UNIVERSITY OF PADOVA

DEPARTMENT OF COMPARATIVE BIOMEDICINE AND FOOD SCIENCE

DOCTORAL COURSE IN VETERINARY SCIENCE

XXIX CYCLE

EFFECTS OF DIETARY SOY ISOFLAVONES ON RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

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PUBLICATIONS ASSOCIATED WITH THIS THESIS

Journal Articles

Pastore M.R., Negrato E., Poltronieri C., Barion G., Messina M., Tulli F., Ballarin C., Maccatrozzo L., Radaelli G., Bertotto D., 2018. Effects of dietary soy isoflavones on estrogenic activity, health and growth in rainbow trout, *Oncorhynchus mykiss*. Aquaculture Research, 49, 1469–1479. https://doi.org/10.1111/are.13602

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Conferences

Pastore M.R., Bertotto D., Poltronieri C., Negrato E., Messina M., Tulli F., Radaelli G. *In vivo* effects of dietary isoflavones in rainbow trout (*Oncorhynchus mykiss*). Aquaculture Europe 2016 Edinburgh, Scotland 20-23 September 2016.

ABSTRACT

A balanced diet and an appropriate feeding are the two most important requirements of aquaculture. Research on feed, quality control and biological evaluation are important to formulate correct diets because without correct and suitable feeding, fish are unable to stay healthy and productive. Furthermore, quality and composition of feeds deeply affect the nutritional and organoleptic characteristics of the final product. In the last years, fish meal has been gradually and partially substituted by plant-based products, because of their improved sustainability and lower costs.

Soybean meal is the principal vegetable protein source, due to its high protein content, good amino acid profile, high digestibility, low cost, consistency and availability. Soybean meal contains isoflavones which are polyphenolic compounds (genistein, daidzein, glycitein) that, as phytoestrogens, may act as estrogen receptor agonists or antagonists causing biological activity on fish in addiction to possible changes on the fillet quality. The potential effects of isoflavones may affect the soybean meal utilization as an alternative protein source in aquaculture feed. In the meantime, it is important, for consumer safety, to evaluate the potential amount of the isoflavones in edible tissue and their antioxidant effect that could enhance the product shelf life.

Our objective was to examine, on the one hand, the effect of dietary isoflavones on growth, reproduction and health in rainbow trout (*Oncorhynchus mykiss*), and on the other hand, on the quality of fish food.

Rainbow trout were fed three experimental diets containing different concentrations of isoflavones (0, 500, 1500 ppm) for 70 days. Growth, estrogenic activity expressed by vitellogenin (VTG) protein levels in plasma and liver, plasma 17β-estradiol (E_2) levels, gonadal development, state of stress and histological changes in various tissues were evaluated in a subsample of the animals at the end of feeding trial. Complementary analyses on growth performance, proximate composition,

lipid oxidation and isoflavone deposition were carried out on the remaining part of the animals for each treatment, after being stored at 4 °C for 1 and 7 days.

Neither growth performance nor the relative mRNA levels of the liver Insulin Growh Factor I (*Igf*-I) were influenced by different levels of dietary isoflavones. Plasma and liver VTG protein levels and plasma E2 were unaffected by the treatments and the correlation between E2 plasma levels and VTG densitometry values was significant ($p < 0.05$). The fish gonadosomatic index (GSI) did not significantly differ among the three experimental groups but correlated with plasma VTG densitometry values ($p < 0.05$). Plasma, muscle and fin cortisol concentrations fell within the normal welfare range and were not correlated to isoflavone levels. Histologically, the distal intestine showed a normal morphology with well-differentiated enterocytes, as well as the liver showed normal hepatocytes. A supranuclear accumulation of lipid droplets in enterocytes and some lipid droplets in hepatocytes were observed in all the tested groups, suggesting an impact of basal dietary lipid on transport/metabolism of fat in the fish.

Inclusion and different levels of isoflavones in diets did not affect the performance of trout used for final product analysis either. Indeed, trout showed similar body weight at caught, 337 g on average, and an average gutted body weight of 299 g. Biometric indexes, skin and fillet colour, rheological characteristics, proximate composition and fatty acids profile of fillet were not affected by the different dietary treatments. Otherwise, time of storage reduced fillet yield (56.4 *vs*. 53.6%; $p < 0.01$), and skin lightness (59.2 *vs*. 51.5; $p < 0.001$); red index moved to more negative values ($p < 0.001$) and yellow index decreased (6.99 *vs*. 5.07; $p < 0.001$). Fillet pH (6.22 *vs*. 6.34; $p < 0.001$) and lightness increased (38 *vs*. 43.6; $p < 0.001$) while yellow index (6.20 *vs*. 4.52; $p < 0.001$) and shear force decreased (0.94 *vs*. 0.80 g kg^{-1}; $p < 0.001$). According to the time of storage, trout fillet showed an increase of water, a loss of crude protein and an increase of total volatile basic nitrogen content (19.3 *vs*. 21.2 mg 100 g^{-1}; $p < 0.001$). As a result of PUFAs omega-6 decrease (13.1% *vs*. 12.7%; $p < 0.05$), omega-3/omega-6 ratio increased (1.05 *vs*. 1.20; $p < 0.05$) during storage. The results evidenced

an accumulation of isoflavones on trout fillets, even if isoflavone concentration in fillets was not significantly influenced by the content of dietary isoflavones in both storage times.

Lipid peroxidation expressed as thiobarbituric acid reactive substances (TBARS) levels in fillet on day 1 of refrigeration was significantly higher than that at day 7 ($p < 0.05$) but no difference resulted among groups fed different isoflavone levels, evidencing no effect of feed soy isoflavones on fillet lipid oxidation.

Overall, these results suggest that the isoflavone tested doses do not compromise rainbow trout reproduction, growth and health; although a moderate transfer of isoflavones from diet to fillet was observed, quality, technological and nutritional characteristics and lipid oxidation, were not affected by dietary treatment. Our data demonstrate that, with regard to isoflavone contents and their potential effects, soybean meal can replace fish meal in rainbow trout diets at a high level with no negative effects on fish performance and final product quality.

RIASSUNTO

Una dieta bilanciata e un'appropriata alimentazione sono le due maggiori richieste da parte dell'acquacoltura. Gli studi scientifici sui mangimi, il controllo qualità e la valutazione dell'aspetto biologico sono importanti per formulare una corretta e idonea dieta per le specie ittiche. Senza un'adeguata alimentazione, il pesce non può crescere nei tempi stabiliti e mantenersi in salute, oltre al fatto che la qualità e la composizione dei mangimi influenzano profondamente le caratteristiche nutrizionali e organolettiche del prodotto finale.

Negli ultimi anni, la farina di pesce è stata sostituita gradualmente e parzialmente dai prodotti derivati dalle piante, per il loro basso costo e la loro sostenibilità rispetto alla farina di pesce. La farina di soia è la principale risorsa proteica vegetale per l'alimentazione animale, per il suo elevato contenuto proteico, il buon profilo amminoacidico, l'elevata digeribilità, il costo ridotto, nonché l'ampia disponibilità.

La farina di soia contiene isoflavoni (genisteina, daidzeina, gliciteina) che sono composti polifenolici nonché fitoestrogeni ed in quanto tali possono agire come agonisti o antagonisti dei recettori degli estrogeni endogeni, agendo biologicamente sulla specie ittica e modificando le caratteristiche qualitative del prodotto finale. I potenziali effetti degli isoflavoni possono influenzare l'utilizzo della farina di soia come risorsa proteica alternativa nei mangimi per pesci. Risulta importante valutare per la sicurezza del consumatore anche il potenziale ammontare degli isoflavoni nel tessuto edibile della specie alimentata con la soia, oltre che il loro effetto antiossidante che potrebbe aumentare la shelf life del prodotto.

Il nostro obiettivo si prefiggeva, da un lato, di esaminare l'effetto degli isoflavoni della soia sulla crescita, la riproduzione e la salute della trota iridea (*Oncorhynchus mykiss*), dall'altro, di valutare la qualità della carne del pesce. Le trote sono state alimentate con tre diete contenenti differenti concentrazioni di isoflavoni (0, 500, 1000 ppm) per un tempo di 70 giorni. Al termine della

prova di alimentazione, una parte delle trote sperimentali sono state utilizzate per valutare la crescita, l'attività estrogenica (espressa in livelli di proteine del tuorlo nel plasma e nel fegato), i livelli plasmatici di estradiolo, lo sviluppo gonadico, lo stato di stress e i cambiamenti istologici nei vari tessuti. Analisi complementari su performance di crescita, composizione prossimale, ossidazione lipidica e deposito degli isoflavoni nel filetto sono state effettuate sulla rimanente parte delle trote, dopo la loro conservazione a 4°C, per 1 e 7 giorni.

I diversi dosaggi di isoflavoni non hanno influenzato né la performance di crescita né le concentrazioni di mRNA del Fattore di Crescita Insulino Simile (*Igf*-I) nel fegato. I livelli della vitellogenina (VTG) in plasma e fegato e di estradiolo plasmatico (E_2) non sono stati condizionati dalle diete utilizzate, inoltre la correlazione tra livello plasmatico di E_2 e i valori densitometrici della VTG si è rivelata significativa ($p < 0.05$). L'indice gonadosomatico (GSI) delle trote non ha mostrato differenze tra i tre gruppi sperimentali ed è risultato correlato con i valori densitometrici della VTG plasmatica ($p < 0.05$). Le concentrazioni di cortisolo rilevate in plasma, muscolo e pinna si sono collocate in un range di valori bassi, compatibili con una condizione di benessere, e non hanno mostrato correlazioni con il livello di isoflavoni nelle diete. Dal punto di vista istologico, l'intestino distale presentava una morfologia normale con enterociti ben differenziati, così come il fegato che, a sua volta, mostrava epatociti normali. In tutti i gruppi testati, si è evidenziato un accumulo sopranucleare di gocce lipidiche che suggerisce un effetto dei lipidi della dieta basale sul trasporto/metabolismo dei grassi nel pesce.

L'inclusione di differenti livelli di isoflavoni nelle diete non ha influenzato nemmeno la performance delle trote utilizzate per l'analisi del prodotto finale in momenti diversi durante la conservazione. Infatti, le trote mostravano un simile peso del corpo alla cattura, con una media di 337 g, ed una media del peso del corpo eviscerato di 229 g. Gli indici biometrici, il colore della pelle e del filetto, le caratteristiche reologiche, la composizione chimica e il profilo acidico dei grassi del filetto non sono stati modificati dai diversi trattamenti alimentari. D'altro canto, il tempo di conservazione ha ridotto significativamente la resa dei filetti (56.4 *vs*. 53.6%; $p < 0.01$) e la luminosità

della pelle (59.2 *vs* 51.5; $p < 0.001$), l'indice del rosso ha mostrato valori più negativi ($p < 0{,}001$) e l'indice del giallo è diminuito (6.99 *vs*. 5.07; $p < 0.001$). Il pH del filetto (6.22 *vs*. 6.34; $p < 0.001$) e la sua luminosità sono aumentati (38 *vs*. 43.6; $p < 0.001$), mentre l'indice del giallo (6.20 *vs*. 4.52; $p < 0.001$) e lo sforzo di taglio sono diminuiti (0.94 *vs*. 0.80 g kg^{-1}; $p < 0.001$).

Sempre sulla base del tempo di conservazione, il filetto ha mostrato un aumento della percentuale d'acqua, una perdita in proteine ed un aumento del contenuto di azoto basico volatile (19.3 *vs*. 21.2 mg 100 g^{-1}; $p < 0.001$). Durante la conservazione, inoltre, è risultata una diminuzione degli acidi grassi polinsaturi (PUFA) omega-6 (13.1% *vs*. 12.7 %; $p < 0.05$) e un aumento del rapporto omega-3/omega-6 (1.05 *vs*. 1.20; $p < 0.05$). I risultati hanno evidenziato, per entrambi i tempi di conservazione, un accumulo di isoflavoni nei filetti di trota, anche se la concentrazione non è stata influenzata significativamente dal contenuto degli isoflavoni presenti nelle diete. L'ossidazione lipidica, espressa come livelli di TBARS (thiobarbituric acid reactive substances), dei filetti conservati dopo 1 giorno di conservazione a 4 °C, è risultata significativamente più alta che al giorno 7 ($p < 0.05$), ma nessuna differenza si è riscontrata tra i gruppi alimentati con diversi livelli di isoflavoni, evidenziando nessun effetto da parte degli isoflavoni della soia sull'ossidazione lipidica del filetto.

I risultati ottenuti sembrano indicare che gli isoflavoni, alle dosi testate, non compromettano la riproduzione, la crescita e la salute della trota; anche se si è osservato un moderato trasferimento degli isoflavoni dalla dieta al filetto, la qualità, le caratteristiche nutrizionali e l'ossidazione lipidica non risultano influenzati dal trattamento alimentare. In merito al contenuto di isoflavoni nella farina di soia e al loro potenziale effetto, i nostri risultati dimostrano che la farina di pesce può essere sostituita dalla farina di soia nelle diete somministrate alle trote senza aver effetti negativi sulla performance e sulla qualità del prodotto finale.

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CHAPTER 1

GENERAL INTRODUCTION

1.1. Overview of global fish production

World fish production has grown in the last 50 years (Figure 1.1) (FAO, 2016). Fish and shellfish are an important resource for global human food consumption. Global *per capita* fish consumption increased from an average of 9.9 kg in the 1960s to 19.2 kg in 2012 (FAO, 2016). This development has been driven by different factors: population growth, rising incomes and urbanization, in addition to the strong expansion of fish production and more efficient distribution channel (FAO, 2016).

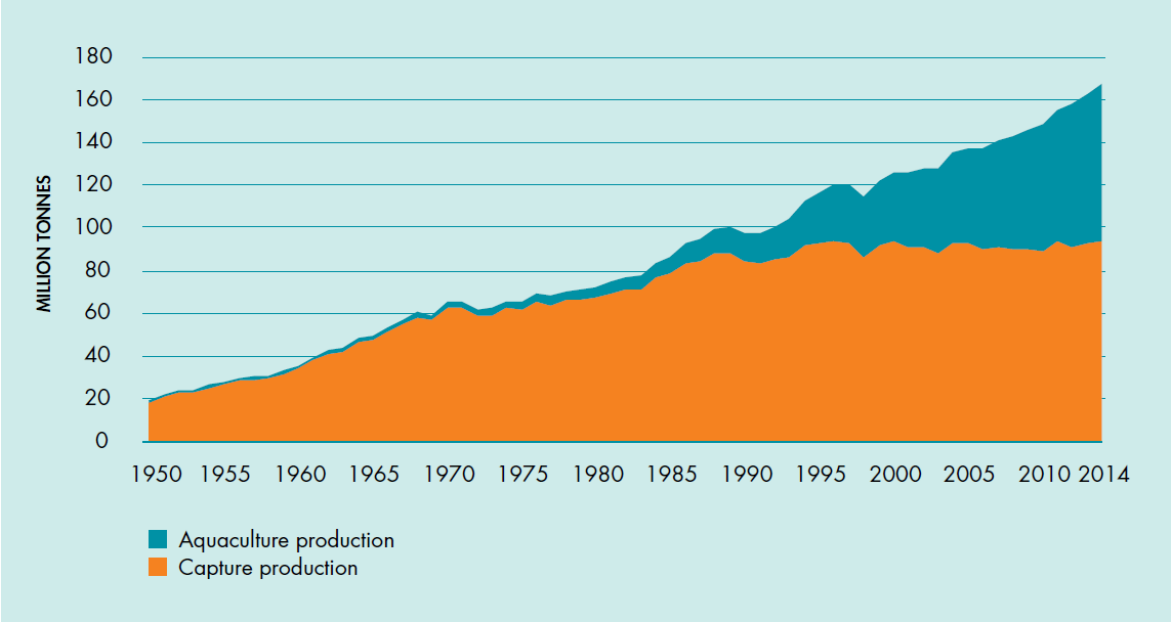

Figure 1.1. World capture fisheries and aquaculture production (FAO, 2016).

Total capture fishery production in 2014 was 93.4 million tonnes, divided in 81.5 million tonnes of marine waters and 11.9 million tonnes of inland waters. China is the major producer for

marine fisheries followed by Indonesia, the United States of America and the Russian Federation (FAO, 2016).

Aquaculture production of aquatic animals in 2014 amounted to 73.8 million tonnes, comprising 49.8 million tonnes of finfish, 16.1 million tonnes of molluscs, 6.9 million tonnes of crustaceans and 7.3 million tonnes of other aquatic animals. Almost all fish produced from aquaculture are destined for human consumption, although by-products may be used for non-food purposes. China accounted for 45.5 million tonnes in 2014, or more than 60% of global fish production from aquaculture, followed by India, Viet Nam, Bangladesh and Egypt (FAO, 2016).

Fish production used for direct human consumption increased from 71% in the 1980s to more than 86% (136 million tonnes) in 2012, with the remainder (21.7 million tonnes) destined to non-food uses (e.g. fish meal and fish oil) (FAO, 2016).

The state of the world's marine fish stocks has not improved overall. FAO (2016) indicated that fish stocks, within biologically sustainable levels, decreased from 90 % in 1974 to 68.6 % in 2013 (FAO, 2016). The increasing trend of marine capture fisheries in the percentage of overexploited, depleted and recovering stocks and the decreasing trend in underexploited and moderately exploited stocks is cause for concern (FAO, 2016). Management actions (e.g. FAO, 2012; EU, 2014), have achieved measurable reductions in exploitation rates in some regions, but a significant fraction of stocks will remain fully fished nonetheless there are further reductions in exploitation rates (Worm *et al*., 2009). Moreover, the use of wild fish in the form of fish meal and fish oil for producing aquaculture feeds, relies on marine species that are renewable, but often overexploited for human use (Klinger and Naylor, 2012).

Fishery capture has reached its potential and the increasing global demand for fishery products suggests that aquaculture must supply the world demand for fish protein into the future (Brugere and Ridler, 2004). In fact, a decline of capture fisheries (Watson and Pauly, 2001; Garcia and Grainger, 2005) associated to an increase of average annual *per capita* consumption (Brugere and Ridler, 2004) induce that the demand on aquaculture production will be even greater. In this context, Cao *et al*.

(2015) state that a key question for the future of the oceans is how aquaculture is going to be developed relieving pressure on wild fisheries.

1.2. Aquaculture and fish feed

World aquaculture production of fish accounted for 44.1% of total production (including for non-food uses) from capture fisheries and aquaculture in 2014, up from 42.1% in 2012 and 31.1% in 2004 (Figure 1.2). All continents have shown a general trend of an increasing share of aquaculture production in total fish production (FAO, 2016).

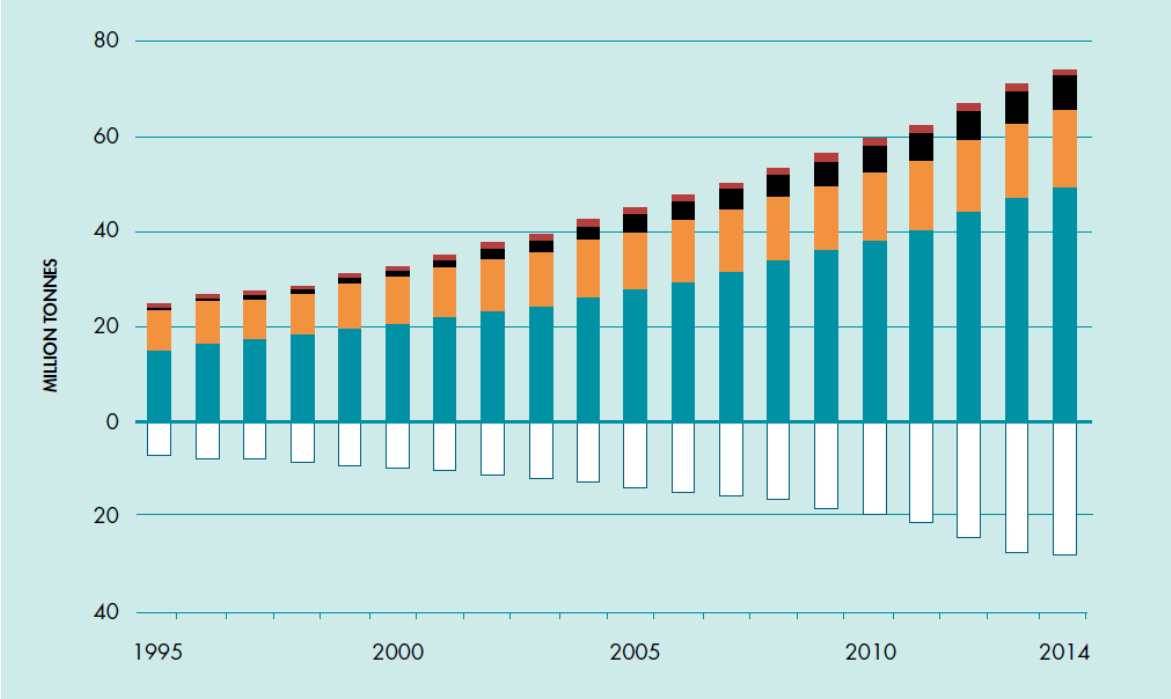

Figure 1.2. World aquaculture production volume and value of aquatic animals and plants (1995-2014) (FAO, 2016).

A significant, but declining, proportion of world fishery production is processed into fish meal and fish oil, used as feed in aquaculture and livestock raising. Fish meal is the crude flour obtained after milling and drying fish or fish parts, while fish oil is liquid obtained through the pressing of the cooked fish. These products can be produced from whole fish, fish remains or other fish by-products

resulting from its processing. Many different species are used for fish meal and fish oil production especially anchoveta, the main groups of species utilized. Fish meal and fish oil production fluctuates according to changes in the catches of these species. Fish meal production peaked in 1994 at 30.1 million tonnes declining in 2014 at 15.8 million tonnes caused by reduced catches of anchoveta. The decreasing fish meal and fish oil production associated to their high prices, makes necessary that alternative sources for fish feed are being explored (FAO, 2016).

Fish meal prices are expected to remain high in the long term because of sustained demand. Increasing request of fish meal from the world of aquaculture may actually increase the exploitation of wild fishery stocks. Fish production using little or no fish meal in feed can reduce wild fish pressure (Naylor *et al*., 1998).

Fish meal is the most important protein for aquaculture feed, providing high quality protein, essential fatty acids, minerals in addition to high palatability (Li *et al*., 2006). Fish generally requires diet containing 25–55% of protein, in base of species and maturity (NRC, 2011). Salmonids have the highest protein demand: 40–50% (or even higher) (Hardy, 1996) and their diet contain about 40–60% of fish meal (Gillund and Myhr, 2010). Furthermore, Essential Amino Acids (EAA) are very important in diet and deficiencies can lead to reduced protein utilization affecting growth and feed efficiency ratio (Anderson *et al*., 1992).

By 2008, 60% of global fish meal supplies were used in aquaculture and a large part of this fish meal has been used to feed salmon, trout ad shrimp, despite the fact that these species are the only 7% of world aquaculture production (Hardy, 2000). In aquaculture, feeding costs represent the highest amount of total expenses, even up to 70% (Thompson *et al*., 2008). In carnivorous species, such as salmonid from 1.35 to 5.16 kg of feed is needed to produce 1.0 kg of farmed fish (Boyd *et al*., 2007); this makes up around 45% of expenses (Meyers, 2009; Martinez-Llorens *et al*., 2008) showing that protein is the costliest component in aquaculture feed (Nguyen *et al*., 2009).

On average, for every 1.0 kg of farmed fish produced, about 0.7 kg of wild fish is consumed (Tacon and Metian, 2009). This average value, however, masks essential differences, indeed the rate

for omnivorous farmed fish, is included in a level from 0.2 to 1.41 kg of wild fish per 1.0 kg of farmed fish, while, the value is higher for carnivorous farmed fish: from 1.35 to 5.16 kg to produce 1.0 kg of farmed fish (Boyd *et al*., 2007).

The limited source of fish meal, the overfishing of wild fish, the increasing demand and expenses have induced researchers to conduct studies on plant protein sources to evaluate the possibility of replacing partially or totally fish meal in diets. Differently from animal protein, plant protein sources are easy to obtain and their cost per unit is generally lower compared to fishmeal (Figure 1.3). Nowadays, fish diets contain portions of soybean meal and corn (Hardy and Tacon, 2002).

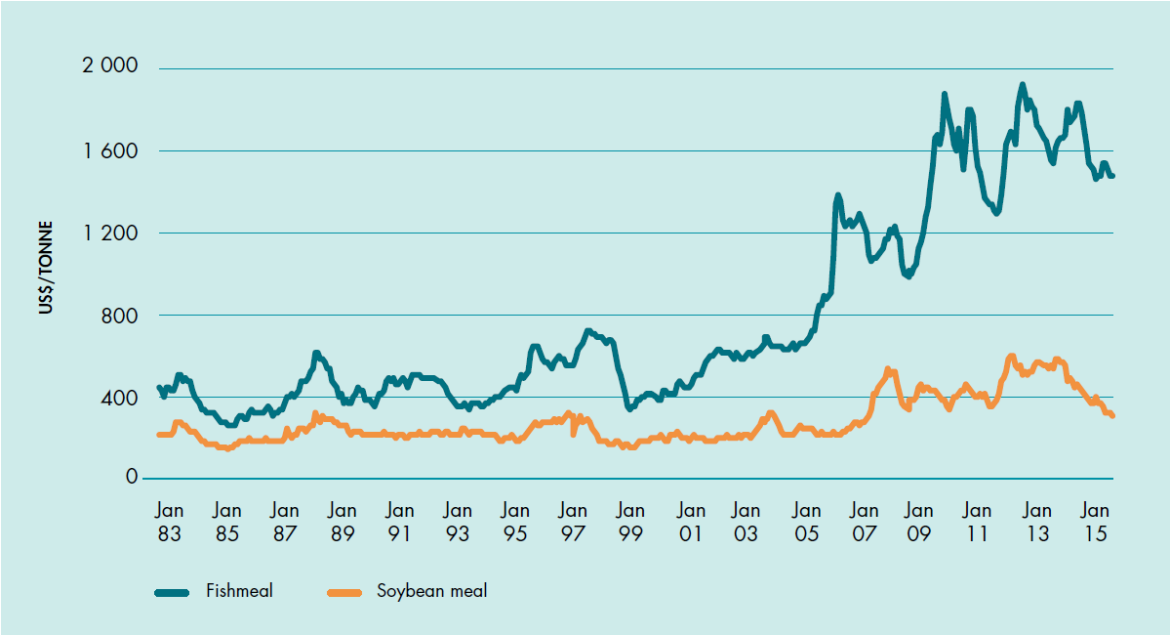

Figure 1.3. Fishmeal and soybean meal price fluctuation from 1983 to 2015 in Germany and Netherland (FAO, 2016).

1.3. Fish meal

Fish meal is produced from whole fish or by product of seafood such as processing waste, viscera or unused parts of fish. It provides a concentrated form of high quality protein and other factors which contribute to feed intake, health and immune function in finfish (Hardy and Tacon,

2002). Fishmeal is generally light brown in colour and is produced by cooking, pressing, drying and milling fresh raw fish and fish trimming.

Fish meals are classified by the type of fish or the species of origin, commonly fish meals include anchovy but also herring and menhaden. They vary in their amino acid profile and proximate compositions (USB, 2008). In fact, depending on the sources, fishmeal may include, referring to dry matter, crude protein (CP) content around 67.4 – 89.6%, 7.3 – 15.7% crude fat, 4.6 – 6.9% lysine and 1.7 – 2.7% methionine (FAO, 2016).

In basis of the source, CP may vary from 500 to 720 g CP kg^{-1} and ash content may vary from 100 to 210 g kg^{-1} on dry basis. The protein quality of an ingredient is dependent on the amino acid composition and digestibility of the ingredient.

The amino acid balance of fish meal is complicated to be achieved and it depends on the species to be fed. Protein efficiency ratio (PER) values indicate the comparing protein quality. The PER values are a measure of the amount of weight gained per unit protein consumed and fish meal values range from 3.1 to 3.7 (Friedman, 1996). Comparing the most commonly used plant ingredients to fish meal, plant proteins are generally lower in CP, have a lower PER and contain a variety of heat-labile and heat-stable secondary compounds which negatively affect diet utilization. Based on high CP and PER values, fish meal is a superior source of protein and other nutrients, particularly for carnivorous fish species (Friedman, 1996).

1.4. Soybean meal as alternative protein

The soybean is a member of the *Fabaceae* family, plants that form root noodles that house nitrogen-fixing soil bacteria (*Rhizobia*) in a symbiotic relationship (Barnes *et al*., 2010). Soybean is often used in crop rotation system to restore the nitrogen in the soil on ground used for agriculture. The soybean, now called *Glycine max*, has had a long history as a domesticated plant, with records of its use as far back as the eleventh century BC in China (Barnes *et al*., 2010).

Soybean products (proteins, oils, and lecithin) have shown results in several aquatic species, indeed some soy products are routinely used in commercial feeds. Soybean products are generally considered over other plant products, due to their high protein content, good amino acid profile, high digestibility, low cost, consistency and availability (Ng *et al*., 2006; Tibaldi *et al*., 2006).

Whole soybeans contain a good amount of protein (35–40% dry bases) and lipid (17–25%) (Wolf, 1970). Beans are crushed into flakes, then the hulls are removed, creating white flake (WF) (Peisker, 2001). Soybean oil is subsequently solvent extracted to leave de-fatted and de-hulled soybean meal (SBM), toasting the flakes then grinding into meal. Soy protein concentrates (SPC) and isolates (SPI) are further refinements of the soybean's valuable protein (Peisker, 2001). SPC can be produced from either WF or SBM. WF or SBM is desolventized, the material is passed through an ethanol extractor, which removes some carbohydrates and antinutritional factors, while concentrating protein and fiber (Swick, 2007). At the end of the process SPC contains 63.6% (wet basis) of protein (NRC, 2011). Soy protein isolate (SPI) is the most purified protein produced in soy processing, which contains about 80.7% (wet basis) protein (NRC, 2011), and is created by dissolving and precipitating proteins from defatted white flake in a pH 6.8 – 10 alkali solution (Lusas and Riaz, 1995). The precipitate is centrifuged to remove fibre, acid precipitated (pH 4.5), and centrifuged again to create a curd. The curd is then spray-dried to create SPI (Lusas and Riaz, 1995).

Soy products can vary in composition depending on growing conditions, genetic strain and processing procedures, moreover soybeans are well known to differ in nutritional characteristics depending on soil type, precipitation, climate and cultivar (Triboï *et al*., 2003; Hungria *et al*., 2000).

The principal soy product used in fish feed is soybean meal (SBM), that has a nutrient composition, referring to dry matter, ranging from 46.9 – 51.2% CP, 1.5 – 4.7% crude fat, 7.1 – 8.4% crude fibre and 6.1 – 7.4% ash (Abimorad *et al*., 2008; Guimaraes *et al*., 2008; Glencross *et al*., 2005; Caine *et al*., 2008). Lysine content is around 2.8 – 4.0% dry basis and methionine is 0.5 – 0.9% (NRC, 2011).

Concentrations of the 10 Essential Amino Acids (EAA) and tyrosine are generally lower in SBM than in fish meal with the exception of cystine, which is present at higher concentrations in SBM. The EAA of concern are lysine, methionine and threonine that may be limiting in soy-based diets for feeding aquatic animals. Concentrations of these EAA increase with processing of soy flakes to SPC and soy protein isolate SPI and approach or exceed those found in fish meal. However, due to the processing costs involved to increase nutrient, these products are not yet economical for large-scale use in aquafeeds. Crude fat and ash concentrations of solvent-extracted SBM and other soy products tend to be lower than those in fish meal, but carbohydrate concentrations tend to be higher (Gatlin *et al*., 2007). Indeed, some factors reducing the SBM value in aquafeed are antinutritional factors (ANFs) which include protease and trypsin inhibitors, phytates, lectins, phytic acid, non-starch polysaccharides, saponins and phytoestrogens.

ANFs are contained in various levels in all plant products, and are created by the plant in defence against herbivorous, insects, pathogens or adverse growing conditions (Herbourne, 1989), but they are inhibitory in carnivorous fishes and must be removed through processing. Soy processing has contributed to beneficial effects on growth and digestibility of herbivorous, omnivorous, and carnivorous fishes (Kim and Kaushik, 1992; Gatlin, 2002). Antinutritional factors could be divided into four groups: factors affecting protein utilisation and digestion, such as protease inhibitors, tannins, lectins; factors affecting mineral utilisation, which include phytates; miscellaneous substances such as phytoestrogens and saponins. ANFs may also be classified according to their ability to withstand thermal processing: they can be either heat stable (saponins, non-starch polysaccharides, phytate, phytoestrogens, protein antigens) or heat labile (trypsin inhibitor, lectins) (van der Peol, 1989; Rumsey *et al*., 1993). Soy processing procedures affect ANFs differently, and their removal can improve digestibility, palatability, and growth performance of fish.

Principal strategies for reducing ANFs and their impact on feeding animal include processing (e.g., heating, fermentation), genetic manipulation (e.g., artificial or natural selection) and diet supplementation (e.g., enzymes) (Gatlin *et al*., 2007). The first processing technologies used to inhibit

ANFs is thermal treatments. However, heat treatment can reduce the level of trypsin inhibitor, but overheating might reduce nutritional value causing destruction of lysine, denaturation of protein and reduction in quality and digestibility of feed (Francis *et al*., 2001; Barrows *et al*., 2007). Heat stable ANFs must be removed using other processes such as alcohol washing, activated carbon exposure, or chromatography.

The carbohydrate fraction of soy is composed of sucrose, oligosaccharides, and non-starch polysaccharides (NSPs; i.e. cellulose, hemicellulose, pectin, β-glucans, and gums) (Krogdahl *et al*., 2005; Gatlin *et al*., 2007; Choct *et al*., 2010). Certain carbohydrates such as hemicelluloses, pectins, and starches are also useful pellet-binding agents (Krogdahl *et al*., 2005). Other carbohydrate fractions such as sucrose, are simple sugars which readily available to fish. Indeed, oligosaccharides and most NSPs are indigestible by most fishes, and considered as ANFs (Refstie *et al*., 1998; Krogdahl *et al*., 2005; Choct et al., 2010). The primary carbohydrate fraction in soy is NSP, which is associated with reduced protein and lipid digestibility (Refstie *et al*., 1999) and with enteritis in salmonids (Yamamoto *et al*., 2008). Oligosaccharides, present in SBM in forms of sucrose, raffinose and stachyose are generally considered as antinutritional (Francis *et al*., 2001) and should be kept at low dietary levels for all carnivorous fishes.

One of the most inhibitory ANFs are protease inhibitors, which disable the activation of digestive enzymes causing a reduction of feed efficiency (Francis *et al*., 2001). Trypsin inhibitors are protease inhibitors that inhibit the activation of two protease enzymes: trypsin and chymotrypsin (Norton, 1991). Trypsin is a digestive enzyme produced in the pancreas, which cleaves peptide bonds of proteins into smaller peptides or amino acids absorbed through the intestine and used for growth (Hedstrom, 2002). Soybeans contain two groups of trypsin inhibitors: the Kunitz trypsin inhibitor and the Bowman-Birk protease inhibitor. Generally typical thermal treatments for SBM reduce most of the trypsin inhibitor activity (TIA) (Francis *et al*., 2001). The Bowman-Birk inhibitor is also more heat-stable than the Kunitz inhibitor (Norton, 1991). Commercial soybean products mostly show trypsin inhibitors (TI) in the range of 2–6 mg g^{-1}. It seems that below the 5 mg g^{-1} level, most cultured

fish are able to compensate for the presence of TI by increasing trypsin production. At the usual levels at which the materials containing protease inhibitors such as commercially available soybean meal are included in fish diets, other antinutritional factors or interactions between them may be more important. For other materials, moist heat treatment (autoclaving for 15 – 30 min) is recommended as a means of reducing the amount of trypsin inhibitors below the critical levels (Francis *et al*., 2001).

Phytate is the major form of phosphorus storage in plants (Han and Wilfred 1988). They can chelate with mineral ions such as Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{3+} and Fe^{3+} resulting in these ions becoming unavailable for consumers (Duffus and Duffus, 1991). Phytates also form phytate–protein complexes, reducing the availability of dietary protein (Richardson *et al*., 1985). Usually phytate is found in SBM at low levels, 10–15 g kg^{-1} (Francis *et al*., 2001). The reduced growth performance of fish fed high phytate diets might be due to various factors: lowered bioavailability of minerals, reduced protein digestibility caused by formation of phytic acid–protein complexes and depressed absorption of nutrients attributed to damage to intestine. Phytates, are concentrated in the outer endosperm of cereal and milling to remove the outer layer of seeds reduces the phytate content of seed. Furthermore, fermentation and heat treatment (autoclaving) has also been shown to deeply reduce the phytic acid (Duffus and Duffus, 1991; Hossain and Jauncey, 1990). Salmonids seem to be able to tolerate dietary levels of phytate in the range of 5–6 g kg^{-1}, and it seems to be advisable to maintain the level of phytates below 5 g kg^{-1} in fish feeds. The addition of minerals such as Zn has been shown to be only partially capable of counteracting the negative effects of dietary phytates (Francis *et al*., 2001).

Soybean lectins, known as soybean agglutinin (SBA), are glycoproteins which are present in soy protein products (Liener, 1994). Even though they are proteins, they are partially resistant to proteolytic degradation in the intestine. Their common biological effects include disruption of the small intestinal metabolism and morphological damage to the villi (Grant, 1991). The observed increase in goblet cell numbers may be the result of hypertrophic mucus production in the intestine when subjected to irritation by lectins and this may impair the enzymatic and absorptive capacity of

the intestine (Francis *et al*., 2001). Lectins can be removed by aqueous heat treatment (100°C for 10 min) or autoclaving (Grant, 1991).

Saponins are heat stable steroid or triterpenoid glycosides including galactose, arabinose, rhamnose, glucose, xylose, and glucuronic acid (Francis *et al*., 2001). The effect of saponins on fish are controversial. Saponins could cause significant intestinal damage, and when dissolved in water can damage the respiratory epithelium of the gills (Bureau *et al*., 1998; Francis *et al*., 2001) but in the other hand an *in vivo* study has shown that saponins in solvent-extracted soybean products did not produce significant negative effects on performance of Atlantic salmon (Krogdahl *et al*., 1995).

Phenolic compounds (i.e. tannins, phytoestrogens) are believed to play an important role in creating adverse organoleptic properties in plant products, as well as affect normal bodily functions of animals (Liener, 1994).

Tannins are compounds of various chemical structures occurring in plant and are classified into hydrolysable and condensed tannins. Their antinutritional effects include interference with the digestive processes either by binding the enzymes or by binding to feed components like proteins or minerals (Liener, 1989). It is noted differences in tolerances of different fish species and differences in the structure of the tannins or their interactions with other components in the diet (Francis *et al*., 2001). Tannins are also known to interact with other antinutrients. Tannins are lower in soybeans than in other legumes, even if recommended methods for the removal of condensed tannins include de-hulling the seeds to remove the tannin rich outer layer, autoclaving or treatment with alkali (Griffiths, 1991).

Phytoestrogens are present at low levels in soybean products but processing can concentrate some isoflavones (Liener, 1994). These compounds can disrupt functions involving reproduction, so careful management of phytoestrogens may be important for brood stock and early life stage feeds.

Heat processing is a mean of inactivating ANFs and improves nutrient retention of raw full-fat or de-fatted SBM. One of the heat treatments is extrusion technology, where the feed is exposed to a high temperature for a relatively short period of time with high pressure (Björk and Asp, 1983).

Extrusion with steam preconditioning is known as wet extrusion in which steam is injected into an extruder barrel, whereas extrusion without steam preconditioning is known as dry extrusion (dry extruded SBM). Authors (Babar *et al*., 1988) reported that steam preconditioning enhances the efficiency of extrusion. In fact, low palatability, reduction of nutrient digestibility and growth had been reported in several studies in which fish meal was totally replaced with SBM, suggesting that dry/wet extruded SBM or extruded full-fat soybean could be used as the main source of dietary protein if supplemented with methionine and lysine (Thompson *et al*., 2007; Goda *et al*., 2007). This conclusion is reached by study in herbivore fish, Tilapia galilaea (*Sarotherodon galilaeus*), that showed higher growth rate, weight gain and fed intake when fed the extruded SBM joined to amino acid, compared to fish meal used as control diet. As regard carnivorous fish, it has been observed that soybean meal supplemented with or without lysine and methionine might replace 20–50% of fish meal without compromising growth or weight gain of rainbow trout (Oliva-Teles *et al*. 1994; Reftie *et al*., 2000). This is consistent with Barrows *et al*. (2007) that observed higher weight gain and feed intake in rainbow trout fed extrusion of solvent-extracted SBM. Atlantic salmon seems more sensitive than rainbow trout to ANF in defatted SBM, because it did not grow at similar rate when fed diets with 37% of dietary protein substituted by defatted SBM in place of fish meal-based diets (Refstie *et al*., 2000).

1.5. Soy isoflavones

Isoflavones are biologically-active, non-nutritive compounds that are present in relatively large amounts in soybean and soy foods. Chemically, these isoflavones belong to a larger group of plant chemicals called flavonoids which are common in many fruits, vegetables and legumes.

Isoflavones stimulate nodule formation (Bellaloui, 2012) in soybean. In deeply, these molecules play major roles in different types of plant–microbe interactions. They are important chemo-attractants and signal molecules for symbiotic bacteria in soybean, and act together with other

flavonoid compounds. The signals released by the soybean that attract the rhizobial bacteria are the isoflavones (Rolfe, 1988). The *nodD* gene encoded proteins of *Rhizobia* have been shown to physically bind to flavonoids and isoflavonoids. This ligand association initiates transcription of the nod operon leading to root nodule formation (Subramanian *et al*., 2004).

Soy isoflavones are also involved as part of defence mechanism of soybean plant against fungi and bacteria (Dakora *et al*., 1996), diseases and in response to environmental stresses such as drought and temperature (Vamerali *et al*., 2012). Soybean seeds contain four groups of isoflavones and twelve distinct compounds i.e., aglycones (daidzein, genistein and glycitein), and their acetyl-, malonyl- and b-glycoside forms (Figure 1.4). The three most abundant isoflavones found in soybeans are malonyl-daidzin, malonyl-genistin and malonyl-glycitin. All isoflavone forms can be expressed like equivalent forms of aglycones and these compounds are typically found in soybeans at a ratio of approximately 1.3:1.0:0.2 (LC Laboratories, 2007). Soy germ has a daidzein/genistein/glycitein ratio of 4:1:3, containing three times more glycitein than genistein (Song *et al*., 1998). Soy flour can be produced from the hypocotyl portion of the soybean, containing a daidzein/genistein/glycitein ratio of 2:1:1 (4290, 1930, and 2050 mg kg^{-1}).

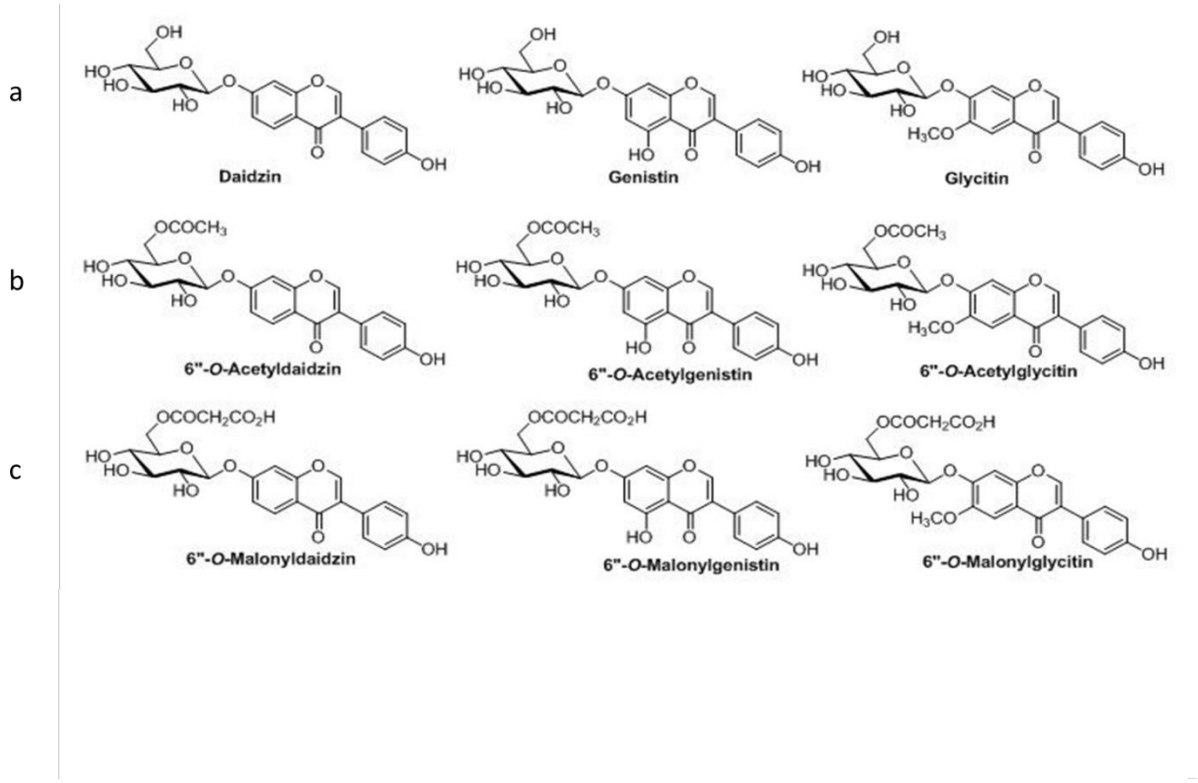

Figure 1.4. Chemical structures of soy isoflavone glycosides: a) b-glycoside, b) acetyl-b-glucoside, c) malonyl-b-glucoside (Kurosu, 2011).

This was a 3-fold increase in isoflavone content relative to a standard soy flour milled from the same batch of beans (0.7:1:0.1: 1000, 1530, and 180 mg kg^{-1}). Daidzein and glycitein are concentrated in the hypocotyl portion of the soybean, whereas genistein is more equally distributed throughout (Wiseman *et al*., 2002).

The isoflavone content of soy foods depends primarily on the isoflavone content of the soybean source. The total isoflavone aglycon and glucoside content of soybeans is generally in the range of 1000–4000 mg kg^{-1}. The USDA-Iowa State University isoflavone database suggests that typical raw, full-fat soy flour contains 712, 968, and 162 mg kg^{-1}, respectively, of daidzein, genistein, and glycitein aglycon equivalents (USDA, 1999). Therefore, soy foods and some foods with soybean additives have the largest concentration of these estrogenic compounds (Nurmi *et al*., 2002; Umphress *et al*., 2005; Wang *et al*., 1994a).

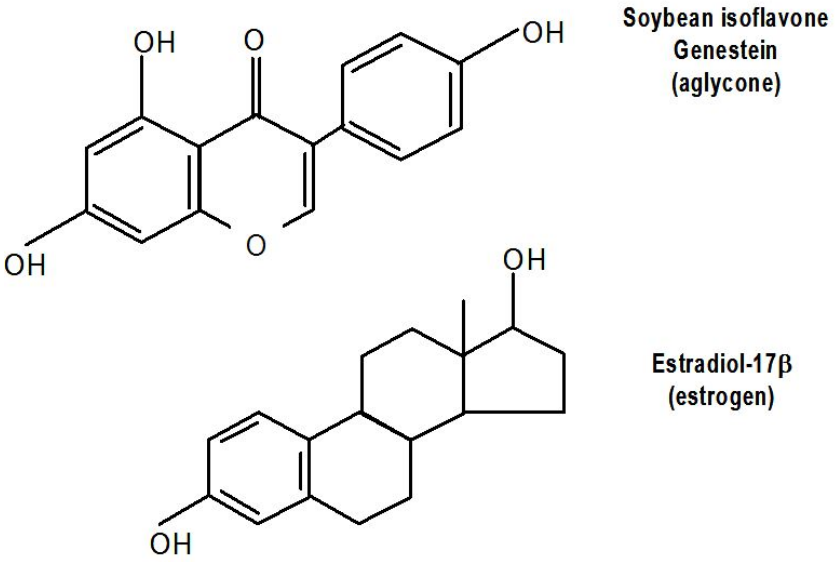

Figure 1.5. The structure of isoflavone aglycone (genistein) and 17β-estradiol showing the similarities between the two molecules (https://infograph.venngage.com/p/99864/infographic-soy-and-environmental-impact).

Distribution of the glucoside forms within soy product is more variable; Japanese soy can contain a 10-fold higher proportion of the malonylglucosides to glucosides than do U.S. soy products (Wang and Murphy, 1994b). Acetylglucosides are generally found at low levels in the intact minimally processed soybean. It is accepted that the malonyl esters are stable in foods but unstable in solution at high temperatures, where they will undergo decarboxylation to the corresponding acetyl ester, and that this degradation is the source of most measured acetylglucosides in soy foods (Horowitz and Asen, 1989). At room temperature, the rate of degradation slows to an acceptable level, whereas heat processing, enzymatic hydrolysis, and fermentation all alter the distribution of isoflavone forms (Wiseman *et al*., 2002).

The chemical structure of isoflavone aglycones is similar to the natural hormone 17β-estradiol (E_2) (Figure 1.5).

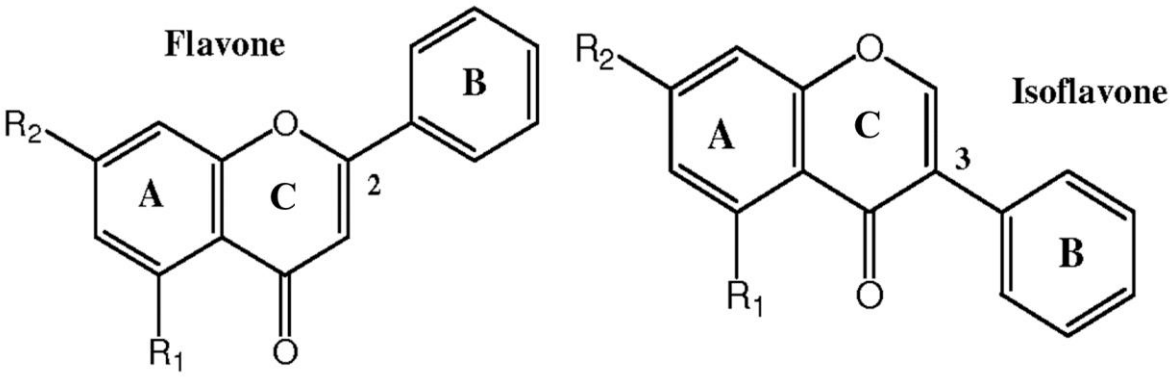

Figure 1.6. Structure comparison of a flavone and isoflavone (Vargas Galdos, 2009).

Isoflavones are flavonoids because of their C6-C3-C6 structure; however, they differ from flavones as the B ring is attached to position 3 of the C ring instead of position 2 (Figure 1.6) (Vargas Galdos, 2009). It is considered that the phenol ring is a key structural element to be able to attach to the estrogen receptors (Leclerq *et al*., 1979) and the flavonoid isomeric configuration increases their similarity to human estrogens (aligned OH groups) which seems to be the foundation of their phytoestrogenic activity (Vaya *et al*., 2004). Isoflavone glucosides have been shown to have similar relative estrogenic potencies to the corresponding aglycons (Farmakalidis and Murphy, 1985). To our knowledge no data are available on the estrogenicity of acetyl- and malonylglucosides, but it is expected that they will have activities similar to those of both the parent aglycons and glucosides. The relative estrogenic potency of the soy isoflavone aglycons is glycitein > genistein > daidzein (Song *et al*., 1999).

1.5.1. Absorption, metabolism and excretion of isoflavones

Isoflavones are consumed with food, absorbed in the gastrointestinal (GI) tract, and finally, excreted in the urine. They are 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 (Turner *et al*., 2003).

Isoflavone aglycones are readily absorbed in the upper small intestine by passive diffusion, peaking in the blood within an hour after being ingested (King *et al*., 1996; Sfakianos *et al*., 1997). In contrast, the β-glucosides (the 6"-O-malonyl- or 6"-O-acetyl-7-O-β-glucosides) are not passively absorbed; however, they are easily hydrolyzed by β-glucosidases, either from intestinal bacteria and the intestinal mucosa (Day *et al*., 2000). This hydrolysis will produce aglycones in the small intestine.

Once the isoflavone aglycones enter enterocyte, they can be glucuronidated by UDP-glucuronyltransferases (King *et al*., 1996) and sulfated by PAPS-sulfotransferases (Ronis *et al*., 2006) or left as aglycones. Glucuronidation and sulfation also occur in the liver (Nakano *et al*., 2004). This can also significantly affect their bioactivity since glucuronides are believed to be pharmacologically inactive (Xu *et al*., 1995). These metabolites are excreted in the bile and are deconjugated in the lower bowel allowing them to be reabsorbed again, creating an enterohepatic circulation (King *et al*., 1996). Isoflavones may be further metabolized by the large intestine's microflora into other metabolites such as equol and O-desmethylangolensin (O-DMA) (Axelson *et al*., 1982).

Studies noted that after absorption most isoflavones are found in urine and plasma as glucuronides, aglycones and sulfates (Adlercreutz *et al*., 1995; Zhang *et al*., 2003). Isoflavones glucuronides represent respectively 60% and 70% of total isoflavones in urine and plasma of women that consistently consume soy milk (Zhang *et al*., 2003). Aglycones only accounted for 5% in urine and 20% in plasma of the total isoflavones. Isoflavones seem to be rapidly glucoronidated 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 (Sfakianos *et al*., 1997).

1.5.2. Mechanisms of action of isoflavones

The binding of isoflavones to mammalian estrogen receptors has been known for over 40 years (Martin *et al*., 1978). Isoflavones can bind estrogen receptor (E_2) acting as agonists or antagonists of the steroid hormone: as agonists, they cause estrogenic effects, as antagonists, they may block or alter estrogen receptors (ER) (Figure 1.7) and prevent estrogenic activity, causing anti-estrogenic effects (Brzezinski and Debi, 1999).

Deeply, soy isoflavones can preferentially bind to and transactivate estrogen receptor, mimicking the effects of estrogen in some tissues and antagonizing (blocking) the effects of estrogen in others (Wang, 2002). Martin *et al*. (1978) suggested that isoflavones may act as anti-estrogens in the presence of high levels of endogenous estrogens.

Soy isoflavones could exert their estrogenic effects by acting as estrogen receptor (ER) agonists, as it has been evidenced in mammals (Kuiper *et al*., 1997; Kuiper *et al*., 1998) and fish (Tollefson *et al*., 2001; Latonnelle *et al*., 2002).

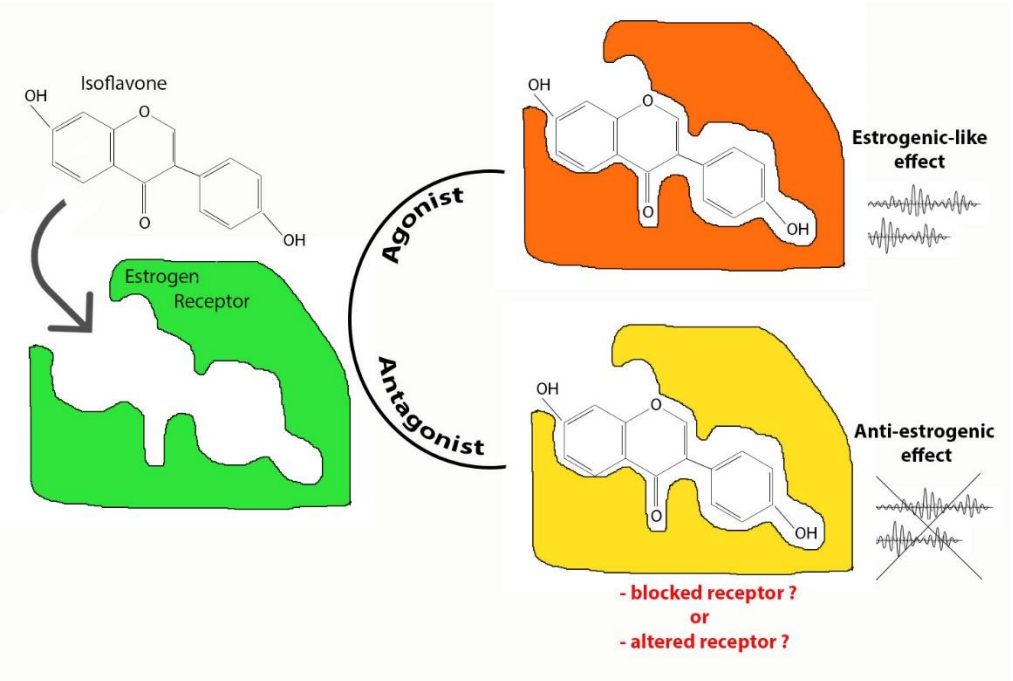

Figure 1.7. Isoflavone action as agonist or antagonist of 17β-estradiol (E_2).

There are numerous reports on genistein estrogenic effects in fish, showing that this molecule can bind to the ER and inducing vitellogenin (egg yolk protein) synthesis by liver in yellow perch *Perca flavescens* (Ko *et al*., 1999) or increasing plasma vitellogenin concentrations in rainbow trout (Pelissero *et al*., 2001), Siberian sturgeon *Acipenser baeri* (Pelissero *et al*., 1991a, b), and juvenile striped bass *Morone saxatilis* (Pollack and Ottinger, 2003) fed isoflavones-based diets.

However, genistein has been showed to be a relatively weak ER agonist in teleosts, binding to the hepatic ER in rainbow trout, Atlantic salmon and Siberian sturgeon with approximately 100–200 times less potency than E_2 (Tollefson *et al*., 2001; Latonnelle *et al*., 2002), indicating that some of the potent estrogenic effects of phytoestrogens would be mediated by other mechanisms besides receptor binding.

Soy isoflavones also have biological activities that are unrelated to their interactions with estrogen receptors. By inhibiting the synthesis and activity of enzymes involved in estrogen metabolism, soy isoflavones may alter the biological activity of endogenous estrogens (Barnes *et al*., 2000). Part of the mechanism by which soy isoflavones could exert estrogenic effects is by increasing the bioavailability of E_2 through inhibition of E_2-metabolizing enzymes in peripheral target tissues, evidenced by estrogenic compound in mammal and fish. Specifically, several hydroxylated compounds have been shown to act as allosteric or competitive inhibitors of the Phase II conjugation reactions that normally inactivate E_2. In the lake trout (*Salvelinus namaycush*), numerous hydroxylated xenoestrogens, (i.e. hydroxylated PCBs and bisphenyl A) inhibited the production of conjugated, water-soluble E_2 metabolites normally produced by the kidney and liver of this species (Jurgella *et al*., 2006). In lake trout, the primary products of liver and kidney E_2 metabolism are water-soluble Phase II conjugates, including E_2-17-glucuronide, E_2-17-sulfate and E_2-3-glucuronide (Jurgella *et al*., 2006).

Moreover, isoflavones might act at the level of the target tissue by inhibiting metabolizing enzymes that normally protect the ER from binding circulating E_2. In the presence of inhibitory

isoflavones, however, this enzymatic protection is lost and the cell becomes responsive to E_2 (and perhaps also to the phytoestrogen itself acting as an ER agonist).

Finally, isoflavones may exert their effects as estrogen antagonists in a high estrogen environment, or they may act as estrogen agonists in a low estrogen environment (Hwang *et al*., 2006). In the same time their effect depends on isoflavones dose, they mainly have agonistic effect if present in very high concentration as evidenced in Harry *et al*. (2005) *in vitro* study.

The different activities and the bioavailability of phytoestrogens (i.e. isoflavones) vary depending on such factors as the form of administration, dosage and individual metabolism (Kelly *et al*., 1995; Xu *et al*., 1995; Wiseman, 1999). Target tissue, concentration dependency, number and type of ER, and the presence or absence of endogenous estrogens also influence the effect of phytoestrogens (Glazier and Bowman, 2001).

Isoflavones, as phytoestrogens, are bioactive compounds and considered antinutrients due to their effects on glucose, lipid, and cholesterol metabolism when ingested with the diet (Krogdal and Bakke, 2015). In mammals and man, the antinutrient or metabolic effects of phytoestrogens have been attributed to their ability to bind to estrogen receptors and other nuclear hormone receptors present in cells of various metabolically important tissues, including intestine and liver (Krogdal and Bakke, 2015). Phytoestrogens are therefore classified among the endocrine disruptors (Bennetts *et al*., 1946) that interfere with regulation of reproduction and the involvement of estrogens in feed intake, lipid metabolism, regulatory action on peroxisome proliferator-activated receptor γ (PPAR γ), and insulin sensitivity. Consequently, phytoestrogens may affect energy utilization as observed in rodents and humans. In rodent and human females, estrogen maintains energy homeostasis via ERα and ERβ, by suppressing energy intake and lipogenesis, enhancing energy expenditure, and ameliorating insulin secretion and sensitivity (Mauvais-Jarvis, 2011). The binding of phytoestrogens to farnesoid X receptor (FXR), liver X receptor (LXR), and/or PPARs explains effects seen on bile acid synthesis, metabolism, and on lipid, glucose, and cholesterol homeostasis, resulting in

hypoglycaemia, hypolipemia, and hypocholesterolemia often observed with the dietary intake of these antinutrients (Caiozzi *et al*., 2012).

Additionally, isoflavones can act as antioxidants *in vitro* and *in vivo*, and they could contribute to the antioxidant status of edible part of reared animals (Chen *et al*., 2011; Ruiz-Larrea *et al*., 1997).

Isoflavone-supplemented diets have been shown to reduce lipid peroxidation and F_2-isoprostane levels, a biomarker of lipid peroxidation, in humans (Wiseman *et al*., 2000) beyond that in few *in vivo* study in fish fed with supplemented isoflavone diet (D'Souza *et al*., 2005; Mai *et al*., 2012).

1.6. Rainbow trout (*Oncorhynchus mykiss*)

The rainbow trout, firstly classified as *Salmo gairdneri* Richardson, 1836, at now has been recognised as a member of the Pacific *Salmonidae*, *Oncorhynchus*, and reclassified as *Oncorhynchus mykiss* Walbaum, 1792 (Kendall, 1988).

The scientific classification and taxonomy of rainbow trout (Starnes, 2003):

Kingdom: *Animalia*

Phylum: *Chordata*

Class: *Actinopterygii*

Order: *Salmoniformes*

Family: *Salmonidae*

Genus: *Oncorhynchus*

Species: *mykiss*

Rainbow trout is a freshwater fish from the *Salmonidae* family. The biological features of rainbow trout include 60 – 66 vertebrae, 3 – 4 dorsal spines, 10 – 12 dorsal soft rays, black, small spots on the back, head and fins but the coloration depends on habitat, size and sexual condition

(Cowx, 2005). Rainbow trout is native to the freshwater rivers and lakes of the West coasts of the North America.

It has been introduced to around 82 countries, generally almost everywhere in the world, because it can tolerate a wide range of environments and its production condition is better than other trout species (Woynarovich *et al*., 2011). Many of these countries were reporting rainbow trout farming production, even if some of them have relatively insignificant production in comparison to the larger systems that are located in Europe, North America, Chile, Japan and Australia (Cowx, 2005). In 2016, the principal producer of rainbow trout in European zone has been Turkey with 100,300 tonnes, followed by Norway 81,715 tonnes and Italy, the least with a production reaching about 38,800 tonnes, divided in 33,800 tonnes of portion size (< 1.2 kg) and in 2,500 tonnes of large rainbow trout (> 1.2 kg) (FEAP, 2017).

Monoculture is the most common practice in rainbow trout culture, and intensive systems are considered necessary in most situations to make the operation economically attractive. A potential site for commercial trout production must have a year-round supply of high quality water (without aeration – 1 l/min/kg of trout without aeration or 5 l/sec/tonne of trout with aeration), that meets a number of criteria: DO_2: near saturation; CO_2: < 2.0 ppm; temperature: 12 – 21ºC; pH: 6.5–8.5; alkalinity (as CaCO3): 10 – 400 mg l^{-1} (Purser and Forteath, 2003).

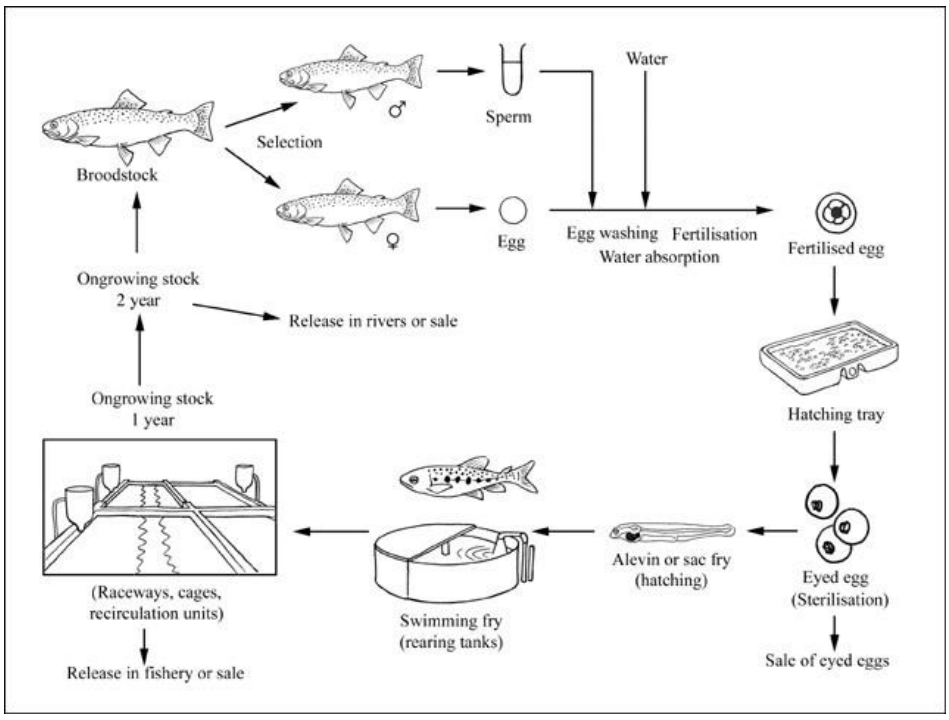

Figure 1.8. Production cycle of rainbow trout, *Oncorhynchus mykiss* (Cowx, 2005).

The entire production cycle of rainbow trout can be resumed in diagram illustrated in Figure1.8. Production of rainbow trout begins with the selection of suitable broodstock in a sex ratio of approximately one male to three females. During the spawning period daily checks for gravidity are undertaken. At maturation, females are removed from the holding unit and gentle pressure is applied along the sides of the fish causing eggs to flow from the urino-genital pore into a collection bowl. More recently, a technologically method has been applied on broodstock sites to be less stressful to fish in addition to be faster and to prevent excessive removal of mucous and scales caused by continual massaging. A low pressure air compressor, a hypodermic needle is inserted about 10 millimetres into the female genital pore between the pelvic fins and air pressure by a low pressure air compressor is insufflated to let out eggs from the body cavity.

Once eggs are collected, milt, extracted from males using the same manual stripping technique, is added to the bowl. Alternatively, where sex reversed females are used, milt extraction is lethal.

For good fertilisation, commercial sites use eggs from more than one female and milt from at least two males. Eggs and milt are mixed and water is added to activate the sperm. The eggs absorb water and become swollen and firm. After, they should be left to incubate in complete darkness. In incubation system, eggs should be supplied with water circulation sufficient to provide enough oxygen and remove suspended particles that may lead to smothering, and subsequent death.

Eggs are incubated undisturbed until the eyed stage is reached, in hatching troughs, vertical flow incubators or hatching jars. Hatching of fertilised eggs is temperature dependent and empty shells should be removed from the holding unit to prevent an accumulation of waste products. Once the yolk-sac is almost fully used, fry swim to the surface and first feeding begins. Feed is introduced until all fry are actively seeking food and the weaning is completed. Once fish reach 8 – 10 cm of length, pass to intensive rearing system.

From post-weaning stage to market size sampling should occur weekly to allow estimations of food conversion ratios, production costs, uniformity and feed strategy. Tank transfers may be necessary as the fish approach market size. Market size, 250 g, is achievable in 9 – 12 months. A small proportion of the stock may be kept as future broodstock. These fish generally exhibit characteristics that appeal to the farmer, processor or consumer.

Rainbow trout spawn between autumn and spring, and mature females are able to produce 2000 eggs/kg body weight (Purser and Forteath, 2003). Males mature at 2 years but majority of females mature at 3 years. Eggs are relatively large in diameter in range of 3 – 7 mm. Most fish only spawn once, from January to May, although selective breeding and photoperiod adjustment has developed hatchery strains that can mature earlier and spawn all year round. Superior characteristic selection is also achieved by cross breeding, increasing growth rates, resistance to disease, and prolificacy, and improving meat quality and taste (Cowx, 2005).

In wild, adult trout feeds aquatic and terrestrial insects, molluscs, crustaceans, fish eggs, minnows and other small fishes, but their most important food is freshwater shrimp, containing the carotenoid pigments responsible for the orange-pink colour in the flesh.

In rearing condition, feeds for rainbow consist of artificial feed, with studied formulation and production process. Feeds for trout have been modified over the years and cooking-extrusion processing of foods now provide compact nutritious pelleted diets for all life stages. Pellets absorb high amounts of added fish oil and permit the production of high-energy feeds with over 16 % fat. Dietary protein levels in feeds have increased from 35 – 45 % and dietary fat levels now exceed 22 % in high energy feeds. Feed formulations for rainbow trout use fish meal, fish oil, grains and other ingredients, but the amount of fish meal has reduced to less than 50 % in recent years by using alternative protein sources such as soybean meal. These high energy diets, are efficiently converted by the rainbow trout, often at food conversion ratios of close to 1:1. Feeding methods vary for production systems. Hand feeding is suitable for small fish eating fine food. Mechanical feeders, driven by electricity or solar power, are frequently used to feed at set intervals depending on fish size, temperature and season. Demand feeders can be used for fish greater than 12 cm (Cowx, 2005).

OUTLINE OF THE THESIS

This thesis presents an *in vivo* feeding trial using a low dose and high dose of soy aglycone isoflavone mix to assess their estrogenic potency, the effect on growth, reproduction and health in *Oncorhynchus mykiss*, and in addition on the quality, freshness and security of food products. Isoflavones, as phytoestrogens, may act as estrogen receptor agonist or antagonist causing biological activity on trout besides possible changes on trout fillet quality. In the main time, it is important to know the amount of the isoflavones in edible tissue for safety consumer and their antioxidant effect that could enhance the shelf life product. Briefly, the thesis in divided in two principal parts: *Chapter 2* and *Chapter 3* that report the results of the *in vivo* feeding trial and are written as scientific paper as already published or under submission.

In *Chapter 1*, a general introduction is provided and background information is given that sets the scene for addressing the research problem.

Chapter 2 describes *in vivo* study performed on rainbow trout fed three experimental diets containing different concentrations of isoflavones (0, 500, 1500 ppm) per 70 days. The isoflavone doses are selected on the base of partial and total supplementation of fishmeal using soybean meal. This *Chapter* contains the obtained results on growth, estrogenic activity, plasma estradiol levels, gonadal development, state of stress and histological evaluation of various selected organs.

In *Chapter 3*, results of isoflavone effect on final product during storage at 4 °C, for 1 and 7 days are reported. More in detail, growth performance, biometric indexes, skin and fillet colour, rheological characteristics, proximate composition, fatty acids profile, lipid oxidation of fillet and isoflavone deposition in tissue are described and discussed.

In *Chapter 4*, overall conclusions are reported on the base of comparisons with the available literature data discussed in the single Chapters.

Appendix 1 contains a pilot study, carried out preliminarily to the *in vivo* study, with the aim to find an eco-friendly procedure to extract isoflavones from soybean seeds.

In *Appendix 2* a new and rapid method for soy isoflavones quantification in rainbow trout muscle is described. The method has been applied to quantify isoflavones deposition in the fillet after feeding trial in *Chapter 3*.

CHAPTER 2

EFFECTS OF DIETARY SOY ISOFLAVONES ON ESTROGENIC ACTIVITY, CORTISOL LEVEL, HEALTH AND GROWTH IN RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

DOI: 10.1111/are.13602

ORIGINAL ARTICLE

WILEY Aquaculture Research

Effects of dietary soy isoflavones on estrogenic activity, cortisol level, health and growth in rainbow trout, *Oncorhynchus mykiss*

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Funding information
University of Padova, Grant/Award Number: prot CPDA123738

Abstract

Soy isoflavones (the phytoestrogens genistein, daidzein and glycitein) may act as estrogen receptor agonists or antagonists. The aim of this study was to examine the effect of dietary isoflavones on growth, reproduction and health in rainbow trout (*Oncorhynchus mykiss*). Rainbow trout fed three experimental diets containing different concentrations of isoflavones (0, 500 and 1,500 ppm). Growth, estrogenic activity, plasma estradiol levels, gonadal development, state of stress and histological changes in selected tissues were evaluated at the end of 70 days. Neither growth performance nor the relative mRNA levels of Insulin Growth Factor I (*igf*-I) in the liver were influenced by different levels of dietary isoflavones. Plasma and liver vitellogenin (VTG) protein levels and plasma 17-β-estradiol (E_2) were unaffected by treatments, although the correlation between plasma levels of E_2 and VTG densitometry values was significant ($p < .05$). The fish gonadosomatic index (GSI) did not significantly differ among the three experimental groups but correlated with plasma VTG densitometry values ($p < .05$). Plasma, muscle and fin cortisol concentrations fell within the normal welfare range and were not correlated with isoflavone levels. Histologically, the distal intestine showed a normal morphology with well-differentiated enterocytes and in the liver hepatocytes were also normal. A supranuclear accumulation of lipid droplets in enterocytes and some lipid droplets in hepatocytes were observed in all tested groups, suggesting an impact of basal dietary lipid on transport/metabolism of fat in the fish. Overall, the present results suggest that the doses of isoflavones tested do not compromise rainbow trout reproduction, growth and health.

KEYWORDS
cortisol, gonadosomatic index, growth, *Oncorhynchus mykiss*, soybean isoflavone, vitellogenin

1 | INTRODUCTION

The increase in seafood consumption has put pressure on traditional fisheries (FAO 2014) and at the same time, fish stocks have been fully exploited limiting the availability of fishmeal, the main protein source in aquaculture diets. The main goal of the aquaculture industry is to guarantee a high quality of feed ingredients to optimize health, growth and reproductive performance of fish, while reducing

production costs. In an effort to reduce fishmeal use in farmed finfish diets, many studies have been conducted to evaluate the use of plant-derived proteins as alternative protein sources (Gatlin et al., 2007). Among vegetable sources, soybean meal (SBM) is frequently used to partially substitute fishmeal because of similar protein content, good amino acid profile, palatability to fishes, availability and low price (Ng, Hanson, Malison, Wentworth & Barry, 2006; Tibaldi et al., 2006). However, SBM contains also phytoestrogens, known as isoflavones, a class of molecules called flavonoids that belong to polyphenols (Barnes et al., 2011). The three most abundant isoflavones found in soybeans are genistein (4′,5,7- trihydroxyisoflavone), daidzein (4′,7-dihydroxyisoflavone) and glycitein (4′,7-dihydroxy-6-methoxyisoflavone), with a ratio of approximately 1.3:1.0:0.2 (LC Laboratories 2007).

Isoflavones are structurally similar to 17-β-estradiol (E_2) and exert several estrogen-like biological effects with possible consequences on health, growth and reproductive function of fish (Latonnelle, Fostier, LeMenn & Bennetau-Pelissero, 2002; Ng et al., 2006). The estrogenic effects of phytoestrogens are well documented in fish (Pelissero et al., 2001). Isoflavones can induce the production of vitellogenin (VTG), a complex protein of the egg yolk, normally secreted by the liver under the induction of estradiol (Wallace, 1985), carried in blood to the ovaries and absorbed by the growing oocytes by linking to specific receptors associated with endocytotic vesicles (Pelissero et al., 2001). VTG is an excellent biomarker for exposure to estrogenic substances, as shown by the presence of VTG in the plasma of males, immature females or juvenile fish when exposed to exogenous estrogens (Cheek et al., 2001; Fossi et al., 2002).

Phytoestrogens can bind to steroid-binding proteins (Dechaud, Ravard, Claustrat, de la Perriere & Pugeat, 1999) and to estrogen receptors (ERs) of target cells (Casanova et al., 1999). A different affinity is noted between E_2 and isoflavones for ER, e.g. genistein affinity is 50 times lower than that of E_2 for rainbow trout ER. Moreover, they exhibit an endocrine-disturbing activity interfering with either steroid metabolism, i.e. aromatization (Chen, Kao & Laughton, 1997; Ng et al., 2006) or the mechanism of action of estrogens, i.e. tyrosine kinase activity (Huang, Fang & Dillon, 1999). As a result, phytoestrogens can induce reproductive disorders (Maclatchy & Van Der Kraak, 1995).

Soy isoflavones are also known as a growth promoters and used for this reason in poultry farming to improve production (Jiang et al., 2007). The effects of these molecules on fish in terms of growth performance and feed utilization are controversial (Ko, Malison & Reed, 1999; Pollack, Ottinger, Sullivan & Woods, 2003; Turker & Bozcaarmutlu, 2009; Ye & Chen, 2008). Growth in fish is regulated by the somatotropic axis principally composed of the growth hormone (GH) and Insulin-like Growth Factors such as IGF-I and IGF-II (Moriyama, Ayson & Kawauchi, 2000).

Most studies on phytoestrogens impact on steroidogenesis have focused on reproductive steroids, but very little is known about the mechanism of phytoestrogens action on glucocorticoids. In vertebrates, natural glucocorticoids such as cortisol regulate development, reproduction, ageing and are a critical factor for a successful response to stress (Holsboer & Barden, 1996; McEwen & Sapolsky, 1995). Any impact on this endocrine axis could potentially affect animal performance. To our knowledge, studies on the effects of phytoestrogens on adrenal cortex functions in fish are very limited, and there is only one in vivo study that indicated that addition of flavonoids in feed impaired cortisol production in rainbow trout (Aluru, Renaud, Leatherland & Vijayan, 2005).

The objective of this study was to evaluate the effects of dietary soy isoflavones at doses potentially found in commercial fish feed, on estrogenic activity, health and growth of adult rainbow trout from the early stage of vitellogenesis to the commercial size. Trout is a suitable model for this study because of its rather long gametogenesis, good historical data regarding its reproductive function (Hoar, Randall & Donaldson, 1983) from a time when soy was not a component of fish feed and as a representative of salmonid fish which are widely farmed.

2 | MATERIALS AND METHODS

2.1 | Experimental diets

A basal diet, containing fish meal and wheat gluten meal as protein source and fish oil as lipid source, was formulated to be adequate for nutritional requirements of *O. mykiss* (NRC, 2011). The ingredient composition and the proximate analysis (i.e. crude protein 45.2%, crude lipid 17.4% and ash 8.3%) of the basal diet are shown in Table 1. A mix of isoflavones based on the relative proportions as analysed in soybean meal (daidzein, 46.65%; genistein, 46.64%; glycitein 6.71%) was added to the basal diet at graded levels (500 and 1500 ppm) to obtain three experimental diets (ISO 0, ISO-Low and ISO-High respectively). Genistein, daidzein and glycitein were obtained from LC Laboratories (Woburn, MA, USA). The isoflavone concentrations of the soybean meal and of the experimental diets were determined by high-performance liquid chromatography (HPLC) equipment (Shimadzu®, Milan, Italy) with a UV diode array detector (SPD-M20A) and a Ultratechsphere C18 analytical column (CIL CLUZEAU, France) (33 mm × 4.6 mm i.d., 1.5 μm particle size), the latter kept at 35°C. The isoflavone concentration of the experimental diets was confirmed as 0 ppm (ISO 0), 523.14 (ISO-L) and 1466.60 ppm (ISO-H) as reported in Table 2.

The diets were manufactured at the pilot feed mill of the Department of Agriculture, Food, Environment and Animal Science of the University of Udine (Italy). All ingredients were ground through a 0.5 mm sieve before final mixing and dry pelleting through a 4.5 mm die. The experimental diets were obtained after cold pelleting (Mod. FT 300, Tenchini s.n.c., Piacenza, Italy). The diets were stored at −20°C until used. The test diets were subjected to proximate analysis according to AOAC (1998).

2.2 | Fish and experimental conditions

The study was approved by the Ethical Committee for Animal Experimentation of the University of Padova (Prot. n. 322114). All animals

TABLE 1 Formulation and proximate composition of basal diet

Ingredient	
Composition (g/kg)	
Chile prime fish meal[a]	400
Wheat gluten meal [b]	215
Wheat starch [b]	160
Fish oil	185
Mineral supplement[c]	4
Vitamin supplement[d]	5
Na lignosulfite	31
Proximate analysis (%)	
Dry matter	90.1
Crude protein	45.2
Crude lipid	17.4
Ash	8.3
n.f.e.[f]	19.2

[a]Vereinigte Fischmehlwerke Cuxhaven GmbH & Co. KG, Cuxhaven, Germany.
[b]Roquette, Lestrem, France.
[c]Composition (% mix): HPO4·2H2O, 78.9; NaCl, 17.65; MgO, 2.725; FeCO3, 0.335; KI, 0.005; ZnSO4·H2O, 0.197; MnSO4·H2O, 0.094; CuSO4·5H2O, 0.027; Na Selenite, 0.067.
[d]Composition (% mix): Thiamine HCl, 0.16; Riboflavin, 0.39; Piridoxine HCl, 0.21; Cyanocobalamine, 0.21; Niacin, 2.12; Calcium pantotenate, 0.63 Folic Acid, 0.10; Biotin Vit H, 1.05; Choline Clorure, 83.99; Myoinositol, 3.15; Stay C® DSM, 4.51; a-tocoferol Vit E, 3.15; Menadione Vit K3, 0.24; Vit A (2,500 IU/kg diet), 0.03; Vit D3 (2,400 IU/kg diet), 0.05.
[f]nitrogen-free extract.

TABLE 2 Final concentration and percentage of isoflavones in the three experimental diets

	Diets		
	ISO 0	ISO-L	ISO-H
ISL final concentration (ppm)	0	523.14	1466.60
% Daidzein	0	46.60	47.31
% Genistein	0	43.15	42.28
% Glycitein	0	10.25	10.41

were handled according to the principles stated by the EC Directive 86/609/EEC regarding the protection of animals used for experimental and other scientific purposes.

A total of 138 rainbow trouts were obtained from a commercial fish farm (Trota Piave Sile, Treviso, Italy). The fish were allocated into 6 groups of 23 individuals (initial mean weight (IBW) of 176.7 ± 0.7 g) kept in flow-through 250-L tanks of the indoor freshwater system at the facilities of the University of Udine, Italy. Fish groups were assigned in duplicate to the three diets according to a completely random design.

After stocking, fish were acclimated for over 3 weeks to the experimental conditions and fed a commercial diet (Supreme 3, Veronesi S.p.A., Verona, Italy) containing no soy derivatives.

Trout were offered the test diets at a fixed daily ration according to feeding tables (1% BW), 6 days a week, over 10 weeks. The actual feed intake per group was recorded daily. An isoflavone intake of 5 μg/g fish/day and 15 μg/g fish/day was estimated for fish fed diet ISO-L and ISO-H respectively. Fish were group weighed every 3 weeks, after a 24 hr fasting and under moderate anaesthesia (50 mg/L tricaine methanesulfonate, MS-222, Sigma Aldrich Co., St. Louis, USA).

The experimental system assured nearly constant and optimal water quality to rainbow trout (water temperature, 12.8 ± 0.8°C; pH, 8.0 ± 0.21, dissolved oxygen, 9.3 ± 0.4 mg/L, NH_4-H 0.03 mg/L; N-NO_2 <0.015 mg/L). Temperature, pH and dissolved oxygen were monitored daily while water ammonia and nitrite concentrations were monitored on weekly basis according to standard methods. A natural day light photoperiod was applied during the trial.

Feed intake as well as any mortality per tank/group of fish were registered on a daily basis. Weight gain (WG) and specific growth rate (SGR) were measured and calculated, respectively, for each group over 70 days.

2.3 | Sample collection

At the end of feeding trial, 22 fish from each experimental group were anaesthetized with tricaine methanesulfonate (200 mg/L MS-222, Sigma Aldrich Co., St. Louis, USA), in water and then weighed. Blood from each fish was withdrawn from the caudal vein using 2 ml syringes and then fish were killed by spinal cord severing. Blood was placed in heparinized tubes on ice and centrifugated (2,000 × *g* at 4°C for 15 min) to separate the plasma. Samples of fin and muscle from the caudal region were collected and stored at −20°C until required for cortisol and estradiol (E_2) analysis by radioimmunoassay (RIA).

Samples of liver and muscle were collected, frozen in liquid nitrogen and stored at −80°C until biomolecular analysis (Western blot, Real-time PCR) could be performed.

2.4 | Vitellogenin analysis and gonadosomatic index

Estrogenic effect was evaluated by measuring VTG in plasma and liver by Western blot followed by densitometric analysis. Liver samples were weighed and homogenized with liquid nitrogen by mortar and pestle. The sample was suspended in 500 μl of 0.125 M Tris-HCl (pH 6.8) buffer containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA), vortexed and centrifuged at 1,500 × *g* at 4°C for 15 min and the suspension was stored at −20°C until analysis. The protein concentration of plasma and liver samples was measured using a bicinchoninic acid kit (BCA Protein Assay Kit, Pierce, Rockford, IL, USA). For the normalization of the Western blots, an equal amount of total protein was used per lane, using bovine serum albumin (BSA) as standard. A total of 4 μg of protein extract in 50 μl of phosphate-buffered saline (PBS) was added to an equal volume of Laemmli Sample buffer 2X (Sigma-Aldrich) and

boiled for 5 min. Five microlitre of liver homogenate (10 μg protein/lane) and 1.5 μl of plasma (3 μg protein/lane) were separated using 8% SDS-polyacrylamide gel electrophoresis under reducing conditions and then transferred to a nitrocellulose filter (GE Healthcare, UK) at 350 mA for 1 hr at 4°C. Filters were treated with blocking solution (10% skim milk, 0.1% Tween-20 in Tris-buffered saline, TBS, pH 7.6) for 1 hr. The membranes were incubated with rabbit anti-VTG polyclonal antiserum (anti-Vtg) diluted 1:500 in blocking buffer, overnight at 4°C. After three washes in TBS, the membranes were next incubated with HRP-labelled goat anti-rabbit IgG diluted 1:50,000 for 1 hr in blocking buffer. All membranes were visualized using Chemiluminescent HRP substrate (Millipore, Billerica, MA, USA).

Band intensity was normalized using an internal standard sample on each gel and loading the same amount of liver homogenate and plasma. The immunoblots were scanned using Image Scanner (Amersham Pharmacia Biotech AB, Uppsala, Sweden) for performing densitometric analyses and the data were quantified by the ImageMaster Total Lab 2.0 Software (Amersham Pharmacia Biotech AB). The integrated density of the band of interest in a rectangular constant area was measured.

The fish gonadosomatic index (GSI) was calculated using the following formula: GSI = [Gonad Weight/Total Tissue Weight] × 100.

2.5 | Steroid concentrations

E_2 and cortisol in plasma, muscle and fin were extracted with diethyl ether, diluted PBS solution and utilized for specific microtitre radioimmunoassays (RIAs) as described by Simontacchi et al. (2009) and Bertotto et al. (2010). In brief, a 96-well microtitre plate (Optiplate, Perkin Elmer Life Sciences) was coated with goat anti-rabbit γ-globulin serum by incubation overnight in this reagent (diluted 1:1,000 in 0.15 mM sodium acetate buffer, pH 9, at 4°C). The plate was washed twice with PBS and incubated overnight at 4°C with the specific antiserum solution (for E2 or cortisol). It was carefully washed with PBS, then standards, reference samples, unknown extracts and ^{3}H tracers were added, and the plate was incubated overnight at 4°C. Lastly, it was washed with PBS, scintillation cocktail (Microscint 20, Perkin Elmer Life Sciences) was added and counted on a beta counter (Top-Count, Perkin Elmer Life Sciences).

The sensitivity of the assay was 3.125 pg well^{-1} and was defined as the dose of hormone at 90% binding (B/B_0).

The anti-17β-estradiol (E_2) serum showed the following cross-reactions: 17-β-estradiol 100%, estrone 2.5%, estriol 0.12%, dehydroepiandrosterone 0.007%, 17-α-estradiol <0.004%, progesterone <0.004%, testosterone <0.004% and androstenedione <0.004%. The anti-cortisol serum showed the following cross-reactions: cortisol 100%, prednisolone 44.3%, 11-deoxycortisol 13.9%, cortisone 4.95%, corticosterone 3.5%, prednisone 2.7%, 17-hydroxyprogesterone 1.0%, 11-deoxycorticosterone 0.3%, dexamethasone 0.1%, progesterone <0.01%, 17-hydroxypregnenolone < 0.01% and pregnenolone <0.01%.

2.6 | Histology

Samples of liver, gonads and distal intestine were fixed by immersion in 4% paraformaldehyde prepared in phosphate-buffered saline (PBS, 0.1 M, pH 7.4) at 4°C overnight, washed in PBS, dehydrated through a graded series of ethanol and embedded in paraffin. Consecutive sections were cut at a thickness of 4 μm using a microtome.

Histopathological analysis of gills, liver, kidney, muscle and gut was carried out. The general morphology of the tissues was evaluated on sections stained with Mayer's haematoxylin and eosin, dehydrated, mounted in Eukitt and examined with an Olympus Vanox photomicroscope (New York Microscope Company, Hicksville, NY, USA). PAS (Periodic Acid Schiff) staining was used to ascertain the steatosis in the liver and the contents of supranuclear vacuoles in intestinal enterocytes.

2.7 | Insulin Growth Factor I (*igf* -I)

2.7.1 | Qualitative RT-PCR

RNA was extracted from 50 mg of liver using TRIZOL Reagent (Gibco-BRL, Gaithersburg, MD, USA) following the manufacturer's protocol. Forty-two samples (N = 14 each experimental group) were treated with DNase I, Amp Grade (Invitrogen) to eliminate DNA contamination. RNA quantity was determined with NanoDrop Spectrophotometer (Thermo Scientific). To assess the integrity and the amount of RNA extracted, agarose gel electrophoresis and spectrophotometric A260/280 readings were performed. Total RNA (1 μg) was retrotranscribed into cDNA.

Single-strand cDNAs were synthesized by using SuperScript II Reverse Transcriptase protocols (Invitrogen, Life technologies, UK) and a mixture of random hexamers as primers (Biotech Ebersberg, Germany). The cDNAs obtained were used as templates for PCR expression analysis. PCRs were carried out to check the primer specificity with the following conditions: 32 cycles at 95°C for 45 s (denaturation), 58°C for 45 s (annealing) and 72°C for 45 s (extension). The primers were designed by using Primers express 3.0 software (Applied Biosystems): *igf*-I forward 5′GTG TGT GGA GAG AGA GGC TTT TA and reverse 5′GTG ACC GCC GTG CAT TGG, designed from *Oncorhynchus mykiss igf*-I sequence (GenBank accession no. M95183) which amplify a 67 bp fragment. To verify the efficiency of the reverse transcription (RT) a fragment of β-*actin* cDNA (180 bp) (GenBank accession no. AF 157514) and of 18S cDNA (131 bp) (GenBank accession no. AC NW_018575822) were amplified with the following primers forward 5′ACC CTG TCC TGC TCA CAG AG and reverse 5′GGG AGT CCA TAA CAA TAC CAG TG; forward 5′GCC CTT CCG TCA ATT CCT TT3′ and reverse 5′AAC GGT GCC AAC TAG CGA TC3′ respectively. PCR products were electrophoresed on a 1.5% agarose gel and visualized under UV light.

2.8 | Relative Quantitative Real-Time PCR

Quantitative real-time RT-PCR was performed to detect the relative expression of *igf*-I among different experimental diets. The

expression analysis was performed on ABI 7500 Real-Time PCR System (Applied Biosystems) in 25 μl reaction volumes containing 1X Power SYBR Green PCR MasterMix (Applied Biosystem), 300 nM forward and reverse primers and 10 ng of cDNA. For all genes (*igf*-I, β-*actin*, 18S) each sample was amplified in triplicates. Data were collected with ABI's 7500 System SDS Software.

For *igf*-I (target gene), β-*actin* assays and 18S (reference genes), fivefold serial dilution of cDNA samples was amplified to assess standard curves and PCR efficiency. Standard curves created on the basis of the linear relationship between the Ct value and the logarithm of cDNA amount showed acceptable slope values (included between −3.7 and −3.1).

To demonstrate that efficiencies of target and reference genes amplifications were approximately equal, a validation experiment was performed calculating the ΔCt = Ct (target gene) − Ct (reference gene) value. The ΔCt was plotted against the logarithm of the concentrations; the absolute value of the slope from the efficiency plot should be less than 0.1.

The experiments were conducted in singleplex using the following PCR conditions: 2 min at 50°C, an activation stage, a first step of denaturation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min, and in the end a dissociation step at 95°C for 15 s, 60°C for 1 min, 95°C for 15 s and 60°C for 15 s.

Baseline and threshold were set manually for each detector generating a threshold cycle (Ct) for each sample. A sample without cDNA template was used to verify that reagents were free of contaminants. Two replicates of each sample for target gene (*igf*-I) and the endogenous controls (β-*actin*, 18S) were amplified. Relative quantification was performed using the comparative ΔΔCt method (ΔCt sample − ΔCt calibrator) and $2^{-\Delta\Delta Ct}$ formula. The values obtained were used to generate the expression plots.

2.9 | Statistical analysis

Data from each treatment were subjected to one-way analysis of variance (ANOVA) or with a GLM nested model designed with diet effects nested within repetition effects.

TABLE 3 Growth performance of rainbow trout fed with graded levels of dietary soybean isoflavones over 70 days

	Diets			
	ISO 0	ISO-L	ISO-H	*p*
Initial body weight (g)	176.5 ± 0.08	177 ± 0.19	176.5 ± 0.09	–
Final body weight (g)	340.9 ± 4.82	344.8 ± 9.50	345.1 ± 9.07	ns
WG (%)[a]	94.7 ± 2.72	92.1 ± 5.35	95.5 ± 5.15	ns
SGR[b]	0.95 ± 0.02	0.92 ± 0.04	0.95 ± 0.04	ns

[a]Weight gain (WG) = (Final body weight–initial body weight) × 100/initial body weight.
[b]SGR: 100 × {(ln final body weight–ln initial body weight)/days}.
ns, not significant.

Steroid concentrations, VTG protein and *igf*-I expression were the dependent variables, whereas diet and repetition were the independent variables. When data were not normally distributed, a logarithmic transformation was applied to normalize the distribution of the data or, alternatively, a non-parametric test was used.

In case overall differences were significant, post hoc test using Statistica (version 10.0, StatSoft Inc., Tulsa, USA) was used to compare mean values among different effects. The level of significant difference was set at $p < .05$.

3 | RESULTS

3.1 | Growth

Feeding the different isoflavone added diets for 70 days did not result in impaired growth performance of rainbow trout. At the end of the experimental trial no significant differences were observed in final body weight (FBW), weight gain (WG) or in specific growth rate (SGR) (ANOVA, F (2, N = 62) = 0.1; 0.15; 0.2, $p > .05$ respectively) (Table 3).

The relative mRNA levels of *igf*-I in liver of trouts fed the different treatments were not influenced by isoflavone levels and they were similar in all groups (ANOVA, F (4, N = 62) = 1.24, $p > .05$; Figure 1).

3.2 | Vitellogenin and estradiol

VTG was found in the plasma of 15, and in the liver of 14, of the 65 fish examined. The number of fish with VTG in plasma and liver was higher in the ISO-H isoflavone group than in the ISO-0 and ISO-L groups (plasma: 8 fish vs. 3 and 4 fish in controls and low-level group, respectively; liver: 7 fish vs. 3 and 4 in control and low-level group, respectively) (Figure 2). Densitometric analysis of VTG abundance in plasma and liver showed values in a range 9936.87 – 793530.6 and 6153.6–457350.5 (pixels), respectively, and was not directly correlated with doses of isoflavones in the diets (One-way ANOVA, F (2, N = 10) = 2.8, $p > .05$).

Plasma E_2 concentrations ranged between 0.16 and 8.97 ng/ml. The mean (±*SE*) of E_2 concentration of each experimental group was 1.20 ± 0.34, 1.88 ± 0.54 and 1.62 ± 0.34 ng/ml for control, ISO-L and ISO-H diets, respectively, and did not differ among groups (ANOVA, F (4, N = 56) = 0.62, $p > .05$). However, the correlation between plasma levels of E_2 and VTG densitometry values was significant (Spearman's correlation, $p < .05$) (Figure 3).

3.3 | Gonadosomatic index and gonadal development

At the end of the experiment, the GSI average was 0.24 ± 0.04%, 0.26 ± 0.06% and 0.31 ± 0.07% for fish fed diet with 0, 500 and 1,500 ppm of isoflavones respectively. The values of GSI did not significantly differ among the three experimental groups (Kruskall–Wallis one-way analysis of variance, H (2, N = 60) = 3.8, $p > .05$) but

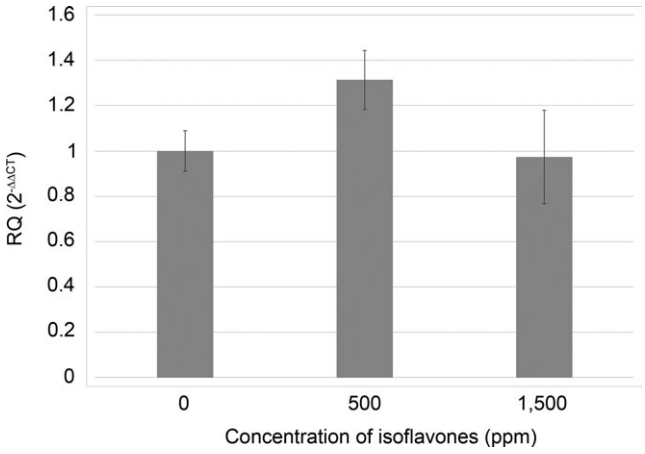

FIGURE 1 *Igf*-I expression in the liver in rainbow trouts fed the test diets over 70 days (0, 500 and 1,500 ppm of isoflavones). Data expressed as mean of every group ± *SE*

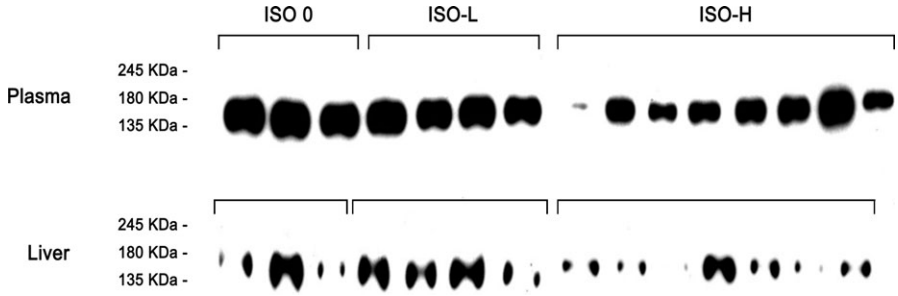

FIGURE 2 Vitellogenin protein detected in plasma and liver in the groups of rainbow trouts fed the three test diets (0, 500 and 1,500 ppm of isoflavones) over 70 days. The bands (180 kDa) represent all plasma and liver samples of the study in which vitellogenin protein is detected by Western blot. Wells contained 3 μg of protein for plasma and 10 μg for liver

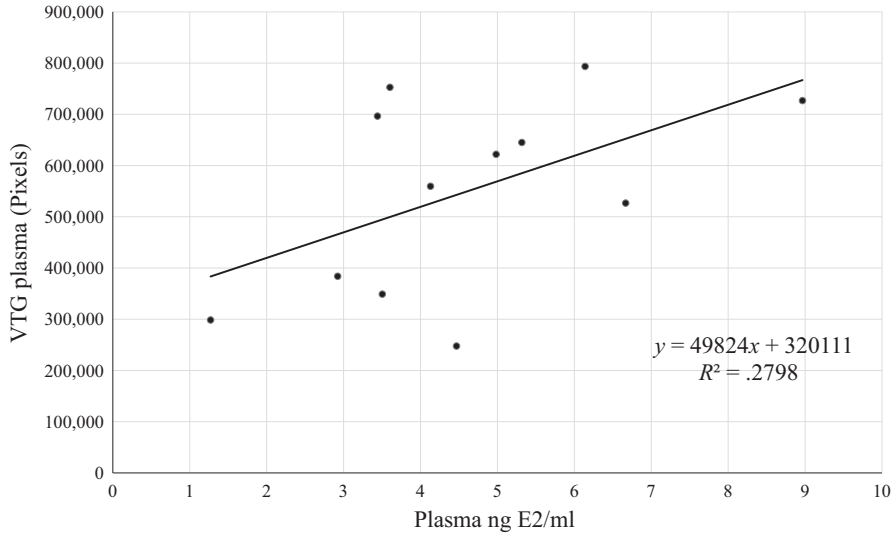

FIGURE 3 Correlation between plasma E2 levels (ng/ml) and VTG densitometry values (pixels). Correlation data were significant (Spearman's correlation, $p < .05$)

was significantly correlated with plasma VTG (Spearman's correlation, $p < .05$).

At the end of feeding trial, females presented oocytes at different stages: in early vitellogenesis and vitellogenesis, only some case in late vitellogenesis. Overall, the analysed ovaries ($N = 24$) were immature in any groups.

3.4 | Cortisol level and fish welfare

The plasma, muscle and fin cortisol average concentrations are shown in Table 4. Statistical analysis did not show any difference among diet groups (ANOVA, F (4, $N = 58$) = 5.7, $p > .05$; ANOVA, F (4, $N = 58$) = 1.45, $p > .05$; ANOVA, F (4, $N = 58$) = 5.11, $p > .05$).

Fish fed the experimental diets showed normal morphology of gills, kidney and muscle. In all the experimental groups, the animals exhibited livers with normal morphology but with a slight degree of steatosis (Figure 4e, 4f).

Histopathological analyses on distal intestine showed a normal morphology and presence of well-differentiated enterocytes with a high number of supranuclear vacuoles (Figure 4a, 4b) and goblet cells (Figure 4c, 4d) in all the fish irrespective of the test diets. PAS (Periodic Acid Schiff) staining of the intestine and liver sections was negative, confirming hepatic steatosis by excluding the presence of glycogen in absorptive vacuoles.

4 | DISCUSSION

The use of soybean meal is a common practice in aquafeed. Soy contains isoflavones that could influence growth, health and reproduction in humans and animals.

In this study, the dietary isoflavone doses were set on the base of possible inclusion of plant protein in aquafeeds and concentrations of isoflavones identified in soybean meal and soybean (Silva & Perrone, 2015). The two isoflavone doses tested (500 and 1,500 ppm) did not significantly influence growth of rainbow trout in terms of final body weight, weight gain or specific growth rate compared with that of control diet animals. Similar results have also been reported in smaller rainbow trout (*O. mykiss*) of 40 g (Pelissero et al., 2001) for a dietary genistein supplementation of 500 and 1,000 ppm, in the yellow perch (*Perca flavescens*) fed a dietary genistein content of 750 ppm (Ko et al., 1999) and in the southern flounder (*Paralichthys lethostigma*) fed experimental diet containing low dose of genistein (100 ppm) (Di Maggio, Kenter, Breton & Berlinsky, 2016), in Nile tilapia (*Oreochromis niloticus*) fed 30 and 300 ppm of dietary genistein (Chen, Wang & Ru, 2016) and in the beluga sturgeon (*Huso huso*) fed different doses of dietary genistein (200, 400, 800 and 1,600 ppm) (Jourdehi et al., 2014). No significant differences were recorded for growth in striped basses (*Morone chrysops*) with high level of genistein (8,000 ppm) in a basal diet (Pollack et al., 2003). By contrast, depressed growth performance was reported in female of yellow perches (*Perca flavescens*), Nile tilapia (*Oreochromis niloticus*), in southern flounders (*Paralichthys lethostigma*) fed different level of dietary genistein (7,500, 3,000 and 1,000 ppm respectively) (Chen et al., 2016; Di Maggio et al., 2016; Ko et al., 1999). Isoflavones as estrogen-like compounds were responsible for the enhancement of growth in juvenile golden pompano (*Trachinotus ovatus*), even if it was used a low dose of genistein (40 ppm) (Zhou et al., 2015).

TABLE 4 The average plasma, muscle and fin cortisol concentrations in rainbow trouts fed the three different diets

	Diets			
Cortisol concentration	ISO 0	ISO-L	ISO-H	*p*
Plasma (ng/ml)	14.32 ± 2.02	31.92 ± 7.31	11.20 ± 2.10	ns
Muscle (ng/g)	0.19 ± 0.02	0.24 ± 0.05	0.3 ± 0.06	ns
Fin (ng/g)	1.11 ± 0.15	2.04 ± 0.42	1.44 ± 0.21	ns

ns, not significant.

GH-enhanced fish growth is mediated by the regulation of IGF levels (Moriyama et al., 2000; Shamblott, Cheng, Bolt & Chen, 1995). IGF-I has a higher correlation with fish growth compared with GH. It has been suggested that soy isoflavones could be regulators of growth hormone and insulin-like growth factors through their estrogen-like activity altering the expression of GH receptor (GHr) or IGF mRNA in fish and other animals (Guo & Zhao, 2005; Han, 1999; Hanson, Kittilson, Martin & Sheridan, 2014).

It was demonstrated that liver *igf*-I mRNA levels in Nile tilapia (*Oreochromis niloticus*) are consistent with body weight gain under feeding conditions (Cruz et al., 2006). Studies on these fish fed diets containing four doses of genistein (0, 30, 300 and 3,000 ppm) showed that, with genistein dietary levels of 3,000 ppm, *igf*-I mRNA expression levels in the liver were significantly lower than in controls and there was no significant difference in the other two groups. These data were confirmed also by significant decreases in growth and IGF-I plasma levels (Chen et al., 2016). Liver *igf*-I mRNA levels in our study did not show significant difference among fish groups and are consistent with the lack of difference in growth (expressed as live weight and growth indices), suggesting no influence of isoflavones on endocrine factors influencing growth at the doses tested.

Soybean isoflavones have estrogenic activity and can bind weakly to estrogen receptors, causing competition between natural estrogens and isoflavones (Zhang, Song, Cunnick, Murphy & Hendrich, 1999), although they may also act as anti-estrogens in the presence of high levels of endogenous estrogens (Martin, Horowitz, Ryan & Mc Guire, 1978). Isoflavones produced large change in plasma vitellogenin levels in the siberian sturgeon (*Acipenser baerii*) (Latonnelle, Le Menn, Kaushik & Bennetau-Pelissero, 2002; Pelissero, Bennetau, Babin, Le Menn & Dunogues, 1991; Pelissero, Le Menn & Kaushick, 1991), in the striped bass (*Morone saxatilis*) (Pollack et al., 2003), in common carp (*Cyprinius carpio*) (Turker & Bozcaarmutlu, 2009) and in rainbow trout (*Oncorhynchus mykiss*) (Pelissero et al., 2001; Pellissero et al., 1991). In this study, no differences in the levels of plasma and liver VTG were found confirming no estrogenic activity of the tested doses of isoflavones on experimental trouts. Vitellogenin detected in plasma and liver of fish in all groups seems due to the circulating endogenous estrogen as shown by the correlation found between plasma E_2 level and VTG densitometry. Previously, the effect of isoflavones on rainbow trout of 600 g body weight fed a low genistein diet (up to 20 ppm) was examined by Latonnelle, Le Menn et al. (2002), and although there was no significant difference, a progressive increase in plasma VTG levels was observed. Other results (Pelissero et al., 2001) have shown that, in the rainbow trout (40 g), only a level of 1000 ppm of genistein in the diet significantly enhanced plasma VTG synthesis in early vitellogenic females. The data found by Pelissero et al. (2001) with doses of isoflavones of 1,000 ppm suggest a dose-dependent effect of these molecules. Estrogenic activity was confirmed in siberian sturgeon (*Acipenser baerii*) both in vivo and in vitro (Pelissero, Bennetau et al., 1991; Pelissero et al., 1993). Striped bass (*Morone saxatilis*) fed

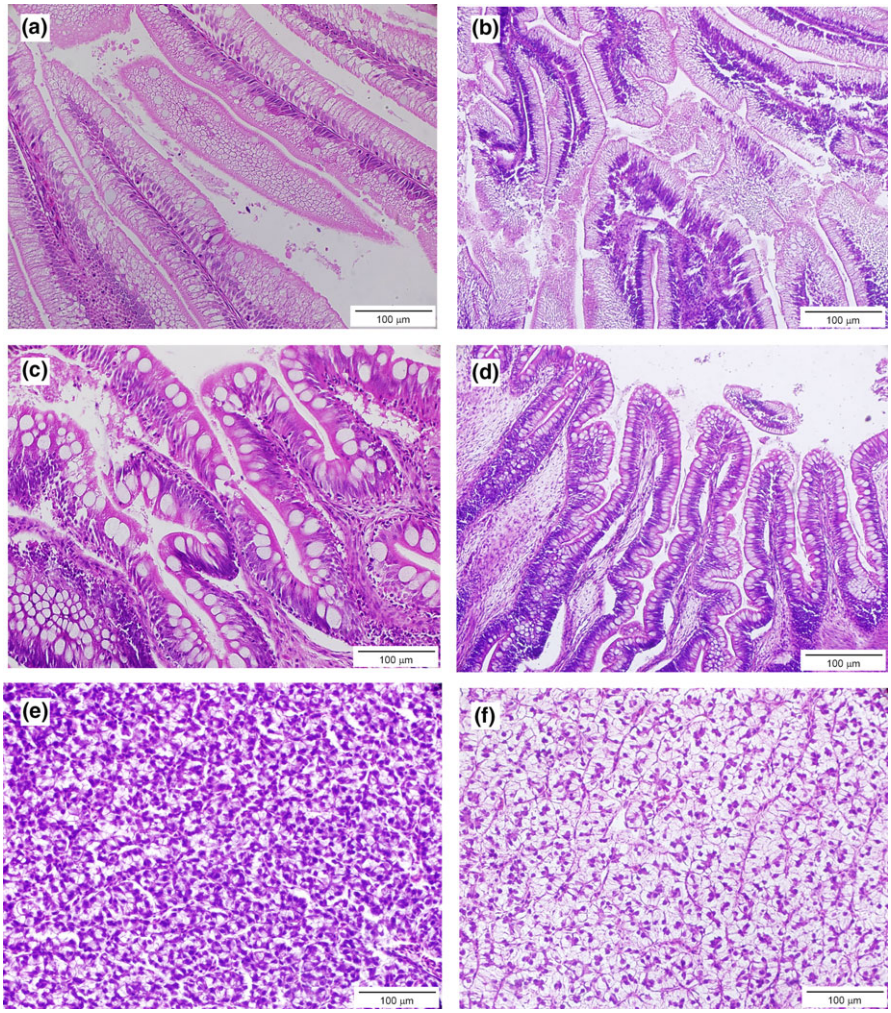

FIGURE 4 Histology of distal intestine (a, b, c, d) and liver (e, f) (H&E) of rainbow trouts fed control diet (a, c, e) and high dose of dietary isoflavones (1,500 ppm) (b, d, f) over 70 days. Distal intestine showed a normal morphology and presence of well-differentiated enterocytes, but a sopranuclear accumulation of lipid droplets (a, b) was observed in enterocytes associated with high number of goblet cells (c, d) with different amount without different. Livers showed normal morphology but with weak amount of lipid droplets (e, f) within hepatocytes, with variable degrees of steatosis

three soy genistein levels (2, 4 and 8 mg/g of diets) had VTG levels that increased at low dose (2 mg/g), decreased at the medium dose (4 mg/g) and raised with highest dose (8 mg/g) (Pollack et al., 2003), showing the controversial effect of genistein, which has been regarded as both an estrogenic agonist and antagonist (Holt, 1998). No estrogenic effect in our results suggest that isoflavones is not acting on the endocrine system of trout neither in stimulatory nor in inhibitory manner probably due to the too low level to exert a classic dose–response effect in this species and size.

Isoflavones could exert their estrogenic effects increasing the circulating endogenous E_2 concentration by disrupting the E_2-inactivating pathways. This mechanism was reported by Ng et al. (2006), who observed that E_2 metabolism was inhibited in a dose-dependent manner by genistein and daidzein, thereby increasing serum steroid hormone levels. In this study, the levels of isoflavones in the diets did not exert their estrogenic effects by increasing the bioavailability of E_2, as E_2 plasma levels were found to be in the physiological range for the developmental stage of the ovaries and were not correlated with isoflavone doses used.

Our results showed that diet supplementation with isoflavones at 500 and 1,500 ppm diets did not improve the gonadal development in rainbow trout. Similarly, the total intake of isoflavones did not result in difference in the GSI of trouts and these values were significantly correlated with plasma VTG densitometry. This result is in agreement with data on Siamese fighting fish (*Betta splendens*) exposed to genistein (Brown, Stevenson, Leonard, Nieves-Puigdoller & Clotfelter, 2014) and results obtained by a study on striped bass (*Morone saxatilis*) in which fish treated with genistein had GSI values similar to those of controls (Pollack et al., 2003). However, genistein and daidzein taken in with the diet had dose- and time-related effects on gonad growth (GSI) in male and female of goldfish (*Carassius auratus*) (Bagheri, Imanpoor & Jafari, 2014).

In this study, the effect of the soy isoflavones on cortisol levels was examined. Isoflavones may alter endocrine functions and, as exogenous compounds that could be present in high dose in feed, may induce stress in fish. Cortisol is a biomarker to evaluate the state of stress in fish (Bertotto et al., 2010; Hasenbein, Fangue, Geist, Komoroske & Connon, 2016) as well as in other vertebrates. At the end of the trial, plasma, muscle and fin cortisol concentrations were not significantly different in the trouts fed different diets. This result indicated that isoflavones, at our doses, did not influence endocrine activity and did not induce a state of stress in fish as the cortisol levels were within the welfare range (Bertotto et al., 2010). Also Aluru et al. (2005) reported that trout fed phytoestrogens had

plasma cortisol levels within unstressed levels in all treatments even if cortisol concentrations among fish fed various flavonoids were significantly different. An in vitro test using head kidney tissue of treated trout indicated no significant effect of flavonoids on basal cortisol production rates compared to fish fed basal diet, but the ACTH-stimulated cortisol production rate was significantly lower with phytoestrogens compared to the control group (Aluru et al., 2005).

The dietary factors responsible for enteropathy and low nutrient utilization of diets are termed anti-nutritional factors and they are also present in soybean seeds. Soyasaponin, phytosterols and isoflavones are considered the most relevant (Krogdahl, Penn, Thorsen, Refstie & Bakke, 2010). Studies are concentrated on the effect of isoflavones on reproductive performance (Brown et al., 2014; Di Maggio et al., 2016; Pelissero et al., 2001; Stevenson, Brown, Montgomery & Clotfelter, 2011), whereas the information to understand their significance on other physiological processes like absorption in fish is incomplete. Atlantic salmon fry fed fishmeal supplemented with isoflavones presented no histological changes in liver and distal intestine. However, the degree of hepatocyte vacuolization was lower than in fish fed diets without isoflavones. Also, isoflavone-supplemented diets induced alterations of digestive enzymes necessary for carbohydrate digestion as confirmed by decreased growth of treated fish (Gu et al., 2014). Our histopathological analysis of rainbow trout fed different level of isoflavones showed normal morphology of intestine and liver, confirming adequate welfare of the fish as in the previous study (Gu et al., 2014), but no effect on their growth. The slight degree of liver steatosis was probably not caused by isoflavones but by the basal diet used in this trial.

In conclusion, neither isoflavone dose (500 and 1,500 ppm) induced estrogenic effects in rainbow trouts. Vitellogenin production was probably stimulated by 17-β-estradiol as secretion of vitellogenin was dose dependent in response to endogenous estrogens. E_2 and cortisol were not influenced by the different levels of isoflavones, indicating no endocrine disruption. Isoflavonic phytoestrogens did not influence reproduction and growth at the tested doses and fish welfare was maintained.

ACKNOWLEDGMENTS

This research was supported by a grant of University of Padova to G Radaelli (Progetto di Ateneo 2012—prot CPDA123738).

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How to cite this article: Pastore MR, Negrato E, Poltronieri C, et al. Effects of dietary soy isoflavones on estrogenic activity, cortisol level, health and growth in rainbow trout, *Oncorhynchus mykiss*. *Aquac Res*. 2018;49:1469–1479. https://doi.org/10.1111/are.13602

CHAPTER 3

DIETARY SOY ISOFLAVONE SUPPLEMENTATION IN RAINBOW TROUT: TISSUE DEPOSITION AND INFLUENCE ON FILLET QUALITY

3.1. Abstract

Fish meal have been substituting partially by soybean meal, containing isoflavones. The estrogenic/antiestrogenic activity of isoflavones may affect fish growth performance and proximate composition of muscle. The potential effects of isoflavones on flesh quality and time of conservation may affect the utilization of soybean meal as an alternative protein source in aquaculture feed. In the main time, it is important, for the consumer safety, to know the isoflavone content in edible tissue and their potential antioxidant effect that could affect the product shelf life.

Rainbow trout fed three experimental diets containing different concentrations of isoflavones (0, 500, 1500 ppm) for 70 days. The levels of isoflavones in diets did not affect trout growth performance during the rearing period. Even biometric indexes, skin and fillet colour, rheological characteristics, proximate composition, fatty acids profile of fillet were not affected by the different dietary treatments. Otherwise, time of storage reduced fillet yield (56.4 *vs*. 5.6 %; $p < 0.01$), and skin lightness (59.2 *vs.* 51.5, $p < 0.01$); red index moved to more negative values (near-zero, however) and yellow index decreased (6.99 *vs.* 5.07, $p < 0.01$). Fillet pH (6.22 *vs.* 6.34, $p < 0.01$) and lightness increased (38 *vs.* 43.6, $p < 0.01$), yellow index (6.20 *vs.* 4.52, $p < 0.01$) and shear force decreased (0.94 *vs.* 0.80 g kg^{-1}, $p < 0.01$). According to the time of storage, trout fillet showed an increase of water, a loss of crude protein and an increase of total volatile basic nitrogen content (19.3 *vs.* 21.2 mg 100 g^{-1}, $p < 0.01$). As a result of PUFAs omega-6 decrease (13.1% *vs.* 12.7%; $p < 0.05$), omega-

3/omega-6 ratio increased (1.05 *vs.* 1.20; $p < 0.05$) during storage.

The fillet analysis evidenced an accumulation of isoflavones influenced both by dietary isoflavone content (0 *vs*. 500 and 1500 ppm; $p < 0.05$) and refrigerated storage time (1 *vs.* 7 days; $p < 0.05$) while fillet lipid oxidation measured by means of thiobarbituric acid reactive substance assay (TBARS) was only affected by the storage time ($p < 0.05$).

In conclusion, although a transfer of isoflavones from diet to fillet was observed, rheological and nutritional characteristics, as well as fillet quality were not affected by dietary treatment.

3.2. Introduction

Aquaculture is one of the fastest growing food-producing sectors (increasing at an average annual growth rate of 6.1% in 2002–2012), supplying approximately 50% of the world's seafood (FAO, 2014). In Europe, the production is stable and high-value species are farmed compared to Asia (STECF, 2014). Rainbow trout (*Oncorhynchus mykiss*) is the most farmed trout all over the world (850,000 tonnes are produced annually, of these 188,000 are produced in UE) (FAO, 2014). UE, Chile, Turkey and Norway are the main producers. In 2014, Italy is the first producer in the UE with 36,800 tonnes, followed by Denmark, France and Poland (FEAP, 2015).

Seafood is extremely healthy and nutritious: it's a vital source of high value proteins and essential nutrients, such as polyunsaturated fatty acids (PUFAs) and has a relatively low fat and connective tissue content (Kris-Etherton *et al*., 2002).

Quality and composition of feeds deeply affect the nutritional, health and organoleptic characteristics of the final product. The formulation of the feed must cover the energetic and nutritional needs of the trout. Over the last 30 years, feeds have become more energetic and richer in lipids, in order to reduce costs of proteins. Fish meal and fish oil have been substituting gradually and partially by plant-based products, because of their improved sustainability and lower costs (Tacon and Metian, 2008). Soybean meal and soy protein concentrate are ingredients currently incorporated in aquaculture feeds as partial replacement of fish ingredients (Tibaldi *et al*., 2006; Watanabe, 2002).

Soy contains anti-nutritional factors, such as lectins, oligosaccharides, saponins and trypsin inhibitors that may exert negative effects on feed digestibility and this limits very high inclusion rates in fish (Krogdahl *et al*., 2010). Soybean-derived ingredients also contain isoflavones, i.e. flavonoids, natural plant compounds with a polyphenolic structure. Genistein, daidzein and glycitein are the major isoflavones in soybean (Barnes *et al*., 2011). These compounds are classified as phytoestrogens because exert an estrogenic-antiestrogenic effect binding estrogen receptors (Ko *et al*., 1999; Latonelle *et al*., 2002; Bennetau-Pelissero *et al*., 2001) and could have potential biological effects on human health (prevention of hormone-related cancers, cardiovascular diseases, osteoporosis and postmenopausal symptoms) (Ko, 2014; Messina, 2014). In fish, they can affect growth (Ko *et al*., 1999; Cleveland and Manor, 2015; Di Maggio *et al*., 2016; Zhou *et al*., 2015) reproduction (Bennetau-Pelissero *et al*., 2001; Ko *et al*., 1999; Ng *et al*., 2006), sexual development (Kiparissis *et al*., 2003), protein turn-over (Cleveland, 2014), immune response and hepatic antioxidant capability (Zhou *et al*., 2015).

Soy isoflavones are also known as a growth promoters and used for this reason in poultry farming to improve production (Jiang *et al*., 2007) but they are also considered as antinutritional factors (Kroghdal *et al*., 2010). The effects of these molecules on fish in terms of growth performance and feed utilization are controversial (Ko *et al*., 1999; Pollack *et al*., 2003; Turker and Bozcaarmutlu, 2009; Ye and Chen, 2008).

Diet influences proximate composition of fish flesh. It is reported that carotenoids, lipid sources, antioxidants, and other components can affect colour, fatty acid profile, texture, and flavour of raised fish (Færgemand *et al*., 1995, Johnsen *et al*., 1991, Nickell *et al*., 1998, Waagbo *et al*., 1993). On the basis of this knowledge, different levels of dietary isoflavones inclusion could produce organoleptic modifications.

Phytochemicals, as isoflavones, are considered 'health promoting' by virtue of their antioxidant activity and positive modulation of the cellular and tissue redox balance (Liu, 2003). This

antioxidant activity may also be for health benefit in fish that in the end leads to a growth increase Chakraborty *et al*., 2014).

Phytochemicals may provide health benefits also as substrates for biochemical reactions, cofactors as well as inhibitors of enzymatic reactions, absorbents/sequestrats that bind to and eliminate undesirable constituents in the intestine, and compounds that enhance the absorption and/or stability of essential nutrients (Holst and Williamson, 2008; Virgili and Marino, 2008). These effects of phytochemicals could promote the histophysiology of fish intestine that plays an important role in the digestive and absorptive functions of the alimentary tract, thereby inducing a significant effect on fish nutrition and growth (El-Bakary and El-Gammal, 2010). Polyphenolic phytochemicals may also act either as elective ligands or ligand mimics that agonize or antagonize cell surface or intracellular receptors (Virgili and Marino, 2008). The estrogen-like effects of genistein may affect proximate composition of fish muscle. Soy isoflavones as other phytoestrogens are involved in the lipid metabolism (Xiao *et al*., 2008) even if, up to date, the results are controversial. Mai *et al*. (2012) showed that the whole-body crude lipid content of *Japanese flounder* decreased significantly with increasing dietary soy isoflavones. Turan and Akyurt (2005) found that administration of red clover, containing isoflvavones, significantly improved the level of protein, lipid and ash in African catfish (*Clarias gariepinus*). Yılmaz *et al*. (2009) had found that the protein content of *C*. *gariepinus* increased with increasing rates of the phytoestrogen mixture. A study on common carp fed dietary red clover did not influence proximate composition contents of fish muscle among all the diet groups (Turan *et al*., 2007). Also, dietary genistein did not affect protein, ash, fat or moisture content of rainbow trout (*Onchorhynchus mykiss*) fillets (D'Souza *et al*., 2005). As previously indicated, there are some evidences that phytoestrogens may affect chemical values; others studies reported an effect of the isoflavones also on quality of fish muscle and particular a well-known astringent taste (Okubo *et al*., 1992). However, recent studies reported no evidence on the sensory quality of fish flesh after the isoflavone dietary inclusion (D'Souza *et al*., 2005; D'Souza, 2006).

Freshness is considered the most important feature of a high quality fish (Gökoğlu and Yerlikaya, 2015). The shelf life of fish can be increase maintaining the cold-chain from the producer to the consumer, as cold delays the negative effect of endogenous and exogenous (microbial) enzyme reactions. Progressive autolysis and spoilage microorganisms produce a lot of metabolites that negatively affect freshness. Lipid oxidation is one of the principal cause of quality loss in seafood, that contains high amounts of polyunsaturated fatty acids. Quality deterioration can be retarded by incorporation of additives with antioxidant properties. The use of synthetic antioxidant, polyphenolic compounds, has long been practiced in retarding lipid oxidation in fish muscle (Maqsood *et al*., 2014). To our knowledge, during the last 2 decades, phytoestrogens (isoflavones i.e.) was been supplemented in feed fish in few trials and tested *in vivo* showing to retard lipid oxidation and potentially increase shelf life (D'Souza *et al*., 2005; Mai *et al*., 2012).

The present study aimed to define the role of dietary isoflavone supplementation in rainbow trout at different levels of inclusion (0, 500, 1500 ppm), for 70 days, focusing on fish growth performance and fillet analysis in terms of rheological and chemical quality, muscle lipid oxidation, freshness and isoflavone deposition 1 and 7 days after storage at 4° C.

3.3. Materials and Methods

3.3.1. Experimental diets

The experimental diets were prepared as described in *Chapter 2*.

3.3.2. The set of samples

The feeding trial is described in *Chapter 2* of this *Thesis*.

At the end of *in vivo* test, 54 fish (9 for each replicate) were collected, slaughtered by immersion in ice slurry and transported immediately to the laboratory in thermally insulated boxes to be stored on ice in a refrigerated room (4 °C) for subsequent analysis. The trouts were analysed in

different times: 27 fish (9 for each experimental group) were evaluated after 1 and 7 days of storage time at 4 °C. Samples of the experimental diets were also collected for the analysis.

3.3.3. Biometric measurements and physicochemical traits of rainbow trout

On the day after collection, the intact fish were weighed to determine the slaughter weight (SW). The colour of the skin was determined at three points on the dorsal side and at two points on the ventral side with a Minolta spectrophotometer CM–508 C (Minolta, Milano, Italy) according to the CIE L*a*b* method (1976) and using the D_{65} light source and a 10° observer. The maximum shear force was recorded on the right side of fillet without skin using LS5 dynamometer (Lloyd Instruments Ltd, Bognor Regis, UK) using the Allo-Kramer (10 blades) probe (load cell: 500 kg; distance between the blades: 5 mm; thickness: 2 mm; cutting speed: 250 mm min^{-1}).

The following biometric measurements were taken on the intact fish (Poli *et al*., 2001): total length, standard length, head length and maximum height. The fish were then dissected, and the carcass, viscera, liver and fillets (with skin) were weighed. The condition factor (100 × live weight/total $length^3$), relative profile (maximum height/total length), cranial index (head length/total length), visceral index (100 × viscera weight/slaughter weight), hepatosomatic index (100 × liver weight/slaughter weight), mesenteric lipid index (100 × mesenteric lipid weight/total weight) dressing percentage (100 × carcass weight/slaughter weight) and fillet yield (100 × fillets weight/ slaughter weight) were calculated.

The colour indexes, as described above for skin, and the final pH were measured at three points on the dorsal side of the fillets taken from the right side of the fish (the right fillets).

3.3.4. Proximate composition, FA and Total Volatile Basic Nitrogen analysis

The left fillets were separated from the skin and minced. Left fillets were divided for different analysis: immediately analysed to evaluate the total volatile basic nitrogen (TVB−N), freeze-dried and stored under vacuum at 4 °C for subsequent analysis of their proximate composition and stored under vacuum at −18 °C for subsequent analysis of their fatty acid profile.

The freeze-dried fillets were analysed with AOAC (2000) methods, to determine the concentrations of dry matter (934.01), ash (967.05) and crude protein (2001.11). Ether extracts of the fillets were analysed with AOAC method (991.36). Ether extracts of the diets were analysed after acid-hydrolysis treatment (European Commission, 1998). The crude fibre content of the diets was analysed with AOAC method 962.09.

The fresh minced fillets were analysed for FA composition. For this purpose, fat was extracted from the samples with accelerated solvent extraction (ASE ®, Dionex, Sunnyvale, CA, Application Note 334). The procedure consisted of two extraction cycles with mixture of hexane/2-propanol (3/2) as a solvent at a temperature of 100 °C, with a heating phase of 5 min and an extraction phase of 1 min.

First, transmethylation was performed on the extracted lipids to determine fatty acid methyl esters (FAMEs) using a solution of 0.5 M sodium methoxide in methanol (1 vol.), a solution of 5 % HCl in methanol, 6 % potassium carbonate in water (Jenkins, 2010). An internal standard (19:0 methyl ester) was added to the extracts prior to methylation. After centrifugation, the supernatant was injected into the split/splitless system of a 2D GC × GC gas chromatograph (Agilent 7890A, Agilent Technologies, Santa Clara, CA) with first column of 75 m × 180 μm (internal diameter) × 0.14 μm film thickness (23348U, Supelco, Bellefonte, PA), and second column of 3.8 m × 250 μm (internal diameter) × 0.25 μm film thickness (J&W 19091−L431, Agilent Technologies). Hydrogen at 0.22 mL min^{-1} and 22 mL min^{-1} was used as the carrier in the first and second column respectively. An oven temperature of 50 °C was held for 2 min, raised to 150 °C at the rate of 50 °C min^{-1}, held for 15 min,

raised to 240 °C (held for 84 min) at the rate of 2 °C min^{-1}. The injector and the detector temperatures were set at 270 and 250 °C respectively. The FAs were identified by comparing their retention times with a standard mixture of 52 fatty acids methyl esters FAMEs (Nu-Chek Prep Inc.). The concentration of individual fatty acids methyl esters (FAMEs) was expressed as a percentage of the total area of eluted FAMEs (known plus unknown). The resulting 2-dimensional chromatograms were analyzed with the comprehensive GC × GC software (Zoex Corp., Houston, TX) to evaluate the cone volume of each FA.

Total volatile basic nitrogen (TVB–N) was determined according to the described method in the EC reference (European Union, 1995). A 10 g sample was extract with 90 ml of perchloric acid 6 %. The sample was homogenized for two minutes with a blender and then filtered. 50 ml of the obtained extract were put in an apparatus for steam distillation. After adding a few drops silicone anti foaming agent, 6.5 ml of sodium hydroxide solution are added to the extract, and steam distillation begins immediately. After the distillation, the contents of the conical flask were titrated with 0.05 N HCl and the total volatile basic nitrogen (TVB–N) was calculated. The results are expressed in mg TVB–N 100 g^{-1} of fish muscle.

3.3.5. Thiobarbituric Acid Reactive Substance Analysis.

The amount of lipid peroxidation in the fillet was assessed by means of thiobarbituric acid-reactive substances (TBARS) according to Vinagre *et al*. (2012). TBARS in one of the most widely used assays for measuring lipid peroxidation end product malondialdehyde (MDA), a reactive aldehyde produced by lipid peroxidation of polyunsaturated fatty acids. TBARS levels were determined in fillets of the trout fed the three isoflavone doses (0 ppm, 500 ppm, 1500 ppm), after 1 and 7 days of refrigerated storage (4°C) to evaluate the potential antioxidant effect of these molecules on edible part of the fish.

The fillet (50 mg) was previously grounded, mixed with Tris HCl 0.125 M pH 6.9 (250 µL), centrifuged at 13,000g at 4°C for 15 min and extracted liquid used for the assay. Fifty microliters of each sample were added to 25 µL of SDS 8.1%, 187.5 µL of trichloroacetic acid (20%, pH 3.5), 187.5 µL of thiobarbituric acid (1%) and 6.25 µL of ethanol cointainig butylated hydroxytoluene 0.8%. To this mixture, 50 µL of Milli–Q grade ultrapure water were added and the eppendorfs were put in a vortex for 30 s. The eppendorfs lids were punctured with a needle and the eppendorfs were incubated in boiling water for 10 min. Straight after, they were placed in ice for a few minutes to cool and 125 µL of Milli–Q grade ultrapure water and 625 µL of n-butanol pyridine (15:1, v/v) were added. Then the eppendorfs were placed in a vortex and centrifuged at 7000 rpm for 5 min. Duplicates of 200 µL of the supernatant of each reaction were put into 96-well microplate and absorbance was read at 530 nm. To quantify the lipid peroxides, an eight-point calibration curve (0–200 µM TMOP) was calculated using tetra-methoxypropane standards. TBARS formation were estimated as micromoles of MDA equivalents per g of fillet.

3.3.6. Soy isoflavones extraction and quantification

The isoflavone content was calculated in muscle of the same specimens (27 trout, 9 for each experimental group, ISO–0, ISO–L and ISO–H) stored 1 and 7 days at 4°C after slaughter.

3.3.6.1. Sample preparation before LC-ESI-MS analysis

All samples were stored at – 80°C pending analysis. The trout muscles were thawed, filleted, chopped into small pieces and homogenized. Five grams of each samples were weighed, placed into a 50 ml centrifuge tube and processed as described in Merlanti *et al*. (2018) (*Appendix 2*).

Isoflavone extraction from rainbow trout muscle was performed with SampliQ Quick Easy Cheap Effective Rugged Safe (QuEChERS) technology which consists of two steps, a salting-out extraction and a dispersive SPE (Solid Phase Extraction) clean-up. Isoflavone reference standards

(Daidzein (De, purity > 99.0%), Genistein (Ge, purity > 99.0%), Glycitein (Gle purity > 99.0%), and 3', 4'-Dimethoxyflavone (Internal Standard, IS, purity > 98.0%); LC Laboratories (USA) were used to obtain standard solutions to comparing obtained data.

3.3.6.2. LC-ESI-MS analysis

Quantitative analysis of IFs was carried out using liquid chromatography coupled with electrospray ionization mass spectrometry (LC-ESI-MS) in the positive ion mode. The chromatographic separation was achieved using an Accela 600 HPLC pump with CTC automatic injector (Thermo Fischer Scientific, San Jose, CA, USA) equipped with a Hypersil Gold (100 x 2.1 mm, 1.9 um) analytical column by Thermo Fisher Scientific (USA). The mass detection was achieved with a Thermo Fischer LTQ XL ion trap mass spectrometer (San Jose, CA, USA), equipped with a heated electrospray ionization (HESI–II) probe. The ESI source was used in a positive mode.

The system was controlled by the X-calibur software (version 2.1), that was also used for the data acquisition and analysis.

3.3.7. Statistical analyses

Biometric measurements, physicochemical traits and chemical analysis of rainbow trout and fillets were analysed by ANOVA with diets and storage time as main factors and with tank (or replications) as a random effect, by PROC MIXED of SAS software (SAS 2013, 9.1 version). Isoflavone and MDA fillet contents were analysed by ANOVA with diets and storage time as main factors followed by Tukey or Bonferroni *post hoc* test to evaluate which specific groups differed (STATISTICA 8.0, StatSoft, Tulsa, OK, USA). Data were log (+1)-transformed if needed and data in the text are reported as mean.

3.4. Results and Discussion

3.4.1. Growth, biometric measurements and physicochemical traits of trout

The different isoflavone doses, added to diets for 70 days, did not affect growth performance as reported in *Chapter* 2 (Pastore *et al*., 2018). At the end of feeding trial the slaughter weight (SW) and carcass weight (CW) were similar in the different groups (Table 3.1). *In vivo* studies using dietary isoflavones reported contradictory results about growth performance (Ko *et al*., 1999; Pollack *et al*., 2003; Turker and Bozcaarmutlu, 2009; Ye and Chen, 2008), probably due to double effects of phytoestrogens as estrogen or anti-estrogen-like substances and/or their antinutrient effect (Krogdahl et *al*., 2015). Moreover, contradictory results from different literatures may be due to the differences of fish species, developmental stages, sex, form and level of soy isoflavones supplementation and dietary protein source, composition of diet, ability to metabolize or utilize a particular additive present in the diet or the level of dietary isoflavones as well as to the timing of trials (Pastore *et al*., 2018; Mai *et al*., 2012).

Biometric traits did not evidence significant differences in relative profile, cranial index, condition factor, hepatosomatic, viscerosomatic and mesenteric lipid index as well as dressing percentage and fillet yield for the dietary treatment. Unexpectedly, the storage time had modified some biometric traits: cranial index ($p < 0.05$), condition factor ($p < 0.05$) and hepatosomatic index ($p < 0.001$) as well as fillet yield ($p < 0.01$) from 56.4 to 53.6%.

The dietary isoflavones did not modify the colour of fish in terms of luminosity, red and yellow indexes, whereas, increasing storage time, L decreased (from 59.2 to 51.5, $p < 0.001$), red index became more negative (from – 0.29 to – 0.87, $p < 0.001$) and yellow index was decreased as well (from 6.99 to 5.07, $p < 0.001$) (Table 3.1).

Table 3.1. Rainbow trout biometric parameters, texture and colour of intact fish and fillets.

	Diets[1] (D)			Storage time (T)		Probability			RSD*
	ISO 0	ISO–L	ISO–H	1d	7d	D	T	DxT	
Rainbow trout (No.)	18	18	18	27	27				
Slaughter weight (SW) (g)	344	333	333	332	340	0.70	0.49	0.07	41.7
Carcass weight (g)	306	296	295	295	302	0.67	0.49	0.04	36.2
Relative profile	0.22	0.22	0.22	0.22	0.22	0.70	0.82	0.99	0.01
Cranial Index	0.19	0.19	0.19	0.19	0.19	0.21	0.04	0.89	0.01
Condition factor	1.20	1.23	1.22	1.20	1.24	0.79	0.03	0.71	0.07
Hepatosomatic Index (% SW)	1.86	1.75	1.77	1.94	1.65	0.91	<0.001	0.29	0.27
Visceral Index (% SW)	10.9	11.1	11.3	11.1	11.1	0.66	0.84	0.36	1.23
Mesenteric lipid Index (% SW)	1.45	1.69	1.89	1.87	1.48	0.22	0.02	0.22	0.57
Dressing percentage (% SW)	89.2	88.9	88.7	88.9	89.0	0.66	0.84	0.36	1.23
Fillet yield (% SW)	55.4	55.6	54.0	56.4	53.6	0.42	<0.01	0.24	3.19
Skin									
L*	54.5	54.9	56.6	59.2	51.5	0.40	<0.001	0.71	4.19
a*	-0.79	-0.41	-0.54	-0.29	-0.87	0.27	<0.001	0.50	0.58
b*	5.67	6.53	5.90	6.99	5.07	0.49	<0.001	0.52	1.98
Fillet									
pH	6.29	6.27	6.29	6.22	6.34	0.78	<0.001	0.19	0.93
L*	41.0	40.7	40.8	38.0	43.6	0.94	<0.001	0.59	2.41
a*	-1.90	-1.85	-1.93	-1.94	-1.85	0.96	0.68	0.06	0.84
b*	5.08	5.31	5.69	6.20	4.52	0.48	<0.001	0.14	1.36
Maximum shear force (kg)	25.5	25.2	23.3	26.1	23.3	0.56	0.03	0.52	4.48
Shear force (kg g^{-1})	0.91	0.87	0.83	0.94	0.80	0.47	<0.001	0.90	0.14

[1]Diet ISO 0 = 0 ppm, ISO–L = 500 ppm, ISO–H = 1500 ppm of isoflavones included in basal diet
* RSD, residual deviation standard

To our knowledge, no research that focused on effect of mix soy isoflavones on fillet colour have been published yet. D'Souza *et al*. (2005) evidenced no influences of the sole dietary genistein administered for a period of 6 and 12 months in fillet *L*, *a* or *b* values in trout. Also in the present study, fillet colour did not change with different dietary isoflavone doses, confirming D'Souza *et al*. (2005) results.

Conversely, the effect of storage time influenced the rheological traits: pH raised from 6.22 (after 1 day of storage at 4°C) to 6.34 (after 7 days of storage) ($p < 0.01$); luminosity index increased (from 38 to 43.6, $p < 0.01$) caused to protein denaturation and lose of capacity of hydric retention; yellow index decreased (from 6.20 to 4.52, $p < 0.01$) suggesting a progressive loss of fillet colour; maximum shear force (kg) and shear force (kg g^{-1}) decreased from 26.1 kg to 23.3 kg ($p < 0.03$) and from 0.94 to 0.80 kg g^{-1} ($p < 0.001$) respectively indicating the progressive loss of structure and gradual progress of *rigor mortis* resolution of fish due to exogenous and endogenous enzyme (Table 3.1).

3.4.2. Proximate composition, Total Volatile Basic Nitrogen and FA profile of trout fillets

Proximate composition of fillet was not influenced by different doses of dietary isoflavones (Table 3.2). The obtained results support data of D'Souza *et al*. (2005): fingerling trout (mean weight of 4.5 g) were fed with different levels of genistein (500, 1000 and 3000 ppm) for 6 and 12 month and diet did not affect protein, ash, fat or moisture content. Similar results have been observed in Japanese flounder fed diets containing 0.08% – 0.64% isoflavone (Chen, 2009) and in juvenile Nile tilapia (*Oreochromis niloticus*) fed diets formulated containing four graded supplements of genistein: 0, 30, 300, and 3000 ppm (Chen *et al*., 2015).

Another study demonstrated that there is no significant effect of dietary isoflavones on crude protein content in Japanese flounder (Mai *et al*., 2012) which was consistent with results of our study.

However, dietary isoflavones can affect crude lipid levels of final product (Mai *et al*., 2012) because as endocrine disruptors they may interfere with regulation of reproduction and the role of

estrogens in feed intake, lipid metabolism, regulatory action on peroxisome proliferator-activated receptor γ (PPARγ), involved in regulation of fatty acid storage (Krogdhal *et al*., 2015). Up to date, the role of soy isoflavones in the lipid metabolism is not fully understood but studies suggest that soy isoflavones may modulate key transcription factors activities, and thereby downstreaming gene expression involved in lipogenesis or lipolysis (Xiao *et al*., 2008; Cao *et al*., 2012). Adipose tissue metabolism is under estrogen control. Soy isoflavones have similar effect even though phytoestrogen have several-fold less estrogenic and antiestrogenic effect than the estradiol. Genistein is antilipogenic and may reduce adipocyte size, at least in part by inhibiting the effects on the lipogenic enzyme LPL and modulating adipose deposition in mice (Naaz *et al*., 2003). Study on fish supported this data, in fact Mai *et al*. (2012) found that the whole-body crude lipid content of Japanese flounder decreased significantly with a high level of genistein (8000 ppm) supplementation in the diet. In our study, different isoflavone doses did not influence lipid level of trout, and these results are consistent with those of Mai *et al*., 2012 involving lower dietary isoflavone levels (1000 and 4000 ppm). It is possible that in fish, effect on endocrine system of isoflavones associated to high isoflavone doses may be partly responsible of control lipid metabolism.

Differently, increasing the storage time, trout fillet showed an increase of water content, a decrease of crude protein and ash and an increase of total volatile basic nitrogen (TVB–N): from 19.3 to 21.2 mg N 100 g-1, indicating a state of fish deterioration (Table 3.2). The content of TVB–N do not compromise commercialization of product in accordance with Regulation (EC) No 853/2004 (European Communities, 2004). No limits is defined for *O*. *mykiss*, but, as an indication, the limit defined for *Salmo salar* is up to the present study detected levels (35 mg of N 100 g^{-1}; European Communities, 2008).

Table 3.2. Proximate composition (% as–fed) and level of total volatile basic nitrogen of the rainbow trout fillets.

	Diets[1] (D)			Storage time (T)		Probability			RSD*
	ISO 0	ISO–L	ISO–H	1d	7d	D	T	DxT	
Rainbow trout (No.)	18	18	18	27	27				
Proximate composition									
Water (%)	76.8	76.4	76.5	76.0	77.1	0.55	<0.001	0.04	0.92
Crude protein (%)	19.0	19.2	19.2	19.4	18.9	0.38	<0.001	0.06	0.41
Ether extract (%)	2.31	2.56	2.52	2.65	2.28	0.57	0.06	0.12	0.69
Ash (%)	1.22	1.22	1.22	1.29	1.15	0.92	<0.001	0.94	0.05
Total volatile basic nitrogen (TVB-N) mg N/ 100 g	20.0	20.3	20.5	19.3	21.2	0.73	<0.001	0.86	1.46

[1]Diet ISO 0 = 0 ppm, ISO–L = 500 ppm, ISO–H = 1500 ppm of isoflavones included in basal diet
* RSD, residual deviation standard

The FA profile of fillets did not change among the three diet fish, but differences due to the two storage times have been evidenced (Table 3.3). In fact, during the storage time, total saturated FAs (SFAs) did not change (28.4% at 1d at 4°C and 28.5% after 7d of storage at 4°C, respectively).

A decrease of monounsaturated fatty acid (MUFAs) was observed after 7d of storage (from 42.3 to 41.1%) but without statistical difference even if p value is approaching to significance level ($p = 0.07$).

The proportion of n – 3 PUFAs raised after 7d of conservation at 4°C (from 13.7 to 15.2%) without difference, the proportion n – 6 statistically decrease during the storage time (from 13.1 to 12.7%; $p < 0.05$). As a consequence, the ratio of n – 3 to n – 6 PUFAs was significantly higher in fish storage for 7 d than 1 d (1.05% *vs*. 1.20%, in fish storage for 1 and 7 d) ($p < 0.05$).

The interaction between diets and storage times had a significant effect ($p < 0.01$) on contents of stearic acid (C18:0) only, belonging to SFAs.

Table 3.3. Fatty acid profile (% of total fatty acid methyl esters) of the diets.

	Diets[1] (D)			Storage time (T)		Probability			RSD*
	ISO 0	ISO–L	ISO–H	1d	7d	D	T	DxT	
Rainbow trout (No.)	18	18	18	27	27				
Fatty acid profile									
C14:0	4.07	3.97	3.92	4.08	3.89	0.53	<0.01	0.98	0.22
C16:0	19.1	18.9	18.5	18.8	18.9	0.38	0.58	0.10	1.00
C17:0	0.33	0.31	0.31	0.31	0.32	0.24	0.27	0.07	0.02
C18:0	4.07	3.95	3.94	3.93	4.04	0.57	0.09	<0.01	0.22
Total SFAs	28.9	28.4	28.0	28.4	28.5	0.27	0.82	0.10	1.35
C16:1 n – 7	5.20	5.30	5.26	5.36	5.15	0.88	0.07	0.64	0.42
C18:1 n – 9	24.6	24.6	24.7	24.9	24.3	0.97	0.12	0.62	1.47
C18:1 n – 7	3.36	3.31	3.33	3.37	3.30	0.71	0.07	0.71	0.14
C20:1 n – 9	2.85	2.79	2.83	2.86	2.79	0.64	0.15	0.76	0.17
C22:1 n – 9	0.37	0.36	0.36	0.36	0.36	0.38	0.91	0.79	0.02
C22:1 n – 11	1.74	1.65	1.68	1.71	1.66	0.30	0.16	0.87	0.13
C24:1 n – 9	0.33	0.30	0.32	0.33	0.30	0.53	0.26	0.58	0.09
Total MUFAs	41.8	41.6	41.7	42.3	41.1	0.96	0.07	0.74	2.31
C18:3 n – 3	1.93	1.93	1.96	1.95	1.93	0.77	0.61	0.34	0.11
C18:4 n – 3	0.60	0.62	0.62	0.61	0.61	0.83	0.91	0.26	0.07
C20:5 n – 3	3.01	3.11	3.07	2.96	3.17	0.90	0.19	0.80	0.57
C22:6 n – 3	6.30	6.88	7.07	6.15	7.35	0.67	0.05	0.63	2.15
PUFAs n – 3	13.9	14.7	14.9	13.7	15.2	0.68	0.07	0.58	2.93
C18:2 n – 6	10.7	10.7	10.7	10.9	10.5	0.99	<0.01	0.89	0.58
C20:4 n – 6	0.54	0.56	0.55	0.52	057	0.76	0.01	0.52	0.07
PUFAs n – 6	12.9	12.9	12.9	13.1	12.7	0.99	0.03	0.66	0.61
Ratio of n – 3 to n – 6 PUFAs	1.08	1.14	1.15	1.05	1.20	0.72	0.03	0.72	0.23
Total PUFAs	29.2	30.0	30.2	29.3	30.4	0.66	0.19	0.48	3.05

[1]Diet ISO 0 = 0 ppm, ISO–L = 500 ppm, ISO–H = 1500 ppm of isoflavones included in basal diet

FAs: fatty acids, SFAs: saturated FAs; MUFAs: monounsaturated FAs; PUFAs: polyunsaturated FAs.

* RSD, residual deviation standard

3.4.3. Lipid peroxidation

Dietary isoflavones had not significant effect on lipid oxidation in the refrigerated grounded fillets. Lipid peroxidation level, determined as concentration of muscle MDA, is showed in Figure 3.1. Even if not statistically different respect to the control, the MDA production was slightly reduced in fillet of 500 and 1500 ppm isoflavones supplemented trout (1day storage) decreasing from 82.27 μM MDA g^{-1}, in the control fish, to 61.44 μM MDA g^{-1} in fillet trout fed 1500 ppm of isoflavones. Thus, MDA production decreased, even if not significantly, with an increase in isoflavone in diet, despite the relationship between tissue isoflavone content and fillet lipid oxidation was nonlinear. The results from TBARS analyses in the present study agree to those of reported by D'Souza *et al*. (2005) in *in vivo* study that showed significantly lower TBARS values in muscle of rainbow trout fed isoflavone (500, 1000, 3000 ppm) harvested at 12 months of growth if compared with muscle of trout fed basal. These results showed that, although dietary genistein levels as low as 500 ppm significantly reduced the extent of lipid oxidation in the resultant fillets.

In *Trachinotus ovatus*, the hepatic MDA content decreased with increasing dietary soy isoflavones up to 40 ppm, and thereafter increased in the other 2 doses (60 and 80 ppm) in the end of feeding trial of 8 weeks (Zhou *et al*., 2015).

Also in other species isoflavones reduce lipid oxidation. Indeed, *in vivo* study in broiler indicates that dietary supplementation with soybean isoflavones can improve meat quality during refrigerated storage by decreasing lipid peroxidation and enhancing oxidative stability and the optimal level of soybean isoflavones was 40 ppm. The concentration of malondialdehyde at 72 h decreased linearly and quadratically with increasing levels of isoflavones indicating that isoflavones supplementation in broilers may reduce lipid oxidation of meat post-mortem (Jiang *et al*., 2014).

Malondialdehyde production significantly decreased in breast muscles of 20, 40 or 80 ppm of isoflavones supplemented chickens indicating that dietary isoflavones could improve meat quality by decreasing lipid peroxidation (Jiang *et al*., 2007).

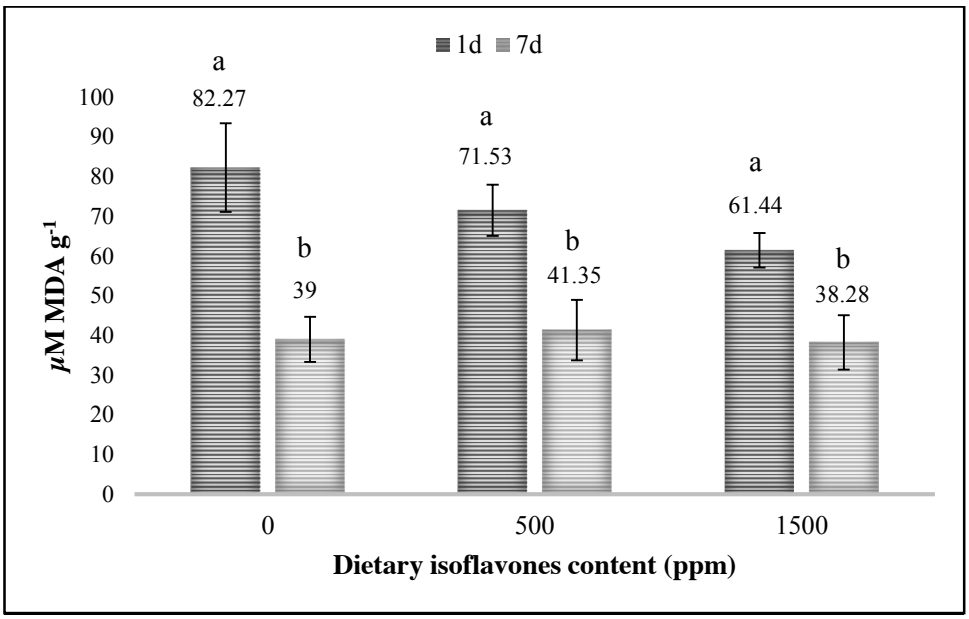

Figure 3.1. TBARS levels in grounded rainbow trout fillets. Values are mean malondialdehyde micromoles per gram of fillet. Error bars represent ±SE (n = 9 per dietary treatment). Different letters indicate statistical difference ($p < 0.05$) of MDA concentration in fillet conserved at 4°C for 1 and 7 days (a, b) based on analysis of variance (ANOVA) followed by a comparison of means with Tukey's HSD test.

Soy isoflavones have been shown to suppress formation of plasma lipid oxidation products *in vivo* (Tikkanen *et al*., 1998; Wiseman *et al*., 2000; Chen, 2001). Yousef *et al*. (2004) reported that supplemental isoflavone significantly decreased concentrations of MDA in plasma and tissues in male rabbits.

It is interesting to highlight that the reduced lipid oxidation observed in fillets from rainbow trout fed isoflavones, could lead to the possibility of an increased shelf life for soybean meal-raised trout. Moreover, MDA content is significantly decreased in muscles of trout for 7d storage respect 1d independent of the isoflavone doses ($p < 0.05$).

3.4.4. Fillet isoflavone deposition

All the groups, from control to the high isoflavone dose diet, exhibited a muscle isoflavone deposition. In fish fed diet without soy isoflavones (control diet: ISO–0), the muscle isoflavone content was significantly different from those of low and high levels of dietary isoflavon fed animals and increased significantly after 1 day of storage at 4°C (3.26 µg kg^{-1} and 13.48 µg kg^{-1} after 1 and 7 days of storage at 4°C, respectively). The muscle isoflavone content significantly increased also in fish fed isoflavone added diets (14.35 µg kg^{-1} and 10.84 µg kg^{-1}; 92.95 µg kg^{-1} and 187.29 µg kg^{-1} in trout fed 500 and 1500 ppm of dietary isoflavones refrigerated for 1 and 7 days, respectively) (Fig. 3.2).

The results evidenced an accumulation of isoflavones in trout fillets influenced by isoflavone dietary contents and by storage times ($p < 0.05$). Our results evidenced an increase in isoflavone deposition in all fillets trouts refrigerated for 7 days. The observed values of isoflavones in muscles was low, compared to D'Souza *et al*. (2005) data of isoflavone (genistein) fillet content.

Fingerling trout fed commercial diets containing 0, 500, 1000 or 3000 ppm of pure genistein for a period of 6 and 12 months showed low level of genistein in muscle as well, but a positive correlation was found between dietary genistein and tissue genistein level in trout harvested at 6 and 12 months (D'Souza *et al*., 2005). This study reported dose-dependent increase in genistein deposition in all dietary genistein levels except at 500 ppm. D'Souza *et al*. (2005) attributed this result to the variability in final fish weight within treatments.

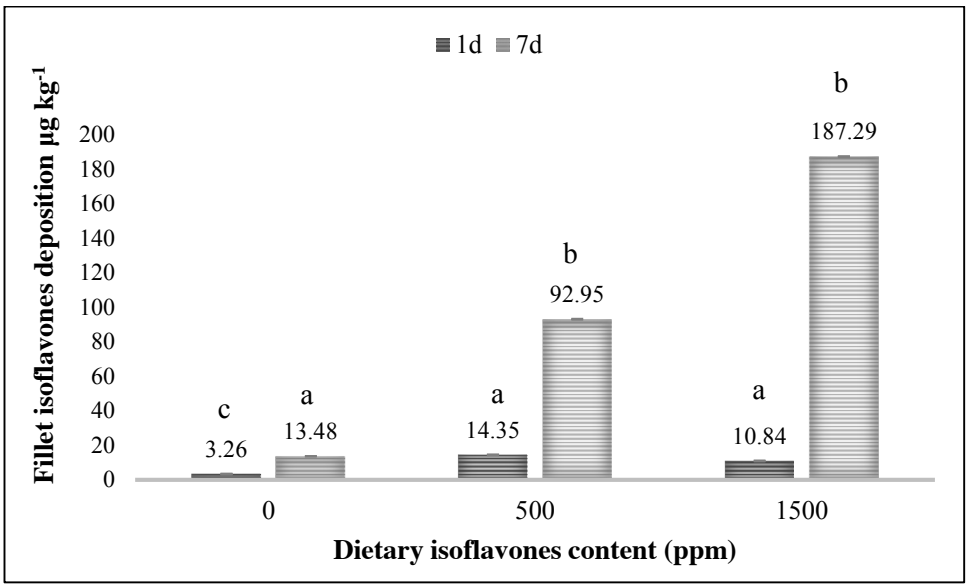

Figure 3.2. Isoflavones deposition means in rainbow trout fillets of two groups refrigerated at 4 °C for 1 and 7 days. Error bars represent ± SE (n = 9 per dietary treatment). Different letters indicate statistical difference ($p < 0.05$) of fillet isoflavones content after 1 and 7 days of conservation (a, b) based on analysis of variance (ANOVA) followed by a comparison of means with Bonferroni test.

The unexpected and interesting phenomenons observed in the present study are the presence of isoflavone also in the control animals beyond an increase of the isoflavones content with increasing of time conservation in all feeding treatments (0, 500, 1500 ppm). Biotransformation of isoflavones from conjugated to aglycone forms may be the reason for the increase associated to storage time in all the groups (included the control samples). Trouts may have been fed with commercial feed containing soy product and all the 12 isoflavones, included the conjugated form, before the feeding trial and deposition of these forms could have changed into aglycones form by enzymatic or bacterial activity during storage.

Although indications of the isoflavone potential to the prevent hormone-related diseases (breast cancer, prostate cancer and diabetes) have been considerable founded (Ko, 2014), in recent years, the relationship between isoflavones and breast cancer has become controversial. In fact, soy

isoflavones exhibit estrogen-like properties under certain experimental conditions and it may stimulate the growth of existing estrogen-sensitive breast tumors (Messina *et al*., 2001).

Moreover, daily intake figures for isoflavones is increasing. Due in part to the use of soya in processed foods, the isoflavone content in consumed foods has increased from the <1 mg day^{-1} estimated in the late 1980s (Clarke *et al*., 2004). Japanese consumers have been assigned intake values from 25 mg day^{-1} (Wakai *et al*., *1999*) to 150-200 mg day^{-1} (Cassidy *et al*., 1994) of total isoflavones, although a figure of 50 mg day^{-1} is probably more realistic (Messina *et al*., 1995).

Our data show that not only in soy deriv product there is presence of isoflavones, but also in unexpected food for the consumer. In our fish the content is low for the short feeding trial and respect soy product derivate (Vanilla soymilk yogurt 183 mg soy isoflavones on kg of product, Wiseman *et al*., 2002) but reared fish for a long time with high content of soybean meal could add isoflavones to daily consumption by consumer beyond that after storage coniugated isoflavones could transform in aglycon forms.

3.5. Conclusions

In the present study, the isoflavone doses did not modify the growth performance and the quality of reared fish as well as the biometric and physicochemical traits. Instead more substantial have been the variation on the whole fish and fillet after 7 days of storage at 4°C. The observed variations were maintained in a range that do not compromise the product acceptability. Dietary isoflavones had no significant effect on lipid oxidation in the refrigerated grounded fillets, even if lipid oxidation decreased with an increase in isoflavone in diet. The obtained resulted confirmed the carry-over of isoflavones from the diet to the fillets during a period of 70 days. Anyway, the high increasing of isoflavone content in fish fillet with increasing of time conservation induce to go deeply on this phenomenon for knowledge and safety of the consumer.

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CHAPTER 4

GENERAL CONCLUSIONS

In conclusion, none of the isoflavone doses (500 and 1500 ppm) induced estrogenic effects in rainbow trouts. Vitellogenin production was probably stimulated by 17β-estradiol (E_2) as secretion of vitellogenin is dose dependent in response to endogenous estrogens. E_2 and cortisol were not influenced by the different levels of isoflavones, indicating no endocrine disruption. Isoflavonic phytoestrogens did not influence reproduction and growth at the tested doses beyond that fish welfare was maintained.

The isoflavone doses did not modify the growth performance and the quality of reared fish as well as the biometric and physicochemical traits. Instead more substantial was the variation on the whole fish and fillet after 7 days of storage at 4°C. The observed variations were maintained in a range that do not compromise the product acceptability. Dietary isoflavones had not significant effect on lipid oxidation in the refrigerated grounded fillets. Moreover, the results confirmed the carry-over of isoflavones from the diet to the fillets during a period of 70 days. The high increase of isoflavones content in fish fillet related to increasing time of conservation induce to go deeply on this phenomenon for knowledge and safety of the consumer.

Overall, these results indicate that the isoflavone tested doses do not compromise rainbow trout reproduction, growth and health; although a moderate transfer of isoflavones from diet to fillet was observed; quality, technological and nutritional characteristics and lipid oxidation, were not affected by dietary treatment. Our data demonstrate that, with regard to isoflavones contents and their potential effects, soybean meal can replace fish meal in rainbow trout diets at high level with no negative effects on fish performance and final product quality.

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Appendix 1

AN ECOFRIENDLY PROCEDURE TO EXTRACT ISOFLAVONES FROM SOYBEAN SEEDS

Journal of Cleaner Production 170 (2018) 1102–1110

Contents lists available at ScienceDirect

Journal of Cleaner Production

journal homepage: www.elsevier.com/locate/jclepro

An ecofriendly procedure to extract isoflavones from soybean seeds

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ARTICLE INFO

Article history:
Received 21 April 2017
Received in revised form
18 August 2017
Accepted 24 September 2017
Available online 25 September 2017

Keywords:
Isoflavones
Water extraction
Anti-nutritional factors
Malonyl forms
Lyophilization

ABSTRACT

A novel procedure for isoflavones extraction from soybean seeds using the "green solvent" water was set-up for its future industrial up-scaling. The optimized processing flow-scheme proposed here consists of a 1:5 flour weight-to-water volume ratio mixed by vertical stirrers for 6 min at 40 °C, with a final 12000-rpm centrifugation, and subsequent lyophilization to ensure stability and easy dosage of the extracts. Results showed that preliminary UV-C light flour irradiation for at least 1 h can improve isoflavones extraction and reduce anti-nutritional factors. Principal component analysis (PCA) and discriminant factorial analysis (DFA) highlighted specific operational conditions, such as extraction volume and time, UV time exposure, and centrifugation speed, for preferential extraction of specific isoflavones. The extraction efficiency varied according to variety choice within a range of 40–73%, possibly due to the relative abundance of the most water-soluble malonyl forms of isoflavones. As the extracts showed promising high stability under freezing and lyophilization conditions, it is concluded that, this eco-friendly extraction approach with can be successfully scalable, helping to save time and energy and obviating at disposal problems in the soy nutraceutical chain.

1. Introduction

Soybean currently plays an important role in food and feed at a global level as the main source of vegetal proteins and essential amino acids, while production has a favorable environmental impact (Tessari et al., 2016; Cassini et al., 2010). Health claims concerning soybean are, however, largely attributed to isoflavones, which commonly bond with proteins in the seeds (Liu et al., 2013).

Isoflavones are recognized as having chemopreventive potential, antioxidant and anti-inflammatory properties, as well as the capacity to modulate steroid hormone levels and receptors (Zhang et al., 2007; Villares et al., 2011). Recently, some authors suggested that isoflavones supplements are also associated with a reduced risk of postmenopausal breast cancer, while some others highlighted their anti-aging activity in dermatology. Interest in soybean isoflavones is increasing in the field of nutrition and preventive medicine, along with the use of botanical or herbal ingredients, to prevent or improve a number of health problems (Boucher et al., 2013; Polito et al., 2012; Gao et al., 2015). Soybean seeds contain four groups of isoflavones and twelve distinct compounds: i.e., aglycones (daidzein, genistein and glycitein), and their acetyl-, malonyl-, and β-glycoside forms. The two latter classes are commonly the highest isoflavones fractions in intact seeds and other soy products (Niamnuy et al., 2011). The biological effects of isoflavones seem to depend mainly on aglycones abundance (Cao et al., 2012), although some author consider it unlikely that aglycones enter the systemic circulation, and report that their percentage in plasma is generally low (Islam et al., 2014). This suggests the need to quantify all types of isoflavones in both seeds and derived foods as their biological activities may differ, and to identify crucial steps in isoflavones degradation during food processing.

Several factors, like genotype-phenotype (Barion et al., 2016; Barion at al., 2010), cultivation environment (Vamerali et al., 2012; Barion et al., 2008a, 2008b), location and post-harvest storage (Lee et al., 2003), play a major role in seed isoflavones accumulation and preservation. It was suggested that high- and low-isoflavones cultivars maintain the same ranking across locations, and that in favorable conditions the same cultivar could present up to a 4-fold increase in isoflavones concentration in the cotyledons,

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https://doi.org/10.1016/j.jclepro.2017.09.218
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the specific class - genistein or diadzein - depending on variety (Berger et al., 2008).

It is possible to identify varieties with stable responses to agricultural management, but there are few suitable genotypes available and they have low isoflavones concentrations (Barion et al., 2010). Concerning soybean processing, the distribution of major isoflavones compounds in soy-based foods depends on the production process (Villares et al., 2011).

Against this background, it is desirable that isoflavones from soybean extracts used as food ingredients have the widest spectrum and highest concentrations as possible, although extraction of all the isoflavones, which vary greatly in polarity, is still a challenging task. Until now, several techniques have been proposed, from classical heat refluxing extraction and Soxhlet and pressurized solvent extraction (Vacek et al., 2008) to modern techniques, such as ultrasonic-assisted (Kaufmann and Christen, 2002) microwave-assisted (Wu et al., 2012) and supercritical-fluid extractions (Pyo et al., 2009).

Pressurized solvent extraction (PSE) has advantages in terms of extraction time, isoflavones yield and reproducibility, as the high pressure and temperature used facilitate solvent penetration into the matrix pores, although the energy costs are high. Given the large particle sizes in plant matrices and the difficulties in applying continuous traditional or PSE methods, a solution may be to introduce ultrasonic techniques into the extraction procedure (Lee and Lin, 2007). The use of ultrasonics for improving extraction yield has greatly advanced since the 1950s, with improvements attributed to a phenomenon called cavitation, produced in the solvent by the passage of an ultrasonic wave (Vinatoru, 2001) usually generated by a transducer, which converts mechanical or electrical energy into high frequency vibrations (Romdhane and Gourdon, 2002). Cavitation bubbles are produced and compressed during the application of ultrasonic waves, allowing greater solvent penetration into the raw material and greater intracellular compound release through disruption of the cell walls, thereby considerably reducing the extraction time (Albu et al., 2004; Rostagno et al., 2003). Continuous microwave-assisted extraction is a viable tool for collecting soybean isoflavones, It can be set an 8 min residence time and 73 °C extraction temperature as optimal parameters (Terigar et al., 2010).

Evaluating supercritical carbon dioxide fluid extraction (SFE) for extracting soybean isoflavones and comparing it with Soxhlet and ultra-sonication methods it was evidenced that, although SFE was the most selective method, maximum isoflavones extraction was achieved with ultra-sonication (Rostagno et al., 2002).

Conventional extraction methods primarily focus on solvent choice - methanol, ethanol and acetonitrile being the main options - with varying proportions of acidified and non-acidified water. The second crucial issue is the use of heat and the procedure for mixing the solvent and raw material in order to increase the solubility and transfer of the compounds sought. In this regard, the literature offers contrasting results. Comparing various ethanol and methanol water mixtures (30–80% v/v) in a pressurized liquid extractor it was reported that the optimal isoflavones yield can be achieved with a 70% ethanol solution (Rostagno et al., 2004) however, a 90% v/v methanol solution is the best for isoflavones extraction from soybean samples (Klejdus et al., 2004). Comparing the extraction efficiency of various aqueous (acidified and non-acidified) mixtures of methanol and acetonitrile and it was reported that 58% v/v non-acidified acetonitrile allowed optimal extraction of isoflavones (Lin and Giusti, 2005). A single step extraction method can markedly underestimate the true isoflavones content. Specifically, protein concentration may influence isoflavones extractability due to stronger protein-polyphenol interactions (Achouri et al., 2005). Whey wastewater, generated by precipitation of soy protein isolates, contains high levels of isoflavones, particularly aglycone forms, which readily bond with proteins; in order to overcome this problem, it was suggested foam fractionation and acidic hydrolysis to collect isoflavones using simple equipment and with low energy consumption and good environmental compatibility (Zhang et al., 2007).

There is, therefore, great scope for improving the methodology of isoflavones extraction from soybean. This study is an attempt to establish an ecofriendly method of extraction from milled seeds using simply water as solvent, and to compare different extraction volumes, temperatures, mixing types and times, sonication times and centrifugation speeds. Extraction parameters were tested on 4 soybean varieties to assess whether isoflavones composition affects the extraction yield.

2. Materials and methods

2.1. Plant materials

Seeds of four soybean varieties, *Luna, Bahia*, *Demetra* and *Hilario*, were collected from field trials at the University of Padua's experimental farm (Legnaro, Padua, Italy) and stored at room temperature until analysis. The varieties belonged to different maturity classes (from 0 to 1+) and had varying chemical compositions (Table 1 SI - Supplementary Information).

2.2. Preparation of soybean flour

The milling conditions were selected on the basis of preliminary trials comparing four types of mill with the seeds of the variety Luna: a) large hammer mill (500 Universal, Peruzzo s.r.l., Curtarolo, Padua, Italy); b) electronic disk mill (ZM 200, Retsch GmbH & Co. KG, Haan, Germany); c) disk rotary type mill (SM100 Confort, Retsch GmbH & Co. KG, Haan, Germany); and d) cooled blade mill (M20, IKA-Werke GmbH & Co. KG, Staufen, Germany).

Non-defatted flours were obtained by grinding (mean value 60 mesh) intact seeds with the four mills following traditional extraction with 7 mL 80% v/v methanol solution (Sigma-Aldrich, Chemie GmbH, Steinheim, Germany) on 0.1-g samples.[12]

2.3. UV light irradiation

For UV light irradiation, the soybean flour was placed in a laminar flow hood (Polaris48, Steril s.p.a., Italy) in a 1-cm thick layer. The apparatus was equipped with a UV-C lamp tube (Germicidal 3′T8 Tube) emitting at a 253.7 nm wavelength with a nominal power of 30 W. The irradiation process was carried out for 30, 60, 120 and 180 min at a constant temperature of 25 °C.

2.4. Experimental conditions of isoflavones extraction

To establish the best solid-to-liquid extraction ratio, several dilutions were tested: 1:5, 1:7, 1:20, 1:50, and 1:70 weight of flour-to-solvent volume (g/mL) ratios using deionized water (obtained with a Millipore-Q purification system, Millipore Corp., New Bedford, MA, USA). The best performing mixture was treated by heating at temperatures of 25 °C or 40 °C for different times (6 min, 2, 5, 18 and 24 h) while constantly stirring the samples. For the purposes of comparison, the solutions were shaken with magnetic, horizontal or vertical stirrers at 70 rpm. We then assessed whether a sonication procedure, applied to the flour-water solution for 2, 4, 8, 10 and 15 min, resulted in comparable isoflavones extraction to non-sonicated samples. Lastly, different centrifugation speeds were compared: 6000, 8000 and 12000 rpm for 12 min in order to separate the extracts from the insoluble fraction (pellet). Three

Table 1
Maturity class and chemical composition (on dry weight, DW) (mean ± SE, n = 3) of 4 soybean varieties and of a freeze-dried water-extract (var. Demetra). Maturity classes 0 and 1 refer, respectively, to 110 and 123 days from sowing to harvest.

Maturity class	Bahia	Demetra	Hilario	Luna
	I+[a]	I	0+[a]	0+[a]
Chemical analysis				
Dry Matter (% FW)	88.7 ± 3.12	84.2 ± 3.21	91.3 ± 2.94	91.7 ± 3.43
Ashes (% DW)	5.47 ± 0.353	4.71 ± 0.324	4.62 ± 0.37	4.94 ± 0.352
Crude protein (% DW)	40.3 ± 1.27	40.3 ± 1.18	39.8 ± 1.26	37.6 ± 1.23
Crude lipids (% DW)	23.4 ± 0.962	21.1 ± 0.955	22.7 ± 0.958	22.3 ± 0.963
Crude fiber (% DW)	4.09 ± 0.604	3.56 ± 0.599	4.74 ± 0.601	4.87 ± 0.607
Nitrogen-free extract (% DW)	26.8 ± 1.57	30.3 ± 1.51	29.8 ± 1.55	28.2 ± 1.54

[a] Tendency towards longer cycle.

replicates were used for each of the extraction procedures for the statistical analyses.

2.5. HPLC identification of soy isoflavones

Soybean isoflavones were analyzed according to Barion et al. (2010). The HPLC equipment (Shimadzu, Milan, Italy) was fitted with a UV diode array detector (SPD-M2=A) and an Ultratech-sphere C18 analytical column (CIL CLUZEAU, France) kept at 35 °C. The mobile phase was 0.25% (v/v) TFA (solvent A) and pure ACN (solvent B). A linear HPLC gradient was used: following 2 μL sample injection, solvent B was kept at 16% for 2 min, then increased gradually to 24% over 2 min, to 32% over 2 min, and to 100% over 3 min, the final rate maintained for a further 1 min. The duration of the analysis was 10 min at a solvent flow rate of 1.3 mL min^{-1}. Genuine standards of aglycone isoflavones (daidzein, genistein, glycitein) and glycosyl forms (daidzin, genistin, glycitin) were obtained from a commercial source (L.C. Laboratories, Woburn, MA, USA). Due to their unstable structure, the malonyl- and acetyl-conjugated forms were not quantified with external standards and their response factor (calibration curve) was calculated from the corresponding glycosyl forms, corrected for molecular mass ratio (Hubert et al., 2005). HPLC-grade (80%) methanol and deionized water (obtained with a Millipore-Q) were used as extracting solvents, the former as reference extractant. Standards were prepared by adding 1 mg of each isoflavone to 0.5 mL dimethyl sulphoxide (DMSO) and brought to 5 mL with HPLC-grade methanol, corresponding to a 0.2 mg mL^{-1} isoflavone concentration. This solution was diluted with methanol into four powers: 80, 50, 20 and 10% (v/v). Standard solutions were analyzed to identify isoflavones, and their retention time (RT) and concentrations. All calibration curves had coefficients of determination >99.4%.

2.6. Determination of anti-nutritional factors

Soybean trypsin inhibitors were detected on flour samples and on the extracts (Liu and Markakis, 1989). Trypsin inhibitory activity was expressed in trypsin units inhibited (TUI) per milligram of dry sample.

2.7. Shelf life of frozen and freeze-dried extracts

The shelf life of isoflavones was monitored by HPLC through weekly assessment of their abundances in frozen (−20 °C) extracts over 30 days. The storage stability of isoflavones was also monitored in freeze-dried extracts: after freeze-drying at −40 °C (Edwards Mini Fast 1700, Crawley, England), the powder was recovered in vials, filled with N_2 gas and stored at −20 °C until analysis.

2.8. Statistical analysis

Every assay was repeated independently at least three times. All values are expressed as the mean ± standard error of three independent experiments. Analysis of variance (ANOVA) was performed using the StatGraphics Centurion XV software (Statpoint, Herndon, VA, USA).

Factorial discriminant analysis (MDA, Multigroup Discriminant) Analysis, with Wilks lambda and Pillai trace tests (Cooley and Lohnes, 1971) and principal component analysis (PCA) were carried out using MS Excel XLSTAT (Addinsoft, Paris, France) to describe the isoflavones profiles extracted from soybean flour with respect to different extraction variables. Before analysis, the data were standardized by subtracting the mean and dividing by the standard deviation within each variable. Multivariate data normality was first verified (Shapiro test) using the public domain software R 2.9.2 (R package mvtnorm; Ihaka and Gentleman, 1996).

3. Results and discussion

A large number of extraction options has been tested with the aim of producing water extracts at maximum isoflavones concentration with minimal costs. The process to carry this out was designed as shown in the flow sheet of Fig. 1, with sequential seed milling, flour UV-C treatment, rotary extraction, centrifugation, filtration and freeze-drying.

A crucial preliminary step in sample preparation was to compare contrasting milling procedures on the variety Luna: large hammer, electronic disk, rotary disk, and cooled blade mills, with traditional methanol extraction. No statistically significant differences between the four types of mill in the total concentration of isoflavones and their profiles were found in the extracts (Fig. 2). The difference between the lowest (rotary disk mill) and highest (cooled blade mill) values was only 9%. Therefore, the large hammer mill, which can work continuously, was considered the most suitable choice for future grain processing on an industrial scale. Conventional extraction methods are based on the use of chemical solvents and sample heating to maximize the solubility of the active principles and speed up mass transfer. The extraction yield depends on several factors, including the type and amount of solvent and its residence time and temperature, and they all need to be optimized. The organic solvents commonly used for recovering soy isoflavones are methanol, ethanol and acetonitrile, with varying percentages of water and with/without acidification (Luthria et al., 2007). Our idea was to set up a simple procedure based on the use of deionized water as solvent, as it is easily available at low cost and does not create any disposal problems. Without any doubt, the replacement of volatile organic compounds (VOCs) by eco-friendly solvents is becoming increasingly important due to the increasing health and environmental concerns as well as economic pressures associated

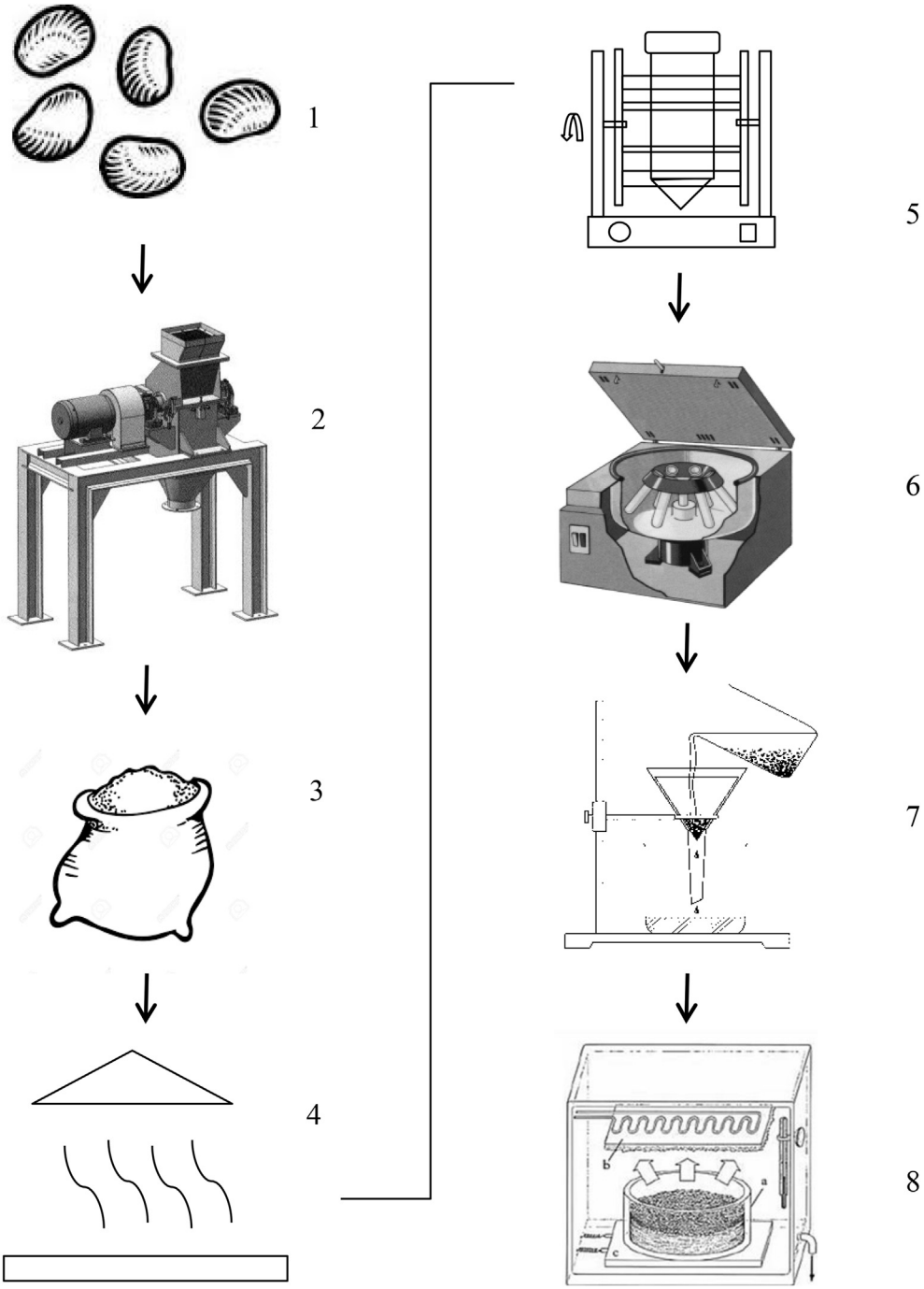

Fig. 1. Flow sheet of the isoflavones extraction process: 1) grains; 2) grinding; 3) flour (60 mesh); 4) UV treatment; 5) water extraction in rotary shaker; 6) centrifugation; 7) filtration; 8) freeze-drying.

with VOCs. As reported by Płotka-Wasylka et al. (2017) water and carbon dioxide which are innocuous and renewable may be included in the group of "green solvents". In addition, also the physicochemical properties of water could be changed by temperature and pressure increasing the possible applications of this solvent. There are enormous attempts on extraction of isoflavones in water extract. Li-Hsun et al. (2004) demonstrated that superheated water extraction is a means of extracting five important isoflavones from defatted soybean flakes, reaching very high yields. On the contrary, Luthria et al. (2007) showed the lowest yields of total isoflavones using superheated pressurized water.

After several preliminary trials, we established an optimal solid-to-solvent ratio of 1:5 (w/v, g/mL) as the minimum volume of water required to soak the flour sample and to obtain a high concentration of isoflavones in the extracts. Compared with weaker dilutions (e.g., 1:7), the 1:5 ratio yielded a higher total concentration of isoflavones (TIC) in the extracts (+21%), although extraction efficiency on a flour weight basis was slightly worse (−11%) (Fig. 3a). In the following steps, extraction efficiency was greater at an ambient temperature of 40 °C than at 25 °C (+11%) (Fig. 3b) and with an extraction time of 0.1 h (Fig. 3c). Extraction was not affected by longer times up to 2 h, but further increases up to 24 h were

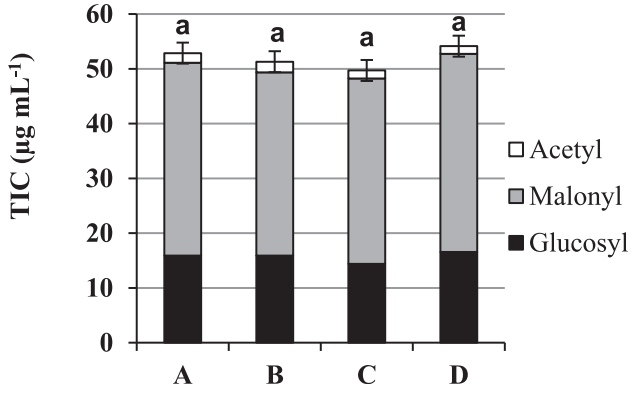

Fig. 2. Effects of different types of mill on total concentrations (mean ± SE, n = 3) and profiles of isoflavones in methanol extracts of soybean variety Luna. A = large hummer mill; B = electronic disk mill; C = rotary disk mill; D = cooled blade mill. TIC = Total isoflavones concentration.

associated with decreased efficiency (−19%). This suggests that considerable energy and time savings are possible, which could also limit bacterial contamination of the extracts and foam formation caused by saponins. As an operating temperature, 40 °C may be considered a mild treatment, easily reached with solar energy and compatible with the sustainability of the extraction procedure (Yatsu et al., 2016). This is in agreement with literature that reported efficient isoflavones extraction from soybean flour at a reduced extraction time and temperature (Terigar et al., 2011).

In order to ensure better contact between the surface of the soy flour particles and the solvent, three different types of shaking were compared: magnetic, horizontal and vertical stirrers. We were able to establish an almost 4-fold greater efficiency of the vertical stirrer, which probably allows thorough mixing of the flour-solvent and more complete isoflavones solubilisation (Fig. 3d).

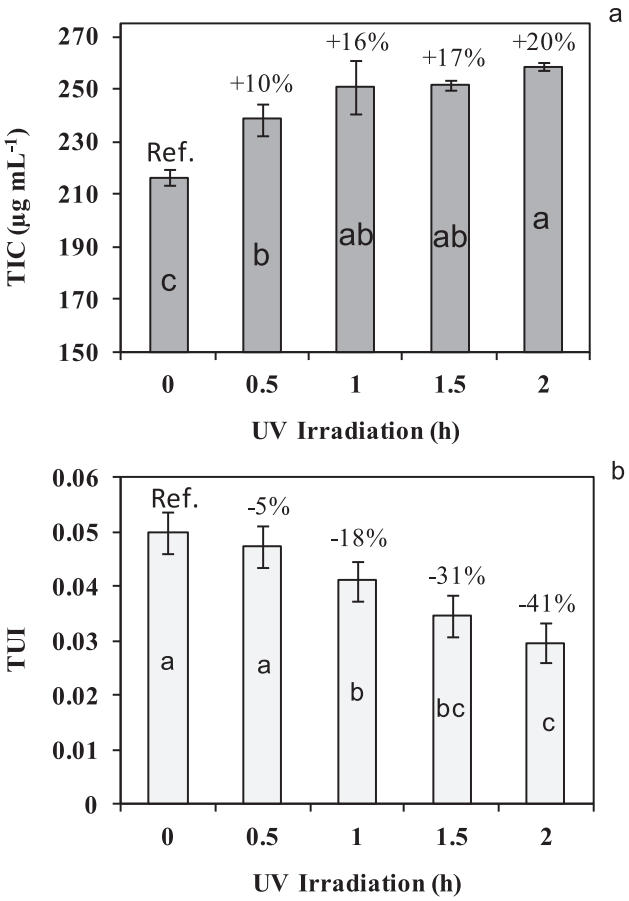

Fig. 4. Effects of UV-C irradiation time on total isoflavones concentration (TIC) (mean ± SE, n=3) (a) and on anti-nutritional factors (b) in soybean extracts. TUI = Trypsin Unit Inhibited per mg of flour. Letters: significant differences among treatments (Newman-Keuls Test, P ≤ 0.05).

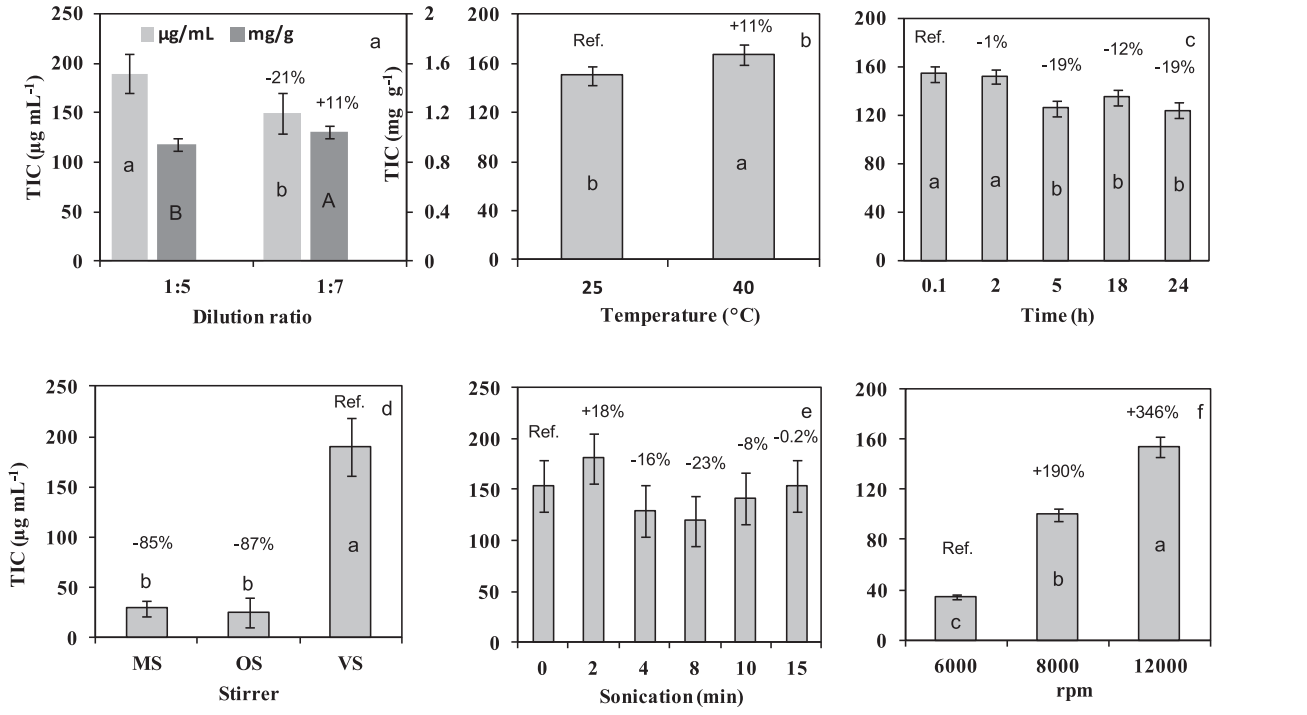

Fig. 3. Effect of extraction conditions on total isoflavones concentration (TIC) (mean ± SE, n = 3) in water extracts (light grey) and on a flour weight basis (dark grey) from soybean variety Luna. Percentage variations refer to the reference (Ref.), the less expensive or less time-consuming option. (a) Dilution ratio (g/mL); (b) Temperature; (c) Stirring time; (d) Kind of stirrer: MS = magnetic stirrer, OS = horizontal stirrer, VS = vertical stirrer; (e) Sonication time; (f) Centrifugation rate. Letters: significant differences among treatments at P < 0.05 (Newman-Keuls test).

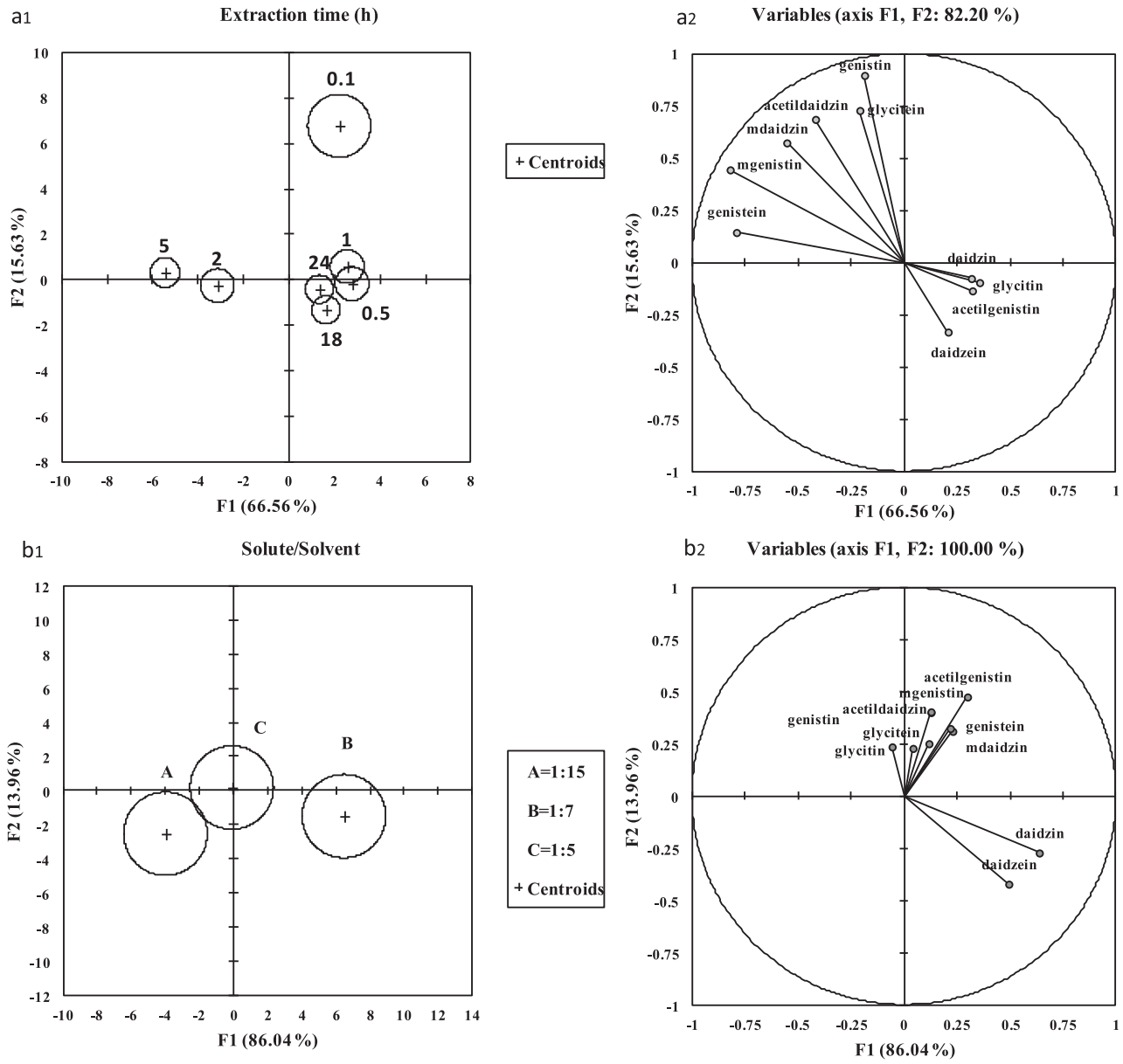

Fig. 5. Discriminant factorial analysis (DFA; a1, b1) and principle components analysis (PCA; a2, b2) of isoflavones profile: aglycones (daidzein, genistein, glycitein), glycosyls (daidzin, genistin, glycitin) and their acetyl and malonyl forms. a1) isodensity groups for extraction times of 0.1, 1, 2, 5, 18 and 24 h; b1) isodensity groups for solute/solvent ratios: A = 1:15, B = 1:7, C = 1:5. Each isodensity group contains 70% of observations.

Improvement to the extractability of isoflavones was then addressed by testing the effect of various operations which, according to the literature, may increase the concentrations of the extracted bioactive compounds (Azmir et al., 2013). Of these, sonication after maceration exhibited variability in the potential extractability of isoflavones, with a 2-min treatment delivering a slight improvement (+18% vs. controls; $P > 0.05$) (Fig. 3e). Isoflavones were finally recovered as a supernatant solution after centrifugation of the water-soy flour suspension. Of three increasing centrifugation speeds, i.e., 6000, 8000 and 12000 rpm for 12 min, the maximum speed gave better results. Compared with the lowest speed, isoflavones yield doubled at 8000 rpm and was 3.5 times higher at 12000 rpm (Fig. 3f, $P < 0.05$).

As an additional treatment, UV-C light irradiation of soy flour resulted in a desirable improvement in isoflavones extraction. A 0.5-h treatment led to a 10% increase in concentration of isoflavones in the extracts, while treatments in the range of 1–2 h led to a 16–20% increase (Fig. 4a). At the same time, increasing UV-C exposure progressively reduced the anti-nutritional content, expressed as TUI (Trypsin Units Inhibited) per mg of flour (Fig. 4b). Trypsin inhibition became significant ($P < 0.05$) when treatment was ≥1 h, the reduction being 18–41% for 1–2 h treatments. This is an interesting result, as the final soy flour suspension separated by centrifugation may be considered a co-product containing high amounts of both isoflavones and proteins (53.1% DW, Table 1 SI) but low anti-nutritional factors.

Considering the large variability in the isoflavones composition of soybean, and with the aim of extracting as much as possible of all the forms, at each extraction step principle component analysis (PCA) together with discriminant factorial analysis (DFA) were

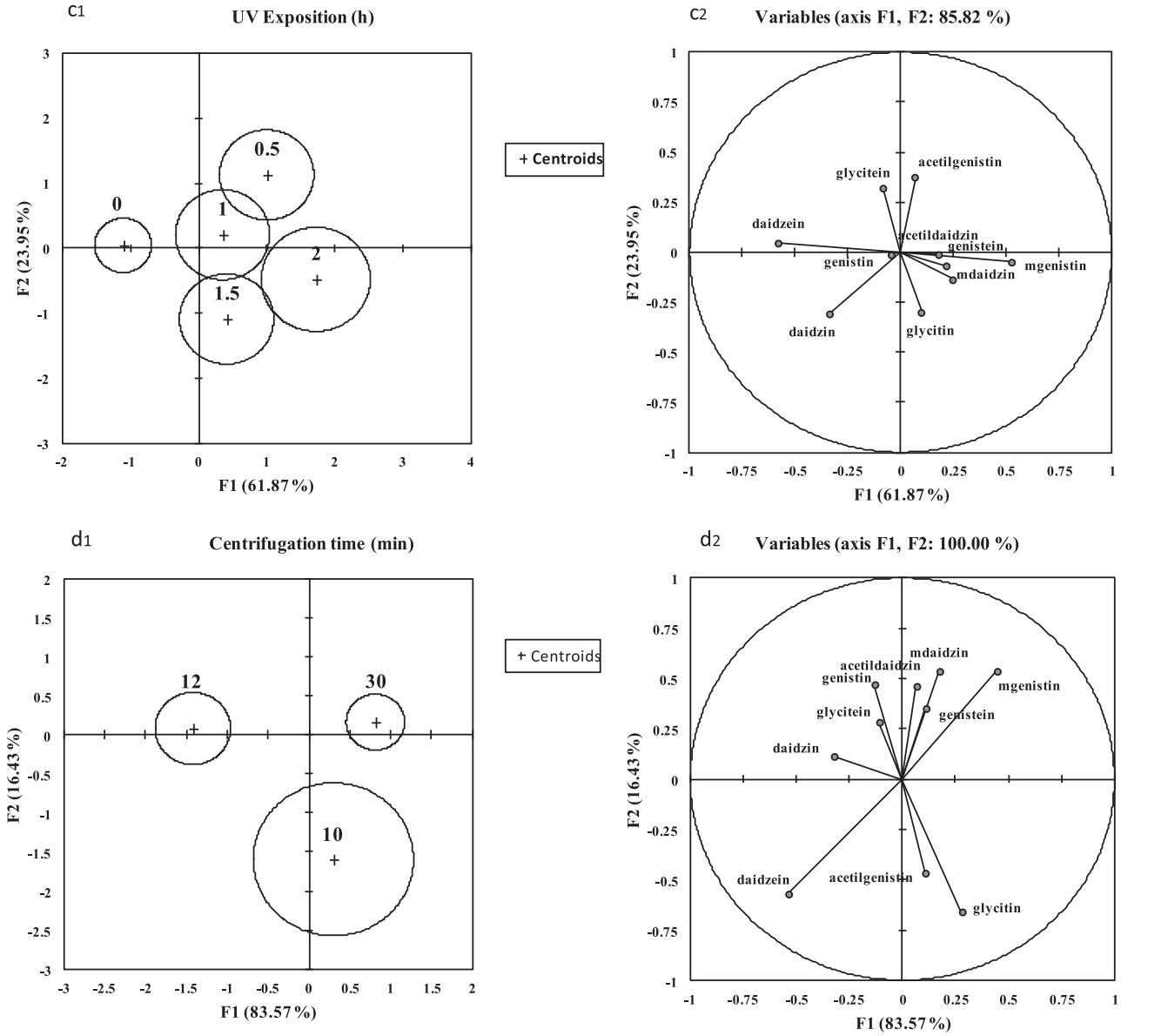

Fig. 6. Discriminant factorial analysis (DFA; c1, d1) and principle components analysis (PCA; c2, d2) of isoflavones profile: aglycones (daidzein, genistein, glycitein), glycosyls (daidzin, genistin, glycitin) and their acetyl and malonyl forms. c1) isodensity groups for UV exposures of 0, 0.5, 1, 1.5 and 2 h; d1) isodensity groups for centrifugation times of: 30, 12, 10 min. Each isodensity group contains 70% of observations.

carried out in order to identify where adjustments could be made to the extraction procedure. Fig. 5a_2-b_2 and Fig. 6c_2-d_2 show the different extents to which the variables contribute to the separation of groups, which are reported in Fig. 5a_1-b_1 and Fig. 6c_1-d_1. Each circle of the DFA represents an isodensity group containing 70% of observations with respect to the specific treatment, with the centroid in the center.

Fig. 5(a_1-a_2) shows that the shorter extraction time (0.1 h), which had high overall extraction efficiency (Fig. 3c), is the best choice for recovering genistin, glycitein, acetyl and malonyl daidzin. However, it seems that working in the range of 0.5–1 h can improve the recovery of daidzin, glycitin and acetilgenistin. Longer extractions (≥18h) lead to good recovery of daidzein, which is known to have greater human health benefits due to its greater bioavailability and lower intestinal microbial degradation compared with genistein (Vergne et al., 2007). Intermediate extraction times of 2 and 5 h are shown on the left side of Fig. 5a_1, and appear to be suitable conditions for recovering genistein and malonylgenistin. However, it is suggested that extraction of daidzin and daidzein may be increased by modifying the extraction volume to a flour-solvent ratio of 1:7 (Fig. 5b_1-b_2), while a 1:5 ratio is suitable for other classes of isoflavones.

UV light exposure for 1 h can play an important role in selectively extracting more malonyl (malonyl genistin, malonyl daidzin) and glycosyl (daidzin, glycitin) forms, whereas a shorter exposure yields higher amounts of aglycones, like daidzein and glycitein, and acetylgenistin (Fig. 6c_1-c_2). Increasing centrifugation time up to 30 min improves malonyl extraction, whereas a centrifugation time of 10 min may shift the profile towards daidzein, glycitin and acetylgenistin, although a 12-min centrifugation improves glycosyl forms, like daidzin and genistin, and the aglycone glycitein (Fig. 6d_1-d_2).

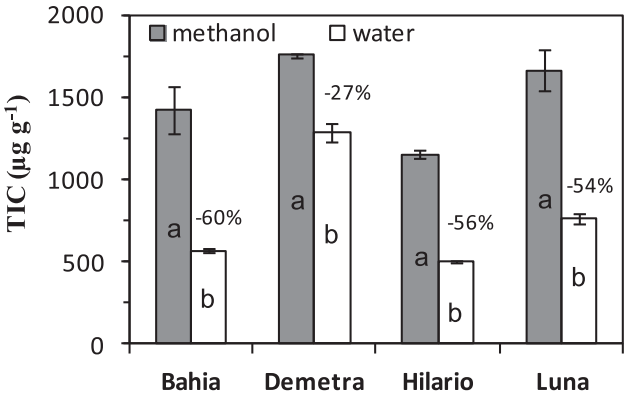

Fig. 7. Comparison of total isoflavones concentrations (TIC) (mean ± SE, n = 3) from aqueous and methanol extracts in 4 soybean varieties. Percentages: variations in water vs. methanol extractions within the same variety. Letters: differences between solvents within the same variety (Newman-Keuls test, $P \leq 0.05$).

These considerations are useful for the selection of specific molecules of isoflavones even if a different approach may be used to achieve a more energy saving method. At this regard, the reduction of extraction time (0.1 h), no UV treatment and a shorter centrifugation time (10 min) is a strategic solution not only for saving energy but also for avoiding the loss of daidzein forms which are probably more sensitive to UV degradation.

In light of these results, the water extraction method proposed here was applied to 4 soybean varieties (see Table 1 SI) and compared with conventional methanol extraction (Fig. 7). Demetra was the highest isoflavone yielding variety with both methanol and aqueous solutions, with a water extraction efficiency of 73% (1287 vs. 1755 g g^{-1} of flour). Our data are in agreement with Li-Hsun et al. (2004) reporting a 74.5% recovery of isoflavones even if their experimental conditions were no energy saving with higher values of water temperature (100 °C), pressure (4196 kPa) and time (2.2 h). Water extraction with the other varieties resulted in a loss of isoflavones about 60% in Bahia, followed by Luna (−54%) and Hilario (−56%). It seems that the drop in the extraction efficiency of water does not depend on the total concentration of isoflavones in flour, but rather on the profile of isoflavones. In fact, we found a higher fraction of malonyl compounds in the variety Demetra

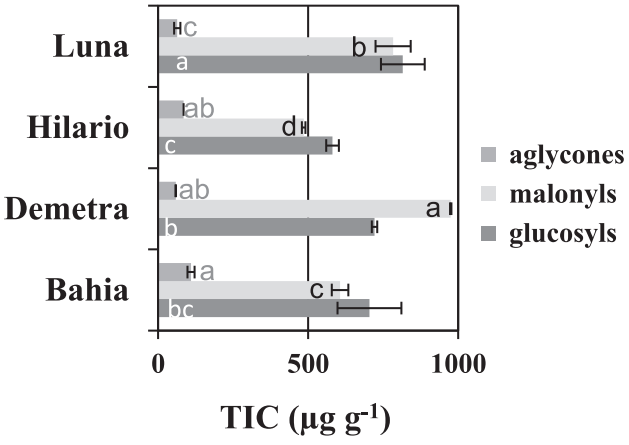

Fig. 8. Comparison of isoflavones profiles TIC (µg g^{-1}, mean ± SE, n = 3) of 4 soybean varieties. Letters: differences among varieties within the same isoflavones class (Newman-Keuls Test, $P \leq 0.05$).

compared with the other ones (Fig. 8). While acetyls were generally present in a trascurable concentration and aglycones are lower than 110 µg g^{-1} with small variation among varieties, variety Demetra had higher malonyls (975 vs. ~620 µg g^{-1} for the other 3 varieties) and higher glycosyls (721 vs. ~700 µg g^{-1}), suggesting that malonyls and glycosyls may have high water solubility, and that their abundance positively affects extraction with our procedure.

In order to completely evaluate the extraction process, we also carried out weekly checks on isoflavones stability in the frozen (−20 °C) extracts over the following 30 days. Results showed a small decrease in isoflavones concentration (about −8%) after one week of storage compared with the initial concentration, while values were stable later on. Since, from a technological point of view, a freeze-dried extract can be more easily managed and stored and is more flexible with regard to dosing at multiple concentrations than the liquid form (Lante et al., 2011), isoflavones stability was also checked in frozen lyophilized powder, but we did not observe any differences between the lyophilized and liquid formulations (data not shown).

4. Conclusion

There is currently great interest in soybean isoflavones for nutraceutical and cosmetic uses due to their health benefits. The overall global nutraceutical market has progressively grown over the last 10 years due to the greater demand for functional foods and beverages and dietary supplements, and the market is expected to further increase over the coming decades. A need has therefore arisen to develop safe and eco-friendly/natural methods to extract these nutraceutical compounds.

It has successfully proposed the use of pure water as a “green solvent” of isoflavones from fine-milled soybean seeds, and suggested specific extraction conditions that save time and energy due to the low water temperature and obviate disposal problems, delivering an expected extraction efficiency of 40–73%, depending on variety. Operational conditions may be varied when a particular class of isoflavones is sought, particularly with respect to extraction volume and time, UV exposure, and centrifugation speed. In addition a different approach may be used to achieve a more energy saving method. At this regard, the reduction of extraction time (0.1 h), no UV treatment and a shorter centrifugation time (10 min) is a strategic solution for saving energy. Moreover, the novel method proposed here with a simple equipment and a good environmental compatibility is easy to applied.

However one of the most important variables to consider is variety selection because total concentration and composition of isoflavones have been found to be strictly genetically controlled, particularly in the embryo axis. Isoflavones are commonly more highly concentrated in the embryo axis than in the cotyledons (~12 mg g^{-1} vs. ~1.5 mg g^{-1}), but the lower weight of the former (~40 times) means they have 5-fold lower amounts of isoflavones. In order to maximize extraction efficiency, soybean varieties with high levels of malonyls should be considered, although possible influences of seed batch aging on the profiles of isoflavones should be further investigated.

Last but not least, the method has been tested on intact seeds, but could also be applied to embryo axes, generally a by-product of soy processing.

Acknowledgments

This work was financially supported by the University of Padua (Project no. CPDA123738, *Estrogenic properties of soybean and its potential in vivo and in vitro effects*). The authors wish to thank Tessa Say for checking the English text.

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Appendix 2

ISOFLAVONES QUANTIFICATION IN RAINBOW TROUT MUSCLE BY QUECHERS TECNIQUE AND LIQUID CHROMATOGRAPHY COUPLED WITH MASS SPECTROMETRY

Journal of Food Composition and Analysis 70 (2018) 114–124

Contents lists available at ScienceDirect

Journal of Food Composition and Analysis

journal homepage: www.elsevier.com/locate/jfca

Original research article

Isoflavones quantification in rainbow trout muscle by QuEChERS tecnique and liquid chromatography coupled with mass spectrometry

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ARTICLE INFO

Keywords:
Isoflavones
Oncorhynchus mykiss
Muscle
QuEChERS
Food analysis
Food composition
Method validation
LC–MS/MS

ABSTRACT

The interest in soy isoflavones, especially genistein and daidzein, has increased since scientific data showing a wide range of biological activities of these phytoestrogens. The aim of the present research was to develop and validate a new analytical method for the identification and quantification of isoflavones for the first time in rainbow trout muscle, by the QuEChERS methodology, followed by liquid chromatography coupled with electrospray ionisation mass spectrometry in positive ion (LC-ESI–MS) analysis. The analytes monitored and quantified were Daidzein, Daidzin, Genistein, Genistin, Glycitein and Glycitin. The method was validated according to the criteria of European Commission Decision 2002/657/EC, and the parameters evaluated included linearity, precision, matrix effect, limit of detection and quantification, absolute recovery and robustness. The limit of quantification was fixed at 0.2 $\mu g\,kg^{-1}$ for all isoflavones, and the absolute recovery ranged from 51.8% to 103.3%. The method was applied to 26 real samples of the rainbow trout muscle, and the correlation between the presence of isoflavones, both free and conjugated forms, in muscle and diet with soybeans and soy derivatives was established. This method is suitable for more accurately determining the human exposure to dietary isoflavones from products of animal origin.

1. Introduction

Isoflavones (IFs) are a subclass of flavonoids, also classified as phytoestrogen compounds, which are of widespread interest in nutritional, medicinal and cosmetic fields (Nemitz et al., 2016; Valls et al., 2009). The primary sources of IFs in the diet are the plants of the *Leguminosae* family, especially soybeans, red clover and many other legumes. In plants, IFs with the highest clinical activity are Genistein (Ge), Daidzein (De) and Glycitein (Gle), the free aglycone forms. In vegetables, IFs are present in different ratios and may be found as aglycones or as glucoconjugated forms. Genistin (Gi), Daidzin (Di) and Glycitin (Gli) are biologically inactive, so they can be hydrolysed to their active free forms (De, Ge and Gle), by the action of the intestinal bacteria or in the human gut (Fig. 1) (Cederroth and Nef, 2009; Hale et al., 2002).

IFs are structurally similar to 17β-oestradiol and bind selectively, but weakly, to the mammalian oestrogen receptors (ER), with a preference for ERβ (Daems et al., 2016). Therefore, the IFs demonstrate multi-biological and pharmacological potential effects in animals and humans, and their dietary intake is associated with the prevention of cardiovascular diseases and other disorders such as diabetes, obesity, breast and prostate cancer, menopausal symptoms, etc. (Cano et al., 2010; Messina, 2010; Siow and Mann, 2010; Rimbach et al., 2008; Cassidy et al., 2006; Cotterchio et al., 2006; Morris et al., 2006; Tronck et al., 2006; Ali et al., 2005; Ali et al., 2004; Ren et al., 2001). Conversely, IFs are also considered as endocrine disruptors (anti-estrogenic activity) with the potential to have adverse health effects; thus, dietary intake of these compounds might have detrimental effects on human health in addition to their benefits (Mortensen et al., 2009; Xiao, 2008).

Many studies focused on the presence of IFs in plant-based foods and many analytical methods and extraction procedure were proposed for their determination in soy-based food, fruits, vegetables, nuts and seeds (Bustamante-Rangel et al., 2014; Bustamante-Rangel et al., 2013; Delgado-Zamarreño et al., 2012; Rostagno et al., 2009; Wanga et al., 2002).

Contrastingly, there is limited information about their concentrations in foods of animal origin. This lack of data can cause an underestimation of the total IFs intake by the consumer.

Abbreviations: IFs, isoflavones; De, Daidzein; Di, Daidzin; Ge, Genistein; Genistin, Gi; Gle, Glycitein; Gli, Glycitin

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https://doi.org/10.1016/j.jfca.2018.04.009
Received 26 June 2017; Received in revised form 12 February 2018; Accepted 19 April 2018
Available online 22 April 2018

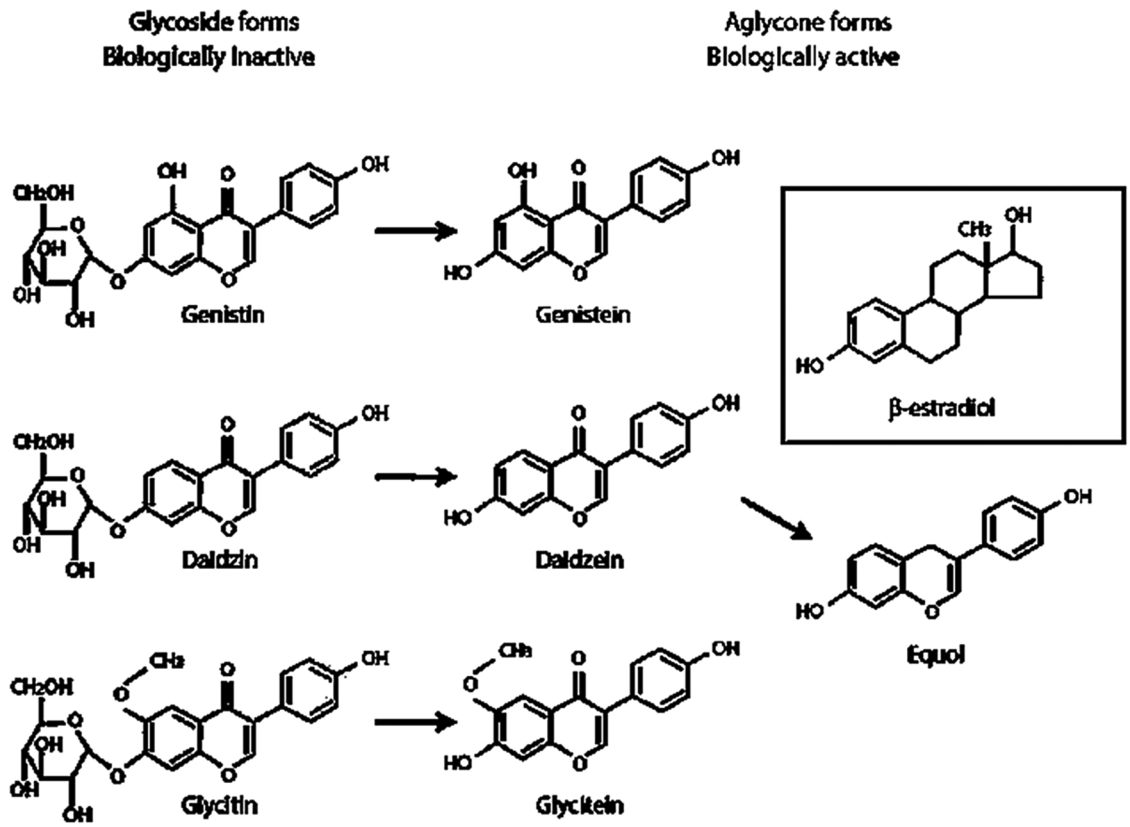

Fig 1. Chemical structure of IFS: glycoside form Genistin, Daidzin and Glycitin; biologically active aglycones: Genistein, Daidzein and Glycitein. Daidzein can be futher metabolized in Equol (Cederroth and Nef, 2009).

Soybeans and soy derivatives (vegetal flours), which contain a high amount of IFs, have been increasingly investigated as healthy ingredients for the formulation of functional foods in the human diet and as a good alternative dietary source of protein in animal (bovine, fish) breeding (Luzzana et al., 2005; Hardy, 1996).

Thus, it is necessary to increase the development of analytical methods to quantify the concentration of IFs in animal products for human consumption, such as milk, eggs, meat and fish.

Some analytical methods were developed and published for the milk matrix regarding the quantification of IFs in foods of animal origin (Daems et al., 2016; Kraicova et al., 2010; Kuhnle et al., 2008; Antignac et al., 2003). The most commonly used protocol provides a liquid–liquid extraction (LLE) followed by a hydrolysis step with β-glucuronidase/sulfatase from *H. pomatia* and a solid phase extraction (SPE) to concentrate the target analyte before instrumental analysis by LC–MS/MS.

Few analytical protocols have been developed for evaluating the IF content in such food products as eggs, meat, and fish. D'Souza et al. (2005) published a method for determining Genistein (the primary isoflavone in soybean) in trout fillet after administering diets containing different amounts of this isoflavone. The protocol comprised an enzymatic digestion step, a step of purification/concentration in SPE, followed by HPLC-DAD analysis.

Later, Kuhnle et al. (2008) investigated the phytoestrogen content in 115 foods of animal origin including milk, milk products, eggs, meat, fish and seafood by extraction with 10% methanol in sodium acetate, hydrolysis with β-glucosidase, solid phase extraction and LC–MS/MS analysis.

Rainbow trout is widely consumed in Europe and Italy, where soy and derivatives are used as logical solutions in the fish meal because they are consistently available, economically attractive and sufficiently palatable to most fish species but there is limited knowledge concerning the effects of soy IFs in this field (Chen et al., 2015; Mai et al., 2012; Yamamoto et al., 2007; Catacutan and Pagador, 2004; Choi et al., 2004; Ko et al., 1999).

This is the first published report concerning a new, sensitive and specific method, suitable for the quantification of both free and conjugated forms of Daidzein (De), Genistein (Ge), Glycitein (Gle), in the muscle of trout. We applied the Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) methodology, for the first time, for extracting IFs from the foods of animal origin.

In fact, so far, this "new" extraction technique was used only for the extraction/purification of isoflavones from legumes (Bustamante-Rangel et al., 2014) and soy-based products (Bustamante-Rangel et al., 2013).

To prevent any change in the original composition of isoflavones present in the sample (a mixture of free or derivative forms) the proposed method, using the QuEChERS technology, allows a significant reduction of the analysis time and sample handling, ensuring more reproducible and reliable results.

Moreover, to reduce the analysis time and simplify the procedure, in this study, we monitored together the aglycones and their glycosylated forms to avoid the hydrolysis step.

Quantitative analysis of IFs was carried out using liquid chromatography coupled with electrospray ionization mass spectrometry (LC-ESI–MS) in the positive ion mode.

The proposed method offers advantages, such as simplicity, especially taking into account the complexity of food matrices, short execution times that permit the reduction in any changes in the real pattern of the IFs in the samples, sensitivity, reproducibility and precision of results.

The aim of this study was to develop a new, faster and easier method, which was more sensitive compared with the existing methods suitable for the determination of isoflavones in trout muscle after the

use of vegetal flours in fish feeds. To verify the applicability and robustness of the validated method, for the first time, real fillets of rainbow trout, fed with different kind of diets, with or without soy and soy derivatives, where analysed.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile (ACN, LC–MS grade) and methanol (MeOH, LC–MS grade) were purchased from Carlo Erba Reagents S.r.l., Milan, Italy; acetic acid (LC–MS grade, purity > 98.0%), hexane (analytical grade, purity > 95.0%), dimethyl sulfoxide (DMSO, analytical grade, purity > 99.9%) were obtained from Sigma-Aldrich, Milan, Italy. Isoflavones reference standards, namely, Daidzein (De, purity > 99.0%), Daidzin (Di, purity > 99.0%), Genistein (Ge, purity > 99.0%), Genistin (Gi, purity > 98.0%), Glycitein (Gle purity > 99.0%), Glycitin (Gli, purity > 99.0%), and 3′,4′-Dimethoxyflavone (Internal Standard, IS, purity > 98.0%) were obtained from LC Laboratories, Woburn, Massachusetts, USA.

Ultrapure water was obtained by a PureLab Classic-Elga system (Veolia Water Technologies, UK).

2.2. Standard solutions

Individual stock solutions of De, Di, Ge, Gi, Gle, Gli and IS were prepared at a concentration of 1000 μg mL^{-1} by dissolving the proper, accurately weighed quantities of each compound in DMSO into volumetric flasks. These solutions were stored at −20 °C in volumetric amber flasks and prepared fresh every 6 months. Solutions of 1 μg mL^{-1} of each analyte, were prepared in a mixture of ACN and H_2O (50:50 v/v) from stock solutions for tuning the ESI source and for MS/MS transition settings.

Working standard solutions (containing all IFs except the IS) used to spike blank muscle samples, were prepared by appropriate dilutions of the concentrated stock standard solutions with a mixture of H_2O:MeOH (50:50, v/v) with 0.3% acetic acid to reach a concentration range of 0.05–12.5 μg mL^{-1}. The internal standard spiking solution was prepared at 2.5 μg mL^{-1} in the same mixture.

2.3. Samples

For method development and validation, 20 rainbow trouts (*Oncorhynchus mykiss)* were purchased from the great retail market.

To verify the applicability and the robustness of the method proposed, 26 rainbow trouts fed with different kind of diets, based on less soy and soy derivatives, were collected from various farms in the Veneto region, Italy.

All samples were stored at −80 °C pending analysis. The trout muscles were thawed, filleted, chopped into small pieces and homogenized. Five grams of each samples were weighed, placed into a 50 mL centrifuge tube and processed as described below.

2.4. Sample preparation

The development of the method of the extraction of IFs from the rainbow trout muscle was conducted by testing a polymer-based sorbent STRATA X-C, 200 mg/6 mL (Phenomenex S.r.l., Bologna, Italy) and a C18 sorbent classical SPE ISOLUTE SCX, 200 mg/6 mL (Biotage, Uppsala, Sweden), compared with the QuEChERS metodologhy. Two different QuEChERS kits obtained from Agilent Technology (Santa Clara, California, USA) was tested: an extraction packets EN method consisting of a salt mixtures with 4 g of $MgSO_4$, 1 g NaCl, 1 g Na citrate and 0.5 g disodium citrate sesquihydrate (citrate buffer); the salt mixture was combined with two different types of dispersive phases (d-SPE): 15 mL clean-up tubes containing 150 mg of C18 and 900 mg of anhydrous $MgSO_4$ and 15 mL clean-up tubes with 150 mg PSA + 45 mg GCB + 900 mg $MgSO_4$.

The homogenized samples, 2.5 and 5 g, were subjected to a preliminary clean up step with hexane and different volumes and composition of pure or aqueous organic solvent (MeOH, ACN, ACN:H_20) before applying the QuEChERS technology. The results obtained from the preliminary tests and the reasons to select QuEChERS as the best technology to obtain sample purification and IFs extraction from the trout muscle, are reported in section 3.1 *Method development and optimization of the extraction procedure*. The validated method of extraction for IFs is shown below.

IF extraction from 5 g of muscle of rainbow trout was performed with QuEChERS technology, which consisted of two steps, a salting-out extraction and a dispersive SPE clean-up.

Before sample preparation, the muscle samples were spiked with 20 μL of IS solution (2.5 μg mL^{-1}). Then, a preliminary clean-up step was applied to remove most of the lipid content before the extraction of IFs: a 10 mL volume of hexane was added to each tube containing the muscle sample (5 g), and the tubes were immediately shaken for 10 min. After centrifugation at 4000 rpm for 10 min at 4 °C, the hexane layer was discarded, and the pellet containing target analytes were subjected to QuEChERS extraction.

The solid residue was mixed with 15 mL of the extraction solvent (H_2O:ACN, 50:50 v/v) and the tubes were agitated for 5 min. Then, an Agilent SampliQ QuEChERS EN extraction salt packet (4 g MgSO4, 1 g NaCl, 1 g Na citrate, 0.5 g disodium citrate sesquihydrate) was added, and the tubes were capped tightly and immediately shaken vigorously for 10 min to prevent agglomeration of the salts. After centrifugation at 4000 rpm for 8 min, to induce phase separation, a 6 mL aliquot of the upper extract (acetonitrile phase) was transferred into an Agilent SampliQ QuEChERS dispersive-SPE tubes. The 15 mL tubes containing 150 mg of C18 and 900 mg of anhydrous $MgSO_4$ were vortexed for 10 min and centrifuged at 4000 rpm for 10 min.

Three mL of the supernatant was transferred into a clean 15 mL tube and evaporated to dryness under a stream of air at 50 °C with a TurboVap evaporator (Zymarck, Hopkinton, Massachusetts, USA). The dry residue was reconstituted in 500 μL of mobile phase (H_2O:MeOH, 50:50 v/v, with 0.3% acetic acid) by vortexing for 3 min. The final extracts were then centrifuged for 10 min at 13000 rpm at 4 °C and transferred to the vials for LC–MS analysis.

A daily calibration curve prepared in the matrix was used to quantify IF contents of all incurred and spiked samples.

2.5. Instrumentation and analytical conditions

All analyses were performed by liquid chromatography with mass spectrometry detection (LC–MS).

The chromatographic separation was achieved using an Accela 600 HPLC pump with CTC automatic injector (Thermo Fischer Scientific, Waltham, Massachusetts, USA) equipped with a Hypersil Gold (100 × 2.1 mm, 1.9 μm) analytical column by Thermo Fisher Scientific (Waltham, Massachusetts, USA).

Chromatographic separation was carried out using a mobile phase consisting of 0.3% acetic acid in H_2O (eluent A) and ACN with 0.3% acetic acid (eluent B). The gradient conditions were from 0 to 6 min ramp linearly from 10 to 50% of eluent B, then from 6 to 10 min ramp

Table 1
Instrument acquisition data for the analysis of isoflavones by LC–MS.

Compound	Precursor ion (*m/z*)	Product ions	CE (%)	Rt (min)
De	255	255 > 237, 227, **199**[a], **137**	40	6.7
Di	417	417 > 255	19	4.5
		255 > 237, 227, **199**[a], **137**	40	
Ge	271	271 > 253, 243, **215**, 187, **153**[a], 145	40	7.8
Gi	433	433 > 271	43	5.4
		271 > 253, 243, **215**, **153**[a]	40	
Gle	285	285 > 270	40	6.8
		270 > **242**[a], 214, **152**	35	
Gli	447	447 > 285	25	4.6
		285 > **270**[a], 257, **229**, 167, 145	43	
IS	283	283 > **268**[a], 265, 247	40	9.2

CE: collision energy; Rt: retention time.
Identifier ions in bold.
[a] product ion used for quantification (quantifier ion).

of eluent B from 50 to 70%, from 10 to 11 min ramp again linearly to reach 90% of eluent B and hold for 1 min. After that, the pump returned to the initial conditions (90% of eluent A and 10% of eluent B) in 0.5 min and hold for 2.5 min to re-equilibrate the system. The flow rate was set at 0.20 mL $min^{-1,}$ and the injection volume was 5 µL. The sample trays were maintained at 4 °C.

The mass detection was achieved with a Thermo Fischer LTQ XL ion trap mass spectrometer (Waltham, Massachusetts, USA), equipped with a heated electrospray ionization (HESI-II) probe.

The system was controlled by the Xcalibur software (version 2.1, Thermo Fisher Scientific, Waltham, Massachusetts, USA), that was also used for data acquisition and analysis.

Standard solutions at 1 µg mL^{-1} of each isoflavone were infused through a syringe pump at 20 µL min^{-1} and introduced into the LC flow via a T-piece before it entered the detector to determine fragmentation patterns, tuning parameters, and MS^2/MS^3 parameters for each analyte. Precursor ions, product ions, collision energies and retention times are shown in Table 1.

The ESI source was used in a positive mode. The mass analyser was set on the full scan monitoring mode. The following optimum tuning parameters were set: sheath gas flow 35 arbitrary units; auxiliary gas flow 6 arbitrary units; ion spray voltage 3.5 kV; capillary temperature 250 °C; capillary voltage 19 V; tube lens 75 V.

Retention time windows for each analyte were checked daily with a mixture containing all isoflavone standards in the mobile phase. Confirmation was achieved by examining the relative ion intensities of two major MS^2 or MS^3 product ions.

2.6. Method validation

The method was validated according to the criteria of European Commission Decision 2002/657/EC (European Commission, Commission decision 2002/657/EC, 2002). The performance of the method was assessed for each analyte through both its qualitative parameters such as specificity, the relative retention time (RRT) and the ion ratio intensities and its quantitative parameters such as linearity, trueness assessed through the evaluation of the apparent recovery, precision (within-day repeatability and within-laboratory reproducibility), matrix effect, limit of detection and quantitation, absolute recovery and robustness.

The optimization and validation of the method were performed using spiked blank samples, due to the unavailability of certified reference material. Therefore, trout muscles collected from 20 different animals with no detectable IFs were pooled and then spiked with 20 µL of IS solution 2.5 µg/mL and proper amounts of working solutions of IFs to obtain calibration samples at different concentrations. Before proceeding with the extraction, to permit the interaction between IFs and tissues, the samples were shaken and allowed to stand in a dark place for 15 min.

2.6.1. Qualitative criteria

The specificity of the method was tested for each analyte. A representative number of blank muscle samples collected from different animals (n = 20) were analysed and the chromatograms were compared with those obtained from the standard solutions, and the muscle samples spiked with IFs, for checking the absence of the potential matrix interference peaks at the retention time of the target analytes.

Confirmation of the identities of IFs was carried out by comparing the chromatographic peak area of the two prominent product ions in MS^2 or MS^3 obtained in muscle samples with those obtained from the calibration standards at comparable concentrations. Identification was considered reliable if the ion intensity ratio, along with the comparison of the chromatographic retention times with those of standards, were within the criteria laid down in the European Commission Decision.

2.6.2. Linearity and sensitivity

Method linearity was evaluated by preparing six different matrix-matched calibration curves on six different days by spiking the blank biological matrix at eight different concentration levels of isoflavones (0.2, 0.5, 1, 2, 5, 10, 20, 50 µg kg^{-1}) before proceeding with the extraction.

Calibration lines were constructed by plotting the ratio of the analyte area to the IS area versus the added concentrations and carrying out a linear regression analysis. Slope and intercept were determined by the square regression method, and the linearity was considered acceptable when the coefficient of correlation (R^2) was above 0.990, and the evaluation of the residual was lower than 15%.

The Limit of Detection (LOD) and the limit of Quantification (LOQ) were determined using the calibration curves in the matrix. For each of the analytes, the LOD values were determined as the concentration corresponding to the blank response plus three times the standard error of the y-intercept. The linear function of the Microsoft Excel 2010 programme was used.

Usually, the Limit of Quantification (LOQ) can be calculated as the blank response plus six times the standard error of the y-intercept, which is two times the LOD. However, in this study, the LOQ values was defined as the smallest measured content of the identified analyte, with a signal-to-noise ratio of at least 10, which was quantified with a precision within 20% and trueness between 80% and 120%, in agreement with the limits reported by the European Commission Decision.

2.6.3. Accuracy considered regarding trueness and precision

The trueness and precision of the method were determined by performing tests on three sets of blank samples fortified with IFs at three different concentrations (six replicates each): 0.5, 5, 50 µg kg^{-1}. Samples were analysed on three different days in the same laboratory, with the same instrument corresponding to 54 samples.

Since no certified reference materials for IFs in the trout muscles are available, the trueness was evaluated by the recovery of the known amount of analytes added to the blank matrices. Therefore, the term of apparent recovery was found suitable for use in place of trueness as we used fortified muscle samples. It was calculated by dividing the mean measured value by the fortification level and multiplying by 100 to

express the results as a percentage. According to 2002/657/EC, the apparent recovery should be between 70 and 110% for the fortification levels between 1.0 and 10.0 μg kg^{-1}, and between 80 and 110% for fortification levels ≥ 10.0 μg kg^{-1}.

The precision of the method was evaluated either in terms of the intra-day (within-run) repeatability (RSDr), the variability of independent test results obtained on the same day (n = 6) with the same method on identical test items, in the same laboratory by the same operator using the same equipment, or in terms of inter-day (between-run) reproducibility (RSDR) and the variability of independent test results (n = 18), obtained by repeating this experiment consecutively for three different days (Karageorgou et al., 2013; Muscarella et al., 2007).

Precision was expressed in terms of imprecision and calculated as the relative standard deviation (RSD in percent) of measured concentrations at each level: RSD% = (standard deviation/mean measured concentration) x 100. The RSD% values for repeatability (RSDr) are acceptable if they are below two-thirds of the value calculated from the Horwitz equation. However, for reproducibility (RSDR), they are acceptable if they are below the values calculated from the Horwitz equation (23% if the concentration is between 100 and 1000 μg kg^{-1} and 16% if the concentrations are higher than 1000 μg kg^{-1}). The Horwitz equation is not applicable to concentrations below 120 μg kg^{-1}, which is the case of the concentration levels considered in this study. Moreover, the repeatability (RSDr) and inter-day reproducibility (RSDR) values should be as low as possible and are considered acceptable if they are below 14.7% and 22%, respectively, as suggested by Thompson (2000).

2.6.4. Absolute recovery and matrix effect

The absolute recovery of all analytes was determined by comparing the analytical results of the extracted isoflavones from fortified trout muscle samples (isoflavones and IS were added before the extraction procedure) with un-extracted standards added at the same concentrations in blank extracts, representing 100% recovery.

Matrix effects were evaluated by calculating the peak area of the analytes in the presence of matrix (analytes added to blank matrix after extraction), to the peak area in absence of matrix (pure solution of the analyte at the same concentration).

Absolute recovery and matrix effect for each analyte were evaluated at three different levels, 0.5, 5, 50 μg kg^{-1} (n = 6). Three sets of samples were used for determination: one consisting of neat standards (set 1), one prepared in a blank matrix extract and spiked after extraction (set 2) and one spiked before extraction (set 3). Absolute recovery (REC%) and matrix effect (ME) were calculated using the following formulas:

$$REC\ (\%) = set\ 3_{area}/set\ 2_{area} \times 100;\ ME = (set\ 2_{area}/IS_{area})/(set\ 1_{area}/IS_{area})$$

2.6.5. Ruggedness

The ruggedness of the method was assessed according to the Youden and Steiner approach (Karageorgou and Samanidou, 2014) by introducing slight variations in potentially critical analytical parameters.

For this purpose, seven reasonable variables were chosen for the sample preparation procedure (volume of hexane; acetonitrile percentage in the mixture H_2O-ACN; shaking and centrifugation time; evaporation temperature of the final extract) and slightly modified with respect to the standard procedure. For each factor, two different conditions were adopted. Eight experiments were carried out for the evaluation of the seven selected factors by using eight aliquots of trout muscle samples spiked at the concentration of 5 μg kg^{-1}. The effect of each factor was calculated by subtracting the mean result obtained from the variable at the high level, and the mean result achieved with the factor at the low level. The standard deviation of the differences was calculated and compared with the values obtained under "within-laboratory" reproducibility conditions.

3. Results and discussion

3.1. Method development and optimization of the extraction procedure

The optimization of the IFs extraction procedure from the rainbow trout muscle was conducted by testing different types of extraction procedures.

In the solid phase extraction, two types of sorbents for SPE were compared: a polymer-based sorbent STRATA X-C, 200 mg/6 mL, and a C18 sorbent ISOLUTE SCX, 200 mg/6 mL. The average absolute recoveries obtained from the two kinds of sorbents were similar: 10–30% for the aglycones form and 70–110% for the glycosides.

Then, an extraction procedure based on the QuEChERS methodology was tested. First, a phase separation was induced by the addition of various pre-weighed salts (extraction packets EN method). The salt mixtures were 4 g of $MgSO_4$ to reduce the volume of the aqueous phase and facilitate the portioning of polar analytes into the organic phase, 1 g NaCl to avoid the presence of interferents in the extracts, and 1 g Na citrate and 0.5 g disodium citrate sesquihydrate (citrate buffer) to provide a pH of approximately 5. This pH value was adequate for the quantitative extraction of isoflavones whose pKa are greater than 9.

Then, a dispersive SPE (d-SPE) was conducted to clean-up interfering compounds that may be co-extracted and affect the determination of IFs. Two different types of dispersive phases were tested: 15 mL clean-up tubes containing 150 mg of C18 and 900 mg of anhydrous $MgSO_4$ and 15 mL clean-up tubes with 150 mg PSA + 45 mg GCB + 900 mg $MgSO_4$.

The 15 mL clean-up tubes containing 150 mg of C18 and 900 mg of anhydrous $MgSO_4$, as dispersive phase guaranteed the best results. Good absolute recoveries were obtained for all the target analytes (from 51 to 103.3%) and were higher than those obtained with the SPE columns and with 150 mg PSA + 45 mg GCB + 900 mg $MgSO_4$ dispersive phase (IF aglycones average recoveries: 18.17–50.75%; IF glucosides average recoveries: 2.70–24.73%).

Furthermore, the protocol was optimized for testing two different amounts of homogenized samples (2.5 and 5 g) and the different types and volumes of organic solvents (MeOH, hexane, ACN, ACN:H_20) for sample extraction before applying the QuEChERS technology.

The best extraction yields were achieved when 5 g of the rainbow filet was used and the extraction was performed first with 10 mL of hexane to remove fat from the samples and with 15 mL of ACN:H_20 (50:50, v/v) after discarding the organic layer. Therefore, the results from the muscle matrix showed that IF extraction with pure solvents provided low yields; the addition of water improved the extraction efficiency of all compounds, and ACN with H_2O was more efficient than other solvents, since IFs aglycones are insoluble in water (Rostagno et al., 2009; Murphy et al., 2002).

The QuEChERS protocol used in this study was similar to that reported for IFs extraction from soy-based foods (Bustamante-Rangel et al., 2013), but the quantification limit (LOQ) obtained for trout muscle (0.2 μg/kg) was much lower than that obtained for soy-based foods (LOQs ranged from 0.69 μg/L to 6.6 μg/L).

The application of QuEChERS technology for IF extraction from

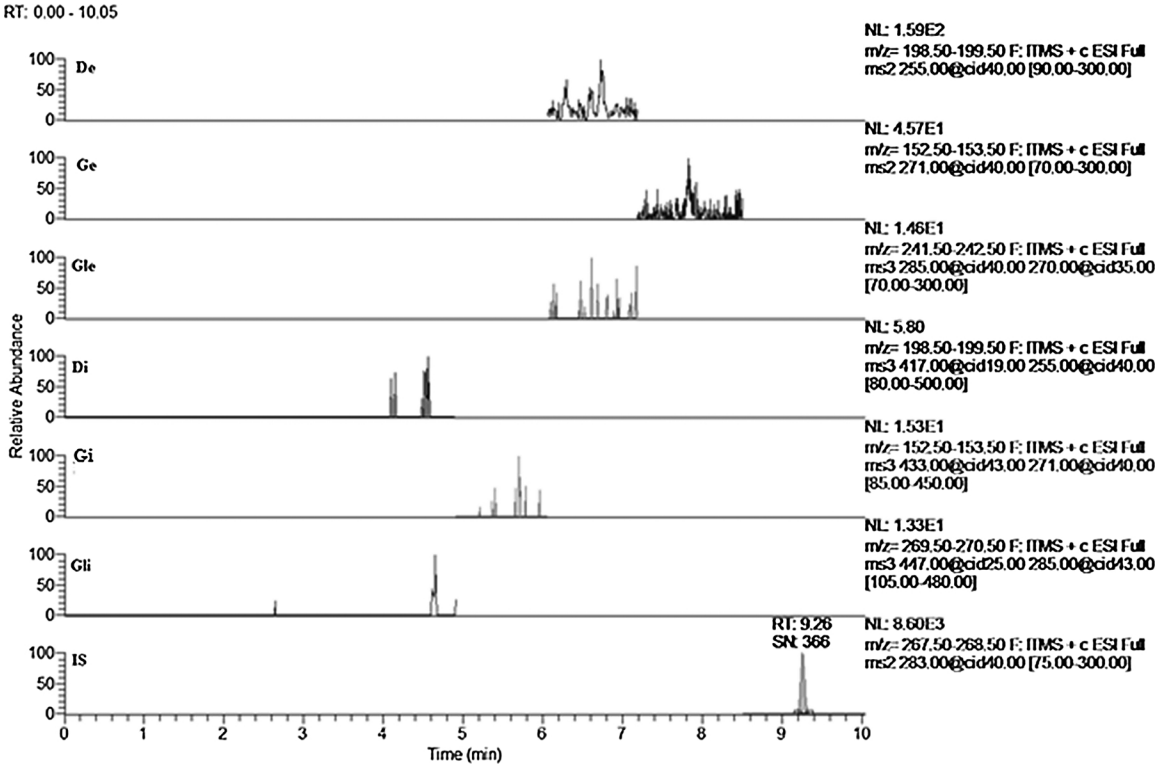

Fig. 2. Chromatogram of IFs obtained from a blank sample of rainbow trout.

Table 2
Confirmation Data: identification criteria obtained for IFs in muscle samples according to the European decision 2002/657/EC. Ion ratio% calculated as identifier ion intensity/quantifier ion intensity x 100 (mean values were reported).

Compound	Ion ratio% Sample	Ion ratio% Standard (tolerance)	RRT sample	RRT Standard (tolerance)
De	67	67 (± 20%)	0.73	0.72 (± 2.5%)
Di	66	65 (± 20%)	0.49	0.49 (± 2.5%)
Ge	80	75 (± 20%)	0.84	0.84 (± 2.5%)
Gi	82	83 (± 20%)	0.58	0.58 (± 2.5%)
Gle	11	11 (± 30%)	0.73	0.74 (± 2.5%)
Gli	18	18 (± 30%)	0.50	0.50 (± 2.5%)

RRT: Relative Retention Time.

trout muscle provided greater sensitivity than that of the existing methods for quantification of IFs in products of animal origin.

The protocol proposed by Kuhnle et al. (2008) on phytoestrogen content, provides a deconjugated step followed by a solid phase extraction and the LOQ is 1.5 µg/100 g of dry weight while D'Souza et al. (2005) proposed a method in HPLC-DAD only for the genistein content in the trout muscle, but the LOQ was not reported.

3.2. Validation results

The specificity was assessed by comparing the chromatograms of blank muscle samples collected from 20 different animals with those of the corresponding spiked samples to test for endogenous interference.

Table 3
Linear range regression data for the calibration curve and LOD calculated for the different isoflavones (LOQ limit of quantification is the lowest level of the linear range).

Compound	Range µg/kg	Slope (± SD)	Intercept (± SD)	R^2	LOD
De	0.2–50	0.093 ± 0.006	0.031 ± 0.010	1	0.098
Di	0.2–50	0.008 ± 0.001	0.003 ± 0.000	1	0.003
Ge	0.2–50	0.019 ± 0.001	0.004 ± 0.002	1	0.006
Gi	0.2–50	0.013 ± 0.012	0.002 ± 0.001	1	0.009
Gle	0.2–50	0.045 ± 0.003	0.011 ± 0.008	0.999	0.057
Gli	0.2–50	0.050 ± 0.004	0.006 ± 0.002	0.999	0.057

SD: standard deviation; LOD: limit of detection.

No significant endogenous interfering peaks were evident at the retention time typical of all studied compounds, indicating that the method was selective as no interfering substances were present in the biological matrix (Fig. 2).

For all the analytes, the identity was confirmed according to 2002/657/EC considering two product ions for each isoflavone − the quantifier and the identifier ions. The intensity ratio between the two ions (ion ratio) was calculated (in percentage) for each isoflavone in the matrix matched calibration samples and the standard samples. The mean values for the ion ratios are reported in Table 2: all ion ratios of samples, compared with those of standard solutions, were within the recommended tolerance (20% for De, Di, Ge and Gi and 30% for Gle and Gli). Moreover, the matching of the relative retention time, which was the ratio of the chromatographic retention time of the analyte to that of the internal standard, was calculated in samples and standard solutions. The relative retention time of the analyte corresponded to

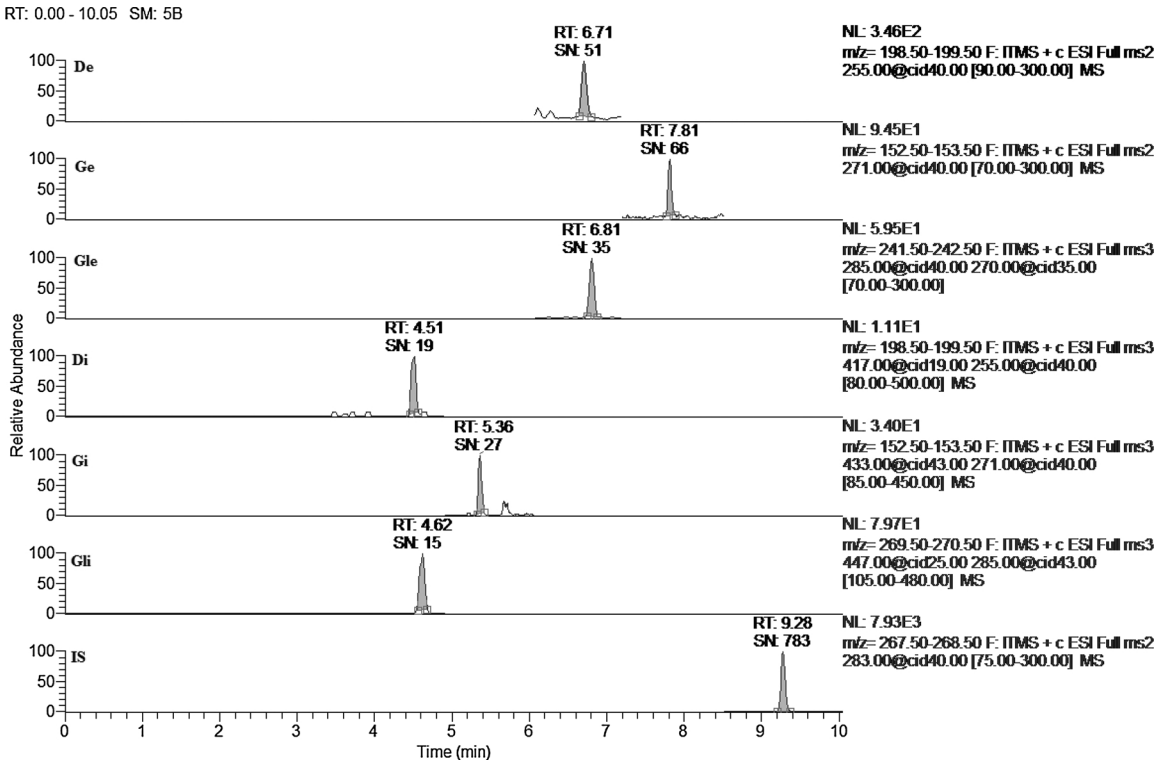

Fig. 3. Chromatogram of IFs obtained from a sample spiked with the lowest concentration of the calibration curves with a signal-to-noise ratio of at least 10 (LOQ: limit of quantification 0.2 µg/kg).

that of the calibration solution at a tolerance of ± 2.5% in all samples. The comparison of the relative intensities and the relative retention times assured peak identity.

The linearity of the calibrations curves in the matrix was checked on six different days. The ratio of the peak area of the analyte to the peak area of the internal standard was plotted versus the added concentrations and a least-squares linear regression, weighed by the reciprocal of the concentrations, was applied to generate the calibration curves. Good linearity was observed within the concentration ranges considered for all isoflavones (0.2–50 µg kg^{-1}) in the matrix, since the calculated determination coefficients R^2 were always > 0.99. Moreover, for all analytes, the residuals were ≤15% at each calibration point. The slopes of the different calibration curves did not vary considerably, and the intercepts were near to theoretical zero value, demonstrating an excellent reliability of the measuring system (see Table 3). The results of the back-calculated concentrations over the validated range of 0.2–50 µg kg^{-1} for all analytes were acceptable. The mean trueness and precision, expressed as RSD%, were respectively in

Table 4
Within-day repeatability (RSDr, n = 6), within-laboratory reproducibility (RSDR, n = 18) expressed as relative standard deviation (%) and apparent recovery (%) obtained by spiking the blank trout muscle with isoflavones at three concentration levels (0.5, 5, 50 µg/kg).

Compound	N_C µg/kg	Day 1 (n = 6)			Day 2 (n = 6)			Day 3 (n = 6)			Inter-day (n = 18)		
		Mean found µg/kg	RSDr (%)	Apparent recovery (%)	Mean found µg/kg	RSDr (%)	Apparent recovery (%)	Mean found µg/kg	RSDr (%)	Apparent Recovery (%)	Mean found µg/kg	RSDR (%)	Apparent recovery (%)
De	**0.5**	0.52	8.19	103.33	0.48	10.16	95.07	0.39	10.37	78.77	0.46	14.46	92.39
	5	4.98	2.21	99.56	5.02	1.28	100.38	4.97	6.08	99.49	4.99	3.59	99.81
	50	51.45	5.28	102.90	46.72	11.34	93.44	48.43	10.92	96.86	48.87	9.75	97.73
Di	**0.5**	0.49	10.44	97.50	0.48	12.67	95.85	0.52	7.51	103.93	0.50	10.32	99.10
	5	4.85	5.54	96.99	5.50	9.54	109.93	4.75	5.31	95.05	5.03	9.66	100.66
	50	46.47	7.83	92.94	50.42	2.85	100.84	49.18	9.14	98.36	48.69	7.50	97.38
Ge	**0.5**	0.54	4.08	107.10	0.49	9.99	98.83	0.52	9.46	103.00	0.51	8.37	102.98
	5	5.02	1.10	100.35	5.00	2.34	99.97	4.66	8.50	93.19	4.89	5.77	97.83
	50	50.61	3.98	101.23	49.99	3.51	99.97	46.07	10.38	92.14	48.89	7.41	97.78
Gi	**0.5**	0.51	8.65	101.02	0.52	6.14	104.17	0.50	9.14	99.35	0.51	7.82	101.51
	5	5.06	2.08	101.11	5.11	3.02	102.22	5.20	7.59	103.98	5.12	4.77	102.44
	50	51.22	10.50	102.44	50.20	3.13	100.39	49.94	2.75	99.88	50.45	6.30	100.91
Gle	**0.5**	0.53	5.80	106.57	0.47	6.61	94.17	0.46	9.32	91.30	0.49	9.77	97.34
	5	4.90	5.04	98.03	4.86	4.25	97.20	4.97	3.23	99.33	4.91	4.08	98.19
	50	51.21	4.31	102.41	49.88	4.80	99.77	52.17	4.22	104.34	51.09	4.58	102.17
Gli	**0.5**	0.53	3.73	106.00	0.49	7.79	98.00	0.49	4.80	97.13	0.50	6.68	100.38
	5	5.03	1.13	100.63	5.11	2.84	102.10	5.04	3.98	100.74	5.06	2.80	101.16
	50	50.83	7.19	101.67	48.99	3.29	97.99	46.69	10.78	93.39	48.84	7.98	97.68

N_C: Nominal concentration.

Table 5
Absolute recovery (REC) and matrix effect (ME) of the IFs obtained by spiking the blank trout muscle with isoflavones at three concentration levels (0.5, 5, 50 µg/kg).

Compound	N_Cµg/kg	REC% ± SD n = 6	ME ± SD n = 6
De	**0.5**	93.8 ± 6.3	$1.2 \pm 1.2 \times 10^{-1}$
	5	80.0 ± 7.1	$0.9 \pm 8.0 \times 10^{-2}$
	50	94.6 ± 6.9	$0.8 \pm 1.0 \times 10^{-2}$
Di	**0.5**	65.2 ± 5.4	$0.8 \pm 1.2 \times 10^{-1}$
	5	51.8 ± 13.7	$0.8 \pm 7.0 \times 10^{-2}$
	50	56.8 ± 7.1	$0.8 \pm 2.0 \times 10^{-2}$
Ge	**0.5**	103.3 ± 3.3	$1.0 \pm 7.0 \times 10^{-2}$
	5	92.2 ± 8.5	$0.8 \pm 9.0 \times 10^{-2}$
	50	93.9 ± 7.2	$0.8 \pm 1.0 \times 10^{-2}$
Gi	**0.5**	71.1 ± 12.4	$0.8 \pm 5.0 \times 10^{-2}$
	5	80.5 ± 7.9	$0.8 \pm 8.0 \times 10^{-2}$
	50	67.9 ± 4.1	$0.8 \pm 2.0 \times 10^{-2}$
Gle	**0.5**	92.2 ± 5.3	$0.9 \pm 3.0 \times 10^{-2}$
	5	88.4 ± 1.6	$0.9 \pm 9.0 \times 10^{-2}$
	50	97.9 ± 5.2	$0.8 \pm 1.0 \times 10^{-2}$
Gli	**0.5**	81.7 ± 10.9	$0.8 \pm 7.0 \times 10^{-2}$
	5	83.7 ± 8.1	$0.8 \pm 8.0 \times 10^{-2}$
	50	71.3 ± 7.4	$0.8 \pm 0.8 \times 10^{-2}$

N_C: Nominal concentration; SD: Standard deviation.

the range of 98.8–104.7%, 2.5–14.1% for De, from 96.3–107.3%, 3.9–14.9% for Di, from 90.1–103.5%, 3.7–10.8% for Ge, from 94.2–104.5%, 2.2–17.2% for Gi, from 90.6–116.7%, 2.8–12.3% for Gle and from 97.5–105.2%, 2.1–8.6% for Gli.

To quantify the unknown samples, a calibration curve was freshly prepared every day of the analysis, and the sample concentrations were back-calculated from the calibration curve.

The LOD values are reported in Table 3.

The LOQ was defined as the lowest concentration of the calibration curve that could be measured with a precision within 20%, and the trueness between 80% and 120%: it was fixed at $0.2\,\mu g\,kg^{-1}$ for all isoflavones. The accuracy and precision of the LOQ were determined by analysing six replicates of the sample at the LOQ concentration (Fig. 3). The accuracy and CV% were respectively, 99.5% and 12.1% for De, 102.2% and 14.9% for Di, 103.5% and 10.3% for Ge, 95.9% and 17.2% for Gi, 116.7% and 11.5% for Gle and 97.5% and 7.2% for Gli.

The trueness, the apparent recovery of the developed method, ranged from 78.77–109.93% for all isoflavones, which was in agreement with the limits established by the Commission Decision 2002/657/EC.

The precision of the method was evaluated at three different levels of fortification by calculating the RSD% of the isoflavone concentrations under within-day repeatability conditions (RSDr, calculated from

Table 6
Diet composition and analytical constituents of the complete commercial fish feed administered during the breeding of rainbow trout used to verify the method on field (Vicenza, Italy). The diet components in bold are based on soy or soy derivatives.

Diet	Diet Composition	Analytical Constituents	Samples n°
A	**Soybean meal, soybean oil,** fish meal, corn gluten, blood products, **peas**, rapeseed meal, wheat meal, fish oil, **soybean protein concentrate**, guar germ meal.	Crude protein 41%; rude fat 24%; rude fibre 2.4%; crude ash 7.5%; phosphorus 0.66%; sodium 0.66%; calcium 0.21%.	**10**
B	Fish meal, **soybean meal**, corn gluten, fish oil, **soybean oil**, rapeseed meal.	Crude protein 41%; crude fat 24%; crude fibre 2.4%; crude ash 7.5%; phosphorus 0.66%; sodium 0.66%; calcium 0.21%.	**9**
C	Fish meal, rapeseed meal, wheat meal, fish oil, blood products, corn gluten, hydrolysed protein, sunflower protein concentration	Crude protein 41%; crude fat 31%; carbohydrates 14%; fibre 1%; ash7%; phosphorus 0.9%; sodium 0.2%; calcium 0.21%.	**7**

Table 7
Results of LC–MS/MS analysis of real samples: free, conjugated forms and total IFs concentration in the muscle of rainbow trout fed with different diets A, B and C.

	Group	n1	n2	n3	n4	n5	n6	n7	n8	n9	n10	Mean ± SD
Deµg/kg	A	2.86	5.29	5.51	6.45	9.75	9.71	4.13	6.22	4.67	5.38	6 ± 2.22
	B	2.61	3.56	10.33	2.55	7.75	2.05	1.72	3.31	3.24		4.12 ± 2.92
	C	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD				
Diµg/kg	A	2.27	4.70	7.25	6.46	16.78	10.06	6.03	23.54	5.67	8.89	9.16 ± 6.38
	B	4.76	7.78	26.07	6.68	4.72	5.77	4.13	8.22	2.25		7.82 ± 7.09
	C	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD				
Geµg/kg	A	3.27	5.73	5.93	7.15	9.86	8.30	4.29	6.41	4.80	4.95	6.07 ± 1.96
	B	3.06	3.65	11.18	3.02	6.42	2.32	1.99	3.43	3.05		4.23 ± 2.89
	C	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD				
Gi µg/kg	A	1.80	3.16	4.50	3.26	11.32	5.35	3.89	11.93	3.42	4.64	5.33 ± 3.46
	B	3.90	6.71	20.67	4.96	4.12	4.36	2.79	6.11	1.69		6.14 ± 5.66
	C	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD				
Gle µg/kg	A	0.40	0.75	0.68	0.94	1.20	1.32	0.54	1.25	0.50	0.76	0.83 ± 0.33
	B	0.54	0.43	0.53	0.27	0.98	nf	nf	0.28	0.44		0.49 ± 0.24
	C	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD				
Gli µg/kg	A	0.44	0.95	1.45	1.24	2.42	2.10	0.91	3.37	1.04	1.54	1.55 ± 0.86
	B	0.84	1.30	4.79	1.20	0.61	1.08	0.92	1.64	0.41		1.42 ± 1.31
	C	0.40	< LOD	0.20	0.27	0.51	< LOD	< LOD				0.35 ± 0.14
ΣIFs µg/kg	A	11.03	20.59	25.31	25.50	51.32	36.84	19.79	52.72	20.10	26.15	**28.94 ± 13.80***
	B	15.71	23.42	73.57	18.67	24.60	15.59	11.56	23.00	11.08		**24.13 ± 19.20***
	C	0.40	< LOD	0.20	0.27	0.51	< LOD	< LOD				**0.35 ± 0.14**

n: sample number; SD: standard deviation; Σ: total IFs concentration; LOD: limit of detection; *: one-way analysis of variance (Sidak's multiple comparisons test $p < 0.05$ A *vs* C; B *vs* C).

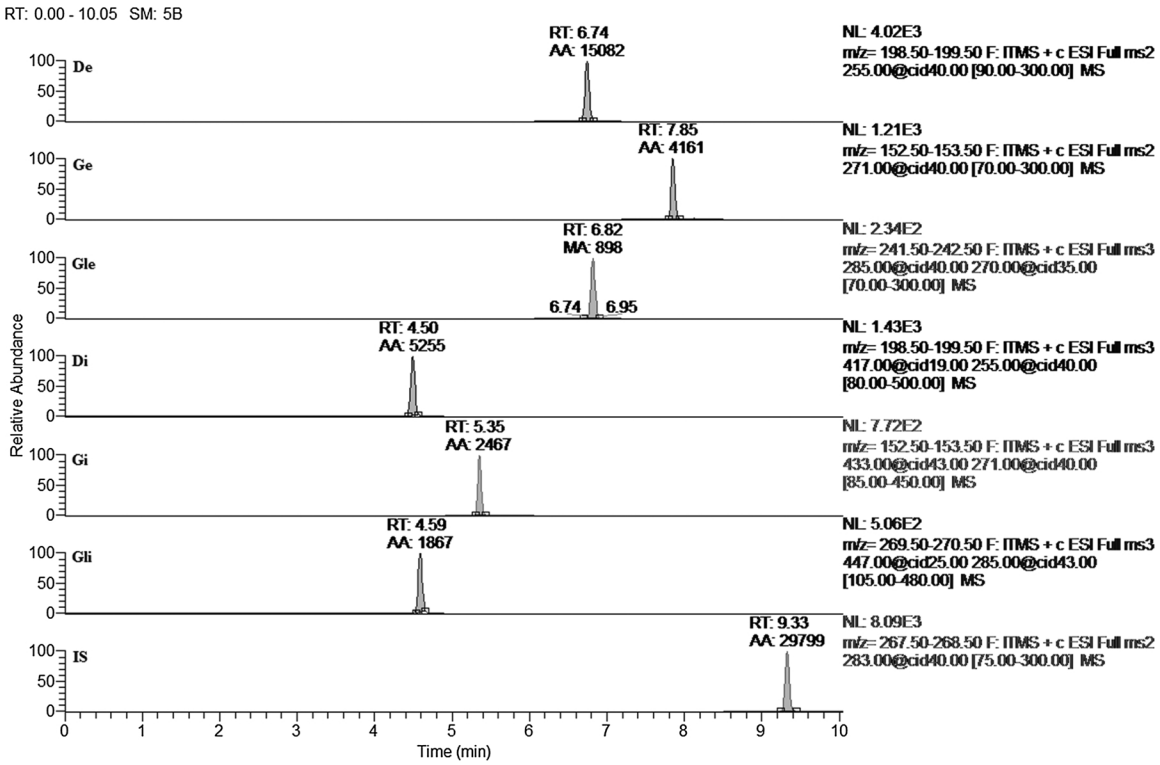

Fig. 4. Chromatogram of IFs obtained from an extracted muscle of an animal of group A.

six replicated samples analysed on one day), and under within-laboratory reproducibility conditions (RSDR, calculated from batches of 18 samples analysed on three different days by different operators). The results, listed in Table 4, reveal that the method was very precise, with RSDr and RSDR, respectively, ≤11.34% and ≤14.46% for De, ≤12.67% and ≤10.32% for Di, ≤10.38% and ≤8.37% for Ge, ≤10.50% and ≤7.82% for Gi, ≤9.32% and ≤9.77% for Gle and ≤10.78% and ≤7.98% for Gli.

The absolute recovery evaluated in six replicates at three different concentrations was in the range 80.0–94.6% for De, 51.8-65.2% for Di, 92.2-103.3% for Ge, 67.9-80.5% for Gi, 88.4-97.9% for Gle, 71.3-83.7% for Gli (Table 5). The matrix effects ranged from 0.8 to 1.2 indicating that the analytes were only slightly influenced by the tissue due to optimized sample clean-up procedures and optimized chromatography conditions (Table 5).

The results of the ruggedness test indicated that the method was not affected by slight variations of some critical factors in the sample preparation procedure and could be considered acceptably robust.

3.3. Application to real samples

To verify the applicability and the robustness of the validated method, 26 real samples of rainbow trout muscle were extracted by QuEChERS and analysed, in duplicates, by LC–MS/MS. The new method was able to identify and quantify IFs in the muscle of animals fed with diets A and B, based on soy and/or soy derivatives (Table 6). Moreover, it was possible to correlate the occurrence of IFs to the high or low presence of soy and its derivatives in the feeding; the results are reported in Table 7. The method permitted the evaluation of the single concentration of IFs, both as aglycones and conjugated forms, without spending time for deconjugation step (Fig. 4); in muscle of the animals of group A and B the most representative IFs were Di > De = Ge > Gi > Gli > Gle. In animals fed with diet C, free of soy or derivatives, the IFs De, Di, Ge, Gi and Gle were not found. The only IF checked in the muscles of 4, out of 7 animals, was Gli, at an average concentration of 0.35 ± 0.14 μg/kg. A one-way analysis of variance (ANOVA) with the GraphPad Prism software version 6 (GraphPad Company, S. Diego, California, USA) was conducted to track statistically differences between the total mean concentrations of IFs in muscle of trout fed with or without soy. A Sidak's paired-samples *t*-test was applied to determine the effect of the presence of soy and its derivatives in the feeding, on the total amount of IFs in muscle. There was a significant difference between the IFs total content of both groups A and B (Table 7), respectively of 28.93 ± 13.80 μg/kg and 24.13 ± 19.20 μg/kg, when compared to the group C, fed with a diet free of soy (0,35 ± 0.14 μg/kg). No statistically evidence existed between the total mean concentration of IFs in muscle of animals of group A and B and therefore no effect attributable to a greater or lesser presence of soy in the diet.

The method allowed to correlate and evaluate the presence of IFs in the muscle of rainbow trout, both aglycone and glucoconjugated forms, a very important result since conjugated IFs can act as sources of aglycones and may have bioactivity.

4. Conclusions

There is an urgent need for validated, reliable, precise, time- and resources saving analytical methods, which can be applied to different types of the matrix for the quantification of IFs in food. Thus, it is essential to have precise information on their distribution to understand

better their role in preventing disease and/or their possible detrimental effects on human health.

The proposed analytical method allows the qualitative identification and quantitative determination of the main isoflavones, present in soy and soy-based feed, in the muscle of rainbow trout. In the present study, the QuEChERS extraction technology was applied followed by LC–MS/MS analysis.

This method for the quantification of IFs in the muscle of rainbow trout has been developed and fully validated, for the first time, according to Decision 2002/657/CE. The method proved to be simple, fast, efficient, precise, reproducible, and accurate as the validation results obtained fully satisfied the requirements of the reference Decision.

This reliable LC-MS/MS method is faster and more sensitive than the current procedures proposed in the literature with a limit of quantification (LOQ) determined for all IFs equal to 0.2 μg/kg.

Method applicability to real samples was also demonstrated by analysing 26 real samples in duplicates. The presence of both free and aglycone forms of IFs was checked in the muscle at low concentration levels after a single sample extraction and analysis, without the addition of any deconjugation procedure. The method applied on real samples made it possible to correlate the presence of IFs in the muscle of rainbow trout, fed a diet containing soybeans and soy derivatives, to more accurately assess the human exposure.

Acknowledgements

The authors thank the health authorities and Dr. Stefano Ferrarini of the Department of Prevention ULSS 8 Berica, Viale Camisano n. 61/68 Vicenza (Italy) for their kind collaboration and some breeders for their kind supply of rainbow trout samples.

The project was support by the University of Padova (Italy) to Prof. G. Radaelli (CPDA123738) and to Dott.ssa R. Merlanti (60A08-8930/13).

The authors declare no competing financial interest and any conflict of interest.

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