ELSEVIER

PII: S0308-8146(98)00037-5

Food Chemistry, Vol. 63, No. 4, pp. 505–512, 1998

Printed in Great Britain
0308-8146/98 $19.00 + 0.00

Effects of extrusion and conventional processing methods on protein and antinutritional factor contents in pea seeds

R. Alonso,* E. Orúe & F. Marzo

Physiology and Animal Nutrition Lab., ETSIA, Universidad Pública de Navarra, Campus Arrosadía s/n, 31006 Pamplona, Spain

(Received 21 November 1997; revised version received 5 January 1998; accepted 5 January 1998)

The effects of high temperature short time (HTST) treatment compared with other conventional processes on protein, phytic acid, condensed tannins, polyphenols, trypsin, chymotrypsin and α-amylase inhibitor activities and haemagglutinating activities in Renata, Solara and Ballet pea seeds were investigated. Ballet cultivar showed highest protein, phytic acid, tannin, polyphenol contents and trypsin and chymotrypsin inhibitory activities. All pea cultivars contained trypsin- and chymotrypsin-inhibiting activity and lectins but only Solara had α-amylase inhibitory activity. Under extrusion conditions (148°C, 25% moisture and 100 rpm) this thermal processing method was the most effective in condensed tannin, trypsin, chymotrypsin, α-amylase inhibitors and haemagglutinating activity reduction, without modifying protein content as occurs by dehulling, soaking and germination treatments. Trypsin and chymotrypsin inhibitors and haemagglutinating activities in peas were more readily abolished by extrusion treatment than was chymotrypsin inhibitory activity.

INTRODUCTION

Pea seeds are important sources of energy and protein, used in many parts of the world, both for animal and human nutrition (Savage and Deo, 1989). Despite the nutritional potential of peas as an economic source of significant amounts of proteins, carbohydrates, vitamins and some minerals including trace minerals, the utilization of this legume has been limited, due to the presence of certain antinutritional factors (Gatel and Grosjean, 1990). Among these are, phytic acid, condensed tannins, polyphenols, protease inhibitors (trypsin and chymotrypsin), α-amylase inhibitors and lectins, which reduce the nutritional quality of the protein.

Attempts to increase the utilization of legumes have employed a wide range of processing techniques such as germination, dehulling, cooking, roasting, autoclaving, fermentation and recently extrusion cooking (Conan and Carré, 1989; Van der Poel, 1990; Almeida *et al.*, 1991; Kim and Barbeau, 1991; Gujska and Khan, 1991; Bishnoi and Khetarpaul, 1994; Frías *et al.*, 1995; Bau *et al.*, 1997; Wang *et al.*, 1997). Extrusion cooking application in legume processing has developed quickly during the last decade, and can now be considered as a technology in its own right. Legume extrusion cooking would allow reduction of antinutritional factor levels and improvement of nutritional quality at a cost lower than other heating systems (such as baking, autoclaving, etc.) due to a more efficient use of energy and better process control, with greater production capacities and absence of process effluents (Reimerdes, 1990). In contrast to traditional legume processing methods that are not automated, with low manufacturing capacities and are highly labour-intensive, extrusion cooking is an extremely versatile process with respect to ingredient selection and shapes and the textures of legume-based foods that can be produced (Cheftel, 1990).

This paper reports the effects which some of these conventional processes have on the protein content, phytic acid, condensed tannins, polyphenols, trypsin, chymotrypsin and α-amylase inhibitors, and haemagglutinating activity of the pea seeds compared with extrusion cooking, in order to develop a simple processing regime that would facilitate utilization as well as improve food value of this legume.

*To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials

Pisum sativum L. seeds (cv. Renata, Solara and Ballet) cultivated in Navarra (Spain), were employed for all determinations. After processing the seeds, 30 kg, for each cultivar, were ground and sieved to 0.5 mm diameter particle size, using a SKI 100 Restch granulator mill and then stored at 4°C until analysis. All chemicals and reagents were purchased from Aldrich Chemical Co Inc. (Milwaukee, WI, USA) and Sigma Chemical Co (St. Louis, MO, USA).

Processing

Dehulling

The seed hulls were mechanically removed in a Culatti mill (DFH 48 LL) at low speed in a few seconds.

Soaking

Pea seeds were soaked in the dark in double-deionized water (1:5 w/v) for 12 h at 30°C in an incubator (Heraeus Vötsch HPS 500). After this, seeds were dried at 50°C in an assisted air circulation oven (Memmert ULM 400).

Germination

Weighed samples of peas were pretreated with 10% mercuric chloride solution to remove surface contamination. Seeds were rinsed three times with autoclaved bideionized water. Grains were transferred to a Petri dish lined with wet filter paper and germinated in an aired and dark incubator (Heraeus Vötsch HPS 500) for 24, 48 or 72 h at 25°C. Sodium azide at a concentration of 0.01% was added to autoclaved bideionized water to prevent microbial growth. During germination, bideionized water (containing sodium azide) was sprinkled on seeds every 12 h. Germinated seeds (the degree of germination was 98% ± 1) were dried in an air oven (Memmert ULM 400) at 50°C overnight.

Extrusion

Pea flour, finely ground (0.5 mm), was processed in a Clextral X-5 model BC 45 twin-screw extruder (F-42100 Firminy, France). The screws were 5.55 cm in diameter and had an overall active length of 50 cm. The extruder was operated at 100 rpm and the feeder was set to deliver 350 g min^{-1}. Water was adjusted in both conditioning cylinder and extrusion barrel using metering pumps with variable settings (calibrated in kg h^{-1}). Moisture content in the extruder barrel was constant at 25%. Extrusion temperature, by iron-constant thermocouple at the outlet die, was 148°C. The extrudates were allowed to cool to room temperature and then were newly ground to pass a 0.5 mm sieve.

Analytical methods

Moisture content

Moisture contents of samples were determined by AOAC method 925.10 (AOAC, 1990).

Crude protein

Total nitrogen of flour was determined according to the micro-Kjeldahl method (AOAC, 1990) using a nitrogen autoanalyzer (Tecator Kjeltec Auto Analyzer Model 1030) and crude protein content was calculated (% $N \times 6.25$).

Phytic acid

Phytic acid was extracted in seed samples with 2.4% HCl (1:20 w/v). The obtained creamy mixture was centrifuged at 17 300 ***g*** (Sorvall® RC-5B) for 30 min at 15°C and the supernatants collected. Glass barrel Econo-columns 0.7×15 cm (BioRad Laboratories), vertically clamped and filled with 0.5 g of AG 1×4 anion exchange resin, were used as the most appropriate for both accuracy and rapidity in phytate purification. To assess total phytic acid content, the simple procedure based on the reaction between ferric chloride and sulfosalicylic acid was followed (Frühbeck *et al.*, 1995).

Condensed tannins

Condensed tannins were extracted with absolute methanol (Salunkhe *et al.*, 1990) for 20 min with mechanical shaking at room temperature and centrifuged at 5000 ***g*** at 15°C for 15 min. Aliquots were immediately analyzed for tannin using the 0.5% vanillin assay (Broadhurst and Jones, 1978).

Polyphenols

The method of the Association of Official Agricultural Chemists with some modifications (Christensen, 1974) was used. Total phenols were extracted in a sample of 1 g flour with 75 ml of water. An internal standard curve was prepared by adding 10 ml of 0–0.01% tannic acid to the flasks, respectively. The contents were heated for 30 min at 70°C with constant shaking. Clear supernatants were collected after centrifuging the contents at 2500 ***g*** for 15 min and filtering them. Polyphenols were determined using the Folin-Denis reagent.

Trypsin inhibitors

Trypsin inhibitor was determined as described by Kakade *et al.* (1974), using α-N-benzoyl-DL-arginine-*p*-nitroanilidehydrochloride (BAPNA) as the substrate for trypsin, with some modifications (Valdebouze *et al.*, 1980). One gram of finely ground sample was extracted with 10 ml of 0.15 M phosphate buffer pH 8.1 at 4°C overnight. Extracts (200 μl) were incubated with 250 μl of trypsin solution (0.004% trypsin in 0.025 M glycine HCl buffer) and diluted to 1 ml with pH 8.1 buffer phosphate. A 2.5 ml of 0.001 M BAPNA solution in pH 8.1 buffer phosphate, previously warmed to 37°C, was

added. Trypsin inhibitor activity (TIA), expressed as trypsin inhibitor units/mg sample, was calculated from the absorbance read at 410 nm against a reagent blank. One trypsin unit was defined as the increase by 0.01 absorbance unit at 410 nm of the reaction mixture.

Chymotrypsin inhibitors

The enzyme inhibitory activity was determined in extracts as described by Decker (1977) and Sathe and Salunkhe (1981). Seed meal is extracted by stirring in Tris HCl buffer, pH 7.6, overnight at 4°C (sample:buffer 1:10 (w:v)). Sample extracts (50 μl) were incubated with 100 μl of chymotrypsin solution (0.005% chymotrypsin in Tris HCl buffer pH 7.6) and diluted to 1 ml with pH 7.8 Tris HCl buffer. 2.5 ml of 0.001 M benzoyl-L-tyrosine ethyl ester (BTEE), previously warmed to 30°C was added and mixed. Changes in absorbance at 256 nm were recorded immediately after substrate addition. One chymotrypsin unit was defined as the increase by 0.01 absorbance unit at 256 nm of the reaction mixture.

α-Amylase inhibitors

α-Amylase inhibitor activity was evaluated according to the method of Deshpande *et al.* (1982). A 1-g sample was extracted with 10 ml of deionized water for 12 h at 4°C and the supernatants were tested for α-amylase inhibitory activity: 0.25 ml of sample solution containing the inhibitor was incubated with 0.25 ml of α-amylase enzyme solution (0.003% in 0.2 M sodium phosphate buffer, pH 7.0, and containing 0.006 M NaCl) for 15 min at 37°C. To this mixture was added 0.5 ml of 1% starch solution (preincubated at 37°C). At the end of 3 min, the reaction was stopped by the addition of 2 ml of dinitrosalicylic acid reagent and heating in a boiling water bath for 10 min. The absorbance was recorded at 540 nm. One unit of enzyme activity was defined as that which liberates, from soluble starch, one micromole of reducing groups (calculated as maltose) per min at 37°C and pH 7.0 under the specified conditions. One unit of α-amylase activity inhibited was defined as one α-amylase inhibitory unit.

Haemagglutinating activity

Haemagglutinating activity was measured by a serial dilution procedure using rabbit blood cells trypsin-treated as described by Grant *et al.* (1983) with some modifications. One gram of pea flour was mixed with 20 ml of 0.04 M sodium borate buffer, pH 8.0 for 16 h at 150 rpm and 4°C in an orbital shaker. After centrifugation (30 000 ***g*** at 4°C for 20 min), the clear supernatant fractions were tested for haemagglutinating activity. The supernatant fractions were serially diluted and mixed with an equal volume of rabbit diluted erythrocytes. Cells in each dilution are resuspended by agitation and the degree of agglutination assessed by microscope. One unit of haemagglutinating activity (HU) was defined as that present in the last dilution giving 50% agglutination of the blood cells.

Statistical analysis

The results were subjected to analysis of variance, and ANOVA test was used to determinate the significant differences among means followed by Fisher's least significant difference (LSD) (Miller and Miller, 1993). The statistically significant difference was defined as $p < 0.05$. To compare percent of antinutritional factor reduction data were angular transformed before ANOVA test application.

RESULTS AND DISCUSSION

Protein content

The results for crude protein content of Renata, Solara and Ballet cultivars as a function of processing are summarized in Table 1. The two-factor (cultivar and treatment) ANOVA for statistical analysis of the protein content are reported in Table 2. Pea protein contents are distributed over a very wide range, 13.3–27.1% protein on a dry, whole seed basis with a mean protein content of 20.7% and a standard deviation of 3.1 (Reichert and McKenzie, 1982). Our data are in line with this result. Comparing cultivars, raw seeds of Ballet had significantly more protein content than those of Renata ($p = 0.0030$) and Solara ($p = 0.0022$). Total protein content increased significantly ($p < 0.0001$) in dehulled peas compared to raw seeds. Proteins are characteristically present in the cotyledon fraction; therefore, the removal of the seed coats causes an increase in the relative protein content. Also a significant increase was observed after 48 h ($p = 0.0023$) and 72 h ($p < 0.0001$) of germination compared to raw seeds. The apparent increase in protein can be attributed to the utilization of carbohydrates as energy sources for the developing sprouts (Dagnia *et al.*, 1992; Donangelo *et al.*, 1995). This agrees well with the pronounced increase of the digestibility of pea starch found by Bishnoi and Khetarpaul (1993) after 48 h germination. An increase in digestibility upon germination is expected because of the pre-digestion of starch by amylolytic enzymes. The decrease in total carbohydrates during germination for energy production may be associated with an increase in crude protein.

Phytic acid

Data on phytic acid contents of raw and processed peas are summarized in Table 3. Phytic acid contents of raw seeds ranged from 1.19 to 1.33 g 100 g^{-1}. Comparing cultivars, Ballet had significantly ($p < 0.0001$) less phytic acid than did any of the others cultivars investigated. Dehulling significantly increased ($p < 0.0001$) the percentage of phytic acid, compared to the content in whole seeds. The mean percent increase in phytic acid content after dehulling was approximately 11%. The

Table 1. Protein content (g 100 g^{-1} DM) in raw, dehulled, soaked, germined and extruded seeds of *Pisum sativum* L.

Treatment	Protein content		
	Renata	Solara	Ballet
Raw seeds	19.0 ± 0.47^{a}	18.8 ± 0.64^{a}	$21.6 \pm 0.06^{a,b}$
Dehulling	20.8 ± 0.17^{b}	$19.1 \pm 0.22^{a,b}$	23.1 ± 0.77^{c}
Soaking	18.9 ± 0.21^{a}	18.9 ± 0.22^{a}	21.1 ± 0.25^{a}
Germination 24 h	19.4 ± 0.10^{a}	$19.5 \pm 0.17^{a,b,c}$	$22.2 \pm 0.04^{b,c}$
Germination 48 h	$19.7 \pm 0.62^{a,c}$	$19.9 \pm 0.14^{b,c}$	$22.5 \pm 0.04^{b,c}$
Germination 72 h	$20.5 \pm 0.53^{b,c}$	20.2 ± 0.27^{c}	22.8 ± 0.14^{c}
Extrusion	19.0 ± 0.15^{a}	$19.0 \pm 0.26^{a,b}$	$21.8 \pm 0.28^{a,b}$

Results are the mean of ten determinations ± SD.
Means within the same column followed by same letter are not statistically significant using one-way ANOVA test ($p < 0.05$).

Table 2. Statistical analysis of protein content of three pea cultivars (*Pisum sativum* L., cv. Renata, Solara and Ballet) determined either in raw or processed seeds[a]

Comparison	DF[b]	$\sum$ squares	MS[c]	*F*	*P*
Single factors					
Cultivar	2	134.5	67.2	144.1	< 0.0001
Processing	6	26.7	4.4	9.5	< 0.0001
Paired interactions:					
Cultivar-processing	12	6.5	0.5	1.2	0.3304
Residual	63	29.4	0.5		

[a]Two-way ANOVA.
[b]DF: degrees of freedom.
[c]MS: mean square.

mean in relative contents of phytic acid after dehulling may be attributed to the fact that this factor is characteristically present in the cotyledons of peas and, the seed coat contributes a substantial portion to the total seed weight. Phytates were significantly ($p < 0.0001$) reduced in all cultivars after soaking or germination. This reduction in phytic acid has been reported by different workers as a result of a increase in phytase activity (Eskin and Wiebe, 1983; Beal and Mehta, 1985; Sripriya *et al.*, 1997; Bau *et al.*, 1997). Twenty four-hours- and 48 h-germinated cv. Solara had significantly more reduction in phytate than cv. Renata ($p < 0.0001$ and $P < 0.0001$, respectively) and Ballet ($p = 0.0008$ and $P = 0.0025$, respectively). However, after 72 h of germination, Solara and Ballet showed a similar rate of phytate reduction ($p = 0.1673$) and Renata had less reduction in phytic acid than Solara ($p < 0.0001$) or Ballet ($p < 0.0001$). Thus, varietal characteristics are important factors influencing phytic acid reduction (Tabekhia and Luh, 1980). The phenomenon may be related to less rapid water penetration and slower germination in the Renata cultivar, attributable perhaps to their phytase activity or a seed coat structure, which hinders water penetration. Kader (1995) found that rate of water absorption by faba beans (*Vicia faba* L.) correlated with the size and density of beans, even though these correlations were relatively low. In all cases a gradual reduction was observed between 24 h and 72 h of germination.

Extrusion cooking caused a significant reduction ($p < 0.0001$) in phytic acid content compared to raw seeds. Other analyses performed using the high performance liquid chromatography (HPLC) method (Sandberg *et al.*, 1987; Bullock *et al.*, 1993) showed that, during extrusion cooking or other thermal treatments, such as autoclaving, part of the inositol hexaphosphate was hydrolyzed to penta- and tetraphosphates.

Condensed tannins and polyphenols

Raw seeds of cultivar Ballet had significantly ($p < 0.0001$) more condensed tannins than Renata and Solara. No differences ($p = 0.2617$) were observed between Renata and Solara cultivars. Dehulling and germination caused similar effects on tannin reduction. Extrusion cooking became the more effective treatment in the reduction (83.6%) of tannin content. This drastic reduction ($p < 0.0001$) might be due to destruction of condensed tannins at high temperature. Similar effects were found with other thermal treatments (Rani and Hira, 1993). Thereby the molecular structure is changed and therefore, it can no longer be detected by the method which was used. Thermal treatments such as steaming, reconstitution or extrusion, imply a qualitative change in the chemical structure of tannins.

Table 3. Phytic acid (g 100 g^{-1} DM), condensed tannin (mg eq cat 100 g^{-1} DM) and polyphenol (mg 100 g^{-1} DM) contents in raw, dehulled, soaked, germinated and extruded seeds of *Pisum sativum* L.

Treatment	Phytic acid			Tannins			Polyphenols		
	Renata	Solara	Ballet	Renata	Solara	Ballet	Renata	Solara	Ballet
Raw seeds	1.31 ± 0.01^{a}	1.33 ± 0.01^{a}	1.19 ± 0.01^{a}	13.5 ± 0.62^{a}	14.5 ± 0.65^{a}	23.8 ± 0.67^{a}	37 ± 4^{a}	39 ± 5^{a}	50 ± 9^{a}
Dehulling	1.49 ± 0.02^{b}	1.50 ± 0.01^{b}	1.27 ± 0.01^{b}	$12.0 \pm 0.51^{b,c}$	11.5 ± 0.50^{b}	$17.0 \pm 1.14^{b,c}$	34 ± 2^{a}	$29 \pm 3^{a,b}$	39 ± 3^{a}
Soaking	1.25 ± 0.01^{c}	1.18 ± 0.01^{c}	1.12 ± 0.01^{c}	$13.0 \pm 0.35^{a,b}$	11.1 ± 0.31^{b}	17.4 ± 0.41^{c}	33 ± 1^{a}	34 ± 3^{a}	39 ± 4^{a}
Germination 24 h	$1.23 \pm 0.01^{c,d}$	0.97 ± 0.01^{d}	1.01 ± 0.03^{d}	$12.7 \pm 0.41^{a,b,c}$	11.0 ± 0.33^{b}	$16.9 \pm 1.24^{b,c}$	$29 \pm 3^{a,b}$	$29 \pm 4^{a,b}$	19 ± 2^{b}
Germination 48 h	1.19 ± 0.02^{d}	0.88 ± 0.02^{e}	0.87 ± 0.01^{e}	11.7 ± 0.46^{c}	10.9 ± 0.31^{b}	$15.6 \pm 0.29^{b,c}$	$23 \pm 2^{b,c}$	$19 \pm 2^{b,c}$	18 ± 1^{b}
Germination 72 h	1.05 ± 0.01^{e}	0.76 ± 0.01^{f}	0.66 ± 0.01^{f}	11.7 ± 0.49^{c}	10.8 ± 0.43^{b}	15.2 ± 0.61^{b}	19 ± 3^{c}	12 ± 2^{c}	13 ± 1^{b}
Extrusion	$1.21 \pm 0.03^{c,d}$	1.15 ± 0.01^{g}	1.12 ± 0.02^{c}	2.23 ± 0.07^{d}	2.54 ± 0.25^{c}	2.34 ± 0.18^{d}	$25 \pm 1^{b,c}$	23 ± 2^{b}	23 ± 3^{b}

Results are the mean of ten determinations ± SD.
Means within the same column followed by same letter are not statistically significant using one-way ANOVA test ($p < 0.05$).

Heat-modified tannins could have a lower extractability due to a certain degree of polymerization (Van der Poel *et al.*, 1991). In addition, they can be destroyed or the chemical reactivity can be changed after heat-processing (Barroga *et al.*, 1985).

No differences ($p = 0.4012$) were observed in polyphenol contents between the three cultivars studied. When the results obtained in dehulled seeds are compared with the germinated ones, it would seem that most of the polyphenols in Renata, Solara and Ballet are not located in the seed-coat; hence dehulling of the seed had significantly less effect on their reduction that 24 h ($p = 0.0024$), 48 h ($p < 0.0001$) or 72 h germination ($p < 0.0001$). Seventy-two hour germination was the most effective treatment in the polyphenol reduction. Different authors (Rao and Deosthale, 1982; Savelkoul *et al.*, 1992) have suggested that enzymatic degradation was responsible for the loss of polyphenols during germination.

Trypsin, chymotrypsin and α-amylase inhibitors

The mean trypsin inhibitor activity (TIA) in raw peas was found to be significantly higher in Ballet than in Renata ($p < 0.0001$) and Solara ($p < 0.0001$). The content of this protease inhibitor was higher in cv. Renata than in cv. Solara ($p = 0.0015$). The TIA in this study falls within the range of the published results (Valdebouze *et al.*, 1980; Borowska and Kozlowska, 1981; Bertrand *et al.*, 1982; Bishnoi and Khetarpaul, 1994). Only in Solara and Ballet did soaking significantly reduce ($p < 0.0001$) this antinutritional factor. Gatfield (1980) investigated the effect of soaking upon the trypsin inhibitor activity of legumes. It was found that legumes lost a certain amount of inhibitor activity as a result of leaching during soaking. A decrease in TIA of germinated field peas has been reported. The percent of decrease in TIA due to the germination process was significantly different in the three cultivars studied at 24 h ($p = 0.0408$), 48 h ($p < 0.0001$) and 72 h ($p = 0.0090$). There was about 58% reduction in TIA after 72 h germination in Renata, 70% in Solara and 76% in Ballet. But extrusion was the most effective ($p < 0.0001$) method for reducing TIA when compared with the other treatments. About 95% reduction was caused by extrusion processing. As Adams (1991) suggests, reactions involving deamidation splitting of covalent bonds, such as hydrolysis of peptide bonds at aspartic acid residues, and interchange or destruction of disulfide bonds, might be involved in the thermal inactivation.

Among the cultivars of peas studied, Ballet had the highest ($p < 0.0001$) chymotrypsin inhibitory activity (CIA). No differences ($p = 0.2084$) were found between Renata and Solara cultivars. These data agree with those found by other researchers (Griffiths, 1984; Vinh and Dworschák, 1986). Dehulling, soaking and germination caused similar effects in TIA and CIA. The results of Griffiths (1984) indicate that trypsin inhibitor content, was independent of protein content. However, in the case of our cultivars, a good statistically significant correlation was found between TIA and protein content ($r = 0.710$; $p < 0.0001$) and between CIA and protein content ($r = 0.846$; $p < 0.0001$). Also, a strong correlation ($r = 0.871$; $p < 0.0001$) was found between TIA and CIA values. This suggests the possible presence of a double-headed trypsin-chymotrypsin inhibitor belonging to the Bowman-Birk family characterized in other pea cultivars (Domoney *et al.*, 1993; Ferrasson *et al.*, 1995, 1997).

From Table 4 it can be seen, too, that in extruded seeds the activity of trypsin inhibitors is much smaller than that of chymotrypsin inhibitors. Similar data were also reported in the literature (Vinh and Dworschák, 1986), where the results had been explained in a way that shows that the Kunitz's inhibitor (KI), which affects only trypsin, is much more sensitive to heat, than the Bowman-Birk's inhibitor (BBI) which influences the activities of both trypsin and chymotrypsin. As Rouhana *et al.* (1996), suggest the more compact and rigid structure of BBI and the many internal disulfide links may explain its greater thermal stability.

Only cv. Solara showed α-amylase inhibitory activity (AIA). This agrees with the results obtained by Jaffé *et al.* (1973) in *Pisum sativum* where, from the seven varieties studied, only five had antiamylasic activity (14–80 IU g^{-1}). Removal of the seed coat of the legume caused an increase of AIA in the dry seeds. This increase, like trypsin and chymotrypsin, might be attributed to the concentration of this inhibitor in the cotyledons fractions more than in the seed coats. Soaking reduced the activity of the inhibitor in cv. Solara. This may be due to the leaching in steeping water. Also, germination reduces, as far as 48% (72 h), the antiamylasic activity. Extrusion became the most effective treatment. Amylase inhibitor of the Solara cultivar became completely inactive when seeds were extruded in the aforementioned conditions. As report Shekib *et al.* (1988), the reduction in activity varied enormously in legume seeds on exposure to a thermal treatment.

Haemagglutining activity

Haemagglutinating activities are given in Table 4. According to Lis and Sharon (1986) erythroagglutination by lectin is affected by the molecular properties of lectin, cell surface properties, metabolic state of cells and conditions of assay, such as temperature, cell concentration and mixing. Comparison of processing inactivation of lectin activity, among studies reported, was further complicated by the different seed cultivars, processing practices and lectin assay methods. The determination of haemagglutinating activity is not very precise, but this measurement is a safe method for checking the efficiency of treatments in toxic seeds (Grant *et al.*, 1982). Although there was no detectable

Table 4. Trypsin (IU mg^{-1} DM), chymotrypsin (IU mg^{-1} DM), α-amylase (IU g^{-1} DM) inhibitor contents and haemagglutinating activity (HU/ mg DM) in raw, dehulled, soaked, germinated and extruded seeds of *Pisum sativum* L.

Treatment	Trypsin inhibitors			Chymotrypsin inhibitors			α-amylase inhibitors			Haemag. activity		
	Renata	Solara	Ballet	Renata	Solara	Ballet	Renata	Solara	Ballet	Renata	Solara	Ballet
Raw seeds	$3.80 \pm 0.24^{a,b}$	2.80 ± 0.09^{a}	6.32 ± 0.23^{a}	2.88 ± 0.05^{a}	2.73 ± 0.10^{a}	4.85 ± 0.09^{a}	—	16.8 ± 1.55^{a}	—	5.1 ± 0.0^{a}	6.2 ± 0.0^{a}	6.0 ± 0.0^{a}
Dehulling	4.02 ± 0.08^{a}	2.82 ± 0.06^{a}	6.47 ± 0.08^{a}	2.92 ± 0.03^{a}	2.76 ± 0.05^{a}	4.91 ± 0.12^{a}	—	20.3 ± 0.39^{b}	—	5.1 ± 0.0^{a}	6.2 ± 0.0^{a}	6.0 ± 0.0^{a}
Soaking	$3.74 \pm 0.09^{a,b}$	2.52 ± 0.06^{b}	5.56 ± 0.19^{b}	2.49 ± 0.06^{b}	2.25 ± 0.07^{b}	4.20 ± 0.08^{b}	—	12.2 ± 0.84^{c}	—	5.1 ± 0.0^{a}	6.2 ± 0.0^{a}	6.0 ± 0.0^{a}
Germination 24 h	$3.57 \pm 0.11^{b,c}$	1.88 ± 0.06^{c}	4.64 ± 0.14^{c}	2.38 ± 0.07^{b}	2.20 ± 0.08^{b}	4.09 ± 0.05^{b}	—	11.1 ± 0.14^{c}	—	5.1 ± 0.0^{a}	6.2 ± 0.0^{a}	6.0 ± 0.0^{a}
Germination 48 h	3.32 ± 0.08^{c}	1.18 ± 0.02^{d}	2.81 ± 0.06^{d}	2.01 ± 0.06^{c}	2.12 ± 0.02^{b}	3.27 ± 0.19^{c}	—	$10.2 \pm 0.27^{c,d}$	—	5.1 ± 0.0^{a}	6.2 ± 0.0^{a}	6.0 ± 0.0^{a}
Germination 72 h	2.76 ± 0.11^{d}	0.70 ± 0.03^{e}	1.55 ± 0.09^{e}	1.80 ± 0.05^{d}	1.87 ± 0.05^{c}	2.28 ± 0.08^{d}	—	8.79 ± 0.25^{d}	—	5.1 ± 0.0^{a}	6.2 ± 0.0^{a}	6.0 ± 0.0^{a}
Extrusion	0.19 ± 0.02^{e}	0.16 ± 0.06^{f}	0.34 ± 0.02^{f}	1.05 ± 0.02^{e}	0.96 ± 0.04^{d}	1.68 ± 0.07^{e}	—	0.00 ± 0.00^{e}	—	0.1 ± 0.0^{b}	0.0 ± 0.0^{b}	0.1 ± 0.0^{b}

Results are the mean of ten determinations ± SD.
Means within the same column followed by same letter are not statistically significant using one-way ANOVA test ($p < 0.05$).

Table 5. Statistical analysis of ANFs of three pea cultivars (*Pisum sativum* L., cv. Renata, Solara and Ballet) determined either in raw or processed seeds[a]

Comparison	DF[b]	$\sum$ squares	MS[c]	*F*	*P* <
Single factors					
Cultivar	2	329.6	164.8	347.7	0.0001
ANFs	6	14631.1	2438.5	5144.8	0.0001
Processing	6	1856.6	309.4	652.9	0.0001
Paired interactions:					
Cultivar-ANFs	12	3300.2	275.0	580.2	0.0001
Cultivar-Processing	12	137.2	11.4	24.1	0.0001
ANFs-Processing	36	2236.4	62.1	131.1	0.0001
Multiple interactions:					
cv-ANFs-Processing	72	949.4	13.2	27.8	0.0001
Residual	931	441.3	0.5		

[a]Three-way ANOVA.
[b]DF: degrees of freedom.
[c]MS: mean square.

change in the haemagglutinating activity of dehulled, presoaked and germinated seeds, this activity was clearly sensitive to heat-treatment. In this respect, only extruded seeds showed significant differences (Table 5) in relation to raw pea seeds. Thus, when Renata, Solara and Ballet seeds were extruded, the activity was reduced 98% in Renata and Ballet, and completely eliminated in Solara.

The results clearly show the importance of these investigations for the improvement of nutritional quality of pea seeds and process design in order to minimize undesirable influences of heat treatment or to avoid them totally. Additional studies must be done to evaluate the nutritional quality of pea seeds and to evaluate how different processing techniques affect the nutritive utilization of pea protein and the antinutritional factors that influence their utilization.

ACKNOWLEDGEMENT

This work was supported by grant PB 94-1047 from the Spanish DGICYT and Government of Navarra.

REFERENCES

Adams, J. B. (1991) Review: Enzyme inactivation during heat processing of food-stuffs. *International Journal of Food Science* **26**, 1–20.

Almeida, N. G., Calderón de la Barca, A. M. and Mauro, E. V. (1991) Effect of different heat treatments on the nutritional activity of *Phaseolus vulgaris* (variety Ojo de Cabra) lectin. *Journal of Agricultural and Food Chemistry* **39**, 1627–1630.

AOAC. (1990) *Official Methods of Analysis*, Association of Official Analytical Chemists, Washington.

Barroga, C. F., Laurena, A. C. and Mendoza, M. T. (1985) Polyphenols in mung bean (*Vigna radiata* L., Wilczek): determination and removal. *Journal of Agricultural and Food Chemistry* **33**, 1006–1009.

Bau, H.-M., Villaume, C., Nicolas, J.-P. and Méjean, L. (1997) Effect of germination on chemical composition, biochemical constituents and antinutritional factors of soya bean (*Glycine max*) seeds. *Journal of the Science of Food and Agriculture* **73**, 1–9.

Beal, L. and Mehta, T. (1985) Zinc and phytate distribution in peas. Influence of heat treatment, germination, pH, substrate, and phosphorus on pea phytate and phytase. *Journal of Food Science*, **50**, 96–100, 115.

Bertrand, D., Delort-Laval, J., Melcion, J. P. and Valdebouze, P. (1982) Influence de l'extrusion et de l'infranisation sur les facteurs antinutritionnels et de la valeur alimentaire du pois (*Pisum sativum* L.). *Sciences de Aliments* **2**, 197–202.

Bishnoi, S. and Khetarpaul, N. (1993) Effect of domestic processing and cooking methods on *in-vitro* starch digestibility of different pea cultivars (*Pisum sativum*). *Food Chemistry* **47**, 177–182.

Bishnoi, S. and Khetarpaul, N. (1994) Saponin content and trypsin inhibitor of pea cultivars: effect of domestic processing and cooking methods. *Journal of Food Science and Technology* **31**, 73–76.

Borowska, J. and Kozlowska, H. (1981) Changes in activity of trypsin inhibitors in bean (*Phaseolus vulgaris*) and pea seeds (*Pisum sativum*) during heat treatment. *Acta Alimentaria Polonica* **VII(XXXI)**, 181–188.

Broadhurst, R. B. and Jones, W. J. (1978) Analysis of condensed tannins using acidified vanillin. *Journal of the Science of Food and Agriculture* **29**, 788–792.

Bullock, J. I., Duffin, P. A. and Nolan, K. B. (1993) *In vitro* hydrolysis of phytate at 95°C and the influence of metal ion on the rate. *Journal of the Science of Food and Agriculture* **63**, 261–263.

Cheftel, J. C. (1990) Extrusion cooking: operating principles, research trends and food applications. In *Processing and Quality of Foods. Vol. 1 High Temperature/Short Time (HTST) Processing: Guarantee for High Quality Food with Long Shelflife*, eds P. Zeuthen, J. C. Cheftel, C. Eriksson, T. R. Gormley, P. Linko and K. Paulus, pp. 1.60–1.73. Elsevier Applied Science, London.

Christensen, K. D. (1974) *Improving the Plant Proteins by Nuclear Techniques*. International Atomic Energy Agency, Vienna.

Conan, L. and Carré, B. (1989) Effect of autoclaving on metabolizable energy value of smooth pea seed (*Pisum sativum*) in growing chicks. *Animal Feed Science and Technology* **26**, 337–345.

Dagnia, S. G., Petterson, D. S., Bell, R. R. and Flanagan, F. V. (1992) Germination alters the chemical composition and protein quality of lupin seeds. *Journal of the Science of Food and Agriculture* **60**, 419–423.

Decker, L. A. (1977) *Worthington Enzyme Manual*. Worthington Biochemical Corp., Freehold, NJ.

Deshpande, S. S., Sathe, S. K., Salunkhe, D. K. and Cornforth, D. P. (1982) Effects of dehulling on phytic acid, polyphenols, and enzyme inhibitors of dry beans (*Phaseolus vulgaris* L.). *Journal of Food Science* **47**, 1846–1850.

Domoney, C., Welham, T. and Sidebottom, C. (1993) Purification and characterization of *Pisum* seed trypsin inhibitors. *Journal of Experimental Botany* **261**, 701–709.

Donangelo, C. M., Trugo, L. C., Trugo, N. M. F. and Eggum, B. O. (1995) Effect of germination of legume seeds on chemical composition and on protein and energy utilization in rats. *Food Chemistry* **53**, 23–27.

Eskin, N. A. M. and Wiebe, S. (1983) Changes in phytase activity and phytate during germination of two fababean cultivars. *Journal of Food Science* **48**, 270–271.

Ferrasson, E., Quillien, L. and Gueguen, J. (1995) Amino acid sequence of a Bowman-Birk proteinase inhibitor from pea seeds. *Journal of Protein Chemistry* **14**, 467–475.

Ferrasson, E., Quillien, L. and Gueguen, J. (1997) Proteinase inhibitors from pea seeds: purification and characterization. *Journal of Agricultural and Food Chemistry* **45**, 127–131.

Frías, J., Díaz-Pollan, C., Hedley, C. L. and Vidal-Valverde, C. (1995) Evolution of trypsin inhibitor activity during germination of lentils. *Journal of Agricultural and Food Chemistry* **43**, 2231–2234.

Frühbeck, G., Alonso, R, Marzo, F. and Santidrián, S. (1995) A modified method for indirect quantitative analysis of phytate in foodstuffs. *Analytical Biochemistry*, **72**, 248–254.

Gatel, F. and Grosjean, F. (1990) Composition and nutritive value of peas for pigs: a review of European results. *Livestock Production Science* **26**, 155–175.

Gatfield, I. L. (1980) Loss of dry matter during the industrial preparation of legumes: leaching of trypsin inhibitors from beans during the soaking step. *Lebensmittel-Wissenschaft und-Technologie* **13**, 46–48.

Grant, G., More, L. J., McKenzie, N. H. and Pusztai, A. (1982) The effect of heating on the haemagglutinationg activity and nutritional properties of bean (*Phaseolus vulgaris*) seeds. *Journal of the Science of Food and Agriculture* **33**, 1324–1326.

Grant, G., More, L. J., McKenzie, N. H., Stewart, J. C. and Pusztai, A. (1983) A survey of the nutritional and haemagglutination properties of legume seeds generally available in the UK. *The British Journal of Nutrition* **50**, 207–214.

Griffiths, D. W. (1984) The trypsin and chymotrypsin inhibitor activities of various pea (*Pisum* spp.) and field bean (*Vicia faba*) cultivars. *Journal of the Science of Food and Agriculture* **35**, 481–486.

Gujska, E. and Khan, K. (1991) Feed moisture effects on functional properties, trypsin inhibitor and hemagglutinating activities of extruded bean high starch fractions. *Journal of Food Science* **56**, 443–447.

Jaffé, W. G., Moreno, R. and Wallis, V. (1973) Amylase inhibitors in legume seeds. *Nutrition Reports International* **7**, 169–174.

Kader, Z. M. A. (1995) Study of some factors affecting water absorption by faba beans during soaking. *Food Chemistry* **53**, 235–238.

Kakade, M. L., Rackis, J. J., McGhee, J. E. and Puski, G. (1974) Determination of trypsin inhibitor activity of soy bean products: a collaborative analysis of an improved procedure. *Cereal Chemistry* **51**, 376–382.

Kim, Y. A. and Barbeau, W. E. (1991) Changes in the nutritive value of soy protein concentrate during autoclaving. *Plant Foods for Human Nutrition* **41**, 179–192.

Lis, H. and Sharon, N. (1986) Biological properties of lectins. In *The Lectins: Properties, Functions and Applications in Biology and Medicine*, eds I. E. Liener, N. Sharon and I. J. Goldstein, pp. 265. Academic Press, Orlando.

Miller, J. C. and Miller, J. N. (1993) *Statistics of Analytical Chemistry*. Ellis Horwood Prentice Hall, Analytical Series, London.

Rani, N. and Hira, C. K. (1993) Effect of various treatments on nutritional quality of faba beans (*Vicia faba*). *Journal of Food Science and Technology* **30**, 413–416.

Rao, P. U. and Deosthale, Y. G. (1982) Tannin content of pulses: varietal differences and effects of germination and cooking. *Journal of the Science of Food and Agriculture* **33**, 1013–1016.

Reichert, R. D. and MacKenzie, S. L. (1982) Composition of peas (*Pisum sativum*) varying widely in protein content. *Journal of Agricultural and Food Chemistry* **30**, 312–317.

Reimerdes, E. H. (1990) New impacts for food science and food industry—view from outside. In *Processing and Quality of Foods. Vol. 1 High Temperature/Short Time (HTST) Processing: Guarantee for High Quality Food with Long Shelflife*, eds P. Zeuthen, J. C. Cheftel, C. Eriksson, T. R. Gormley, P. Linko and K. Paulus, pp. 1.4–1.11. Elsevier Applied Science, London.

Rouhana, A., Adler-Nissen, J., Cogan, U. and Frøkiær, H. (1996) Heat inactivation kinetics of trypsin inhibitors during high temperature-short time processing of soymilk. *Journal of Food Science* **61**, 265–269.

Salunkhe, D. K., Chavan, J. K. and Kadam, S. S. (1990) Methods of tannin analysis. In *Dietary Tannins: Consequences and Remedies*. CRC Press Inc., Boca Raton, FL.

Sandberg, A.-S., Andersson, H., Carlsson, N.-G. and Sandström, B. (1987) Degradation products of bran phytate formed during digestion in the human small intestine: effect of extrusion cooking on digestibility. *The Journal of Nutrition* **117**, 2061–2065.

Sathe, S. K. and Salunkhe, D. K. (1981) Studies on trypsin and chymotrypsin inhibitory activities, hemagglutinating activity, and sugars in the Great Northern beans (*Phaseolus vulgaris* L.). *Journal of Food Science* **46**, 626–629.

Savage, G. P. and Deo, S. (1989) The nutritional value of peas (*Pisum sativum*): A literature review. *Nutrition Abstracts and Reviews, (Series A)* **59**, 66–83.

Savelkoul, F. H. M. G. and Van der Poel, A. F. B. (1992) The presence and inactivation of trypsin inhibitors, tannins, lectins and amylase inhibitors in legume seeds during germination. A review. *Plant Foods for Human Nutrition* **42**, 71–85.

Shekib, L. A., El-Iraqui, S. M. and Abo-Bakr, T. M. (1988) Studies on amylase inhibitors in some Egyptian legume seeds. *Plant Foods for Human Nutrition* **38**, 325–332.

Sripriya, G., Antony, U. and Chandra, T. S. (1997) Changes in carbohydrate, free amino acids, organic acids, phytate and HCl extractability of minerals during germination and fermentation of finger millet (*Eleusine coracana*). *Food Chemistry* **58**, 345–350.

Tabekhia, M. M. and Luh, B. S. (1980) Effect of germination, cooking, and canning on phosphorus and phytate retention in dry beans. *Journal of Food Science* **45**, 406–408.

Valdebouze, P., Bergeron, E., Gaborit, T. and Delort-Laval, J. (1980) Content and distribution of trypsin inhibitors and haemaglutinins in some legume seeds. *Canadian Journal of Plant Science* **60**, 695–701.

Van der Poel, A. F. B. (1990) Effect of processing on antinutritional factors and protein nutritional value of dry beans (*Phaseolus vulgaris* L.). A review. *Animal Feed Science and Technology* **29**, 179–208.

Van der Poel, A. F. B., Gravendeel, S. and Boel, H. (1991) Effect of different processing methods on tannin content and *in vitro* protein digestibility of faba bean (*Vicia faba* L.). *Animal Feed Science and Technology* **33**, 49–58.

Vihn, L. T. and Dworschák, D. (1986) Trypsin and chymotrypsin inhibitor activities in plant foods from Vietnam and Hungary. *Nahrung* **30**, 53–58.

Wang, N., Lewis, M. J., Brennan, J. G. and Westby, A. (1997) Effect of processing methods on nutrients and antinutritional factors in cowpea. *Food Chemistry* **58**, 59–68.