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Abstract: Chickpea protein concentrates can be used as food ingredients because of their favorable nutritional and functional properties, and could also supply health promoting isoflavones. The goal of this research was to analyze the isoflavones in chickpea concentrates as compared to the original flour. Protein concentrates were prepared by alkaline extraction and precipitation of protein at the isoelectric pH. HPLC analysis revealed that the concentrates were enriched in isoflavones. Thus, the concentration of total isoflavones was 45 and 10 µg/g in concentrates and flour, respectively. Isoflavones in flour were hydrolyzed to the corresponding aglycones during alkaline extraction. While hydrolyzable derivatives of biochanin A represent 90 % w/w total isoflavones in flour (9 µg/g), biochanin A aglycone was the major isoflavone in concentrates (31 µg/g). Minor components were formononetin, genistein, and the flavonol kaempferol. Thus, chickpea protein concentrates represent an even better source of health-promoting isoflavones than the original chickpea.

Highlights

- Chickpea protein concentrates are potential sources of healthy isoflavones.
- Protein concentrates are enriched in isoflavones as compared to the original flour.
- Derivatives of biochanin A represent 90 % w/w isoflavones in flour (9 μg/g).
- Biochanin A aglycone represents 93 % w/w isoflavones in concentrates (31 μg/g).
- Formononetin and genistein were also present at much lower concentration.

Isoflavones in chickpea (*Cicer arietinum*) protein concentrates

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Abstract

Chickpea protein concentrates can be used as food ingredients because of their favorable nutritional and functional properties, and could also supply health promoting isoflavones. The goal of this research was to analyze the isoflavones in chickpea concentrates as compared to the original flour. Protein concentrates were prepared by alkaline extraction and precipitation of protein at the isoelectric pH. HPLC analysis revealed that the concentrates were enriched in isoflavones. Thus, the concentration of total isoflavones was 45 and 10 μg/g in concentrates and flour, respectively. Isoflavones in flour were hydrolyzed to the corresponding aglycones during alkaline extraction. While hydrolyzable derivatives of biochanin A represent 90 % (w/w) total isoflavones in flour (9 μg/g), biochanin A aglycone was the major isoflavone in concentrates (31 μg/g). Minor components were formononetin, genistein, and the flavonol kaempferol. Thus, chickpea protein concentrates represent an even better source of health-promoting isoflavones than the original chickpea.

Keywords

Isoflavone; chickpea; *Cicer arietinum*; biochanin A; protein concentrate; grain legume.

Chemical compounds studied in this article

Biochanin A (PubChem CID: 5280373); Formononetin (PubChem CID: 5280378); Genistein (PubChem CID: 5280961); Kaempferol (PubChem CID: 5280863).

1. Introduction

The isoflavones are a class of flavonoids characteristic of the Leguminosae family of plants, which includes soybean, pea, fava bean, chickpea, lentil, and other less known grain foods and forage plants. The isoflavones in soybean, namely genistein, daidzein, and glycitein (Figure 1), are responsible for health promoting properties. These include a reduced incidence of cardiovascular disease and cancer, and improvement of menopausal symptoms (Mazur, Duke, Wahala, Rasku, & Adlercreutz, 1998; Messina, 1999). The health benefits of these isoflavones have been extensively studied because soy foods are essential in the diet of most Asian countries and are becoming more and more popular in western countries. In addition, soybean flour and protein concentrates are used in many food products as a source of high quality protein with good functional properties and no saturated fat or cholesterol (Horn-Ross et al., 2000; Wolf, 1970).

Chickpea is not only a staple food in many countries, but it is also an inexpensive source of protein with very good functional properties. Thus, our group (Sánchez-Vioque et al., 1999) and others (Ulloa, Valencia, & Garcia, 1988; Withana-Gamage, Wanasundara, Pietrasik, & Shand, 2011) have described the preparation of chickpea protein concentrates and isolates with physicochemical properties as good as those of soybean isolates and superior to those of pea concentrates (Sánchez-Vioque et al., 1999; Withana-Gamage et al., 2011). These concentrates and isolates have potential health-promoting activities as well (Girón-Calle, Alaiz, & Vioque, 2010; Giron-Calle et al., 2004), although the concentration of isoflavones in soybean is higher (Horn-Ross et al., 2000). Total polyphenol contents in chickpea and soybean are very similar.

Thus, total polyphenol concentrations of 0.5 to 6.8 mg/g and 1.5 to 5.6 mg/g have been reported for different varieties of chickpea (Segev et al., 2010) and soybean (Xu, Yuan, & Chang, 2007), respectively. Colored desi chickpea varieties, typical of India, have a higher content in polyphenols and flavonoids than the beige and cream color seeds that are characteristic of the kabuli varieties, more popular elsewhere (Segev et al., 2010). Blanco Lechoso, Castellano, Pedrosillano, and Venoso Andaluz are the four mayor cultivars traditionally grown in Spain (Del Moral de la Vega, Mejias Guisado, & López Morillo, 1996).

Chickpea is characterized by a high content in biochanin A (Mazur et al., 1998), and a lower content in biochanin B, better known as formononetin (Aguilera et al., 2011; Mazur et al., 1998). Biochanin A and B were named after the Hindi word for chickpea, chana. They have the same chemical structure than genistein and daidzein, respectively, except for having a methylated 4´ hydroxyl group (Stevenson & Aslam, 2006) (Figure 1). Health promoting properties have also been reported for these isoflavones (Cassady et al., 1988; Kole, Giri, Manna, Pal, & Ghosh, 2011; Rathel, Leikert, Vollmar, & Dirsch, 2005). Most interestingly, biochanin A and formononetin can be demethylated to genistein and daidzein, respectively, by intestinal microflora and by the human liver (Tolleson, Doerge, Churchwell, Marques, & Roberts, 2002).

The goal of this work was to analyze the isoflavones in chickpea protein concentrates as compared to the original flour. Changes in the isoflavone composition of soybean during processing for preparation of protein isolates and other food products have been described (Shao et al., 2009; Wang & Murphy, 1996) and recently reviewed (Villares, Rostagno, García-Lafuente,

Guillamón, & Martínez, 2011), but this information cannot be directly extrapolated to chickpea because the overall chemical composition of chickpea and its composition in total and individual isoflavones are different than those of soybean.

2. Materials and methods

2.1. Materials

Chickpea (*Cicer arietinum*) of the Kabuli blanco lechoso variety were a gift from Sociedad Cooperativa Andaluza Campo de Tejada (Escacena del Campo, Huelva, Spain). HPLC grade organic solvents were purchased from Merck (Darmstadt, Germany). Daidzein, quercetin, genistein, kaempferol, formononetin, and biochanin A were purchased from Sigma (St. Louis, MO, USA).

2.2. Protein concentrates.

Protein concentrates were prepared as previously described (Giron-Calle et al., 2004; Sánchez-Vioque et al., 1999). Chickpea seeds were ground using a domestic blender, and the resulting flour was extracted at pH 10.5 under vigorous stirring for 50 min at room temperature. Extracts were centrifuged at 500 g for 10 min, and the pellets were reextracted twice more and pooled together (pH 10.5 pellets). Combined supernatants were taken to the isoelectric point, pH 4.3, and centrifuged at 500 g for 10 min. Supernatants were saved (pH 4.3 supernatants), and the resulting precipitates (protein concentrates)

were washed with water. Protein was determined by amino acid analysis as described (Alaiz, Navarro, Girón, & Vioque, 1992; Yust et al., 2004).

2.3. Polyphenol extraction.

Extraction of polyphenols was carried out as described with modifications (Aguilera et al., 2011). Solid samples were suspended 12% (w/v) in 1% HCl (v/v) in methanol/water 80/20 (v/v) and vigorously shaken for 30 min at room temperature. Extracts were recovered by centrifugation at 3000 g for 10 min and the resulting pellets were reextracted twice more using the same procedure. The combined extracts were extracted three times with ethyl ether and three times more using ethyl acetate. The pH 4.3 supernatants were directly extracted using ethyl ether and ethyl acetate. Organic phases were combined, dried using sodium sulfate, taken to dryness under nitrogen, and resuspended in methanol/water 1/1 (v/v).

2.4. Determination of total polyphenols.

Polyphenols were determined according to (Singleton, Orthofer, & Lamuela-Raventos, 1999) using the Folin-ciocalteu reagent and gallic acid standard.

2.5. Hydrolysis.

Isoflavone glycosides were hydrolyzed by treatment with HCl as described (Dinelli et al., 2006). Extracts were dried under nitrogen, redissolved in acetonitrile/water 4/1 (v/v), 0.1 - 0.6 mg extract/ml, and heated at 85^{o}C for 2 h after addition of HCl (250 μl extracts, 50 μl 12 M HCl). The solution was allowed to cool down and aglycones were extracted using ethyl acetate (3x350 μl). The

resulting solutions were taken to dryness under nitrogen and resuspended in ethanol/water 3/1 (v/v).

2.6. HPLC/UV and HPLC/MS analysis.

Quantification of flavonoids was carried out by reverse phase HPLC and UV detection, using a 5 µm 25 x 4.6 mm Ultrasphere ODS column (Beckman-Coulter, Orange County, California) and a UV detector set at 254 nm. Elution was carried out at 1 mL/min using the following gradient of methanol in water adjusted to pH 3.0 using phosphoric acid: 0 to 70 min linear gradient from 0 to 70% methanol, 70 to 75 min 70% methanol, 75 to 80 min linear gradient to 100% methanol, 80 to 85 min 100% methanol. Response factors were calculated from calibration curves for daidzein, quercetin, genistein, kaempferol, formononetin, and biochanin A standards. The response factor for biochanin A was also used for quantification of biochanin A derivatives that were identified by HPLC/MS as follows.

Identification of the components that did not correspond to any of the standards was carried out by HPLC/MS using a reverse-phase 3 µm 20 x 0.46 cm Mediterranea Sea 18 column (Qmx Laboratories, Essex, UK). Elution was carried out at 1 mL/min using a gradient of methanol in water acidified using 1% (v/v) formic acid. The elution gradient was as follows: 0 to 40 min linear gradient from 0 to 70% methanol, 40 to 55 min 70% methanol, 55 to 60 min linear gradient from 70 to 0% methanol. After monitoring in the UV the eluent went on to an electrospray ionization micrOTOF-Q II mass spectrometer (Bruker Daltonics, Bremen, Germany) operated in negative mode. Detection was recorded in the 50-1500 m/z range. Interface parameters were as follows:

capillary voltage 4.5 kV, nebulization pressure 1.2 bar, and ionization gas flow 8 L/min at 200 ^{o}C. Data was acquired in full scan mode and MS^2 spectra were acquired in auto mode (data dependent acquisition). Target Analysis 1.2 and Hystar 3.2 software packages (Bruker Daltonics, Bremen, Germany) were used for data analysis.

3. Results

Chickpea protein concentrates were prepared as previously described (Giron-Calle et al., 2004) based on (Sánchez-Vioque et al., 1999) (Figure 2). Polyphenols were extracted from the protein concentrates, the original flour, and the two byproducts of the procedure: pH10.5 pellets and 4.3 supernatants. The method that was used for extraction allows for recovery of flavonoid aglycones and glycosides as described for chickpea and lentils (Aguilera et al., 2011; Aguilera et al., 2010). Aliquots of the extracts were subjected to hydrolysis in HCl before analysis in order to release aglycones from glycosides. Reverse phase HPLC with UV detection was used for quantification of individual flavonoids as described in materials and methods.

All the flavonoids identified in chickpea flour were isoflavones except for the flavonol kaempferol (Table 1). No additional isoflavones were found in the extracts corresponding to protein concentrates, pH10.5 pellets, and 4.3 supernatants. Samples of flour extracts were also submitted to HPLC/MS/MS, which provided identification of peaks that did not correspond to any of the available standards. These were biochanin A hexoside and two undetermined biochanin A derivatives and were quantified by applying the response factor

corresponding to biochanin A aglycone (Tables 1 and 2). Figure 3 shows that hydrolysis of flour extracts using HCL released biochanin A aglycone from the three biochanin A derivatives. Hydrolysis also released a small amount of formononetin, and even smaller amounts of genistein and kaempferol.

The relative isoflavone composition in both hydrolyzed and nonhydrolyzed protein concentrates (Figure 4, lower panel) was very similar to that of the hydrolyzed flour extracts. The content in biochanin A was not significantly different in the non-hydrolyzed and hydrolyzed concentrate extracts, 5.1 ± 0.4 and 5.5 ± 1.3 μg/g flour, respectively. Nevertheless, it appears that some glycosides of genistein, formononetin and kaempferol remain in the concentrates, and were released by hydrolysis. The pH 4.3 supernatants and pH 10.5 pellets resulting from the preparation of the protein concentrates had similar biochanin A concentrations, between 0.4 and 0.9 μg/g flour for both hydrolyzed and non-hydrolyzed samples. Nevertheless, the concentration of genistein, kaempferol and formononetin in the pH 4.3 supernatants was higher than in the pH 10.5 pellets.

Table 3 facilitates comparison of the total polyphenols and biochanin A contents in flour, protein concentrates, pH 10.5 pellets, and pH 4.3 supernatants. Contents are not only given as concentration in the different fractions (μg/g), but they are also referred to original flour (μg/g flour) in order to facilitate comparison between fractions, and to allow for calculation of recovery (% content in flour). Concentrates had more than twice the content in polyphenols than flour (Table 3, first column). The concentration in pH 4.3 supernatants was also higher than in flour, while the concentration in the pH 10.5 pellets was much lower. Concerning recovery (Table 3, fifth column),

almost half the polyphenol content in flour was recovered in the concentrates, implying that these polyphenols were co-extracted with proteins at pH 10.5, and later co-precipitated with proteins at pH 4.3. Polyphenols remaining in the pH 4.3 supernatant represent a 21 % recovery; these polyphenols may be in solution or bound to albumins, which represent the major protein components in the pH 4.3 supernatant. Only 8 % of the polyphenols in flour were recovered in the pH 10.5 pellet, indicating that most polyphenols were effectively extracted at pH 10.5.

Biochanin A enrichment in concentrates was even higher than the enrichment in total polyphenols. Thus, protein concentrates had almost four times more biochanin A than flour, representing 64 % recovery from flour. On the other hand, the pH 4.3 supernatant, and especially the pH 10.5 pellet, had much lower concentrations of biochanin A, representing recoveries of only 8 and 10 %, respectively.

4. Discussion

It was already reported more than thirty years ago (Dziedzic & Dick, 1982) (Sharma, 1979) that biochanin A and formononetin were the mayor isoflavones in chickpea. Only a few more analyses of the flavonoid composition of chickpeas have followed, and unfortunately description of the varieties that were used in these analyses was frequently absent or rather vague. In addition, most of these analyses were carried out in acid-hydrolyzed samples and glycosides were not determined. Biochanin A at between 8 and 31 μg/g, and

lower amounts of formononetin, genistein and daidzein, were found in several undetermined chickpea varieties from commercial sources in the USA (Horn-Ross et al., 2000; Mazur et al., 1998). Biochanin A at 15 μg/g was also found in a Californian variety of garbanzo beans (Franke, Custer, Cerna, & Narala, 1994). Analysis of an undetermined variety from the USA revealed biochanin A, formononetin, and daidzein at 25, 4 and 6 μg/g, respectively (Nakamura et al., 2001). One more analysis of chickpea bought in Singapore revealed 19 and 1 μg/g biochanin A and formononetin, respectively (Wu et al., 2012).

Much more informative was the analysis of Sinaloa and Castellano varieties carried out by Aguilera and coworkers (Aguilera et al., 2011), showing the presence of biochanin A and formononetin as aglycones, glucosides, and undetermined derivatives. Concentrations of total biochanin A and formononetin in the dry seeds were between 7 and 29 μg/g. Our study represents the first analysis of the flavonoid composition of the blanco lechoso chickpea, and shows that this variety is characterized by very high levels of biochanin A, and lower levels of formononetin, genistein, and the flavonol kaempferol. This isoflavone profile is consistent with previous reports and would be most similar to the Castellano variety as described by Aguilera and coworkers (Aguilera et al., 2011). Despite having an isoflavone content consistent with previous reports, the total polyphenols concentration in Blanco Lechoso chickpeas is rather low. This might be due to the fact that the highest polyphenol concentration in the seeds is found in the skin (Sreerama, Sashikala, & Pratape, 2010), which is rather thin in the Blanco Lechoso variety, best known for its smoothness and creaminess in the mouth. Interestingly, soaking, cooking, and industrial dehydration greatly affects the isoflavone profile, and this effect

depends on the chickpea variety (Aguilera et al., 2011). Thus, cooking and dehydration released high amounts of biochanin A from glycosides in Sinaloa seeds, but completely eliminated this aglycone in the Castellano variety. Wu et al. (2012) also described that biochanin A aglycone was the mayor isoflavone in chickpea seeds, but germination rapidly and dramatically increased the total amount and distribution of isoflavones.

Our results clearly show that preparation of chickpea protein concentrates by alkaline extraction and precipitation at the isoelectric pH releases the isoflavones in chickpea, mostly biochanin A, in its aglycone form. Most importantly, these aglycones remain to a great extent bound to the globulins that form the concentrate (Sánchez-Vioque et al., 1999), so that more than half the biochanin A present in chickpea flour is recovered in the concentrates, resulting in a concentration almost four times higher than in flour. Thus, the enrichment in biochanin A is even higher than the enrichment in protein, which in our hands goes from 24 % (w/w) protein in flour to 75 % (w/w) protein in concentrates. Further washing the protein concentrates with polar solvents leads to protein concentrations up to 95 %, but it also leads to depletion of bioactive isoflavones (Giron-Calle et al., 2004) as was also described for soy isolates (Coward, Smith, Kirk, & Barnes, 1998; Wang & Murphy, 1994). Therefore, protein isolates, defined as protein preparations with a concentration of protein of at least 90 % (w/w) (Wolf, 1970), may be desirable for their higher content in protein, but lose the health-promoting properties of isoflavones.

The fact that the isoflavones in the pH 10.5 pellets and pH 4.3 supernatants were already found as aglycones indicates that hydrolysis occurs

early on in the process, during alkaline extraction. Thus, most of the isoflavone glycosides, which are stored in vacuoles (Dixon & Pasinetti, 2010; Zhao & Dixon, 2010) would be hydrolyzed, and the resulting less hydrophilic aglycones would bind to globulins and carried along into protein concentrates. Therefore, a relatively mild alkaline treatment, i.e. pH 10.5 for 50 min at room temperature, is sufficient to hydrolyze most isoflavone glycosides in chickpea flour, even though harsher treatments are usually followed for quantitative chemical hydrolysis of glycosides. Although pH 10.5 and room temperature are far from the optimum pH and temperature of glycosidases, it cannot be discarded that endogenous glycosidases might have a role on the release of aglycones during protein extraction (Barbosa, Lajolo, & Genovese, 2006).

Soybeans are the archetype of protein-rich seeds that can be used as sources of protein ingredients for the food industry. Chickpeas share with soybeans a high content in protein and the presence of healthy isoflavones, but they have lower oil and isoflavone concentration, and a different isoflavone profile as well. Our results are consistent with reports on the effect of soybean processing for production of protein concentrates as far as pointing out that alkaline extraction cause hydrolysis of isoflavone glycosides. Nevertheless, while chickpea concentrates are enriched in isoflavones and retain half the isoflavones in flour, soybean concentrates suffer losses of isoflavones as compared to flour. Thus, analysis of a number of commercial soy products revealed that protein concentrates and isolates had between 10 times less and the same total isoflavone concentration than soy flour (Eldridge, 1982; Wang & Murphy, 1994). In addition, soy protein concentrates, although enriched in aglycones, still retain isoflavone glycosides so that these glycosides are still

more abundant than aglycones in the isolate (Wang & Murphy, 1994), and some isolates hardly have any aglycone at all (Coward et al., 1998). Nevertheless, total isoflavone concentration in soy flour is much higher than in chickpea, about 2000 μg/g, so that soy concentrates and isolates still have higher isoflavone content. Production of a soy protein isolate in laboratory conditions yielded similar isoflavones concentrations in the final product and in the original flour (1353 vs. 1512 μg/g), representing 26 % of the isoflavones in flour. Again, aglycones were more abundant in the isolate than in the original flour, but glycosides were still more abundant than aglycones in the isolate (Wang, Ma, Pagadala, Sherrard, & Krishnan, 1998). It has been shown that low temperature and softer centrifugation to separate the isoelectric protein precipitate facilitates retantion of isoflavones in soy protein isolates (Barbosa et al., 2006; Lin, Krishnan, & Wang, 2006).

The present study shows that chickpea protein concentrates represent a "hidden source" (Horn-Ross et al., 2000) of health-promoting isoflavones. These concentrates are enriched in isoflavones as compared to chickpea flour, as opposed to soybean protein concentrates that suffer higher losses of isoflavones during their preparation. This might be due to the relatively soft conditions that were used for preparation of the chickpea concentrates, but could also be due to the different nature of chickpea and soybean flours. Thus, soybean flour has a higher concentration in isoflavones, which could lead to a lower yield of co-precipitation/adsorption with the storage proteins that form the concentrates. In addition, while chickpea flour is used as it is, soybean flour is used as defatted meal for preparation of protein concentrates, which could limit co-precipitation of the isoflavones with proteins as well.

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7. Figure captions.

Figure 1. Major flavonoids in soybean and chickpea.

Figure 2. Preparation of chickpea protein concentrates. Protein contents are shown as % (w/w), yield refers to dry matter mass balance % (w/w).

Figure 3. Flavonoids in chickpea flour before and after acid hydrolysis. Samples were analyzed by reverse-phase HPLC with detection at 254 nm. Data represent mean and standard error of the mean (n=3).

Figure 4. Flavonoids in chickpea protein concentrate and byproducts (pH 4.3 supernatant and pH 10.5 pellet) before and after acid hydrolysis. Samples were analyzed by reverse-phase HPLC with detection at 254 nm. Concentration was referred to the amount of flour from which these fractions were prepared in order to facilitate comparison among different fractions in Figures 3 and 4. Data represent mean and standard error of the mean (n=3).

Table 1

Table 1. Flavonoids in chickpea flour (non-hydrolyzed).

Polyphenol	Retention Time (min)	Response Factor ($x10^{-9}$)[a]	Concentration (μg/g flour)[b]
biochanin A hexoside	54.1	136[c]	1.62 ± 0.29
biochanin A derivative	57.4	136[c]	3.31 ± 0.63
genistein	58.8	157	0.01 ± 0.01
biochanin A derivative	59.9	136[c]	5.25 ± 0.46
kaempferol	61.9	339	0.09 ± 0.07
formononetin	64.9	174	0.02 ± 0.01
biochanin A	70.3	136	0.78 ± 0.52

a Slope of the calibration curve using external standards:

Concentration (μg/20 μl) = Response Factor x Area (au).

b Average ± standard error of the mean, n=3.

c Identified by HPLC/MS (see table 2). Same response factor than biochanin A was used for quantification.

Table 2

Table 2. Biochanin A derivatives identified by HPLC/MS/MS.

Polyphenol	molecular formula	[M-H] (m/z)	MS/MS (m/z)
biochanin A hexoside	$C_{22}H_{22}O_{10}$	445.11	283/268
biochanin A derivative	$(C_{16}H_{12}O_5)^a$	(283.06)	268
biochanin A derivative	$(C_{16}H_{12}O_5)^a$	(283.06)	268/239
biochanin A	$C_{16}H_{12}O_5$	283.06	268/239

a largest ion detected.

Table 3

Table 3. Total polyphenol and biochanin A concentration in chickpea flour, protein concentrate, and byproducts (pH 4.3 supernatant and pH 10.5 pellet) resulting from preparation of the concentrates.

	Concentration (μg/g) [a]		Concentration (μg/g flour) [a]		Recovery (% flour)	
	Polyphenols [b]	Biochanin A[c]	Polyphenols [b]	Biochanin A[c]	Polyphenols	Biochanin A[c]
Flour	74.4 ± 8.6	8.6 ± 3.1	74.4 ± 8.6	8.6 ± 3.1	100.0	100.0
Concentrate	182.7 ± 45.2	30.7 ± 7.3	32.7 ± 8.1	5.5 ± 1.3	43.9	64.3
Pellet pH 10.5	9.2 ± 1.8	1.3 ± 0.3	6.2 ± 1.2	0.9 ± 0.2	8.3	10.5
Supernatant pH 4.3	104.8 ± 13.7	4.8 ± 0.7	15.3 ± 2.0	0.7 ± 0.1	20.6	7.8
Unaccounted for					7.2	17.4

[a] Mean ± standard error of the mean, n=3.
[b] Gallic acid equivalents.
[c] Biochanin A in hydrolyzed samples.

Figure 1

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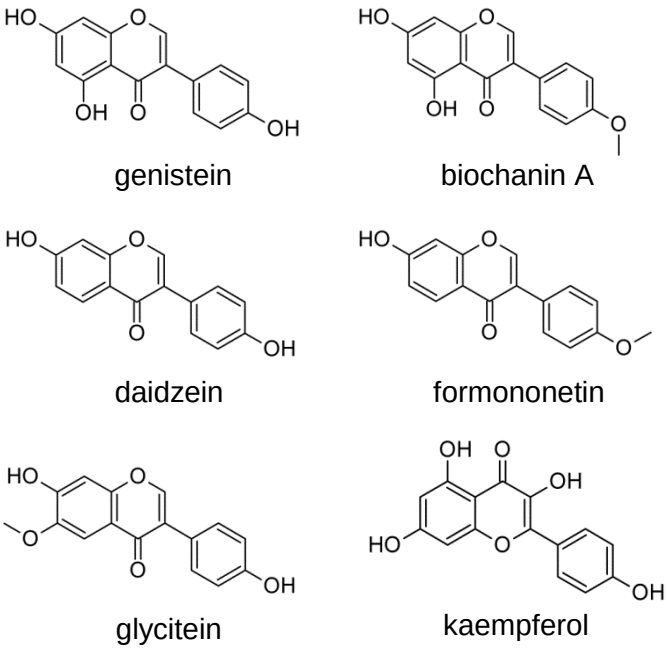

Figure 2

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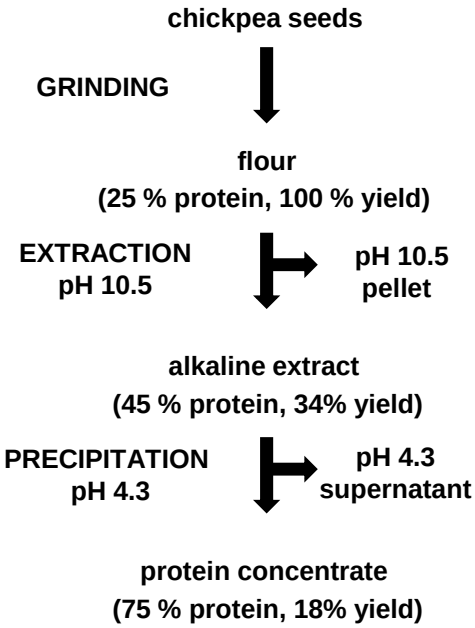

Figure 3

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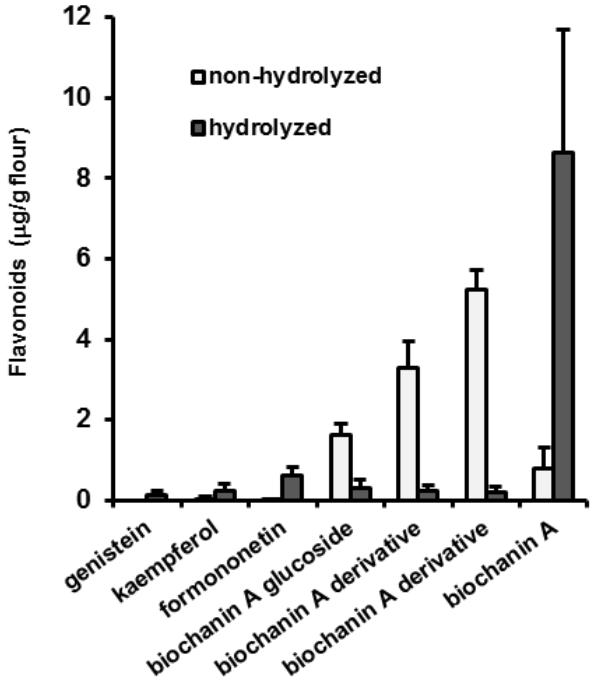

Figure 4

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