is Estradiol-17β (**Figure 2.3**). This estrogen

is produced in the ovaries and acts through the estrogen receptors ERα and ERβ (2).

Isoflavone activity is related to their capability to use these same estrogen receptors. As

previously noted, genistein has a very similar structure to estradiol in many aspects.

These similarities aid in isoflavone binding to the active site of the receptors. The main

similarity is the intermolecular distances between the hydroxyl groups; these groups

determine the hydrogen bond interaction with the amino acids of the receptors (12). ERα

is the receptor that is related to tissue proliferation and therefore considered to be crucial

in cancerous development (31). Finally, it is important to consider that if a hormone

treatment includes not only estrogens but also progestin, it has been shown to reduce

colon cancer (32). Genistein has shown to have around thirty times more affinity to ERβ

than ERα (12).

Equol (**Figure 2.3**) is a derivate from daidzein that is obtained by microflora

degradation in the large intestine. It is said that its affinity to ERs is as strong as that of

genistein but that it has a stronger transcription activity, especially in ERα (33). It has

also been shown that equol takes longer than other isoflavones to appear in human urine

after the consumption of a single dose of an isoflavone containing soy food; this suggests

that equol is produced in the large intestine, contrary to the rest of isoflavones that can

also be absorbed in the small intestine (26). However, equol's appearance in human urine

analysis is related to soy consumption, since the human body does not produce significant

amounts (19). Further research has confirmed that equol can only be produced by

intestinal microflora; since germ free animals did not show any production (34) and the

absence of equol in soy-based formula fed infants who inherently lack active microflora do not produce equol (35).

Finally, since equol production in the intestine is largely dependent on microflora, and this varies in humans, it is not produced by every individual. It is said that an average of only 30% of women can convert daidzein into equol because of the specific microflora requirements (36). Some researchers have indicated that isoflavone containing soy foods and extracts will have higher benefits in equol producing individuals (19, 26).

2.1.4. Isoflavones in Health and Diseases

It has been widely noted that individuals from countries with high dietary content of soy foods are less likely to develop mammalian cancer. This has been stated since Japanese immigrants into western civilization and their children have developed mammalian cancer as often as original inhabitants of the western countries (6)(42, 43). Over the years, isoflavone effects on many different chronic diseases have been studied as well as their activity regarding women's health.

Isoflavones, as many other flavonoids, have been shown to have antioxidant activities in vitro and in vivo. Wei et al (1995) (37), found genistein to be a very potent inhibitor of hydrogen peroxide production in 12-O-tetradecanonylphorbol-13-acetate-activated HL-60 cells. The study also showed that genistein increases the activity of antioxidant enzymes (37, 38). Additionally, isoflavones have been shown to have inhibition properties of protein kinases and DNA topoisomerases (39), as well as, antiproliferative (40) and antiangiogenic (41) properties.

2.1.4.1. Isoflavones and Women's Health

Lu (1996) (44)showed that ingestion of 36oz of soy milk each day can reduce 17β estradiol levels, luteal phase progesterone levels, and dehydroepiandrosterone sulfate levels, as well as, increase the length of the menstrual period. The study also hypothesized that isoflavones may protect women from hormone dependent cancers by lengthening the menstrual cycle; therefore, reducing exposure to estrogen.

It has also been mentioned that a women's age may not be a factor in isoflavone bioavailability. A study indicated that after ingestion of soy nuts, the fate of isoflavones was not influenced by a woman's age or menopausal status and their bioavailability was similar (26).

2.1.4.2. Isoflavones and Chronic Diseases

2.1.4.2.1. Osteoporosis

Phytoestrogens have been considered as the main factor by which Asian women develop significantly less bone loss compared to Western women (49). It has been hypothesized that isoflavones are the reason by which bones have increased calcium retention; therefore, increasing bone mineral density (50). Previously, there has been uncertainty regarding the main reason for the bone loss reduction after consumption of soy protein isolates, it was not clear if it should be attributed to isoflavones, until Alekel et al. (2000) (51)confirmed that it was isoflavones and not the isolate overall that cause the beneficial effect.

2.1.4.2.2. Cardiovascular Disease

Isoflavones have been continuously studied regarding their capability to reduce LDL cholesterol which is attributed as the main reason for cardiovascular diseases (52, 53). Additionally, isoflavones have not only been able to lower LDL cholesterol, but also increased HDL cholesterol, inhibited lipid peroxidation and lowered blood pressure (29).

2.1.4.2.3. Cancer

As mentioned before, there is consistent evidence supporting the fact that Asian women have lesser risk of developing breast cancer because of their increased consumption of soy-based foods (42, 43). Also, it has been mentioned that pre-pubertal consumption of soy based foods can act as a cancer preventive measure (54). However, there is conflicting information regarding this important issue. Some researchers, as mentioned before, have found evidence of benefits of isoflavones regarding mammary cancer (55, 56). There have also been findings of no correlation between isoflavone consumption and cancer risk (57) and increased risk because of increases in the density of the mammary gland tissue based on the content of O-DMA (58).

2.1.4.3. Isoflavones and Safety

It has been long noted that isoflavones play a role in women's health and chronic diseases. These are not the only motives for which isoflavones have been studied. Infant formulas have long been scrutinized for their high isoflavone levels because these

phytochemicals at such elevated concentrations at an age where the developmental stage is just beginning may have permanent effects in life (45).

Results in the concentration of isoflavones in infant formula powders have varied. Setchell et al. (1998) (46) and Murphy et al. (1997) (47) obtained values of 316μg/g and 232 μg/g, respectively. According to Miniello et al. (2003)(45)based on human weight, the average consumption of a 4 month old is six to eleven times higher than the necessary concentration to produce hormonal menstrual effects on adult women. However, there has not been any correlation between isoflavone content and negative effects in infants. The main concern regarding these formulas is the developmental effects that the consistent and high doses of isoflavones may have on infants. However, a study developed by Strom et al (2001) (48) indicated that there were no main differences in the comparison of the adult results of infants fed breast milk and soy-based formulas during infancy.

2.2. Isoflavone Sources

2.2.1. Soybean (*Glicine max*)

2.2.1.1. Soybean History

Soybean is one of the oldest crops in the Far East (1). It originated in Southeast Asia and was first domesticated by Chinese farmers around 1100 BC. By the first century AD, soybeans were grown in Japan and many other countries (59) Soybean is mentioned to be one of the most important cultivated legumes throughout Asia. Asians have used them as important sources of dietary protein and oil (1).

Soybeans were reported to have been planted by a colonist in the British colony of Georgia in 1765. It 1770 it is said that Benjamin Franklin sent soybeans to his home to be planted in his garden. In Europe and British colonies soy sauce arrived before soybean seeds. Nowadays, soybean is still considered to be a very important source of protein in Asian nations and is an important food and industrial product in Asian countries.

2.2.1.2. Isoflavone Content in Soybeans

Total isoflavones contents in Japanese varieties grown in two different years (1991 and 1992) were estimated to be from 2041 to 2343 µg/g and from 1261-1417µg/g, respectively (9). These values have to be taken only as reference since it has to be kept in mind that there are many factors that may vary the concentration of isoflavones in soybeans. Several researchers have obtained differences in isoflavone contents in various cultivars, in different years, several environments, as well as, in separate locations (9, 60, 61) Harsh environments seem to be positively correlated with isoflavone concentration (61); this is confirmed by Dixon (1999)(59) who states that isoflavones are synthesized in response to different environmental stresses.

The isoflavone concentration also varies within the soybeans seed. The different anatomical parts of soybeans have different isoflavone contents. Ledridge and Kwolek (1983) (62) stated that cotyledons contain 80 to 90% of total soybean isoflavones and hypocotyls contain the difference. However, hypocotyls have a higher concentration than cotyledons on a weight basis (60). Genistein has been found to be more abundant than daidzein, and daidzein more abundant than glycitein in different varieties of soybeans (9). Additionally, according to Kudou et al. (1991) (63) glycitein can only be found in

hypocotyls. Regarding the isoflavone isomer profile found in soybeans, malonyl derivatives seem to be more abundant, followed by β-glucosides, acetyl derivatives and very small concentrations of free isoflavones (aglycones) (62).

2.2.1.3. Isoflavone Content and Profiles in Soy Foods and Soy Additives.

As soybean isoflavones have different concentrations within cultivars and many other factors, soy foods which are subjected to different processes, not only have total isoflavone content variations but also, different isoflavone profiles. Soy flour, which undergoes limited processing, has 80% malonyl β-glucosides, and 15% β-glucosides and very limited concentrations of acetyl glucosides and aglycones (64).

Umphress et al. (2003) (8)found an average soy flour total isoflavone value of 1.06 mg/g. Tofu, subjected to a soaking step where glycosidase activity is expected, contains 37% malonyl β-glucosides, and 25% β-glucosides and 37% aglycones (65), with total isoflavone values of 0.53 mg/g (9). Tempeh undergoes stronger enzymatic activity, resulting in increased values of aglycones. It has 35% malonyl β-glucosides and only 17% β-glucosides, but, it has 50% aglycones. Total isoflavone are estimated at 0.86mg/g (9, 65). Textured vegetable protein has 50% malonyl forms, 32% β-glucosides and 20% acetyl β-glucosides. Dry heat is regarded as the reason for the transfer of malonyl glucosides to acetyl glucosides (65). Soy germ contains much higher levels of isoflavones compared to the previous foods and additives, up to 10 times higher. Also, according to Song et al. (2003) (66) the aglycone base profile is different; daidzein

is the most abundant followed by glycitein and finally genistein. The concentrations are estimated to be 9.22 mg/g, 2.67mg/g and 7.27 mg/g for daidzein, glycitein and genistein respectively.

2.2.2.   Red Clover (*Trifolium platense*)

Red clover is a second identified source of isoflavones with relevant concentrations. There is a total of 31 different isoflavones found in red clover. The free isoflavones (aglycones) found are daidzein, formononetin, genistein, pseudobaptigenin, glycitein, calycosin, prunetin, biochanin A, irilone and pratensein, the remainder of the isoflavones are aglycone derivatives (67). Out of the aglycones mentioned above, genistein, biochanin A, formononetin and daidzein are the most abundant (68, 69). Red clover has been recently studied because of its consumption by sheep and apparent effects on their reproductive systems (68).

2.2.3.   Soybeans in Animal Feed

It is well known that soybeans and soybean foods are mainly consumed in Asian countries; the main reason for this consumption is that soybeans provide foods with unconventional flavors not widely accepted in Western cultures. However, as mentioned previously in this review, the health benefits of isoflavones have been described and should make it desirable to increase soybean/isoflavone consumption.

The American Soybean Association website (www.soystats.com) states that U.S. soy meal production has increased to a total of 43.8 million metric tons, of which 32 million is use to feed livestock (70, 71).

Steps have been taken to study the transfer of isoflavones from the feed into animal tissues (68, 72), as well as, poultry eggs (73-75).

2.2.4. Eggs as a Nutrient Carrier

Studies have shown that an eggs composition can be modified by changing the contents of poultry feed (76). According to Sim and Sungwoo (2002) (77) designer eggs can be created to increase the consumption of nutritionally beneficial compounds without significantly modifying a person's dietary regimen. This has driven egg producers to put it into practice; they have been able to modify the eggs content to include beneficial nutrients. Previous studies have proved that this nutrient transfer can also be applied to isoflavones. Lin and Giusti (2003) (75) showed how feeding Japanese quail with dietary genistein increased the levels of this isoflavone in their egg yolk. Saitoh et al. (2001) (74) confirmed that these compounds can be transferred from the feed into the egg yolk of chickens. It would be interesting to determine if regular 25% soy meal concentrations can be sufficient to yield isoflavones in commercial eggs. It would also be of interest to determine the ratio of

this transfer and the transfer into other hen tissues, such as heart and liver, which are consumed in several different countries.

2.2.5. Isoflavones in Animal Tissues

Isoflavone's antioxidant activity has been speculated to have effects on increased performance and meat quality in animals. Jiang et al. (2007) (78) showed that in male broilers (chickens) adding 40 or 80mg of isoflavones/kg to the feed. Muscle antioxidant

enzyme activity and antioxidant status were increased, and leaner muscle was obtained. It has also been reported that soybean isoflavones can in fact suppress plasma lipid oxidation products in vivo (79, 80). D'Souza et al. (2005) tested soy isoflavone antioxidant activity in rainbow trout finding the same results (72). Similar studies have shown that increased isoflavones in the feed result in increased growth rate and carcass muscling (81) Also, Payne et al. (2001) (82) indicated that increased isoflavones in the feed increased carcass leanness and decreased carcass fat. Overall, most animal isoflavone research has been directed to the effects that these estrogenic compounds may have on the animal. Extensive attention has not been focused on the final isoflavone contents and their potential transfer into humans after consumption of tissues containing these substances. Urpi-Sarda et al. (2008) (68) analyzed the isoflavone content of different tissues in ewes after consumption of red clover silage and found significant concentrations in livers, kidneys and hearts; however, this study was mainly focused on "clover disease" which had been attributed to clover isoflavone consumption. It would be important to analyze these organs (liver, kidney and heart) in hens, to determine if the isoflavone levels can be in fact high enough to be considered relevant as sources of isoflavones for humans that may have consumed them.

CHAPTER 3

3. IDENTIFICATION AND QUANTIFICATION OF SOY ISOFLAVONES AND THE ISOFLAVONE METABOLITE EQUOL IN COMMERCIAL EGGS

3.1. Abstract

Isoflavones, mainly found in soybeans, have been widely studied because of their phytoestrogenic activity. They have been associated to many health benefits, such as cancer prevention, reduction in menopausal symptoms, bone mass increases, among others. Isoflavone contents have been evaluated in many different food products, mainly soy foods and foods with soy additives, as well as, in animal products due to their transfer from the diet. Hen eggs are widely consumed and could become an additional source of isoflavones. The objective of this study was the identification and quantification of soybean isoflavones and equol, a more bioactive metabolite of soy phytoestrogens, in 18 different commercial eggs with different claims from 9 brands. Egg yolk samples were incubated with β-glucuronidase to better account for the different isoflavone aglycones and metabolites. Isoflavones were extracted with 80% methanol and purified by using

C18 solid phase extraction. The extracted samples were analyzed by HPLC-PDA for quantification and MS for identification. Total isoflavone content ranged from 33μg to 139μg/100g of egg yolk (~2.20 μg to 9.26 μg/egg). There were no significant differences on isoflavone content among most of the samples with different types of claims, except for eggs high in omega 3 fatty acids. This may be due to the partial replacement of soybean meal for omega-3 rich materials in the poultry diet. The results showed that soy isoflavones, as well as, the more biologically active phytoestrogen equol can be found in commercial egg products in different concentrations. Therefore, eggs constitute an unexpected additional source of phytoestrogens in the human diet.

Keywords: isoflavones, egg yolk, commercial brands, equol

3.2. Introduction

Isoflavone consumption through the ingestion of soy foods has been extensively studied for their effects on women's health and on chronic diseases, such as cancer, osteoporosis, cardiovascular diseases, among others (27, 43, 53). It has been reported that genistein's hormonal activity is mainly related to its structural similarity to the ovary synthesized estrogen, estradiol 17-β (2). This similarity and capability to bind to estrogen receptors is based upon the presence of a phenol ring and the positioning of the structures' –OH groups which allows it to bind to the receptor's aminoacidic active sites (12). Isoflavones have also been attributed non-hormonal activity, such as their antioxidant capabilities, along with many other compounds from the flavonoid family (37, 72, 78).

Dietary isoflavones found in soybeans are mainly the glycosidic forms of three base aglycones (daidzein, genistein and glycitein). After consumption isoflavones are initially hydrolyzed and absorbed in the small intestine (17, 18) into the liver where most are glucuronidated (70%) (23, 24). Research has shown that a more estrogenic isoflavone, equol, a daidzein metabolite, is formed and absorbed in the large intestine (34). Overall, isoflavone absorption depends greatly on the individuals' microflora. It has been shown that some women take longer to hydrolyze isoflavone glucosides than others (83) and an average of only 30% of women can produce equol (19).

The American Soybean Association (70, 71) has shown that soybean meal production in the world has grown significantly. The United States alone produces up to 48 million metric tons a year. From this total production, 80% is directed toward animal feed, from which 50% is derived to poultry feed.

There are several studies world wide that have proved that isoflavones can be transferred from the feed into the tissues of animals. A European study analyzed the transfer of red clover silage isoflavones into sheep tissues owing to concerns about their potentially negative reproductive effects in these animals (clover disease) (68). In the United States, Lin and Giusti (2004) (75) studied the transfer of isoflavones into the egg yolk of Japanese quails. In Japan, Saitoh et al. (2001,2004) (73, 74) studied the transfer of these well known compounds into the egg yolk of chickens. In these studies, only Saitoh et al. (2004) (73) has analyzed the presence of equol in the yolk.

Based on the benefits associated with isoflavone consumption, there has been considerable effort to increase soy consumption in Western countries' diets with limited results due to the perceived off flavor provided by soy products. For many years, chicken

eggs have been used to transfer nutrients lacking in the human diet (77). However, even though some individuals would need isoflavones in the diet, others may not (6). The transfer of these relevant compounds into the egg can be an unwanted source of them. It has to be considered soybean meal is used as part of poultry feed , which is a carrier for isoflavones.

The objectives of this study were to quantify the isoflavone content of commercial eggs from different brands and analyze the effect of product claims in these isoflavone contents. In addition an evaluation was made of the presence and concentration of the more estrogenic isoflavone metabolite equol.

3.3. Materials and Methods

3.3.1. Chemicals and Enzyme

Genistein and daidzein standards for HPLC-MS identification were purchased from LC Laboratories (Woburn, MA.). Standard purity for isoflavones was 99%. Formononetin, used as internal standard was purchased from Acros Organics (Morris

Plains, NJ). Acetonitrile, methanol (99.9%) and water were HPLC-MS grade and were obtained from Fisher Scientific (Fair Lawn, NJ). Sodium acetate, hexane and acetic acid were also obtained from Fisher Scientific. β-glucuronidase Type HP-2 from *Helix pomatia* aqueous solution with 102,000 units/mL was purchased from Sigma Aldrich (St. Louis, MO).

3.3.2. Experimental Design

Commercial fresh eggs from 9 brands and with different claims, for a total of 18 different egg samples, were purchased from 3 local grocery stores in Columbus, Ohio,. From these samples, 2 were regular white eggs and 2 regular brown eggs (claim free), 4 of them had organic claims, 8 were eggs from cage-free hens, 4 had vitamin E claims, 11 indicated vegetable fed hens, 1 was pasteurized, and 9 had elevated omega-3 values (one of these had up to 660mg, another 350mg, compared to an average of 100 from the other 7 samples, as reported in their labels) as presented in **Table 3.1**.

3.3.3. Isoflavone Extraction

The isoflavone extraction method from the egg yolks was modified from Lin & Giusti (2003) and Saitoh et al. (2001). Isoflavones were extracted from 5g of egg yolks. Isoflavones were first hydrolyzed by mixing the sample with 10 mL of 0.1M sodium acetate buffer in a 125mL flask, along with 75uL of enzyme. Samples were held overnight at 37ºC in a Fisher Scientific (Fair Lawn, NJ) Isotemp incubator. After incubation, 30mL of 100% MeOH was added to obtain a final concentration of 75% MeOH. Isoflavones were then extracted by stirring for 2 h on a Fisher Scientific isotemp digital stirrer. Extracts were centrifuged in a Fisher Scientific Centrific ® Centrifuge at 10,000 rpm for 20 min. Supernatants were collected and precipitates were re-dissolved in 10mL of 75% MeOH. Both supernatants were mixed, 30mL of

water were added and then extracted with 30mL of hexane. Samples were then mixed and centrifuged to separate the hexane layer with the remaining lipids from the initial extraction. The MeOH/water layer was collected and concentrated on a Buchi Rotavapor® at 40 ºC. The samples were purified by passing them through Sep-Pak® C-18 cartridges (Waters Corporation, Milford, MA.) using a C-18 extraction kit. Samples were purified by washing the column first with 10% acetonitrile in water to remove the more polar compounds and isoflavones were recovered from the cartridge with 80% methanol and a ratio of 80/20 ethyl acetate/MeOH. Purified samples were concentrated in the Rotavapor®. Finally, samples were taken to 2mL, filtered through a 0.45µm syringe polypropylene filter and injected into the HPLC system.

3.3.4. HPLC Analysis

A Shimadzu Prominence® LC-20 A_5 reverse phase high pressure liquid chromatography system coupled with a Shimadzu Prominence® SPD-M20-A diode array detector, a Shimadzu Prominence® SIL20-AC auto sampler and a Shimadzu LCMS Solution software was used. Sample elution was obtained by use of a Waters Corporation 4.6 x150mm Symmetry® 3.5 µm C-18 column. Spectral data was obtained from 200 to 450nm and isoflavone elution was monitored at 254 nm. Two different gradients were used to separate all compounds. The gradients included an aqueous solvent A (0.1% acetic acid, 5% acetonitrile in water) and a organic solvent B (0.1% acetic acid in acetonitrile)

	Brands	Type or Claim
Sample 1	B 1	Organic, 100mg Ω3
Sample 2	B 1	Cage Free, vegetable fed, 100mg Ω3
Sample 3	B 2	Regular white
Sample 4	B 3	High vitamin E, vegetable fed, 110mg Ω3
Sample 5	B 4	Regular white
Sample 6	B 1	High vitamin E, vegetable fed, 660mg Ω3
Sample 7	B 2	Regular brown
Sample 8	B 4	Regular brown
Sample 9	B 3	Cage free, high vitamin E, vegetable fed; 100mg Ω3
Sample 10	B 3	Organic, high vitamin E, vegetable fed, 100mg Ω3
Sample 11	B 5	Vegetable fed, 350mg Ω3
Sample 12	B 6	Cage free
Sample 13	B 6	Organic, cage free
Sample 14	B 5	Cage free, vegetable fed
Sample 15	B 7	Cage free, vegetable fed, 100mg Ω3
Sample 16	B 7	Organic, cage free, vegetable fed, 100mg Ω3
Sample 17	B 8	Cage free, vegetable fed
Sample 18	B 9	Vegetable fed, pasteurized.

Table 3.1: Types and claims of different commercial egg samples..

Daidzein separation: To better separate daidzein from other small quantities of unknown compounds the following gradient was used. After the injection of 150µL of sample the linear gradient started from 15% B to 20%B from 0-2 min, 20 to 21% B from 2-20 min, 21-35 B from 20-24 min, maintained at 35%B from 24-32 min and finally it was returned to 15%B from 32-34 min.

Genistein and equol separation: Genistein and equol eluted at approximately the same time. A small variation of the gradient recommended by Saitoh et al. (2004) (73) was applied. After the injection of 150µL of sample the linear gradient started from 15%B and was taken to 27.5%B in 5 min., 27.5 to 31% B from 5-30 min and returned to 15%B at 31 min.

3.3.5. Statistical Analyses

Statistical analyses of the results were performed using the Microsoft Excel 97 statistical package. The experiment was carried out using a complete random block design (CRBD), where each sample was run in triplicate. Each egg brand replicate was extracted and analyzed on different weeks. A new set of egg samples was purchased for each replication. All 18 samples were run within a week. Each set of a dozen eggs was considered to be one experimental unit.

3.4. Results and Discussion

3.4.1. Method Optimization for Isoflavone Extraction from the Egg Yolk of Laying Hens.

The method used for the extraction and purification of isoflavones from the egg yolk was optimized. During the purification step, isoflavone recovery, tested with an internal standard, from the C-18 column with 80% methanol was not considered to be efficient enough (as low as 25%). The inclusion of a washing step with 10% acetonitrile along with a final recovery step with 80/20 Ethyl acetate/ MeOH increased final internal standard recovery from 50 to 75%.

3.4.2. Quantification of Isoflavones and Equol in Commercial Fresh Eggs.

As recommended by Saitoh et al. (2001,2004) and Lin & Giusti (2004) (73-75), all samples were subjected to β-glucuronidase treatment to hydrolyze metabolites into their respective aglycones to increase their final aglycones concentration As isoflavones are ingested, they are hydrolyzed before being absorbed (17, 18). Once absorbed, these compounds are usually glucuronidated or sulfated (23, 24); therefore, hydrolization of compounds resulted in the increased concentration of the isoflavone aglycones.

Based on the compounds retention times, spectral characteristics (**Figure 3.1**) and mass spectrometric data; genistein, daidzein and equol were identified. Total isoflavone

content was obtained with the aid of standard curves developed using isoflavone and equol standards.

Total sample isoflavone contents varied from 33μg to 139μg/100g of egg yolk (**Figure 3.2**). The total isoflavone average of all samples tested was 93μg/100g of egg yolk. In previous studies, where hens were treated with purified isoflavones, their transfer into the egg yolk was monitored (74), They found that hens fed 124mg of total isoflavones per 100g of feed transferred up to 60μg isoflavones/100g of yolk; while feed concentrations of 180mg and 530mg resulted on 180μg and 360μg of isoflavones/100g of yolk, respectively (73). A regression analysis of this data, determined that a final content of 93μg of isoflavones /100g of yolk required a feed isoflavone concentration of 115mg/100g. This value is high compared to a 16% soybean meal (~37mg of isoflavones/100g feed); it suggests that the soybean meal isoflavone concentrations used in the feed of laying hens which provided the eggs for this study were higher than the ones used by Saitoh et al. (2004) (74).

Equol was found in all egg samples tested and the concentrations varied from 8μg/100g to 47μg/100g. On average, equol concentration comprised 30% of total isoflavone values. This does not agree with the results reported by Saitoh et al. (2004) (73). In that study they stated that equol values were higher than any other isoflavone found and that it comprised more than half of total isoflavones. However, in this present study, each replication was comprised of one carton of eggs (12 individual eggs), Four randomly picked eggs were mixed to better correlate isoflavone contents with specific egg brands; therefore, it can not be stated that all hens were equol producers. Setchell et al. (2002)(19) mentions that only 30% of women can produce equol based on their

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intestinal microflora. This may also be true for poultry. The large standard deviations of equol concentration may suggest strong variations in the equol producing capabilities of poultry. This would support the hypothesis that hens, like humans, have different equol production capabilities.
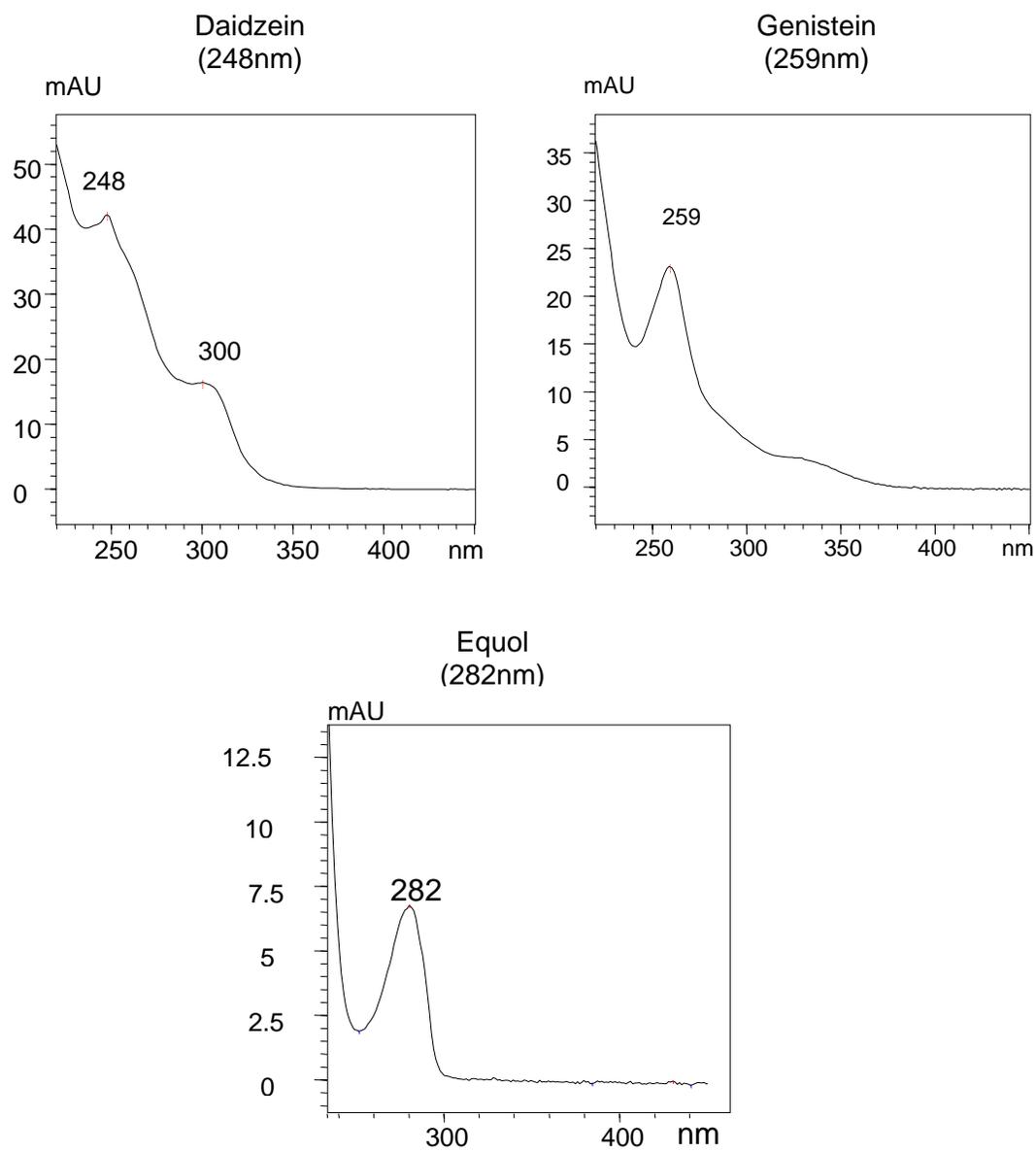
Figure 3.1. Spectral characteristics of daidzein, genistein and equol.
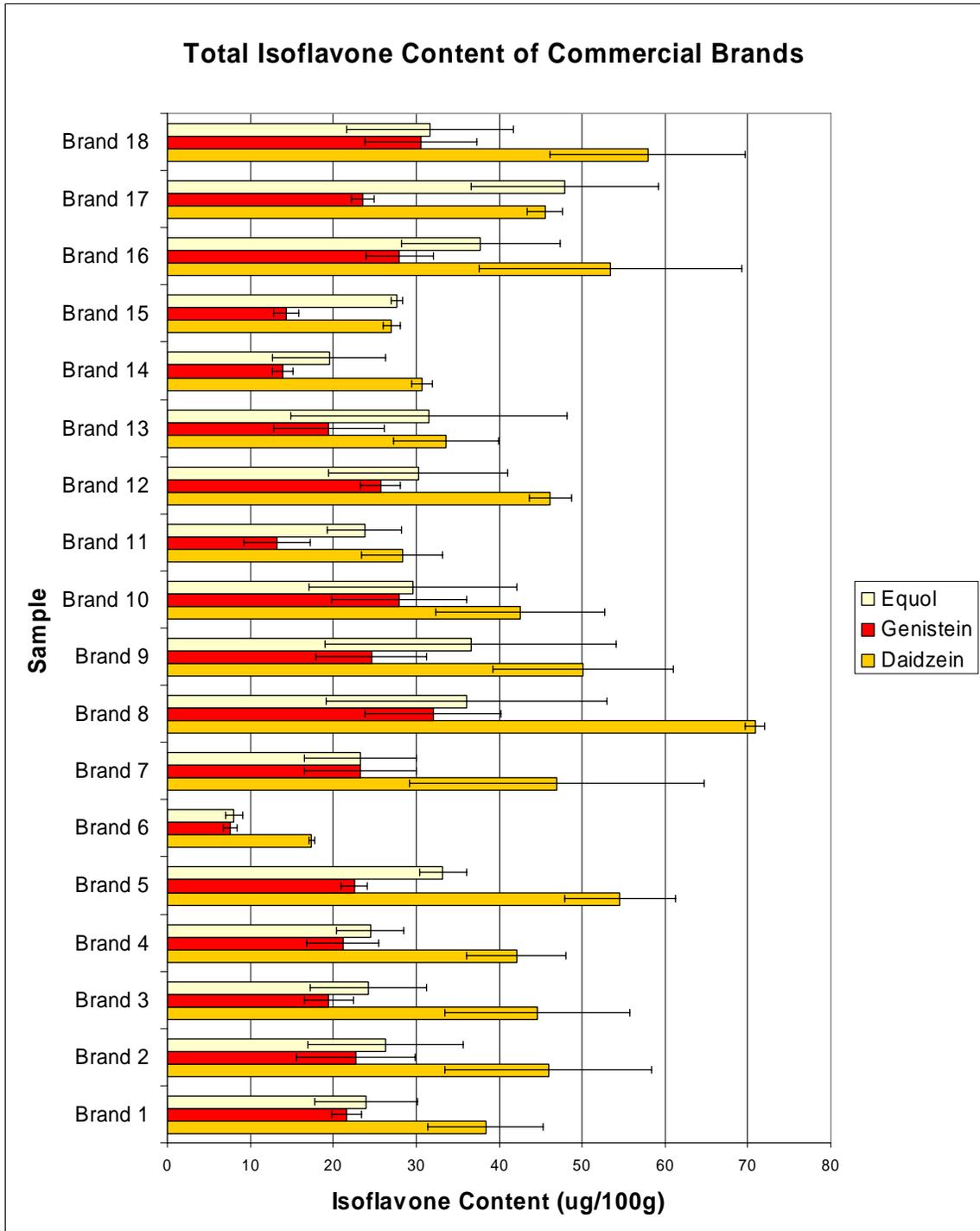
Figure 3.2. Equol, genistein and daidzein content in branded commercial eggs.

3.4.3.   Isoflavone and Equol Content of Eggs with Different Claims

Eighteen different fresh egg products were analyzed (**Table 3.1**). The claims found on the labels varied from hen feeding (vegetable fed hens), to laying hen living conditions (cage free), to increased nutrient content (high omega-3, high vitamin E), to final product processing (pasteurized). As can be observed in **Table 3.1**, some products had several different claims, and the eggs also included white and brown types.

Daidzein, genistein and equol concentrations are presented in **Table 3.2**. and the isoflavone profiles are shown in **Figure 3.3**. Isoflavone concentrations within different claims varied from 33 to 120 μg of isoflavones/100g of yolk. Overall, the results for the various claims did not show any statistical differences. However, high omega-3 values showed lower concentrations.

Eggs with claims of omega-3 fatty acids concentrations higher than 600 mg showed lower concentrations when compared to other egg samples. This is explained by the replacement of soybean meal in the feed to increase omega-3 values to such elevated concentrations. The main contributors of omega-3 fatty acids in poultry feed are flax seed and fish oils. It is reported that flax seed could replace soybean meal in up to 2 to 3 % of feed without any side effects (88); however, obtained values suggest higher replacement of soybean meal. This indicates that fish oils might also have been used as a soybean meal substitute.

	Daidzein (µg/g)		Genistein (µg/g)		Equol (µg/g)		Total Isoflavones (µg/g)	
	Avg.	St Dev.	Avg.	St Dev.	Avg.	St Dev.	Avg.	St Dev.
Organic	41.97 ± 8.48		24.24 ± 4.36		30.73 ± 5.69		96.94 ± 12.84	
Cage Free	41.56 ± 9.73		21.54 ± 5.20		32.19 ± 8.59		95.29 ± 14.93	
Regular White	49.59 ± 7.07		21.00 ± 2.16		28.76 ± 6.33		99.35 ± 9.23	
Regular Brown	58.92 ± 16.94		27.66 ± 6.16		29.68 ± 9.04		116.26 ± 23.10	
Omega 3 (100-350g)	40.98 ± 9.47		21.70 ± 5.53		28.77 ± 5.57		91.45 ± 15.00	
Omega 3 (>600g)	17.42 ± 0.00		7.57 ± 0.00		8.05 ± 0.00		33.03 ± 0.00	
High Vitamin E	38.05 ± 14.24		20.31 ± 8.93		24.67 ± 12.15		83.03 ± 23.17	
Vegetable Fed Hens	40.10 ± 12.57		20.68 ± 7.38		28.48 ± 10.40		89.26 ± 19.95	
Pasteurized	57.91 ± 0.00		30.52 ± 0.00		31.65 ± 0.00		120.09 ± 0.00	

Table 3.2 Isoflavone and equol content in eggs with different claims.
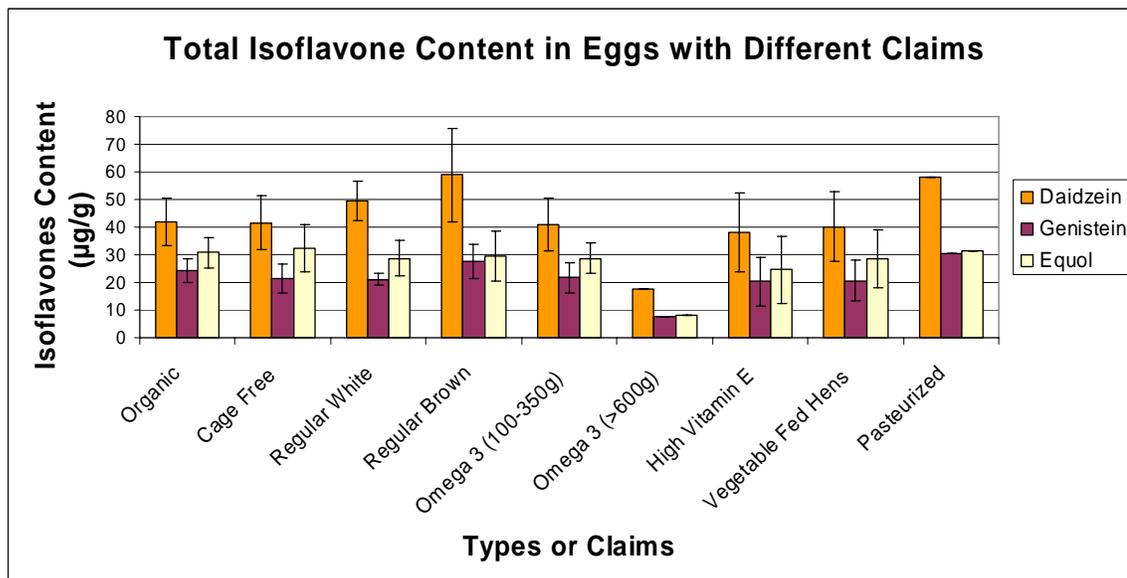
Figure 3.3. Isoflavone content in eggs with different claims

3.5. Conclusion

In summary, isoflavones were found in the egg yolk of all commercial egg samples analyzed in concentrations ranging from 33 to 120 µg of isoflavones/ 100g of egg yolk. Equol, a potent metabolite of daidzein, was present in all samples analyzed; however, total concentrations should not be assigned to a specific hen since the results were obtained from a blend of several eggs from within an egg carton. Fresh eggs with claims to have very high (>600g) omega-3 values, showed significantly lower values of isoflavones when compared to all other samples analyzed probably due to partial replacement of soybean with flax seed or fish oils. Finally, this research project demonstrated that, although in limited concentrations, eggs constitute an additional unexpected source of isoflavones in human diet.

CHAPTER 4

4. DETERMINATION OF ISOFLAVONE TRANSFER FROM THE FEED , EQUOL PRODUCTION AND TOTAL ACCUMULATION IN HEN EGGS AND SPECIFIC TISSUES

4.1. Abstract

Isoflavones are phytoestrogens with potential health benefits. However in some instances their health benefits have been challenged. Consumers are becoming more selective in regard to their dietary intake: some seek soy enhancements, while others prefer foods free of estrogenic compounds. The objective of this research was to evaluate the transfer and accumulation of isoflavones and a metabolite, equol, from different poultry feed formulations into hen eggs and tissues. Hens were subjected to 3 different dietary interventions: a soy free diet, regular soy based diet (25% soybean meal) and a diet enhanced with isoflavones (25% soybean meal plus 5g of 10% isoflavone soy germ /100g feed). Forty eight laying hens were subjected to the dietary interventions for 28 days. Eight cages (two hens per cage) were used per treatment. Eggs were collected over a 28 day period, and evaluated for soy isoflavones and the

isoflavone metabolite, equol. On day thirty, tissues were collected and analyzed.

Isoflavones disappeared within 10 days of soy free diet consumption. Eggs from hens in

the control diet (25% soybean meal) showed an average of 46 µg/100g egg yolk

throughout the study. Finally, a significant increase in isoflavone content was observed in

eggs from hens subjected to the isoflavones enhanced feed with total isoflavone values up

to 998 µg/100g within ten days of treatment. Liver, kidney, heart and muscle isoflavone

contents after the 28 day trial period were 7162 µg/100g , 3355 µg/100g , 272 µg/100g

and 97 µg/100g , respectively. Overall hen health was not affected by any of the

experimental diets. The results showed that isoflavone concentration in hen egg and

tissues can be modulated to produce isoflavone-free eggs or designer eggs by modifying

the diet. This information will be very useful for the industry and consumers concerned

about estrogenic compounds in the diet.

Keywords: animal tissues, isoflavones, equol, transfer.

4.2. Introduction

Isoflavones, main consumed phytoestrogens, are considered relevant in the

treatment of several chronic diseases, such as cancer, cardiovascular disease, and

osteoporosis, among others (27, 43, 53). Isoflavones have also been associated with

negative effects in reproductive functions, mainly in animal studies (68, 69). Isoflavone

hormonal activity is mainly based on their weak interaction with an

estrogen receptor (ER) (2).This receptor is known to transport the ovary-produced

estradiol, which has several similar structural characteristics to those of isoflavones

(12). Isoflavones have also been related to non-hormonal effects such as antioxidant activities, which has created interest not only in humans, but also, in animals (78-80).

There have been twelve main isoflavones found in soybeans and they are all derived from three isoflavone aglycones; genistein, daidzein and glycitein (10) The derivative and aglycone profiles of these isoflavones in different soy foods and ingredients vary depending on the processes they are subjected to (9, 65). However, isoflavones extracted after human and animal consumption will present completely different profiles (19). Isoflavones are hydrolyzed in the intestine, absorbed, and later glucuronidated, sulfated or left as aglycones (20). Most researchers initially hydrolyze isoflavones extracted from human fluids and tissues into aglycones to increase final concentration of the analytes (73-75). An additional compound, equol, is a more estrogenic form of isoflavone (73); however, it has been reported that only 30% of women can produce equol (26).

A few studies have reported the transfer of isoflavones from the feed into the egg yolk of poultry (73-75). Lin and Giusti (2004) (75) analyzed the transfer of encapsulated genistein and genistin into the egg yolks of Japanese quail. Saitoh (2001,2004) (73, 74) studied the transfer of isoflavones from the feed into chicken plasma, egg yolks and isoflavone effects on chicken cholesterol levels. However, they did not determine isoflavone transfer into the organs of the chickens. Also, some studies have analyzed the chemical and physiological effects of soy consumption through the feed in poultry, mainly broiler chickens (78). Increasing isoflavone consumption from 10mg to 20mg /Kg of diet increased the weight gain of chickens. Also, isoflavone ingestion of 40 to 80mg/kg of feed gave increased breast muscle antioxidant activity (78). Other studies, mostly with

pigs, showed that increased isoflavones in the feed result in increased growth rate and carcass muscling(81). Also, Payne et al. (2001) (84) indicated that increased isoflavones in the feed increased carcass leanness and decreased carcass fat. Finally, Jiang et al (2007) (78) indicated that increased isoflavones affected the color of broiler carcasses. All these studies have shown interest in adding isoflavones to the feed of chickens for their appearance and physiological effects but they have not considered analyzing the final isoflavone content of the tissues which may increase isoflavone consumption by humans.

For these reasons, the objective of our research was to monitor the transfer of isoflavones into the egg yolk of laying hens over four weeks of dietary intervention. In addition, the transfer of isoflavones into the liver, heart, muscle and kidneys of the hens were measured. Many of these tissues have considerable amounts of isoflavones after consumption of a source of these substances (68).

4.3. Materials and Methods

4.3.1. Chemicals

Daidzein, glycitein and genistein standards for HPLC-MS identification were purchased from LC Laboratories (Woburn, MA.). Standard purity for isoflavones was 99%. β-glucuronidase Type HP-2 from *Helix pomatia* aqueous solution with 102,000 units/mL was purchased from Sigma Aldrich (St. Louis, MI). Formononetin, used as internal standard was purchased from Acros Organics (Morris Plains, NJ)

Acetonitrile, methanol and water were HPLC-MS grade and were obtained from Fisher Scientific (Fair Lawn, NJ). Sodium acetate, hexane and acetic acid were also obtained from Fisher Scientific.

4.3.2. Animal Trial

Fifty six laying hens were used in this research project. Initially, eight hens on the regular 25% soybean meal feed diet were used as a baseline. For this baseline period, eggs were collected for 5 days and their egg yolks were analyzed. At the end of the baseline period, hens were euthanized by cervical dislocation and tissues (liver, kidney, heart & muscle) were collected. Forty eight laying hens were utilized and divided in three experimental groups. They were randomly distributed into 24 cages properly equipped for laying hens, 2 hens per cage. Of the twenty four cages, eight were given a 25% soybean meal feed; this group was used as the control group. Eight cages were assigned an isoflavones enhanced feed to monitor the effects isoflavone increases. Finally, the remaining eight cages were assigned Cocofeed ® a soy free feed to monitor isoflavone reduction. Cocofeed ®, a coconut based soy free feed was provided by Tropical Traditions (Oklahoma City, OK). The soy free feed was minimally modified to better provide all hens with similar nutrients (0.2% methionine, 3mg/kg riboflavin and 5mg/kg of pantothenic acid were added). The regular 25% soybean meal feed was used as a control and was not modified. Soy germ was donated for the enhanced isoflavone feed by Frutarom (Minneapolis, MN). The isoflavone enhanced feed was prepared by adding 5g of Soylife ® 10 soy germ to every 100g of the regular 25% soybean meal feed. Large batches of the three experimental diets were produced to last for the entirety of the

dietary intervention. The isoflavone concentrations in the feed were measured by the HPLC method mentioned by Lin (2004) (75)

Eggs were collected at the end of each day, but samples were analyzed on days 1, 4, 7, 10, 14, 21, and 28. After the 28 day trial period, hens were euthanized by cervical dislocation and tissues were collected. Each laying hen had approximately eighty square inches of floor space which is considered adequate for laying hens.

4.3.3.   Isoflavone Extraction from the Feed

The method reported by Umpphress et al. (8) was used to quantify isoflavones in the three experimental diets. One gram of feed was weighed and extracted with 10mL of 80% methanol. The samples were placed on a Rotamix® (ATR Biotech, Laurel, MD) for 1 h. The mixture was centrifuged for 20 min and supernatants were collected. The precipitates were re-dissolved in 80% MeOH and placed on the Rotamix® for an additional 30 min. Samples were re-centrifuged and supernatants were pooled. The extracted volume was evaporated to <2mL in a roto evaporator at 40ºC. 10mL of sodium acetate (0.1M, pH 5) and 75µL of β-glucuronidase were added to extracts. The solution was incubated overnight at 37 ºC. Samples were finally purified by a C-18 purification kit (Burdick & Jackson, Muskegon, MI), evaporated and taken to 2mL before injection into the HPLC. Each sample was run in triplicate.

4.3.4. Isoflavone Extraction from the Egg Yolks

Eggs were collected on a daily basis. Eggs yolks from four hens (2 cages) represented one replicate and were mixed and extracted. All samples were subjected to β-glucuronidase treatment to hydrolyze metabolites into their respective aglycones as recommended by Lin and Giusti (2004)(75) and Saitoh et al. (2001,2004) (73, 74),. This would elevate the aglycone concentration in the final samples by avoiding the different isoflavone derivatives (glucuronides and sulfates).

The isoflavone extraction method from the egg yolks was modified from Lin & Giusti (2004) (75) and Saitoh et al. (2001) (73). Isoflavones were extracted by weighing 5g of egg yolks in a 125mL flask, 10 mL of 0.1M sodium acetate buffer was added along with 75μL of enzyme. Samples were left overnight at 37ºC in a Fisher Scientific (Fair Lawn, NJ) Isotemp incubator. After incubation, 30mL of 100% MeOH was added to obtain a final concentration of 75% MeOH. Samples were extracted on a Fisher Scientific isotemp digital stirrer for 2 h. Extracts were centrifuged in a Fisher Scientific Centrific ® Centrifuge at 10,000 rpm for 20 min. Supernatants were collected and precipitates were re-dissolved in 10mL of 75% MeOH. Both supernatants were pooled; 30mL of water was added and lipids were extracted with 30mL of hexane. Solvents were mixed and centrifuged to separate the hexane layer from the remaining lipids from the initial extraction. The MeOH/water layer was collected and concentrated on a Buchi Rotavapor® (New Castle, DE) at 40 ºC. The sample was purified through a C-18 extraction kit by Burdick and Jackson

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(Muskegon, MI). C-18 cartridges were obtained from Waters Corporation (Milford, MA.)

Samples were purified by using 10% acetonitrile in water and recovered with 80%

methanol and a ratio of 80/20 ethyl acetate/MeOH. Purified samples were concentrated in

the Rotavapor®. Finally, extracts were added 20% MeOH until to 2mL, filtered through a

0.45μm syringe polypropylene filter and injected into the HPLC system.

4.3.5. Isoflavone Extraction From Poultry Tissues

The isoflavone extraction process used by Umphress et al. (8) was used. Tissues

were kept frozen at -80ºC from collection until extraction. A few grams of tissue sample

was frozen by liquid nitrogen and blended. One gram of the pulverized sample was

weighed and extracted with 10mL of 80% methanol. The samples were placed on a

Rotamix® (ATR Biotech, Laurel, MD) for one hour. The mixture was centrifuged for 20

minutes and supernatants were collected. The precipitates were re-dissolved in 80%

MeOH and placed on the Rotamix® for 30 minutes. Samples were re-centrifuged and

supernatants were pooled. The extracted volume was evaporated to <2mL in a roto

evaporator at 40ºC. 10mL of sodium acetate (0.1M, pH 5) were added to extracts and

75μL of β-glucuronidase. The samples were then incubated overnight at 37 ºC. The

samples were finally purified by a C-18 purification kit, evaporated and 20% MeOH was

added until 2mL before injection into the HPLC.

4.3.6. HPLC Analyses

A Shimadzu Prominence® LC-20 A$_5$ reverse phase high pressure liquid chromatography system coupled with a Shimadzu Prominence® SPD-M20-A diode array detector, a Shimadzu Prominence® SIL20-AC auto sampler and a Shimadzu LCMS Solution software was used. Sample elution was obtained by use of a Waters Corporation 4.6 x150mm Symmetry® 3.5 μm C-18 column. Elution was monitored at 254nm for daidzein, genistein and glycitein and at 282 for equol. Spectral data was obtained from 200 to 450nm. The gradient used to separate all four compounds of interest was a very slight modification from the method proposed by Saitoh et al. (2001): 15%B from 0-1 min, 15-27.5%B from 1-5 min, 27.5 to 32.5% from 5-40min and 32.5 to 15% from 40-41 min..

4.3.7. Statistical Analysis

Statistical analysis was developed by using Microsoft Excel's 97 statistical package. Data is presented as averages ± standard deviations. Two cages (four hens) were considered to be one experimental unit, in order to reduce sample variability.

4.4. Results and Discussion

4.4.1. Isoflavone Content of Experimental Feeds.

Three different feeds were used in this study. Isoflavone compositions of the experimental diets are presented in **Table 4.1.** As previously presented by Song et al

(2003) (66) soy germ glycitein concentration is much higher than that of genistein. Total isoflavone concentrations in their study averaged 2g/100g of soy germ. Glycitein comprises almost 40% of total isoflavones (66). According to Saitoh et al (2001) (74), a 16% soybean meal contains an average of 37 mg of isoflavones / 100g of feed.

The results showed that the soy free feed had very small concentrations of isoflavones. This can be explained by the findings of Liggins et al. (2000) (89) who reported low isoflavone concentrations in fresh coconut with values of 12.8 and 18.5 µg/100g of dry fruit for daidzein and genistein respectively. The 25% regular soybean meal feed contained 77mg of isoflavones / 100g of feed. The isoflavone enhanced feed presented 573 mg of isoflavones / 100g of feed.

4.4.2. Initial Isoflavone Concentration in Eggs

Feed consumption averaged 116g/day , 117 g/day, and 112 g/day for the soy free feed, regular feed and isoflavone enhanced feed, respectively. No health abnormalities were noticed on the subjects throughout the experimental period. Egg weights did not show significant differences across the different diets.

Egg yolk samples for one replicate (2 cages = 4 hens) were pooled at the beginning of the extraction process. Yolk samples had daidzein and genistein concentrations of 35.5 ± 0.01µg / 100g of egg yolk and 26.6 ± 2.25µg / 100g of egg yolk, respectively. This provided a total isoflavone concentration of 62.1 ± 2.26µg / 100g of egg yolk. During the baseline period, egg yolk samples did not have quantifiable levels of glycitein or equol.

Isoflavone	Cocofeed ®		Regular		Isoflavone Enhanced		Soylife ®10	
	mg/ 100g	%	mg/ 100g	%	mg/ 100g	%	mg/ 100g	%
Daidzein	3.23 (1.15)	40.2	42.18 (10.95)	54.5	362.97 (73.18)	63.4	5.67 (0.37)	55.3
Glycitein	0.95 (0.54)	11.8	14.78 (4.87)	19.1	181.04 (41.17)	31.6	4.00 (0.93)	39.0
Genistein	3.85 (1.27)	48.0	20.49 (3.85)	26.4	28.83 (9.70)	5.0	0.59 (0.08)	5.7
Total Isoflavones	8.03 (2.95)		77.44 (19.67)		572.85 (124)		10.25 (1.25)	

Table 4.1. Isoflavone content in experimental feeds and Soylife®10. (n = 3). Numbers in parenthesis ( ) are standard deviations.

4.4.3. Isoflavone Transfer from the Feed into Egg Yolks of Laying Hens.

Measurements were taken at 254 nm for the isoflavones daidzein, genistein and glycitein and at 282nm for equol. Equol's absorbance in the UV-range is very limited; however, relevant concentrations were obtained and allowed us to quantify this analyte. The limit of quantitation was considered as 10x the standard deviation of the noise.

On day one, daidzein and genistein were the only isoflavones with quantifiable levels in eggs from hens subjected to all three experimental diets, which agrees with the data presented in the baseline period.

Dietary intervention had a significant impact on the isoflavone concentrations in hen eggs. **Figure 4.1**. shows the egg yolk isoflavone concentration changes through the experimental period. Egg yolks of hens provided with the soy free diet, showed a rapid decrease of isoflavone concentration. From an initial isoflavone content of 52μg ± 0.73/100g it quickly diminished until at day 7, the concentration reached individual aglycone undetectable levels. The control feed isoflavone concentrations (25% soybean meal) remained stable throughout the experimental period averaging 46 ± 7 μg/100g The isoflavone enhanced treatment increased rapidly throughout the first days of the treatment and seemed to reach a plateau right below 1000μg/100g (day 7). These results are different from the ones presented by

Saitoh (2001) (74) where he stated that isoflavone concentrations continued to increase until day 12 and then the value was maintained.

Table 4.2 shows the day 10 profile of the isoflavone content in egg yolks of hens subjected to the dietary intervention. The concentrations presented represent the control feed and the isoflavone enhanced feed. The eggs from hens subjected to the isoflavone enhanced feed had very high values of daidzein and glycitein and lower values of genistein. This correlates well with the feed content since glycitein is more abundant in soy germ than genistein. Also, genistein has been shown to be more metabolized compared to other isoflavones (85,86). No isoflavones were quantifiable in the egg yolks of hens limited to the soy free diet on day 10.

4.4.4.   Isoflavone Transfer into Hen Tissues

This is the first study to show laying hen tissue isoflavone concentrations after dietary supplementation with an isoflavone enhanced feed (**Table 4.3**). The four tissues selected for this analysis, liver, heart, kidney and muscle were chosen because previous reports of isoflavone transfer in ewes (liver, kidney) (68) and because of their role in human diet. These tissues can prove to be significant sources of isoflavones in the diet.
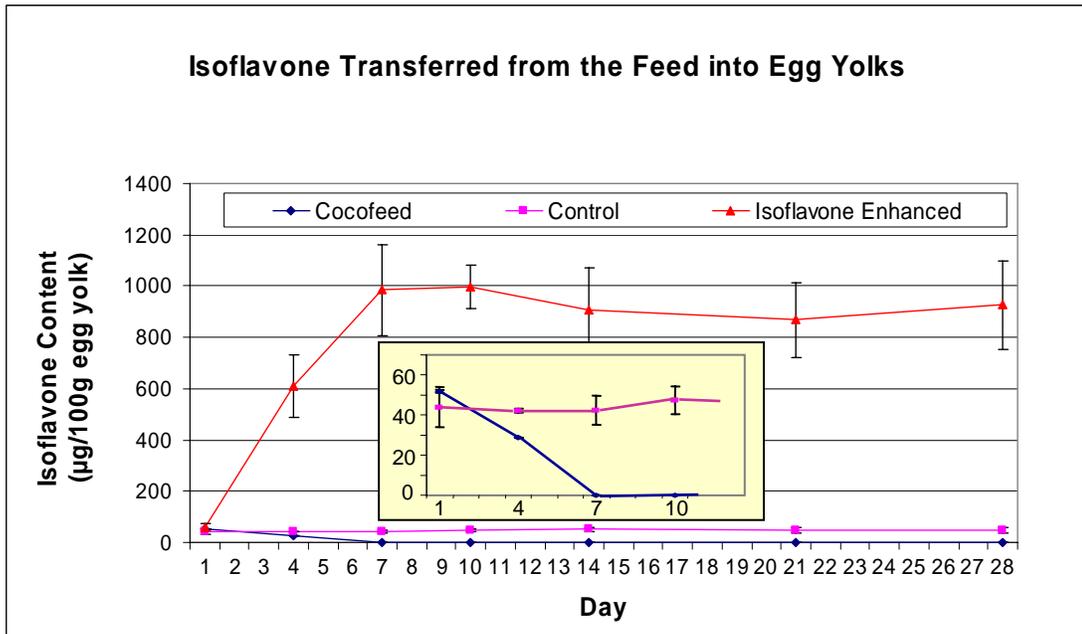
Figure 4.1. Isoflavone transfer from feeds into egg yolks of laying hens over a 4 week

dietary intervention.

Isoflavone	Isoflavone Enhanced Feed (µg / 100g)	Control Feed (µg / 100g)
Daidzein	277.12 ± 30.06	30.87 ± 3.83
Glycitein	194.93 ± 16.81	NQ
Genistein	41.66 ± 5.40	16.45 ± 3.19
Equol	484.23 ± 33.50	NQ
Total Isoflavones	997.94 ± 85.77	47.32 ± 7.02

(NQ = detected but not quantifiable)

Table 4.2. Isoflavone profile in the egg yolks of laying hens after 10 days of dietary intervention.

Isoflavone	Isoflavone Enhanced				Control
	Liver µg/100g	Kidney µg/100g	Heart µg/100g	Muscle µg/100g	Liver µg/100g
Daidzein	3406 (1729.8)	1319 (142.9)	206 (37.7)	97 (29.0)	312 (0.9)
Glycitein	1633 (954.4)	414 (38.5)	66 (7.6)	NQ	137 (8.1)
Genistein	455 (160.7)	147 (20.7)	NQ	NQ	115 (20.6)
Equol	1668 (650.2)	1475 (290.8)	NQ	NQ	255 (120.1)
Total Isoflavones	**7162** (3495.1)	**3355** (492.9)	**272** (45.3)	**97** (29.0)	**819** (150.3)

(NQ = detected but not quantifiable)

Table 4.3: Isoflavone content of tissues of laying hens subjected to the dietary

treatments. Numbers in parenthesis ( ) are standard deviations.

Laying hens subjected to the soy free diet (Cocofeed®) did not show any quantifiable levels of individual isoflavones in their tissues. The control 25% soybean meal feed showed an average of 819 ± 150 µg/100g of total isoflavones in the liver (**Table 4.3**). Of this value, genistein only comprised 14% of the total isoflavones compared to the 26% genistein content in the regular control feed. This is consistent with previously documented extensive bacterial degradation of genistein in comparison with the other present isoflavones (85, 86).

Total isoflavone content in the liver averaged 7162µg/100g and showed large variability. The liver is one of the initial places were isoflavones reside after being absorbed through the intestine (20, 87). Biliary excretion is usually the fate of isoflavones (24); so, increased concentration in the liver should be expected. The large group variability observed, in addition to individual variability, can be attributed to the some hens feeding before tissue collection; therefore liver isoflavone concentration values may be artificially increased in some subjects.

Kidney isoflavone values averaged 3355µg/100g. This tissue, contrary to the data presented by Urpi-Sarda, et al. (2007) (68). In their study regarding ewe red clover consumption, did not contain higher concentrations of isoflavones when compared to the liver. The kidney contained half of the liver isoflavone concentration. Their study presented kidney isoflavone values around 1.25 times that of isoflavones in the liver. However, the high isoflavone content found in the kidney is in agreement with its main purpose of filtering compounds in the body before excretion.

Equol percentages of total isoflavone content varied significantly in the liver and kidney. Equol made up 23% of the total isoflavones in the liver in comparison with 31%

in the kidney. Also, when comparing equol to daidzein contents, equol surpassed

daidzein concentration in the kidney, becoming the most abundant isoflavone in the

tissue. This agrees with the results reported by Urpi-Sarda et al.(2007) (68). Equol is a

daidzein metabolite; therefore, for equol to have larger concentrations, daidzein

concentrations have to diminish. Also, before isoflavones reach the kidney, they may

have been subjected to further degradation than when isoflavones reach the liver, since

isoflavones can be absorbed in the small intestine, before equol is produced (26).

Isoflavone content in the heart and muscle samples were very limited. Genistein

and equol concentrations were less than the quantitation limits, Glycitein was also not

quantifiable in the muscle.

4.5. Conclusions

The results of this study have shown that isoflavones are present in the egg yolks

and tissues of laying hens when the hen is subjected to a regular 25% soybean meal feed

or an isoflavones enhanced feed. Isoflavone concentrations in egg yolks, liver and

kidneys of laying hens can be successfully increased by supplementing the diet with an

isoflavone rich source. Egg yolk isoflavone concentrations reached a maximum between

day 7 and day 10 of the dietary intervention. Equol, the potent isoflavone metabolite of

daidzein, was the most abundant isoflavone in the egg yolk and kidneys of laying hens. In

the kidney, equol concentration was similar to that of daidzein. From tissues analyzed,

the highest levels of isoflavone transfer were found

in the liver followed by the kidney. A soy free diet can result in hen eggs and tissues

without any quantifiable levels of isoflavones. Finally, this study demonstrated that

isoflavone concentrations in egg yolks can be modulated to produce designer eggs having an additional unexpected source of isoflavones.

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