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Polyphenol release during domestic processing and simulated digestion of beans (*Phaseolus vulgaris* and *Vicia faba*) and their anti-inflammatory effects in RAW264.7 macrophages.

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The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

Publications

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Abstract

Phaseolus vulgaris and *Vicia faba* beans contain polyphenols that are associated with chronic disease prevention; yet, their benefits may be limited by their bioaccessibility. Bean polyphenol bioaccessibility is unknown and information about their biological effects is scarce. Therefore, to assess these parameters, the polyphenols released after and optimized *in vitro* digestion method were determined and the anti-inflammatory effect of three *P. vulgaris* Mexican beans (Negro Jamapa, Marcela and Azufrado) was tested in RAW 264.7 macrophages. Enzymatic digestion extracted around twenty times more polyphenols compared to domestic processing and acid hydrolysis. Starch digestion released four times more polyphenols compared to protein digestion. The main polyphenols released during digestion were conjugates of catechin, sinapic acid, and *p*-coumaric acid. Domestic processing mainly released flavonoids which are likely to come from the bean hull. All the beans presented a slightly variable polyphenolic profile. Sinapic acid and Negro Jamapa bean exhibited an anti-inflammatory effect by reducing inflammatory related target genes (IL6 and TNFα for sinapic acid and IL6, iNOS and IL1β for Negro Jamapa bean). However, Marcela and Azufrado beans showed a pro-inflammatory effect.

Bean polyphenols bioaccessibility appears to be higher than previously reported and appears to be limited by interactions with starch. This is the first time that starch-polyophenol interaction has been observed in beans. More studies are needed to understand the function of this interaction in plants. This study showed that polyphenol composition in beans is variable, which reflects in the different outcomes obtained during the cell culture experiments. Hence, different beans might exert diverse positive or negative health benefits. More studies are required

to know the health properties of different beans in order to know which are more recommended to consume.

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Abbreviations

AMG…….. amyloglucosidase

DMEM……. Dulbecco's Modified Eagle Medium

DMSO……. Dimethyl sulfoxide

DNS…….. 3,5-Dinitrosalicylic acid

FRAP ……Ferric Reducing Antioxidant Power

FW…….. fresh weight

GAE ……..gallic acid equivalents

GSH ……. ……glutathione

Hba1……. ……..hemoglobin, alpha 1

HDL ……high density lipoprotein

HO1……Heme oxygenase 1

HPAEC-DAD……. high-performance anion-exchange chromatography coupled with a diode array detector

HPLC……. high performance liquid chormatography

IL1β…… interleukin 1 β

IL6…… interleukin 6

iNOS ……inducible nitric oxide synthase

LCMS ……liquid chromatography–mass spectrometry

LDL ……..low density lipoprotein

LPS…….. lipopolysaccharide

masl……..…… meters above sea level

MS ……mass spectrometry

NADPH ….....nicotinamide adenine dinucleotide phosphate

NF-κB ……nuclear factor kappa B

Nrf2……..……. nuclear factor erythroid 2-related factor 2

ROS …….……reactive oxygen species

TEAC…… Trolox equivalent antioxidant capacity

TNFα ……..tumor necrosis factor α

TPC……..…… total polyphenolic content

Chapter 1

1.1. Origin of common and fava beans

Phaseolus vulgaris was domesticated over 6,000 years ago in Mexico and Central America and is cultivated in Latin America, Africa, Europe, China, the Middle East, and North America (Salinas et al., 1988). Common beans have been cultivated in Mexico since pre-hispanic times; *P. vulgaris* is believed to have been domesticated in Mexico and Central America over 6,000 years ago (Souza et al., 1994). This legume originates from wild *P. vulgaris*, an indeterminate plant, distributed from Mexico to Argentina in midaltitude, neotropical and subtropical regions (Gepts and Debouck, 1991). *P. vulgaris* is normally grown in areas with altitudes that range from 800 to 2200 masl and can develop in diverse environments; for instance, in Mexico, this legume can be cultivated in semiarid regions such as the ones found in the state of Durango (<350 mm precipitation per annum), as well as subtropical regions such as the ones found in the state of Chiapas (>2000 mm precipitation per annum) (Toro-Chica et al., 1990). The fact that *P. vulgaris* can grow in different environments suggests that this plant has advantageous adaptation traits and a rich genetic diversity (Acosta-Gallegos et al., 2007).

In 2012, the total bean production in the world was estimated at 2,3598,102 tonnes (FAO, 2012). The top ten bean producing countries in the world are shown in table 1.1. According to the Secretariat of Agriculture, Livestock, Rural Development, Fisheries and Food of Mexico (SAGARPA, 2009) the main producing states in Mexico are: Zacatecas (25.4%), Sinaloa (15.6%), Durango (13.3%), Chihuahua (11.3%), Nayarit (7.3%), and Chiapas (6.7%).

Table 1.1. Top ten bean (*P. vulgaris*) producing countries

Country	Tonnes per annum
1. Myanmar	3,900,000
2. India	3,630,000
3. Brazil	2,794,854
4. China, mainland	1,450,000
5. United States of America	1,448,090
6. United Republic of Tanzania	1,199,267
7. Mexico	1,080,857
8. Kenya	613,902
9. Ethiopia	463,009
10. Rwanda	432,857

Source: (FAO, 2012)

Not much of the background of *Vicia faba* (or fava bean) is known and its origin is still debated. It is believed that it originated in the Near East (Afghanistan to India) and that it has been cultivated since Neolithic times; a wild progenitor of the fava bean has not been found (Tanno and Willcox, 2006). Most of the consumption of this legume is for animal feed rather than for humans.

1.2. Consumption of beans in the world and in Mexico

The world consumption of beans is approximately 17 millions of tons, of which 57.7% are consumed in five countries: India (24.7%), Brazil (19.0%), United States (5.5%), Mexico (5.%) and Tanzania (3.3%) (FAO, 2012).

Common beans are an important part of the Latin American diet in and their consumption comprises 87% of the total pulses consumed in these countries. Beans are essential in these countries because they are part of the traditional Latin American cuisine, the income level of a large population and for their nutritional content (beans are considered the “poor man’s meat”) (Leterme and Muũoz, 2002). In Mexico, beans represent up to 15% of the total diet (Osorio-Díaz *et al.*, 2003).

1.3. Nutritional and health benefits of beans

Beans are an important legume and nutritional source in many countries of the world (Porch *et al.*, 2013). The common bean is a basic and traditional food in Latin America, especially in Mexico, Central America, the Andean Zone, Brazil and the Caribbean (Romero-Arenas *et al*., 2013, Du *et al*., 2014). Several human studies have shown the beneficial effect of beans on human health (table 1.2). Beans have gained attention due to their high nutrient content (fibre, protein, carbohydrates and minerals) and their consumption has been associated with prevention of the chronic diseases development (Dinelli *et al.*, 2006).

Table 1.3 shows the proximate composition of common beans. The main macronutrients present in common beans are protein (20.15 ± 1.28 g/100 g) and carbohydrates (60.53 ± 3.50 g/100g). Fava beans contain 51%-68% of carbohydrates, 20%-41% of protein, 15%-30% of dietary fibre and only 1.2%-4% of lipids (Turco et al.,2016).

Several studies that evaluate the nutritional aspects of beans focus on their protein content and the presence of anti-nutrients in these legumes. Recently, the consumption of beans has been generally associated with health benefits because they are an important part of vegetarian and Mediterranean-

style diets; the possible health benefits of bean consumption may be due to their low saturated fat and their high fibre content, essential nutrients and phytochemicals (Messina, 2014).

Evidence suggests that the bean consumption reduces serum cholesterol and stimulates weight loss. Anderson et al. (1984) showed that total serum cholesterol was reduced by 19% in 10 hypercholesterolemic male subjects that consumed 115 g/day (dry weight) of cooked navy or pinto beans for 21 days. In the same study, it was observed that the subjects subjected to a bean diet lost 0.3 kg per week. A similar study performed by the same group of researchers (Anderson et al., 1990) observed a 10.4% reduction of serum cholesterol in 24 hypercholesterolemic male subjects after consuming 120-160 g/day of canned beans for 21 days; in addition, the subjects lost 1.0-1.5 kg during the study. Winham et al. (2007) observed that daily consumption of 85 g of pinto beans for 8 weeks significantly lowered serum total cholesterol by 8.4% in 16 insulin resistant adult subjects. Winham and Hutchins (2007) observed a reduction of total cholesterol of 5.6% in 23 hypercholesterolemic adult subjects that consumed ½ cup of baked beans for 8 weeks.

Most of the putative prevention of chronic diseases exerted by bean consumption has been attributed to dietary fibre; however, the protective effect might be associated with the polyphenols present in this legume (Oomah et al., 2006). A diet containing polyphenols is recommended for the prevention of diseases since they have been shown to scavenge reactive oxygen species (ROS) and therefore protect cellular components from any damage produced by oxidative stress (Jonfia-Essien et al., 2008, Bellion et al., 2008)

Table 1.2. Human studies focused on different health conditions in which bean consumption is involved, characteristics of each study and final outcome.

Study	Condition evaluated	No. of subjects	Age of subjects	Condition of subjects	Gender of subjects	Type of bean	Amount of beans	Duration of study	Outcome
Mollard et al., 2012	Metabolic syndrome.	40	33-55	Overweight and obese	Mixed	Navy beans (together with other pulses).	Five cups per week	8 weeks	Consuming pulses *ad libitum* (yellow peas, chickpeas, lentils and navy beans) reduced energy intakes, waist circumference, systolic blood pressure, HbA1c, improved blood glucose control, decreased insulin resistance, decreased postprandial C-peptide, increased fasting plasma leptin and increased HDL.
Finley et al., 2007	Lipid profiles in blood of healthy humans.	80	18-55	40 with premetabolic syndrome and 40 healthy	Mixed	Pinto bean	130 g per day (as an entrée)	12 weeks	Beans lowered serum cholesterol, (HLD cholesterol and LDL cholesterol).
Bornet et al., 1987	Type 2-diabetes	18	Not specified.	Type 2 diabetic	Mixed	Type of beans not specified (together with bread, potato spaghetti, rice and lentils).	Not specified (diets comprised a total of 50g of carbohy-drate).	3 days	A diet that includes beans showed an effect the insulinemic and glycaemic indexes of diabetic subjects.

Thompson et al., 2012	Type 2-diabetes	17	35-70	Type 2 diabetic	Mixed	Pinto, black and red kidney (all eaten together with rice)	Not specified (diets comprised a total of 50g of carbohy-drate).	150 minutes	Pinto and black beans reduces the glycaemic response in comparison to rice alone.
Winham and Hutchins, 2007	Hyperchole sterolemia	17	22- 70	Hypercholest-erolemic subjects.	Mixed	Baked beans (navy beans).	½ cup per day.	8 weeks	Consumption of baked bean can reduce serum Total cholesterol in hypercholesterolemic adults.

Table 1.3. Proximate analysis of different varieties of common beans (*P. vulgaris*).

Study	Parameter						
	Bean varieties	**Moisture (g/100g)**	**Ash (g/100g)**	**Crude fat (g/100g)**	**Crude protein (g/100g)**	**Carbohydrate (g/100g)**	**Crude fibre (g/100 g)**
Fan and Beta et al. (2016)							
	Dimeta	9.66 ± 0.03	3.57 ± 0.03	2.99 ± 0.16	19.43 ± 0.58	64.35 ± 0.36	n. d.
	Napitita	9.00 ± 0.09	3.61 ± 0.01	1.80 ± 0.04	21.32 ± 1.50	64.28 ± 1.61	n. d.
	Nanyati	8.58 ± 0.11	3.77 ± 0.00	1.70 ± 0.12	18.82 ± 0.73	67.14 ± 0.73	n. d.
Shimelis and Rakshit (2005)							
	Roba	11.01 ± 0.3	3.93 ± 0.06	2.44 ± 0.01	20.55 ± 0.21	56.53 ± 0.19	5.53 ±0.05
	Gobirasha	9.48 ± 0.05	3.84 ± 0.00	2.82 ± 0.01	17.96 ± 0.39	60.69 ± 0.47	5.22 ± 0.26
	Beshbesh	10.00 ± 0.02	3.12 ± 0.05	3.02 ± 0.07	20.44 ± 0.05	58.23 ± 0.21	5.18 ± 0.12
	Gofta	9.68 ± 0.03	3.36 ± 0.01	2.80 ±0.01	20.04 ± 0.04	59.46 ± 0.04	4.66 ± 0.06
	Awash	10.51 ± 0.13	4.26 ± 0.16	1.44 ± 0.04	21.95 ± 0.05	57.14 ± 0.05	4.70 ± 0.01
	Mexican	9.48 ± 0.01	2.86 ± 0.01	2.71 ± 0.03	22.07 ± 0.02	56.97 ± 0.19	5.95 ± 0.20
	Redwolaita	9.51 ± 0.05	3.27 ± 0.01	2.67 ± 0.01	19.48 ± 0.14	59.46 ±0.09	5.61 ± 0.01
	Tabor	9.08 ± 0.09	3.52 ± 0.03	1.27 ± 0.01	19.63 ± 0.01	61.56 ± 0.15	4.94 ± 0.03
	Average	**9.63 ± 0.68**	**3.56 ± 0.40**	**2.33 ± 0.65**	**20.15 ± 1.28**	**60.53 ± 3.50**	**5.22 ± 0.45**

Several studies regarding the polyphenolic content and antioxidant activities of several Mexican beans have been published (Mejıa et al., 1999, Diaz-Batalla et al., 2006, Guevara-Lara et al., 2006, Espinosa-Alonso et al., 2006, Rocha-Guzman et al., 2013). Most of these studies have been performed with beans originating from Central Mexico. However, information on the phenolic content and antioxidant potential of beans harvested in Western Mexico is scarce, even though some of the main producing states are in this area. In the state of Nayarit, one of the main producers of Western Mexico, bean is the main crop that is cultivated. Due to the importance of beans in Nayarit and the scarcity of information regarding their polyphenolic content, a study is necessary to generate information about their antioxidant activity and their potential benefits that their consumption might have on human health. Information about bioaccessibility and biological effect of bean polyphenols is also scarce.

1.4 Polyphenols

Polyphenols are a common group of plant metabolites found in all vegetative organs, flowers and fruits; thousands of molecules with a polyphenolic structure (possessing one or more hydroxyl groups on aromatic rings) have been identified in edible plants (Manach et al., 2004). They are secondary metabolites that help plants protect themselves against predators and abiotic stress signals such as temperature (heat and cold), salinity, drought, flood, radiation, chemical stress (mineral salts, toxins, pollutants and heavy metals) and mechanical stress (wind and soil movement) (Ferrazzano et al., 2011). Polyphenols have benzene rings that contain hydroxyl groups; these compounds are derived from the metabolism of shikimic acid (Figure 1.1) (Munin and Edwards-Levy, 2011, Heleno et al., 2015). Polyphenols are highly

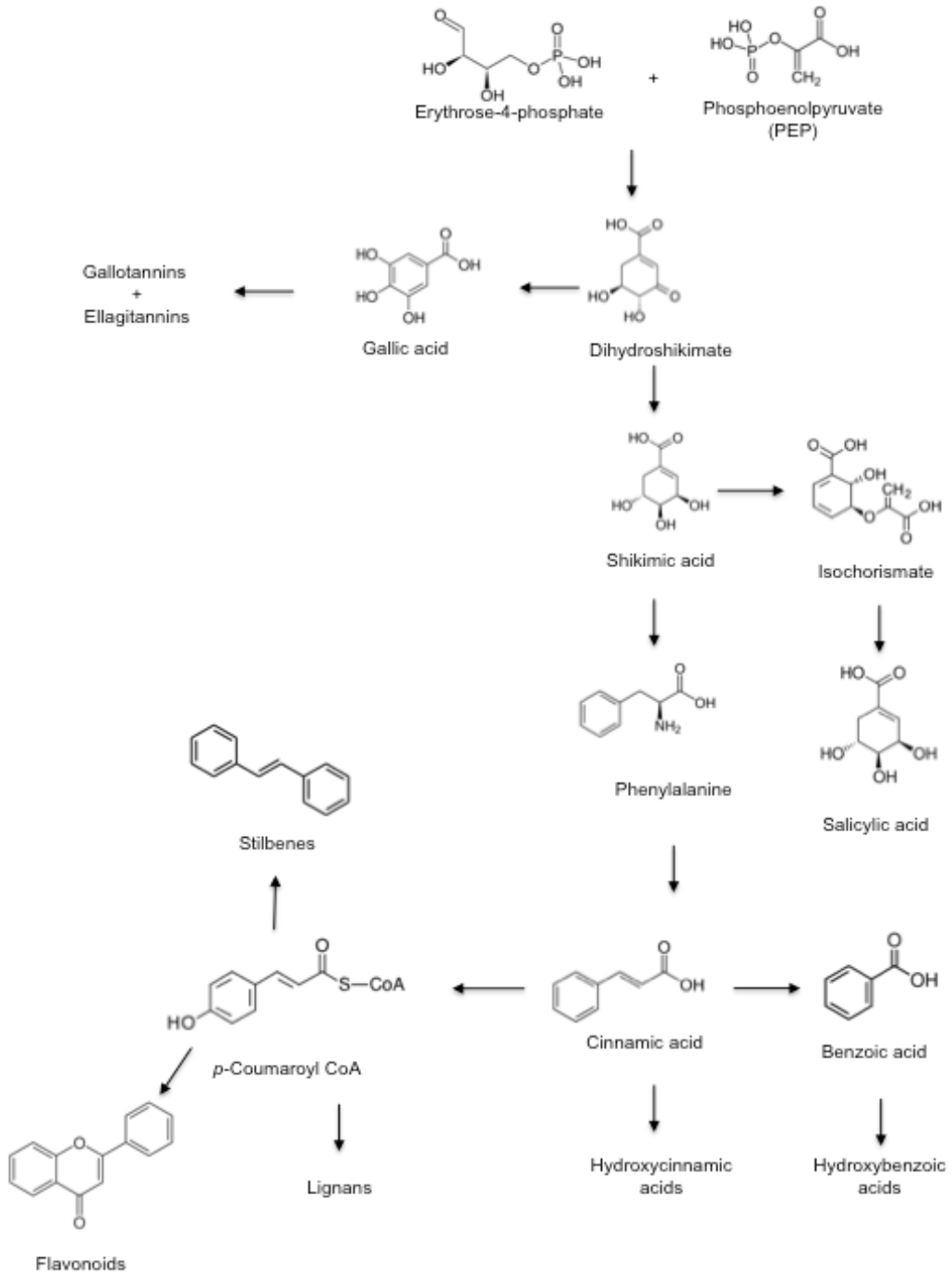
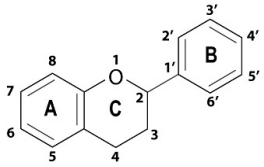


Figure 1.1. Summary of the biosynthesis of different types of polyphenols by the shikimic acid pathway (Heleno et al.,2015)

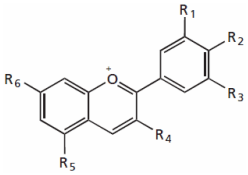
Table 1.3 The different categories of polyphenols, examples and their source (Rao et al., 2012).

Category	Subclass	Example	Source
Flavonoids	Anthocyanins	Cyanidin	Berries, purple cabbage, beets, grape seed extract and red wine.
	Flavanols	Catechin	White, green and black teas.
		Theflavins	Black teas
		Proanthocyanidins	Chocolate and fruits.
		Hesperidin	Citrus fruits.
	Flavanones	Naringenin	Citrus fruits.
		Silybin	Blessed milk thistle.
	Flavonols	Quercetin	Red and yellow onions, tea, wine, apples, cranberries, buckwheat, beans.
	Flavones	Apigenin	Chamomile, celery and parsley.
		Tangertin	Tangenine and other citrus peels.
		Luteolin	Celery and thyme.
	Isoflavones	Genistein	Soy, alfalfa, sprouts, red clover, chickpeas, peanuts and other legumes.
Stilbenes		Resveratrol	Grapes and red wine.
Lignans		Secoisolaiciresinol	Flaxseeds.
Phenolic acids	Hydroxycinnamic acids	Caffeic acid	Common beans and coffee beans.
	Hydroxybenzoic acids	Gallic acid	Gallnuts, sumac, witch hazel, tea leaves and oak bark.

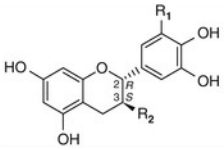
Flavonoids



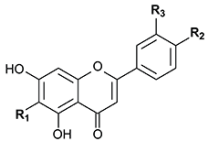
General structure of flavanoids



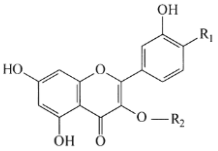
Anthocyanidins



Flavan-3-diols



Flavanones



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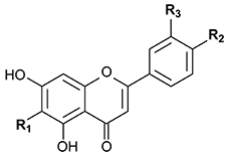
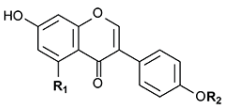
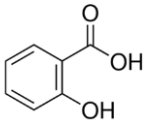


Flavones

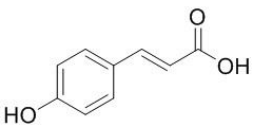


Isoflavones

Phenolic acids

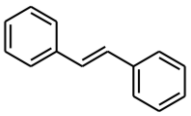


Hydroxybenzoic acids



Hydroxycinnamic acids

Stilbenes



Lignans

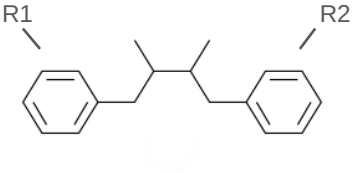


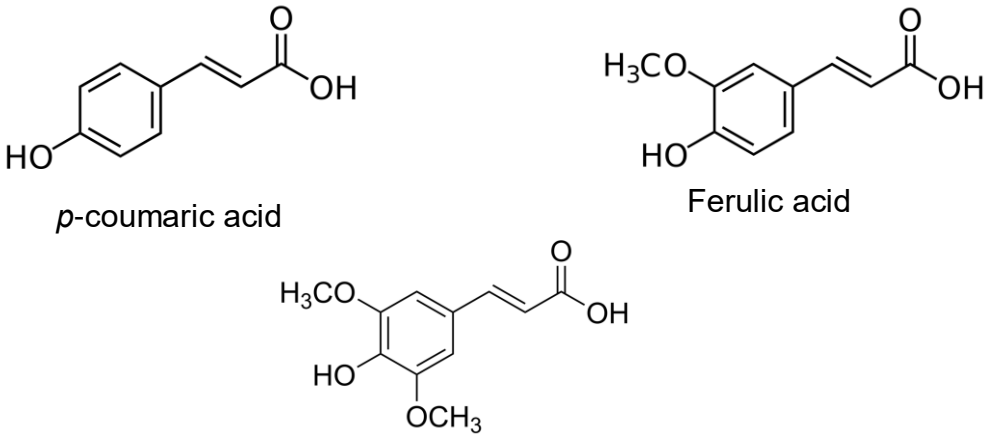
Figure 1.2. Chemical structures of different categories of polyphenols (Rao et al., 2012).

diverse and comprise several sub-groups of phenolic compounds (Tsao, 2010). Different types of polyphenols, structure and sources are listed in table 1.4 and figure 1.2.

1.4.2. Polyphenols in beans

P. vulgaris contains phenolic acids and flavonoids (Korus et al., 2007). Although some publications have reported anti-nutritional aspects of seed polyphenols, other studies have shown positive effects of these molecules on human health (anti-carcinogenic and antioxidant properties *In vitro* and *In vivo*) (Hangen and Bennink, 2002, Anton et al., 2008). Polyphenols in grains are found mainly associated with fibre or the cell wall and can pass through the stomach and small intestine to the colon, being released later by bacterial fermentation (Faller et al., 2012). In general, the most important phenolic compounds found in beans are hydroxycinnamic acids (*p*-coumaric, ferulic, cinnamic, and sinapic), flavonoids (catechin, epicatechin, quercetin, and kaemferol) and condensed tannins (procyanidins), and anthocyanins (delphinidins, cyanidins, and malvidins) (Luthria and Pastor-Corrales, 2006, Xu and Chang, 2008). Some examples are shown in figure 1.2. Studies that have identified and quantified bean polyphenols are shown in table 1.4 (Luthria and Pastor-Corrales, 2006, Drumm et al., 1990, Madhujith et al., 2004).

According to the phenol explorer database (phenol-explorer.eu) the composition of polyphenols varies not only between common and fava beans, but also between cultivars of the same bean species. White common beans (*P. vulgaris*) contain hydroxycinnamic acids (5-O-caffeoylquinic, ferulic and sinapic acid), flavanols (catechin, epicatechin and procyanidins) and flavonols (keampferol) and isoflavones. Black beans (*P. vulgaris*) contain hydroxycinnamic acids (5-O-caffeoylquinic, ferulic and sinapic acid),



p-coumaric acid

Ferulic acid

Sinapic acid

Figure 1.3. The main hydroxycinnamic acids present in beans: a) *p*-coumaric acid; b) ferulic acid; c) sinapic acid.

Table 1.4. Phenolic acids identified and quantified in different cultivars of *P. vulgaris* beans.

Author	Bean	Vanillic acid (mg/100g)	*p*-coumaric acid (mg/100g)	Ferulic acid (mg/100g)	Sinapic acid (mg/100g)	Cinnamic acid (mg/100g)	Caffeic acid (mg/100g)
Luthria and Pastor Corrales, 2007	Pinto Bean (cv. Maverick)	ND	4.5 ± 0.3	22.9 ± 2.1	8.5 ± 0.4	ND	ND
	Pinto bean (cv. Buster)	ND	4.5 ± 0.1	16.0 ± 0.3	9.0 ± 0.3	ND	ND
	Pinto bean (cv. Othello)	ND	5.6 ± 0.3	15.2 ± 1.2	5.9 ± 0.2	ND	ND
	Great (cv. Norstar)	ND	4.0 ± 0.4	17.0 ± 2.3	9.4 ± 2.1	ND	ND
	Northern (cv. Matterhorn)	ND	6.3 ± 9	17.2 ± 0.6	9.0 ± 1.4	ND	ND
	Navy (cv. Vista)	ND	12.4 ± 2.5	26.6 ± 2.3	9.2 ± 1.7	ND	ND
	Black bean (cv. T-39)	ND	11.6 ± 0.6	25.5 ± 1.6	9.0 ± 0.7	ND	1.1 ± 0.1
	Black bean (cv. Jaguar)	ND	7.0 ±1.4	11.7 ± 2.5	5.7 ± 0.7	ND	ND
	Black bean (cv. Eclipse)	ND	9.8 ± 0.4	24.7 ± 0.3	6.8 ± 0.2	ND	1.1 ± 0.1
	Dark red kidney (cv. Red Hawk)	ND	1.8 ± 0.1	15.3 ± 1.1	3.8 ± 0.3	ND	ND

Author	Bean	Vanillic acid (mg/100g)	*p*-coumaric acid (mg/100g)	Ferulic acid (mg/100g)	Sinapic acid (mg/100g)	Cinnamic acid (mg/100g)	Caffeic acid (mg/100g)
	Dark red kidney (cv. Red Hawk)	ND	1.8 ± 0.1	15.3 ± 1.1	3.8 ± 0.3	ND	ND
	Light red kidney (cv. Cal Early)	ND	7.0 ± 0.2	14.8 ± 0.5	5.7 ± 0.2	ND	ND
	Red Mexican (cv. UI 239)	ND	5.8 ± 0.1	17.4 ± 0.6	5.4 ± 0.2	ND	ND
	Cranberry (cv. Taylor Cranberry)	ND	1.7 ± 0.1	14 ± 1.1	3.5 ± 0.3	ND	ND
	Pink (cv. UI 537)	ND	6.8 ± 0.7	19.4 ± 1.7	8.2 ± 0.5	ND	ND
	Alubia (CV. Beluga)	ND	5.3 ± 0.1	10.6 ± 0.7	4.0 ± 0.3	ND	ND
Drumm et. al., 1990	Navy	ND	1.91	1.18	0.94	2.90	ND
	Dark red kidney	ND	1.46	0.72	0.64	4.56	ND
	Pinto	ND	1.17	0.46	1.79	3.25	ND
	Black turtle soup	ND	0.93	Traces	1.42	4.11	ND
	Navy	ND	2.24	3.67	1.18	4.91	ND
	Dark red kidney	ND	2.10	6.84	2.00	2.44	ND
	Pinto	ND	2.34	7.18	3.05	3.97	ND

Author	Bean	Vanillic acid (mg/100g)	*p*-coumaric acid (mg/100g)	Ferulic acid (mg/100g)	Sinapic acid (mg/100g)	Cinnamic acid (mg/100g)	Caffeic acid (mg/100g)
Drumm et. al., 1990	Black turtle soup	ND	2.06	6.12	3.07	3.34	ND
	Navy	ND	1.04	Traces	1.05	4.54	ND
	Dark red kidney	ND	0.77	0,29	1.28	1.24	ND
	Pinto	ND	0.41	Traces	1.45	2.73	ND
Madhujith et. al., 2004	Red	ND	12.06 ± .11	1.40 ± 0.04	2.83 ± 0.03	ND	1.55 ± 0.03
	Brown	ND	2.09 ± 0.04	1.20 ± 0.01	0.87 ± 0.03	ND	ND
	Black	2.54 ± 0.02	0.96 ± 0.04	1.46 ± 0.02	2.21 ± 0.03	ND	ND
	White	ND	1.52 ± 0.04	12.17 ± 0.09	6.03 ± 0.02	ND	ND

anthocyanins (cyaniding, delphinidin, malvidin, pelargonidin, peonidin and petunidin), flavonols (kaempferol derivatives) and isoflavonoids (daidzein and genistein). Fava beans (*V. faba*) contain hydroxycinnamic acids (ferulic and *p*-coumaric acids) and flavanols. Further details about the polyphenol quantification in beans are shown in Chapter 4 (table 4.10).

1.5 Effect of domestic processing on polyphenolic content of legumes

Industrial and domestic preparation of food of vegetable origin normally leads to the decrease of antioxidants, including polyphenols; this is due to the fact that these compounds are relatively unstable to thermal processing (Nikoli et al., 1999). A decrease in the antioxidant capacity of food could also be due because polyphenols may act as reactants of the Maillard reaction during thermal processing (Djilas and Milic, 1994).

Beans are hard seeds when uncooked and cannot be consumed raw; hence, in order to consume them, a previous cooking method is needed. (Rocha-Guzman et al., 2005). Processing methods used for bean preparation to achieve desirable sensory properties are soaking, boiling, pressure cooking and roasting (Xu and Chang, 2008; Siah et al., 2014).

Rocha-Guzman et al. (2007) studied the effect of pressure cooking (121 °C) on the polyphenolic content of the seed coat and cotyledon of three types of common beans (Pinto Villa, Bayo Victoria and Flor de Mayo). The cooking time for Pinto Villa was 7 minutes, 60 minutes for Bayo Victoria and 5 minutes for Flor de Mayo. Results showed that the polyphenol content in the seed coat decreased around 90% or more upon pressure cooking. The Bayo Victoria bean presented a higher amount of polyphenols in the cooked cotyledon in comparison to the raw cotyledon. This could have been caused to a possible

lixiviation process during cooking. The lixiviation phenomenon increases at higher temperatures and could have stimulated the transportation of polyphenols from the seed coat to the cooking water and cotyledons (Rocha-Guzman et al., 2007). An increase of polyphenol content in the cooked cotyledon has also been observed in cooked legumes (Vidal-Valverde et al., 1994).

Barroga et al. (1985) studied the effect of soaking, cooking and germination on the polyphenol content of 10 mung bean (*Vigna radiata*) cultivars. The soaking method used in the mentioned study consisted of soaking in water for 16 h. The cooking method used in the same study consisted on boiling (100°C) for 30 minutes (with no previous soaking) and frying for 10 minutes. Germination lasted up to 5 days at room temperature. Results showed that soaking reduced the polyphenolic content by 24-50%, cooking by 73%, frying by 17% and germination by 36%.

Satwadhar et al. (1981) studied the effect of cooking on the polyphenol content of moth bean (*Vigna aconitifolia*). In the mentioned study, cooking was performed by boiling (100°C) for 10, 15 and 80 minutes. It was observed that the polyphenol content decreased by 50% after 10 minutes of boiling. No further loss of polyphenols was observed after 15 and 80 minutes of boiling.

Jood et al. (1987) evaluated the polyphenol losses upon soaking, cooking and pressure cooking of chickpeas (*Cicer arietinum*) and black gram beans (*Vigna mungo*). Soaking reduced the polyphenol content by 4-8% in chickpea and by 4-7% in black gram bean. Cooking reduced the amount of polyphenols by 9-12% and 11-15% in chickpea and in black gram bean, respectively. Pressure cooking of these samples lead to a greater decrease in

the polyphenol content of the legumes (21-31% for chickpea and 22-30% for black gram).

Results of the previously discussed studies might indicate that temperature could have a greater impact on polyphenol stability in comparison to cooking time, since pressure cooking (higher temperatures for shorter times) resulted in a greater loss of polyphenols than traditional cooking. Yet, this behaviour was not observed during the canning process of green beans) (Jiratanan and Liu, 2004). In this study, green beans were subjected to 115°C for 10, 20 and 40 minutes. A 40% polyphenol decrease was shown after 10 minutes of thermal processing. No further loss was observed at 20 minutes of processing. Yet, a significant increase in the total polyphenolic content was reported upon 40 minutes of processing (around 5% increase). However, this behaviour did not enhance the antioxidant activity of the green beans, since no significant differences for this parameter was observed at the three times evaluated.

Lopez et al. (2013) studied the effect of cooking on the phenolic composition of dark common beans (*P. vulgaris*). In this study, the main polyphenols identified in raw beans were anthocyanins. Yet, upon cooking, the most abundant polyphenols were hydroxycinnamic acids. Cyanidin and pelargonidin glucoside were the most abundant anthocyanins in both, raw and cooked bean. After boiling, the anthocyanin content decreased by 68% and flavanones decreased by 53%. The most abundant flavanols found were quercetin and kaempferol that decreased by 44% and 5.82% upon cooking, respectively. Of the most abundant hydroxycinnamic acids identified, ferulic acid decreased by 48%, sinapic acid by 56% and *p*-coumaric acid by 60%.

1.6 Bioavailability and degradation of phenolic compounds during digestion

Bioavailability is the magnitude in which a consumed nutrient is able to be used for physiological functions. Factors that affect polyphenol bioavailability are interactions of the compound with the food matrix, the chemical structure and glycolisation of the polyphenol (Terao et al., 2008). In addition, the digestion and absorption of nutrients in the gut is the main rate-limiting factor of bioavailability (Ekmekcioglu, 2002). Since polyphenols are often present in foods as glycosides, esters and polymers, they generally need to undergo enzymatic hydrolysis in the digestive tract or be metabolized by the gut microflora before they can be absorbed. The effects of polyphenols in the body depend on the results that metabolism has on their chemical structure, as well as their absorption (Tarko et al., 2013).

Some studies have found that glycosylated polyphenols may be hydrolysed to their respective aglycones by β-glucosidase and esterases present in saliva during the first stage of digestion (Yang et al., 1999, Walle et al., 2005). During stomach digestion, the polyphenols that are absorbed are aglycones (Crespy et al., 2002).

Glycosylated polyphenols reach the small intestine where they may be hydrolysed to an aglycone by lactase phlorizin hydrolase (LPH) (Day et al., 2000). The resulting aglycones are then absorbed in the erythrocytes present in the microvilli of the small intestine. The remaining glycosylated polyphenols will reach the large intestine where they are metabolized by microbial enzymes of the microflora (Hollman, 2004; Thilakarathna and Rupasinghe, 2013).

The resulting aglycone will be transported across the enterocyte of the small intestine or the colon and subsequently reach the liver. Flavonoids will undergo phase II metabolism in both the enterocyte and the liver. Aglycones will be metabolized by UDP- glucuronosyltransferases (UGTs), sulfotransferases (SULTs) and catechol-O-methyltransferase (COMT) producing glucuronidated, sulfated and methylated metabolites, respectively (Manach et al., 2003, Matsumoto et al. 2004, Mullen et al., 2008, Brett et al., 2009, Brand et al., 2010). These metabolites will then be distributed throughout the plasma, and will be excreted either through the urine or the bile. Significant amounts may be found in biological fluids such as plasma and urine (Matsumoto et al., 2004, Bredsdorff et al., 2010).

The aglycones may be further hydrolysed by the microflora to form phenolic acids that are later absorbed and could eventually conjugate (Mullen et al., 2008). The absorption and metabolism processes are summarised in figure 1.4.

Bacterial hydrolysis by the microflora may decrease or increase the antioxidant activity of food. For example, in a study performed by Schneider et. al. (1999), two phenotypically different types of bacteria were isolated from human faeces. Both were grown on quercetin-3-glucoside, which was used as an energy source. One of the types of bacteria isolated was identified as *Enterococcus casseliflavus*. These bacteria metabolized the sugar of the phenolic glycoside, but did not degrade the aglycone any further. Justesen et al. (2001) demonstrated that a polyphenolic aglycone is better absorbed than the glycoside form; hence the degradation of polyphenols by *Enterococcus casseliflavus* may enhance the biological activity of polyphenols found in food (Justesen and Knuthsen, 2001). Schneider et. al. (1999) also isolated

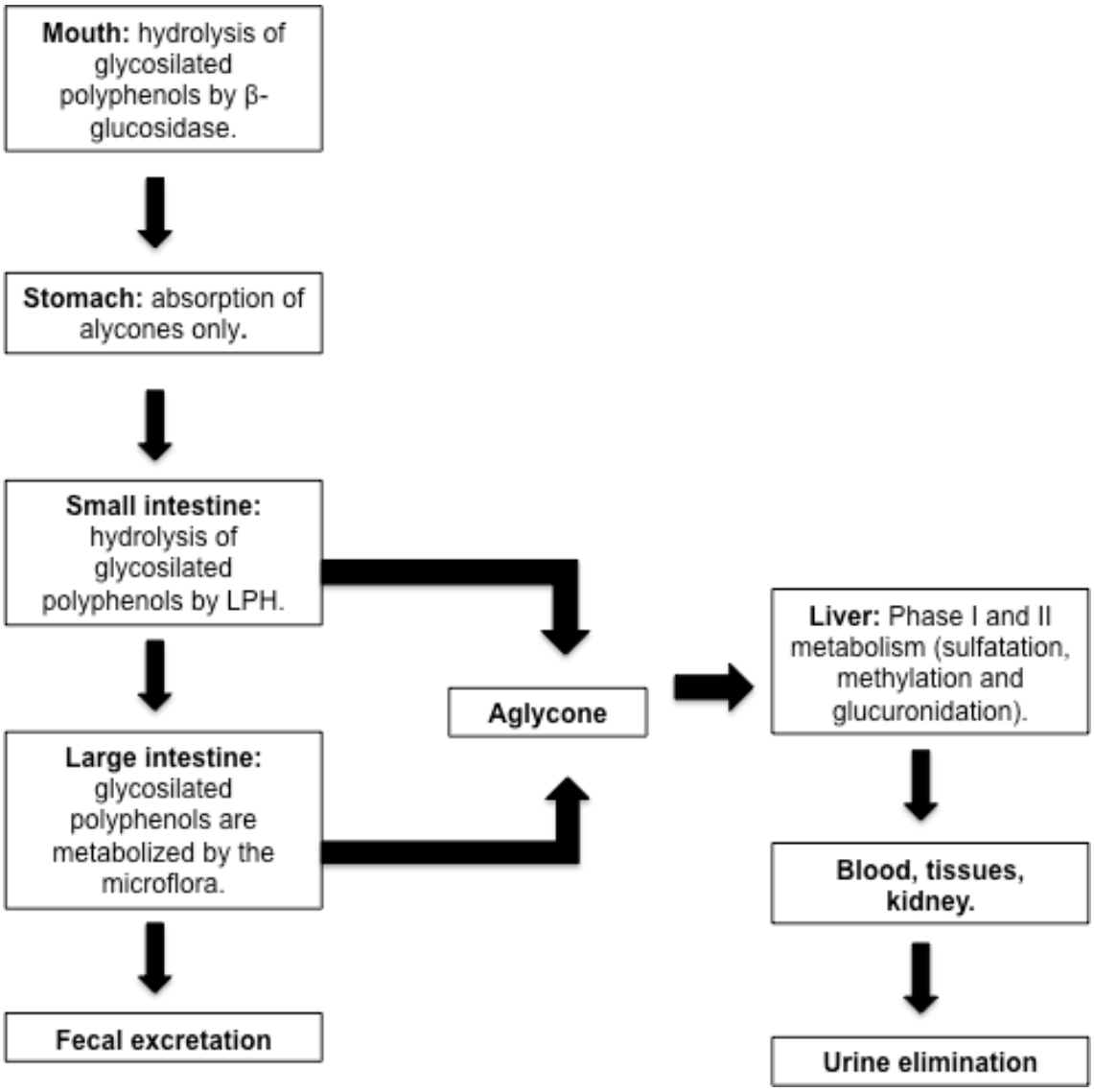


Figure 1.4. Metabolism of polyphenols *in vivo*. Glycosilated polyphenols may be hydrolysed by lactase phlorizin hydrolyse (LPH). The aglycone diffuses into the enterocytes present in the small intestine and are absorbed. The polyphenols that were not absorbed are hydrolysed by the microflora present in the colon and released as aglycones.

Eubacterium ramulus which was found to hydrolyse the phenolic ring of polyphenols. In this case, due to the degradation of the phenolic ring, the antioxidant capacity of polyphenols may be lost.

McDougall *et al.* (2007) suggest that polyphenols bind to food matrices during digestion, which protects them from degradation (McDougall et al., 2007). Many methods, both *in vivo* and *in vitro*, are used to assess the rates and the degrees to which these substances are digested and absorbed. *In vivo* methods are more reliable, although they have various limitations (they are expensive and slow); for this reason, *in vitro* models may be used to simulate the function of human digestion (Tarko et al., 2013).

1.7. Phenolic interactions with food matrix and extraction from plant tissue

Polyphenols may exist in the food matrix in a free, esterified, glycosylated or polymerized form (Robbins and Bean, 2004). In addition, they are not uniformly distributed in plant tissue and may co-exist as complexes with proteins, carbohydrates, lipids or other plant components such as fibre (Jakobek, 2015). For example, phenolic acids may form ether and ester linkages with arabinoxylans in fibre (Akin and Rigsby, 2008). Another factor that makes extraction hard is the significant variation in the polarity of phenolic acids (Luthria and Pastor-Corrales, 2006). Hence, it is very likely that the amount of polyphenols might be underestimated when using polyphenol solvent extraction. When enzymes hydrolyse food macronutrients (such as proteins and carbohydrates), the polyphenols associated to them are liberated. As a result, mayor bean compounds digestion may liberate polyphenols.

Starch degradation can be accomplished by using enzymes such as α-amylase and amyloglucosidase, which hydrolyse α(1,4) and α(1,6)-glycosidic bonds respectively, keeping the configuration of the anomeric carbon of the sugar intact (Piparo *et al.*, 2008). Studies focused on starch digestibility of common beans are shown in table 1.6.

Consumption of dietary antioxidants may enhance cell homeostasis (Song *et al.*, 2010). Thus, attention has been paid to the antioxidant content of foods that are frequently consumed (Lopez-Alarcon and Denicola, 2013).

1.7.1. Effect of interaction with lipids

It has been suggested that polyphenols interact with lipids and have a potential positive role on the fat absorption process (Shishikura et al., 2006; Uchiyama et al., 2011). This interaction may also lead to the protection of polyphenols during digestion allowing them to be absorbed further in the gastrointestinal tract. In addition, polyphenols may prevent the formation of lipid peroxidation products that could be potentially harmful (Jakobek, 2015). During mastication, lipids are transformed to emulsions and later travel to the gastrointestinal tract where these emulsions are exposed to components that may further enhance the emulsification process; emulsion properties such as droplet size and surface area have an influence on lipase activity (Shishikura et al., 2006). Since lipase breaks down lipids into smaller molecules (lipolysis) that can later be absorbed, any molecule that could improve the emulsion properties or lipase activity could directly affect fat absorption in the gastrointestinal tract (Jakobek, 2015). Molecules that interact with emulsions may be inside the water phase, in the oil droplet or the interfacial region, depending on the polarity of the molecules (non-polar molecules would be in the oil droplet, polar molecules in

Table 1.5. Studies focused on starch digestibility of common beans using different enzymes and incubation conditions.

Author	Bean type (*P. vulgaris*)	Type of α-amylase	Reaction time (h)	Degree of hydrolysis (%)	Units of enzyme used	Temperature °C
Yanez-Farias et. al., 1997	Pinto (raw)	Porcine	1	62	n/a	37
Hoover and Sosulski, 1985	Navy (raw)	Porcine	6	32	2,500 u/ g sample	37
	Northern bean (raw)	Porcine	6	29	3,500 U/ g sample	37
	Black (raw)	Porcine	6	34.8	5,500 U/ g sample	37
	Pinto (raw)	Porcine	6	22.2	5,200 U/ g sample	37
	Kidney (raw)	Porcine	6	27	1,000 units per milligram of protein	37
Rosenthal and Nakamura, 1972	Pinto (raw)	*Bacillus subtilis,*	6	62	2% of α-amylase	50
Socorro et al., 1989	Black (raw)	Porcine, human, and bovine	1 to 2	Porcine: 49.5; Human: 66.1; Bovine: 30.1	8 FIP amylase Units per ml solution (96 % w/w)	n/a
Du et. al., 2014	Pinto (raw)	Pancreatin (source n/a) with amyloglucosidase	25	2	n/a	37
	Red Kidney (raw)	Pancreatin (source n/a) with amyloglucosidase	27.9	2	n/a	37

Author	Bean type (*P. vulgaris*)	Type of α-amylase	Reaction time (h)	Degree of hydrolysis (%)	Units of enzyme used	Temperature °C
Du et. al., 2014	Black (raw)	Pancreatin (source n/a) with amyloglucosidase	21.7	2	n/a	37
	Navy (raw)	Pancreatin (source n/a) with amyloglucosidase	22.6	2	n/a	37
	Pinto (raw)	Pancreatin (source n/a) with amyloglucosidase	90.1	2	n/a	37
	Red Kidney (raw)	Pancreatin (source n/a) with amyloglucosidase	89	2	n/a	37
	Black (raw)	Pancreatin (source n/a) with amyloglucosidase	90.1	2	n/a	37
	Navy (raw)	Pancreatin (source n/a) with amyloglucosidase	89.9	2	n/a	37

the water phase and amphiphilic molecules in the interfacial region). In this way, some molecules may change the surface and size of the emulsion. Shishikura et al. (2006) performed an *in vitro* study with an emulsion model that consisted of olive oil, phosphatidylcholine and bile salt (to emulate the intestinal conditions) in order to determine the effect of green and black tea polyphenols on the emulsification properties. Results showed that the tea polyphenols increased the droplet size of the emulsion. The increase of droplet size may affect the interaction of this with lipase, inhibiting lipolysis and further fat absorption. Uchiyama et al. (2011) studied the effect of black tea polyphenols *in vivo* using diet-induced obese rats. Their results showed that black tea polyphenols inhibited lipase activity, reduced intestinal lipid absorption and decreased the triglyceride levels in the plasma. Sugiyama et al. (2007) studied the effects of procyanidins and apple polyphenols on the *in vitro* lipase activity and the *in vivo* triglyceride absorption using mice and human subjects. They found that the polyphenols studied inhibited lipase activity and decreased triglyceride content in the plasma.

Lipids may suffer oxidation in the stomach leading to the formation of potentially harmful lipid peroxidation products. Due to their antioxidant properties, polyphenols may have a positive effect by reacting with the lipid peroxidation products. (Lorrain et al., 2010; Lorraine et al., 2012; Gorelik et al., 2013). Gorelik et al. (2013) found that red wine prevented the oxidation of LDL (low density lipoproteins) by lipid peroxidation products (like malondialdehyde) in rats. This effect may be due to the antioxidant properties of red wine polyphenols (Gorelik et al., 2013). Lorrain et al. (2010, 2012) observed an inhibition of lipid peroxidation *in vitro* in a sunflower oil emulsion model by

quercetin, rutin, catechin, caffeic acid and cholorogenic acid.

As mentioned before, the lipid-polyphenol interaction may also have a protective effect on polyphenols by “capturing” them and enhancing their absorption in the lower parts of the gastrointestinal tract. Ortega et al. (2009) studied the effect of digestion and bioaccessibility of cocoa polyphenols using an *in vitro* digestion model. For this purpose, cocoa liquor (50% fat) and cocoa powder (15% fat) were assessed. Their results showed that the high fat content in cocoa liquor might have a protective effect on cocoa polyphenols due to an enhanced micellarization of the emulsion, which could improve the stability of polyphenols during digestion.

1.7.2. Effect of interaction with proteins

The interactions between proteins and polyphenols may have several biological effects. For example, astringency results from the interaction of tannins with salivary proteins. Astringency is defined as a drying-mouth feeling oral sensation. It results specifically from the association of proanthocyanidins and salivary proteins, which lead to the formation of aggregates that can reduce saliva lubrication (De Freitas and Mateus, 2001).

Protein-polyphenol interaction may also have an effect on protein structure, affecting their quality and functionality. It is believed that this type of interaction may have a negative effect on the bioaccessibility of certain aminoacids (Jakobek et al.,2015). Rawel et al. (2002) found that phenolic acids and flavonoids interacted with soy proteins, reducing the bioaccessibility of lysine, tryptophan and cysteine in an *in vitro* digestion assay. This could indicate that polyphenols may decrease the nutritional quality of proteins. However,

Petzke et al. (2005) found that chlorogenic acid did not produce an amino acid deficiency present in β-lactoglobulin in an *in vivo* assay performed with rats. More studies are required in order to determine if polyphenols affect the bioaccessibility and bioavailability of protein amino acids.

Other studies have focused on the effect that the protein-polyphenol interaction has on polyphenol bioavailability. Van her Hof et al. (1998) found that the addition of milk in green and black tea did not impact the bioavailability of tea catechins in a study performed with human subjects. Yet, Duarte and Farah (2011) found a different outcome in another human study, since their results that when milk was added to coffee, the bioavailability of coffee polyphenols was reduced.

Polyphenols may also interact with digestive enzymes, affecting the digestion of proteins. This effect was observed *in vitro* with chlorogenic acid and whey protein (Petzke et al., 2005). Kroll and Rawel (2001) also observed that polyphenols decreased myoglobin digestion in an *in vitro* digestion study. The decrease in protein digestibility could be detrimental to health. Enzyme inhibition by polyphenols may also be beneficial. For example, Piparo et al. (2008) found that flavonoids may inhibit human α-amylase *in vivo*, which could control starch digestion and prevent postprandial glycemia.

Protein-polyphenol interaction could have a protective effect on polyphenols during digestion; polyphenols may be later delivered to the lower parts of the gastrointestinal tract where they are absorbed (Shpigelman et al., 2010). It has also been observed that this complex may preserve the anti-proliferative activity of polyphenols (Von Staszewski et al., 2012). However, negative effects on the biological effect of polyphenols have also been observed

as a result of interaction between proteins and polyphenols. Hasni et al. (2011) found that the antioxidant activity of polyphenols was reduced when these interacted with proteins in an *in vitro* study. The same behaviour was observed in an *in vitro* study performed by Arts et al. (2002) with green and black tea flavonoids and casein.

1.7.3. Effect of interaction with carbohydrates

Polyphenols may bind to carbohydrates having various effects. For instance, carbohydrate-polyphenol interaction may have an impact on polyphenol bioaccessibility and bioavailability. Adam et al. (2002) studied the bioavailability of ferulic acid in rats after consuming whole and white flours (ferulic acid is usually associated to cereal fibre). Results showed that the cereal matrix decreased the bioavailability of polyphenols; this behaviour could be due to the fact that ferulic acid may form cross-links with arabinoxylans and lignins, making their absorption difficult. *In vitro* studies have shown that ferulic acid may be more bioaccessible when the aleurone of grains is ruptured mechanically (Pekkinen et al., 2014; Rosa, Aura, et al., 2013).

Some studies have found that polyphenol bioavailability is increased when these are consumed with high carbohydrate foods. Schramm et al. (2003) found that the bioavailability of flavanols was increased when human subjects consumed bread together with flavanol rich cocoa. The mechanism by which the bioavailability of flavanols is enhanced is still not known (Schramm et al., 2003). This same behaviour has been observed by Serra et al. (2010) in an *in vitro* study with monomeric proanthocyanidins from grape seed extracts; yet, in this same study, it was also observed that the bioavailability of larger proanthocyanidins (dimers and trimers) was not increased during the simulated

in vitro digestion performed. This suggests that the beneficial effect might be only observed with smaller polyphenols.

The carbohydrate-polyphenol complex can exert a protective effect for polyphenols and could lead to an increase of polyphenol bioaccessibility in the colon; when the complex reaches the colon the enzymes and microflora present might release the polyphenols (Palafox-Carlos et al., 2011). The polyphenols released could then be absorbed in the colon or further metabolized by the microflora, as mentioned previously.

When polyphenols arrive to the colon there could be several effects. These molecules can be released and be absorbed in the colon. If polyphenols and carbohydrates reach the colon, these can enhance the growth of the microflora. In addition, the presence of polyphenols in the colon may create an antioxidant environment, leading to possible health benefits (Palafox-Carlos et al., 2011; Saura-Calixto, 2011; MacDonald & Wagner, 2012; Tuohy et al., 2012). Since the carbohydrate-polyphenol complex can stimulate the delivery of polyphenols to the lower parts of the gastrointestinal tract and have a positive effect on polyphenol bioavailability, carbohydrates can be used to encapsulate the polyphenols that could lead to positive health benefits (Jung et al., 2013).

As mentioned before, the interactions of polyphenols with proteins can decrease the bioavailability of certain amino acids, inhibit certain enzymes and produce astringency. The presence of carbohydrates in foods containing polyphenols can avoid the formation of the protein-polyphenol complex. Goncalvez et al. (2011 a) found that the interaction between procyanidin B3 and trypsin can be inhibited by the presence of polygalacturonic acid, gum arabic, pectin and xanthan. Soares et al. (2012a) found that pectin, gum arabic, and

polygalacturonic acid prevented the formation of a complex between grape seed polyphenols and salivary proteins, which could reduce astringency. As a result, the addition of this carbohydrates may decrease a possible unwanted astringency in certain food products.

1.8. Polyphenols as inhibitors of anti-inflammatory activity

Inflammation is defined as a nonspecific immune response that helps the body defend itself against organisms and chemical substances from the environment (Romier et al., 2009). It is caused by physical injury, viral and bacterial infections, burns, chemical irritants, foreign bodies, oxidative stress; defining clinical features of inflammation are known in Latin as rubor (redness), calor (warmth), tumor (swelling) and dolor (pain) (Yoon and Baek, 2005). The main function of this process is to resolve an infection; if this regulation fails, the response may become chronic and the risk of diseases such as atherosclerosis, diabetes, cancer, rheumatoid arthritis or Alzheimer may increase (Arranz et al., 2015).

Inflammation is a mechanism exerted mainly by blood and epithelial cells. Macrophages play a major role during inflammatory and immune response; an excessive activation of these cells may cause extensive damage to tissues (Sommella et al., 2014). Epithelial, endothelial, mesenchymal, and nerve cells exchange regulatory signals via the production of mediators (cytokines, growth factors, adhesion molecules, etc.), which facilitate and amplify cell interactions and inflammation (Adams et al., 2006).

Polyphenols may reduce inflammation by inhibiting the arachidonic acid dependent pathway; the final products of this pathway are prostaglandins and leukotrienes that participate in the vasodilatation involved in the inflammation process. Polyphenols inhibit this pathway by supressing some enzymes that are

involved in this process, such as cyclooxygenases (COX2), lipoxygenase (LOX) and phospholipase (PLA_2). (Yoon and Baek, 2005).

Another known mechanism in which polyphenols exert an anti-inflammatory effect is by the modulation of the nuclear factor-kappa B (NF-kB) pathway (Rahman et al., 2006). The inhibition of NFκB is generally thought as a useful strategy for the treatment of inflammatory disorders (Karin et al., 2004). This pathway can be activated by oxidative stress (UV light), lipopolysaccharides (LPS) (which bind to toll-like receptors) and cytokines (Karunaweera et al., 2015). The latter are defined as small secreted proteins that have an effect on the interactions and communications between cells; these proteins can be used as markers to monitor inflammation processes (Zhang et al., 2007).

NFκB is a protein complex that is inactive in the cytoplasm of most cell types; it is a dimer conformed by two subunits known as RELA (p65), and NFκB1 (p50) (Miyamoto, 2011). NFκB stays inactive in the cytoplasm when the dimer is bound to the IκB protein, known as the NFκB inhibitor. The pathway is activated when cytokines and LPS bind to specific toll-like receptors, which leads to a biochemical cascade that activates kinases such as the IκB kinase (IKK). Once activated, IKK phosphorylates IκB. When IκB is phosphorylated, this unit detaches from the NFκB dimer, activating the protein complex. Once the NFκB dimer is in an active form, it migrates to the nucleus where it binds inflammatory responsive elements. A scheme of this biochemical pathway is shown in figure 1.5.

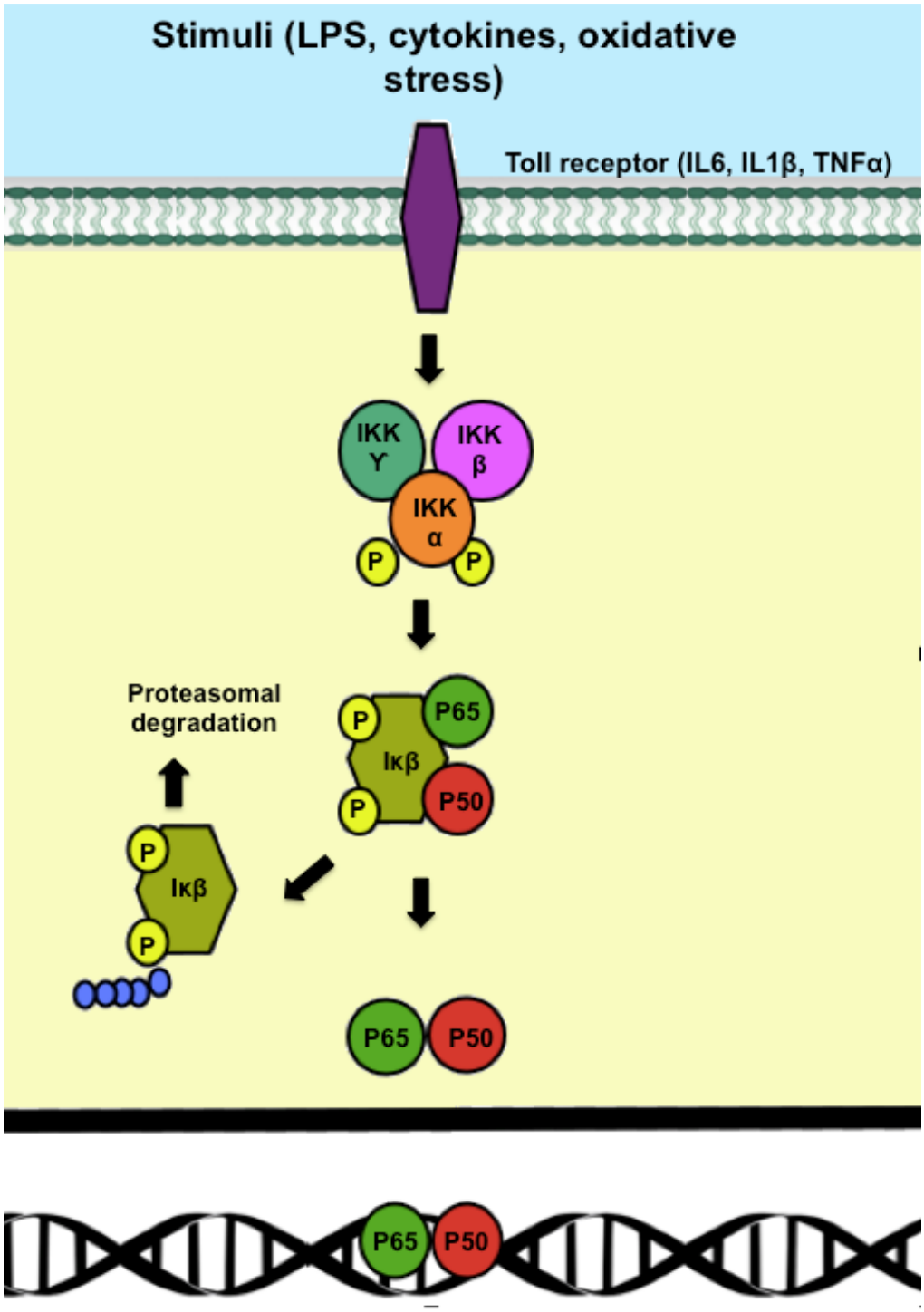


Figure 1.5. Scheme of the NFκB pathway.. The pathway is activates by LPS, cytokines and oxidative stress LPS and cytokines bind to toll receptors in the cell membrane, which stimulates the phosphorylation of IKK (comprised by IKKα, IKKβ and IKKY). The activation of IKK stimulates the phosphorylation of NFκB (comprised by IκB, P50 and P65). IκB detaches from P50 and P65 and. migrates by ubiquitinization to the proteasome, where it is degraded. P50 and P65 migrate to the nucleus where it stimulates the transcription of inflammatory cytokines (IL6, IL1β, TNFα and iNOS).

Once activated, IκB is released and is further degraded in the proteasome; the dimer formed by p65 and p50 is later phosphorylated and it binds to specific DNA sequences that are present in promoters of target genes that encode for proteins that are involved in the inflammation process (Shrech et al., 1992; Bours et al., 2000, Aggarwal and Shishodia, 2004). The pro-inflammatory agents that are induced during this pathway include cytokines (e.g., IL-1, IL-2, IL-6, and TNFα), chemokines (e.g., IL-8, MIP-1α and MCP-1), adhesion molecules (e.g., ICAM, VCAM, and E-selectin), acute-phase proteins, immune-receptors, growth factors and inducible enzymes such as vascular endothelial growth factor (VEGF), COX-2, matrix metalloproteinases (MMPs) and iNOS (Nam, 2006). Once the NFκB pathway is activated, an autocrine, self-perpetuating response is produced because the proteins produced during this pathway (like COX2, IL6 and TNFα) bind to NFκB cell membrane receptors, which prolongs the activation of the inflammatory process (Bours et al, 2000). The mechanism by which polyphenols inhibit this pathway is not fully known. Karunaweera et al. (2015) found that curcumin (a phenolic acid) may inhibit the phosphorylation of IκB by IKK. Thus, it is likely that other polyphenols might inhibit this pathway in the same way.

A biochemical pathway that is believed to attenuate inflammation is the nuclear factor (erythroid-derived 2)-like 2 (Nrf2). The NrF2 pathway is a biochemical pathway that controls cell homoeostasis in response to oxidative stress (Wardyn et al., 2015). This pathway stimulates the production of antioxidant enzymes such as heme oxygenase1 (HO1); since NrF2 reduces oxidative stress, it is believed that NrF2 attenuates inflammation (Rahman et al., 2006). Hence, a balance between the activities of both biochemical pathways is essential to resolve inflammation in the cell/tissue; an imbalance may cause the

development of diseases such as neurodegeneration, autoimmune disorders and cancer (Ben-Neriah and Karin, 2011).

A few studies have demonstrated that beans may exert an anti-inflammatory effect *in vitro* (by inhibiting and COX-1, COX-2 and LOX) and *in vivo* (by reducing oxidative stress and inflammatory cytokines in rat's colon) (Oomah *et al.* 2010; Zhang et al., 2014). Since beans can be so different in their polyphenolic composition and there is a wide variety of bean cultivars available, more studies that focus on the anti-inflammatory of different beans are required in order to determine if these legumes are beneficial for health.

1.9 Outline of this thesis

1.9.1. Aim

The aim of this project is to study the effect of digestion on the bioaccessibility of bean polyphenols and their anti-inflammatory effect.

1.9.2. Objectives

1. Optimize a simulated *in vitro* digestion method with pancreatic α-amylase to digest bean in order to generate information the effect of digestion and bioaccessibility of their polyphenols.
2. Determine the total polyphenols and identify the individual polyphenolic compounds that were released upon *in vitro* digestion and domestic processing.
3. Evaluate the anti-inflammatory effect of bean extracts of a group of selected samples in RAW 264.7 macrophages.

1.9.3. Hypothesis

In vitro digestion will enhance the bioaccessibility of bean polyphenols by releasing them after the hydrolysis of bean matrix macronutrients. Since the anti-inflammatory effect of some beans has been reported, the bean samples used in this study might show an anti-inflammatory effect *in vitro*.

Chapter 2 Materials and methods

2.1. Materials

2.1.1 Bean samples

Bean samples used for this study are shown in Figure 2.1. Borlotti beans (*P. vulgaris*) were purchased from Waitrose, UK. This beans were used in the first instance to optimize methods while Mexican beans were being sourced. Six Mexican varieties of *P. vulgaris* (Negro Jamapa, Azufrado Higuera, Pinto Saltillo, Marcela, Azufrado and Peruano Bola) were obtained from Villa Hidalgo, Nayarit, Mexico (21°44'36" N; 105°13'51" W). Four UK varieties of *V. faba* (Divine, Tattoo, Wizard and Buzz) were obtained from Wherries and Sons (Bourne, UK; 52.7684° N, 0.3775° W).

2.1.2 Enzymes

The following enzymes were purchased: porcine pancreatic α-amylase (Sigma Aldrich, Gillingham, UK), amyloglucosidase (from *Aspergillus niger,* Megazyme International, Bray, Ireland) and protease (subtilisin A from *Bacillus licheniformis,* Megazyme International, Bray, Ireland).

2.1.3 General reagents

Sodium chloride, sodium carbonate, maleic acid, tris(hydroxymethyl)aminomethane (Tris), ethylenediaminetetraacetic acid (EDTA), sodium metabisulfite, sodium potassium tartrate, 3,5-dinitrosalicylic acid (DNS) and trifluoroacetic acid (TFA) were all purchased from Sigma Aldrich (Gillingham, UK). Miracloth filter was purchased from Millipore Merck (Billerica, USA). Sodium phosphate monobasic anhydrous ($Na_2H_2PO_4$) and sodium phosphate dibasic anhydrous (Na_2HPO_4) were both from Acros Organics

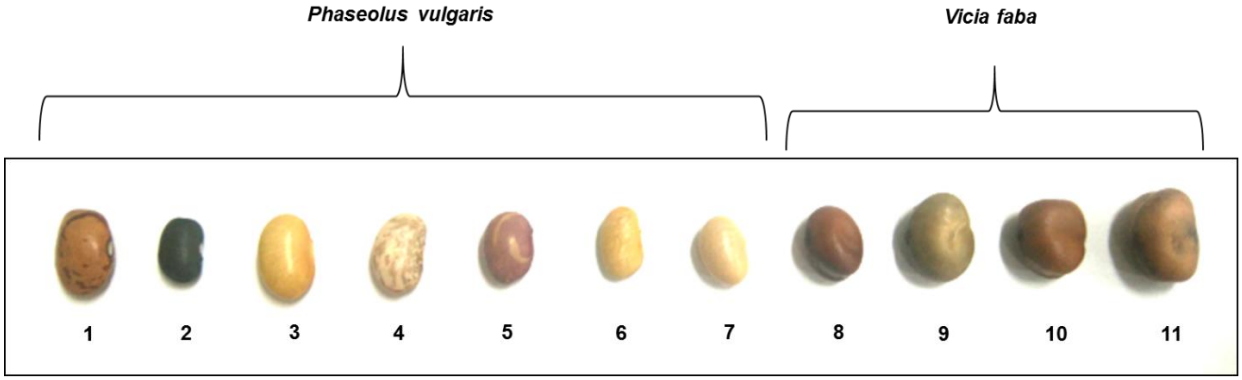


Figure 2.1. *P. vulgaris* and *V. faba* beans used during this study: Borlotti (1), Negro Jamapa (2), Azufrado Higuera (3), Pinto Saltillo (4), Marcela (5), Azufrado(6), Peruano Bola (7), Divine (8), Tattoo (9), Wizard (10), and Buzz (11).

(Loughborough, UK).

2.1.4 General Solvents

Methanol, acetone and ethanol were obtained from VWR International (Lutterworth, UK). All solvents were analytical grade. Sodium hydroxide was purchased from Fischer Scientific (Loughborough, UK).

2.1.5 HPLC solvents

HPLC grade sodium hydroxide solution (50%) was purchased from Fluka (Gillingham, UK). LCMS grade acetonitrile (100%) was obtained from VWR International (Lutterworth, UK).

2.1.6 Standards

The carbohydrate standards used were D-glucose, D-fucose and maltose monohydrate (Sigma Aldrich, Gillingham, UK). The polyphenol standards used were sinapic acid, ferulic acid, chlorogenic acid, *p*-coumaric acid, gallic acid and catechin (Sigma Aldrich, Gillingham, UK).

2.1.7 General Equipment

A pH meter (Hanna Instruments HI-2211 Bench top pH and ORP Meer), a Polytron (IKA T-25 ULTRA-TURRAX Digital High-Speed Homogenizer Systems), a shaking incubator (Grant OLS 200) and a spectrophotometer (Cecil Aquarius CE 7200) were used for general experiments.

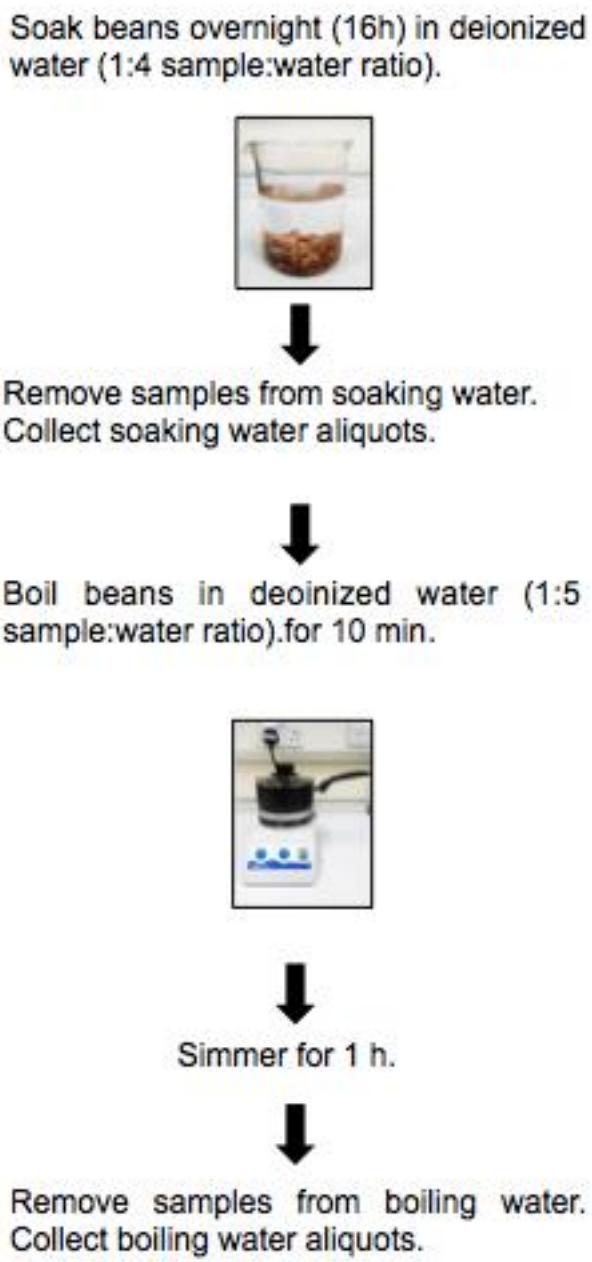


Figure 2.2. Methodology used for bean sample domestic processing.

2.2 Sample preparation

The sample preparation method used in this study is described in figure 2.2. Briefly, beans were first soaked overnight (16h) in deionized water (1:4 sample:water ratio). The soaking water was later removed and aliquots collected. After that, fresh water was added (1:5 sample:water ratio) in order to cook the beans. Samples were boiled for 10 min and then simmered for 1 h. Cooking water aliquots were collected. Cooked beans and aliquots (soaking and cooking water) were kept at -20°C until further analysis.

2.3 Moisture determination of bean samples

To determine the moisture content of the beans, 1 g of cooked bean sample was dried overnight at 80°C and weighed. Samples were dried until no further weight loss was observed. The weight difference before and after drying was considered as the water content in the sample. Results were expressed as % moisture.

2.4 Optimization of bean fibre extraction with pancreatic α-amylase and amyloglucosidase

Borlotti beans were selected in order to establish the optimal bean starch digestion conditions. Borlotti bean is a legume originating from Italy that is commercialized and consumed in the United Kingdom. Few studies about the polysaccharides and polyphenols in Borlotti beans have been performed. As a result, this sample was convenient to standardize the digestion method that was later used for the Mexican and UK bean samples. For this purpose, different experiments were developed in order to establish:

1. optimal incubation time of pancreatic α-amylase with Borlotti bean

sample

2. performance of pancreatic α-amylase in calcium free and calcium enriched buffer
3. optimal amount of pancreatic α-amylase to digest Borlotti bean sample
4. optimal incubation time of amyloglucosidase with Borlotti bean sample

2.4.1 Optimization of the incubation time of pancreatic α-amylase with Borlotti bean sample

Cooked Borlotti beans (1 g) were homogenized for 3 min with a Polytron PT 2500 homogenizer in 40 mL phosphate buffer (20 mM). Afterwards, the sample was digested with 100 U/mL of pancreatic α-amylase. The amount of enzyme used was selected according to the method described by Hoover and Sosulski (1985). Lower pancreatic α-amylase concentrations (50 U and 10 U) were also evaluated. The sample was incubated at 37°C for 2, 4, 6 and 16 h. To stop the starch hydrolysis, the mixture was boiled for 5 minutes to inactivate the enzyme. The fibre obtained was separated by precipitation as described in section 2.5. The amount of reducing sugars found in the supernatant was determined by the DNS assay and by high performance anion exchange chromatography (HPAEC-PAD; described further) in order to know the degree of starch digestion.

2.4.2. Determination of the performance of pancreatic α-amylase in two different buffers

Sodium phosphate (Bruckner, 1955) and sodium maleate buffer (McCleary, 2007) were tested in order to evaluate the performance of pancreatic α-amylase in buffer with and without calcium.

To prepare the sodium phosphate buffer, a 0.1 M sodium phosphate stock solution was first prepared by mixing 550 mL of 0.1 Na_2HPO_4 (di-basic) and 450 mL of 0.1 M $Na_2H_2PO_4$ (mono-basic).. Afterwards, the pH was adjusted to 6.9 and the stock solution was diluted (1:5) to obtain a 20 mM sodium phosphate buffer. Lastly, 35 mg of sodium chloride was added to get a final concentration of 0.6 mM.

Sodium maleate buffer (pH = 6.0) was prepared according to the method described by McCleary (McCleary, 2007). Briefly, 11.6 g of maleic acid were dissolved in 1600 mL of deionized water. The pH was adjusted to 6 with 4 M NaOH solution. Next, 0.6 g of $CaCl_2$ was added. Finally, deionized water was added to obtain a final volume of 2000 mL.

Borlotti bean samples were incubated overnight at 37°C with 100 U/mL of pancreatic α-amylase. Fibre was separated as described in section 2.5. The supernatant was stored at -20°C until further reducing sugar analysis.

2.4.3. Determination of the optimal incubation with amyloglucosidase

For this step, 1 g of Borlotti bean sample in sodium maleate buffer was digested overnight at 37°C with 100 U/mL of pancreatic α-amylase. The enzyme was later inactivated by boiling the sample as mentioned previously. The pH of the mix was later lowered to 4.5 with HCl 1M. Then, 80 U/mL amyloglucosidase was added (amount determined previously for bean starch digestion) and incubated for 0.5, 1 and 2 h at 60°C in a shaking incubator at 105 rpm. Fibre was precipitated and the supernatant obtained was kept at -20°C until further reducing sugar analysis.

2.4.4. Determination of optimal amount of pancreatic α-amylase

Once the optimal incubation time of pancreatic α-amylase with Borlotti bean sample was established, several concentrations of pancreatic α-amylase (100 – 5000 U per mL of bean solution) were tested. The sample was incubated with the different amounts pancreatic α-amylase at 37°C for 16 h. After the incubation, pancreatic α-amylase was inactivated by boiling, fibre obtained and supernatant collected and stored at -20°C for further reducing sugar analysis.

2.4.5 Determination of reducing sugars

To determine the degree of starch hydrolysis the amount of reducing sugars released after the digestion was measured by the DNS method and HPAEC-PAD.

2.4.5.1 Determination of reducing sugars by the DNS method

The supernatant (1 mL) was mixed with water (1 mL) and the DNS solution (1 mL) in a 15 mL centrifuge tube. Tubes were placed in a boiling water bath (100°C) for 15 minutes to stimulate the reaction with the reducing sugars with DNS. The tubes were cooled down after the incubation. Then, 9 mL of water was added to each tube. The absorbance was read at λ= 540 nm.

Two standard curves were constructed using maltose and glucose as standards with concentrations ranging from 0.1 – 4 mg/mL. The concentration of reducing sugars present in the supernatants was calculated with the equations of the curves.

2.4.5.2 Quantification of digested carbohydrates by HPAEC-PAD

To quantify the sugars released after starch hydrolysis, digestion supernatants were analysed by HPAEC-PAD. Separation was performed with a

PA20 column (Dionex, CA, USA) at a flow rate of 0.4 ml/min (ØBro et al., 2004). Before injection, each sample was appropriately diluted in water and passed through 0.45 µm filters. The column was initially washed with 200 mM NaOH and later equilibrated with 60 mM NaOH. The injection volume was 10 µL. The elution program consisted of a linear gradient from 60 mM NaOH to 200 mM NaOH from 0 to 15 min, followed by an isocratic elution with 200 mM NaOH from 15 to 18 min and a decreasing linear gradient to 60 mM NaOH from 18 to 20 min. Finally, an isocratic elution with 60 mM NaOH from 20 to 25 min was performed. The monosaccharides in the supernatants were detected using a pulsed amperometric detector (gold electrode). Samples were diluted in water and fucose was added as internal standard at a final concentration of 5 µg/mL. Standard curves (2 - 10 µg/mL) were constructed using D-glucose and maltose as standards. The concentration of reducing sugars in the samples was calculated with the equation of the curve.

2.5 Recovery of dietary fibre (DF)

Once the optimal starch and protein digestion conditions were selected, all the beans were digested with the method established. After the digestion, fibre was precipitated by adding 4 volumes of ethanol (95%), mixing thoroughly and allowing the precipitate to form at room temperature for 60 min. The fibre was separated from the supernatant using vacuum filtration. The fibre was recovered on a miracloth filter (previously dried and weighted). The undigested residue (fibre) was washed sequentially with two 15 mL portions of 78% (v/v) ethanol, 95% (v/v ethanol and acetone. The Miracloth filter containing the fibre was dried at 80°C overnight, cooled and weighted. Total dietary fibre was calculated with the following equation:

$$\text{Fibre \%} = \frac{\text{final weight miracloth} - \text{initial weight miracloth}}{\text{weight sample}} \text{ X } 100$$

The volume of the supernatants were measured in mL and were subsequently stored at -20°C for further reducing sugar and polyphenol analysis.

2.6 Total starch extraction and determination of digestible and resistant starch

Starch extraction of beans was performed as described by Bustos et al., (2004) with a few modifications. Briefly, 5 g of cooked beans were homogenized in 250 mL of 50 mM Tris (pH 7.5)/10 mM EDTA_0.5 g/L sodium metabisulfite at 4°C and filtered through four layers of cheesecloth. The filtrate was centrifuged at 20,000 X *g* and 4°C for 15 min. The pellet was washed three times using extraction medium, then twice in acetone and centrifuged as mentioned previously. The final pellet was dried overnight at room temperature and weight to calculate the starch yield. Results were expressed as g of starch per 100 g of cooked sample.

In order to determine digestible starch, bean samples were digested with the optimized *in vitro* digestion method. The glucose released after the digestion was measured by HPAEC-PAD (ad described in section 2.4.5.2). Digestible starch was calculated with the formula:

$$\text{Digestible starch} = \text{free glucose} \times 0.9$$

Once digestible starch was calculated, resistant starch was determined with the formula:

$$\text{Resistant starch} = \text{Total starch} - \text{Digestible starch}$$

2.6.1. UV and scanning electron microscopy (SEM) determinations of starch granules

Bean starch was isolated as mentioned previously (total starch extraction section 2.5). For UV microscopy, beans starch were fixed in water and analysed with an Olympus BH2 fluorescence trinocular microscope (Life Solutions, Japan). Pictures of native and gelatinized (from cooked bean) starch were taken with white and UV light.

SEM microscopy was performed as described by Orfila et al. (2001). Briefly, the dry starch was fixed in 2.5% (w/v) glutaraldehyde in 0.1 m sodium phosphate buffer, pH 7.2, for 2 h at 4°C, washed extensively with sodium phosphate buffer and subsequently fixed in 1% (w/v) osmium tetroxide in sodium phosphate buffer for 1 h at 4°C. The starch sample was later washed extensively in sodium phosphate buffer and dehydrated with acetone (10%–100%). Dehydrated fragments were mounted onto metal studs, coated with colloidal gold and viewed using a SEM microscope (CamScan, Leica, Cambridge, UK).

2.7 Polyphenol analysis

Polyphenols were determined from raw bean methanolic extracts, bean fibre hydrolysis, soaking and cooking water and digestion supernatants. Total polyphenol content (TPC) was determined by the Folin-Ciocalteu colorimetric method and were quantified and identified by LCMS analysis.

2.7.1. Extraction of polyphenols from raw beans

Milled raw beans (0.5 g) were placed in a 15 mL centrifuge tube. Afterwards, 10 mL of acidified methanol (97% with 3% trifluoroacetic acid) was added. Samples were vortexed for 1 h and centrifuged (9000 x *g* for 10 min).

The volume of the supernatant was measured (mL) and stored at -20°C for further polyphenol analysis.

2.7.2 Recovery of polyphenols from DF

Bean samples were digested with the optimized digestion method and the undigested material (fibre) was recovered in 50 mL centrifuge tubes. Later, 10 mL of 1N HCl was added and samples incubated at 100°C for 1h to achieve the hydrolysis of polyphenols from cell wall components. After the hydrolysis, samples were centrifuged (9000 x *g* for 10 min). The volume of the supernatant was measured (mL) and stored at -20°C for further polyphenol analysis.

2.7.3 Determination of TPC by the Folin-Ciocalteu assay

TPC content of the different fractions was determined by the Folin-Ciocalteu assay (Singleton and Orthofer, 1999) with a few modifications. Briefly, 50 µL of methanolic extract, cooking water, soaking water or supernatant, were mixed with 50 µL of Folin-Ciocalteu reagent (1 N), 150 µL of Na_2CO_3 (20%, w:v) and 750 µL of water. The mixture was incubated for 30 min in the darkness and the absorbance measured at 765 nm with a double beam spectrophotometer. Results were expressed as mg equivalents of gallic acid/ g dry weight.

2.7.4 Identification and quantification of polyphenols by HPLC-DAD-MS

2.7.4.1 Sample preparation for HPLC-DAD-MS analysis

All analysed samples were precipitated overnight (16 h) at 4°C. with 4 volumes of acetone. After that, samples were centrifuged (9000 *g*, 10 min, 4°C), decanted and supernatants collected. Samples were dried with a Genevac equipment and the pellets obtained were dissolved in solvent A (composition described further). Finally, samples were passed through 0.2 µm filters.

2.7.4.2 Chromatographic conditions

The analysis was performed using a single quad LC-MS Shimadzu LC-2010 HT HPLC coupled with a LCMS-2020 quadrupole mass spectrometer fitted with an electro spray ionisation source used in negative mode, detector -1.80 kV, DL temperature 250°C, nebulizing gas flow and drying gas flow 1.50 and 15 L/min respectively (Milton Keys, UK). The system consisted of a micro vacuum degasser (Prominence Degasser LC-20 A5, Shimadzu), an auto sampler (Prominence Auto Sampler SIL-30 AC, Shimadzu), a column oven (Prominence Column Oven CTO-20 AC, Shimadzu), a controller (Prominence Controller CBM-20 A, Shimadzu), a liquid chromatograph (Prominence Liquid Chromatograph LC-30 AD, Shimadzu), a diode array detector (Prominence Diode Array Detector system SPDM20A, Shimadzu), and an MS detector with electrospray ion source and quadrupole analyser (Liquid Chromatograph Mass Spectrometer LCMS-2020, Shimadzu).

Separation was performed using an Agilent Zorbax Eclipse plus C18 column (4.6 mm x 150 mm, and1.80 micron internal diameter). The column temperature, flow rate and injection volume were 35°C, 0.5 mL/min, and 5 µL, respectively. The solvent system used was Solvent A (0.1% formic acid, 5% acetonitrile and 94.9% water) and Solvent B (0.1% formic acid, 5% water and 94.4% acetonitrile). The elution program consisted of a linear gradient from 0-51 min from 0% to 100% solvent B, isocratic elution from 51.1-56 min with 100% solvent B, linear gradient from 56-56.1 min to 0% solvent B and isocratic elution from 56.1-61 min with 0% solvent B. Detection spectra wavelengths used were 280, 290, 315, 320 and 330 nm. The software used to control the LCMS system and for data processing was Labs solutions (Shimadzu).

2.8 Evaluation on anti-inflammatory activity of bean polyphenols in murine RAW264.7 macrophages

2.8.1 Sample preparation

In order to evaluate the anti-inflammatory effect of beans, four cooked beans, Borlotti, Negro Jamapa, Marcela and Azufrado, were freeze dried and milled. Extracts were prepared by adding 10 mL of methanol (97% + 3% TFA), vortexing vigorously for 1 h, centrifuged (9000 x *g* for 10 min) and decanted. The extraction solvent was later evaporated using a Genevac EZ-2 Series equipment (Ipswich, UK). After that, 100 mg of dried extract were weighted and dissolved in 1 mL of 100% dimethyl sulfoxide (DMSO) in order to prepare a 100 mg/mL stock solution. A 100 mM sinapic acid stock solution was also prepared in DMSO. Stock solutions were stored in aliquots at -20°C until further analysis.

2.8.2 Cell culture and cytotoxicity assays

Murine RAW264.7 macrophages (kindly provided by St. James Hospital) were cultured in Dulbecco's modified Eagle's medium (DMEM), high glucose (4.5 g/l), containing sodium pyruvate (110 mg/l), supplemented with 10% (v/v) foetal bovine serum (non-heat inactivated), 2 mmol/l L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were grown in a humidified incubator at 37°C and 5% CO_2.

The Neutral Red Assay was performed in order to determine if the bean extracts and sinapic acid exerted a cytotoxic effect on of RAW264.7 cells. To achieve this, cells were seeded in 24 well plates (250,000 cells/well) 24 h prior to the experiment. The concentrations of compound and extracts evaluated ranged from 10 – 100 µM for sinapic acid and from 10- 100 µg/mL for the extracts. The stocks were later diluted in DMEM to obtain the desired concentrations (working solutions). Afterwards, 1 mL of the working solutions

was added to each well. Cells were incubated with the compound or extract for 24 h (37°C and 5% CO_2).

Afterwards, the working solutions were replaced with 1 mL/well of Neutral Red working solution (60 µg Neutral Red /ml DMEM) and incubated 2 h. Later, the Neutral Red was removed and cells washed with Phosphate buffered saline (PBS). Bleaching solution (50% ethanol, 49% deionized H_2O, 1% glacial acid) was added (1 mL per well) and the plate was incubated for 15 min on a horizontal shaker. After shaking, 200 µl of bleaching solution was transferred to a 96 well plate. The absorbance was measured with a TECAN spark photometer (Männedorf, Switzerland) at λ = 540 nm. Finally, the viability of treated cells was calculated in percentage of control cells (DMEM medium control). All determinations were done by triplicate.

2.8.3 Optimization of LPS concentration

In order to determine an optimal concentration of LPS to induce inflammation in the RAW264.7 cells, concentrations that ranged from 10-1000 ng/mL of this compound were evaluated. LPS from two different sources (*Escherichia coli* and *Salmonella enterica* from Sigma-Aldrich) were tested. LPS stock solutions (1 mg/ml) were prepared in PBS. The stocks were stored at -20°C until further use.

The LPS stock solutions were dissolved in DMEM in order to prepare LPS working solutions with the concentrations aimed. RAW264.7 cells were seeded in 12 well plates 24 h prior to the experiment. Later, medium was replaced with LPS working solution (2 mL/well) and incubated for 1 h (37°C and 5% CO_2). After incubation, supernatants were collected from each well,

transferred to 1.5 mL Eppendorf tubes and stored at -80°C until further analysis. All determinations were done by triplicate.

2.8.4 NO measurement in cell supernatants using Griess assay

The Griess assay was performed in order to measure the NO production by the cells. First, 100µL supernatant was pipetted into wells of a 96 well plate. Next, 100µL of 1X Griess reagent (Sigma G4410) were added to each well. The plate was incubated in the dark at room temperature for 15 min. The absorbance was measured at λ= 540 nm with a TECAN Spark 10M (Männedorf, Switzerland) plate reader. NO concentration was determined with the equation of the curve constructed previously with $NaNO_2$ (Sigma N2252; MW 69.0) and expressed as NO µM. All determinations were done by triplicate.

2.8.5 Cell treatment and RNA isolation

RAW264.7 cells were seeded in 12 well plates (400,000 cells/well) 24 h before the experiment. Working solutions of sinapic acid and bean extracts were prepared by diluting the stocks in DMEM. The concentrations evaluated ranged from 1 – 100 µM for sinapic acid and 10 – 100 µg/mL for the bean extracts. Working solution (2 mL) was added to each well. After 1 h of incubation (37°C and 5% CO_2), LPS was added to induce an inflammatory response in the cells. After that, cells were incubated for 6 h under the same conditions mentioned previously. Medium was discarded and each well washed with PBS. Finally, 600 µL of Trizure reagent was added per well. The plates were stored at -80°C until further analysis.

The 12 well plates were later thawed. Cells were resuspended in Trizure by pipetting and transferred to 2 mL RNA/DNA free Eppendorf tubes. Then, 100

μL of Chloroform (100%) was added to each tube and vortexed vigorously. Samples were incubated 2-3 at room temperature and centrifuged (12,000 x g for 15 minutes at 2-8°C) and the upper chloroform layers were transferred to 2 mL RNA/DNA free Eppendorf tubes. Afterwards, 300 μL were added to the chloroform layer. Samples were shaken vigorously by hand and incubated 10 min at room temperature. Then, samples were centrifuged (12,000 x g, 10min, 2-8°C) and supernatant decanted and discarded. The pellet (cells) was washed with 600 μL of ethanol (75%), samples vortexed gently and centrifuged (7500 x g for 5min at 2-8°C). Supernatants were decanted and 25 μL of DEPC-water was added to dissolve the RNA. The Eppendorf tubes were flicked to dissolve the isolated RNA. Finally, RNA concentration was determined using a TECAN Spark 10M (Männedorf, Switzerland) reader and results were expressed in ng/mL. Samples were stored at -80°C until further analysis.

2.8.6 cDNA synthesis

RNA samples were thawed and stored in ice. In 0.5ml RNAse/DNAse free tubes, 500 ng of RNA were pipetted and DEPC-water was added to get a final volume of 7.5 μL. A mastermix consisting of 1 part of iScript RT enzyme and 4 parts of 5x iScript buffer was prepared separately. After that, 2.5 μL of the mastermix was added to each sample. The reaction protocol consisted on the following incubations: 5 min at 25°C, 30 min at 42°C and 5 min at 85°C. Samples were diluted 1:20 by adding 190 μL of DEPC-water and stored at -20°C until further analysis.

2.8.7 Quantitative real time PCR (qPCR)

The Bioline, SensiMix SYBR High-ROX Kit (London, UK) was used for this determination. The primers used corresponded to the following target

genes: interleukin 6 (IL-6), interleukin 1 beta (IL1β), nitric oxide synthase (iNOS), tumour necrosis factor alpha (TNFα) and heme oxygenase 1 (HO1). The housekeeper gene used was beta actin (ACBT). Initially, a primer test was performed by preparing a series of cDNA dilutions as shown in Table 2.1.

Table 2.1. Primer test dilutions for qPCR analysis serial tenfold dilutions.

Dilution	1:10	1:100	1:1,000	1:10,000	1:100,000	0
cDNA (µl)	3	3	3	3	3	0
Water (µl)	27	27	27	27	27	30

The mastermix prepared consisted of 10 parts of DEPC-water, 1 part of primer forward, 1 part of primer reverse and 20 parts of 2X SensiMix buffer. Mastermix was protected from the light. Later, 16 µl of mastermix plus 4 µl of each sample was added to each well of a 96 well PCR plate. Next, the plate was briefly centrifuged and an adhesive film was placed over it. The cycling conditions used for the qPCR run consisted of the following:

- enzyme activation step: 95°C for 10 min)
- cycling (40x): 95°C for 15sec, 57°C for 15sec and 72°C for 15sec.
- melt curve (95°C for 15 sec, 60 °C for 60 sec and 95 °C for 15 sec)

Each cDNA sample was ran in duplicate. Results were expressed as the ratio of target gene:housekeeper gene as compared to the negative control (cells incubated with no sample and stimulated with LPS). The target genes tested and their sequence is shown in table 2.2.

In order to determine the change in expression, the $\Delta\Delta C_T$ method of relative quantification was used. To achieve this, the resulting threshold cycle (C_T) values of the target mRNAs to the C_T values of the internal control β-actine

in the same samples ($\Delta C_T = C_{T\ \text{Target}} - C_{T\ \beta\text{-actine}}$) were normalized. The fold change in the expression was then obtained ($2^{-\Delta\Delta CT}$).

2.9 Statistical analysis

All results are presented as mean of triplicates ± standard deviation of the mean. All statistical analysis was performed using SPSS 24.0 statistical package. For digestion experiments and polyphenol determination, statistical differences between means of different treatment groups were detected by one-way ANOVA, followed by multiple comparisons using the Tukey test ($P < 0.05$). For cell culture experiments, statistical differences were first detected by one/way ANOVA followed the Dunett test ($P < 0.05$) in order to compare samples with controls.

Table 2.2. Target genes tested in this study and their forward (5'-3') and reverse (3'-5') sequence.

Gene name	Forward sequence (5'-3')	Reverse sequence (5'-3')
b-actin	CCTCTATGCCAACACAGTGC	CCTGCTTGCTGATCCACATC
IL-6	AGTTGCCTTCTTGGGACTGA	CAGAATTGCCATTGCACAAC
IL-1b	CAGGCAGGCAGTATCACTCA	AGCTCATATGGGTCCGACAG
TNF-a	GTGCCTATGTCTCAGCCTCT	AGTTGGTTGTCTTTGAGATCCA
iNOS	GCAGCCTGTGAGACCTTTG	GCATTGGAAGTGAAGCGTTTC
HO-1	GAGCCTGAATCGAGCAGAAC	AGCCTTCTCTGGACACCTGA

Chapter 3

Optimization of an *in vitro* digestion method with pancreatic α-amylase and determination of the total polyphenols released in *P. vulgaris* and *V. faba* upon simulated digestion

Summary

- An *In vitro* digestion method with pancreatic α-amylase was optimized using Borlotti bean. The optimal pancreatic α-amylase concentration to digest Borlotti bean starch was 250 U/mL, which is similar to the established INFOGEST consensus for simulated digestion (200 U/mL).
- Enzymatic digestion extracted around twenty times more polyphenols compared to soaking, cooking and acid hydrolysis.
- Four times more polyphenols were extracted following starch digestion compared to protein digestion.
- *V. faba* presented higher polyphenol bioaccessibility (44.12 mg gallic acid equivalents (GAE) / g sample) compared to *P. vulgaris* (34.24 mg GAE / g sample).
- The bioaccessibility of bean polyphenols appears to be higher than previously reported and it might be limited by interactions with starch.

3.1 Introduction

Beans display great genetic diversity (Foyer et al., 2016) which is reflected in their varied polyphenol composition. It has been reported that beans contain hydroxycinnamic acids (p-coumaric, ferulic, cinnamic, and sinapic acids), flavonoids (catechin, epicatechin, and quercetin), condensed tannins (procyanidins) and anthocyanins (delphinidins, cyanidins, and malvidins) (Luthria and Pastor-Corrales, 2006, Lin et al., 2008, Xu and Chang, 2008, Lopez-Alarcon and Denicola, 2013, Chen et al., 2015). Polyphenols have been associated with a reduced risk of a number of chronic diseases, including cancer, cardiovascular disease and neurodegenerative disorders (Vauzour et al., 2010). However, their health benefits may be greatly limited by low bioaccessibility and bioavailability.

Even though the polyphenolic profile of beans has been extensively studied, information on the bioaccessibility of these molecules is scarce and available data is inconclusive. Bioaccessibility is defined as the quantity of a specific compound which is released from the food matrix and is available to the intestinal cells for potential absorption; this term is key to establish the nutritional efficiency of food (Fernández-García et al., 2009). Chen et al. (2015) observed an increase in polyphenol bioaccessibility after pressure cooking of raw cranberry beans but their release was limited (20% of amount released by cooking) upon *in vitro* digestion with pancreatin, amyloglucosidase and invertase. LaParra et al. (2008) performed an *in vitro* digestion of white, red and black beans using porcine pepsin, pancreatin and bile extract; the amount of polyphenols solubilized with the enzyme treatment was up to 91% of the levels extracted with acidified methanol (LaParra et al., 2008). Faller et al. (2012) compared pepsin and pancreatin digestion with acetone extraction of feijoada (a

traditional Brazilian meal containing black beans, rice, kale, cassava flour, and orange), finding no differences between the two extraction procedures (Faller et al., 2012). Wootton-Beard et al. (2011) found that *in vitro* digestion with pepsin followed by pancreatin enhanced the release of polyphenols, antioxidant and scavenging capacities in different vegetable juices compared to undigested samples, with varying effects depending on the juice (Wootton-Beard et al., 2011). Miranda et al. (2013) found that there was a higher release of polyphenols after simulated gastrointestinal digestion of boiled potatoes compared to solvent extraction (Miranda et al., 2013). In this same experiment, up to five times more chlorogenic acid was released during the gastric phase compared to undigested sample, while caffeic acid was mostly released during the intestinal phase (Miranda et al., 2013). Most studies involving the release of polyphenols upon *in* vitro digestion have used combinations of digestive enzymes. To our knowledge, there are no studies that have investigated the release of polyphenols after digesting starch and protein separately.

3.2 Methodology

The methodology used for this section is described in Chapter 2 (sections 2.2-2.7.3).

3.3. Aim and objective

3.3.1. Aim

The aim of this section of the study is to quantify the total polyphenols released upon *in vitro* digestion of beans and domestic processing.

3.3.2. Objectives

1. Optimize an *in vitro* digestion method with pancreatic α-amylase using Borlotti beans.

2. Quantify the total polyphenols released during soaking and cooking of beans.
3. Quantify the total polyphenols released after *in vitro* starch and protein digestion of the samples.
4. Hydrolyse the undigested residue to quantify the total polyphenols that were not released during the domestic processing nor *in vitro* digestion

3.4 Results

3.4.1 Optimization of bean fibre extraction with pancreatic α-amylase and amyloglucosidase (AMG)

The first step of this experiment consisted on optimizing an *in vitro* digestion method with porcine pancreatic α-amylase in order to digest as much starch as possible. In order to achieve this, a cooked Borlotti bean sample was used to determine the conditions of the digestion with pancreatic α-amylase and amyloglucosidase (AMG). The optimized method was further used to digest the rest of the bean samples studied. The conditions optimized for pancreatic α-amylase are mentioned in table 3.1.

Table 3.1. Digestion conditions tested for the optimization of the *in vitro* starch digestion method with pancreatic α-amylase.

Condition optimized	
Incubation time with pancreatic α-amylase	2, 4, 6 and 16 h
Buffers	Sodium maleate (60 mM with CaCl 2 mM) and phoshapte (20 mM with NaCl 0.6 mM)
Pancreatic α-amylase concentration	1-1000 U/mL
Incubation time with AMG	30, 60 and 120 minutes

During the optimization of this method, the degree of starch digestion was firstly determined by measuring the reducing sugars released in the

supernatants by the DNS assay. When using the DNS spectrophotometric assay, the results obtained were higher than expected. The digestion supernatants and the pancreatic α- amylase preparation were later analysed by HPAEC-PAD chromatography (Figure 3.1). It was observed that the enzyme preparation contained lactose; this monosaccharide was interfering with the DNS results obtained previously. Hence, for the DNS results, a blank containing the same amount of enzyme powder was prepared to eliminate the interference produced by the lactose present in the enzyme preparation. The absorbance values of the blank was later subtracted from the final DNS values

No significant differences were observed in the degree of starch digestion when incubating the sample with pancreatic α-amylase at different times (2, 4, 6 and 16 h). For this initial stage, the amount of pancreatic α-amylase tested ranged from 10-100 U/mL. The concentration of enzyme used did have a significant effect on the degree of starch digestion. The efficiency of pancreatic α-amylase showed a dose/response effect at the concentrations tested. Yet, when the digestion was performed with pancreatic α-amylase followed by AMG, results showed that time had an impact on the degree of starch digestion. Results indicated that the maximum starch digestion was achieved when incubating overnight (16 h) with pancreatic α-amylase, followed by AMG (30 U/mL) digestion.

After the incubation time was established, the next step was to determine the optimal concentration of pancreatic α-amylase to use. As mentioned in table 3.1, the enzyme concentrations tested at this stage ranged from 100-1000 U/mL. The maximum starch digestion degree was observed when using 250 U/mL. As shown in figure 3.1, after digesting the samples with this established

amount of pancreatic α-amylase and AMG, all the starch was digested, since no oligosaccharides were detected and the only sugar identified was glucose.

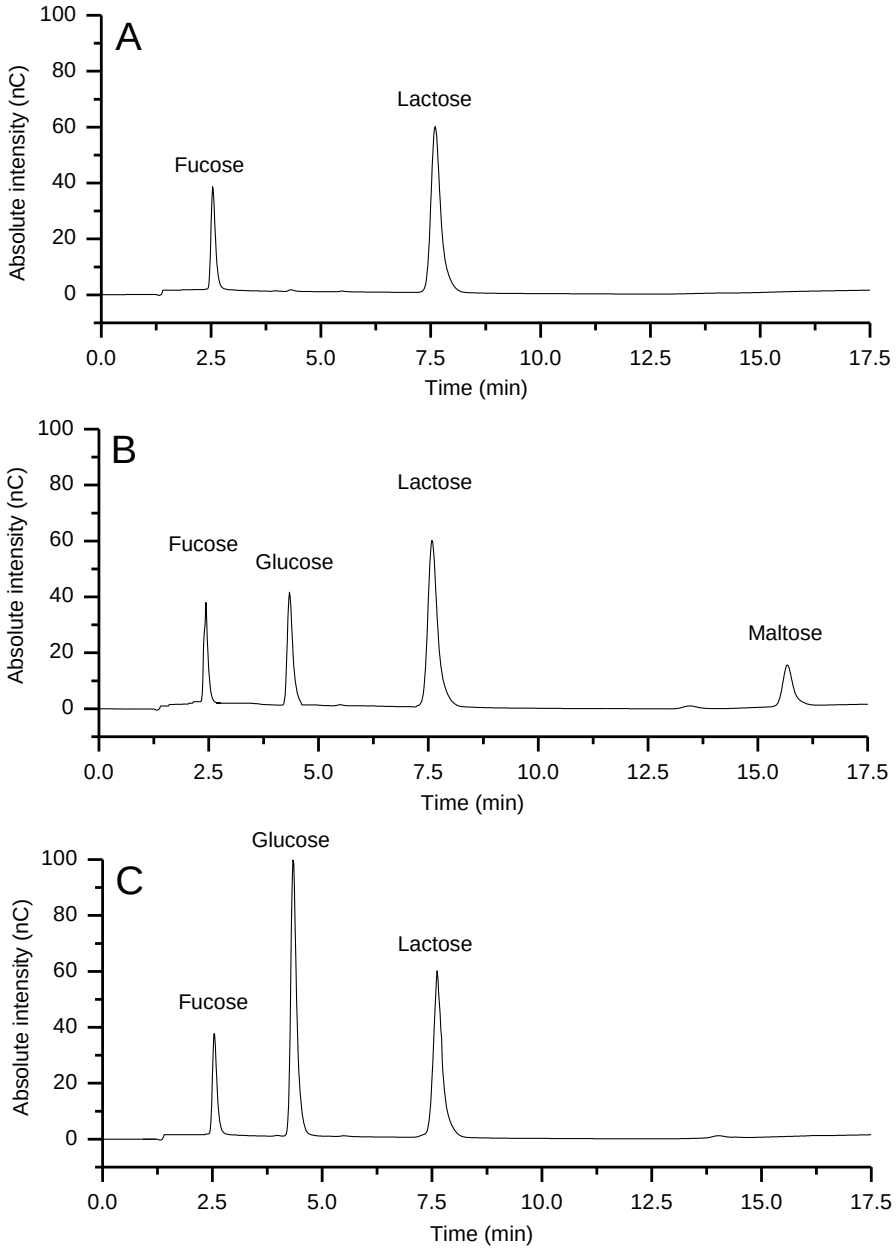


Figure 3.1. Chromatograms obtained from digestion supernatants: a) blank prepared with 250 U of enzyme and no sample; b) sample digested with 250 U/mL of pancreatic α-amylase; c) sample digested with 250 U/mL of pancreatic α-amylase followed by 30 U/mL AMG.

After starch hydrolysis with pancreatic α-amylase, the sample was incubated with AMG for three different times (30, 60 and 120 minutes) in order to optimize the incubation time of the bean sample with AMG. The results obtained showed that the starch hydrolysis reached a peak at 60 minutes, with no further increase at 120 min. Hence, the incubation time with this enzyme was established at 60 min.

Figure 3.2 shows the optimized *in vitro* digestion method that was achieved after establishing the conditions previously mentioned. After digestion with pancreatic α-amylase followed by AMG, the final digestion product was only glucose. This indicates that the starch digestion has reached an end, as only this monomer was detected and no other disaccharides or oligosaccharides were detected. The optimal amount of pancreatic α-amylase used to digest bean starch was 250 U/mL, as no further increase in the starch digestion degree was observed with higher concentrations of this enzyme. This pancreatic α-amylase concentration was used for further experiments.

3.4.2 Recovery of fibre from Borlotti beans after starch digestion

After Borlotti bean was digested with the optimized method, the undigested residue (fibre) presented a beige colour (figure 3.3). This indicated the possible presence of polyphenols bound to this phase. The final weight of fibre obtained from 1 g of cooked sample was 22.5 ± 01.5**%.**

3.4.3 Starch and fibre content in *P. vulgaris* and *V. faba*

Total starch, starch digestibility and fibre content varied significantly between the bean samples (table 3.2). The total starch content in *P. vulgaris* samples ranged from 35.00 ± 1.72 g / 100 g of dry sample to 42.30 ± 1.19 g / 100 g of dry. The *V. faba* samples had starch contents

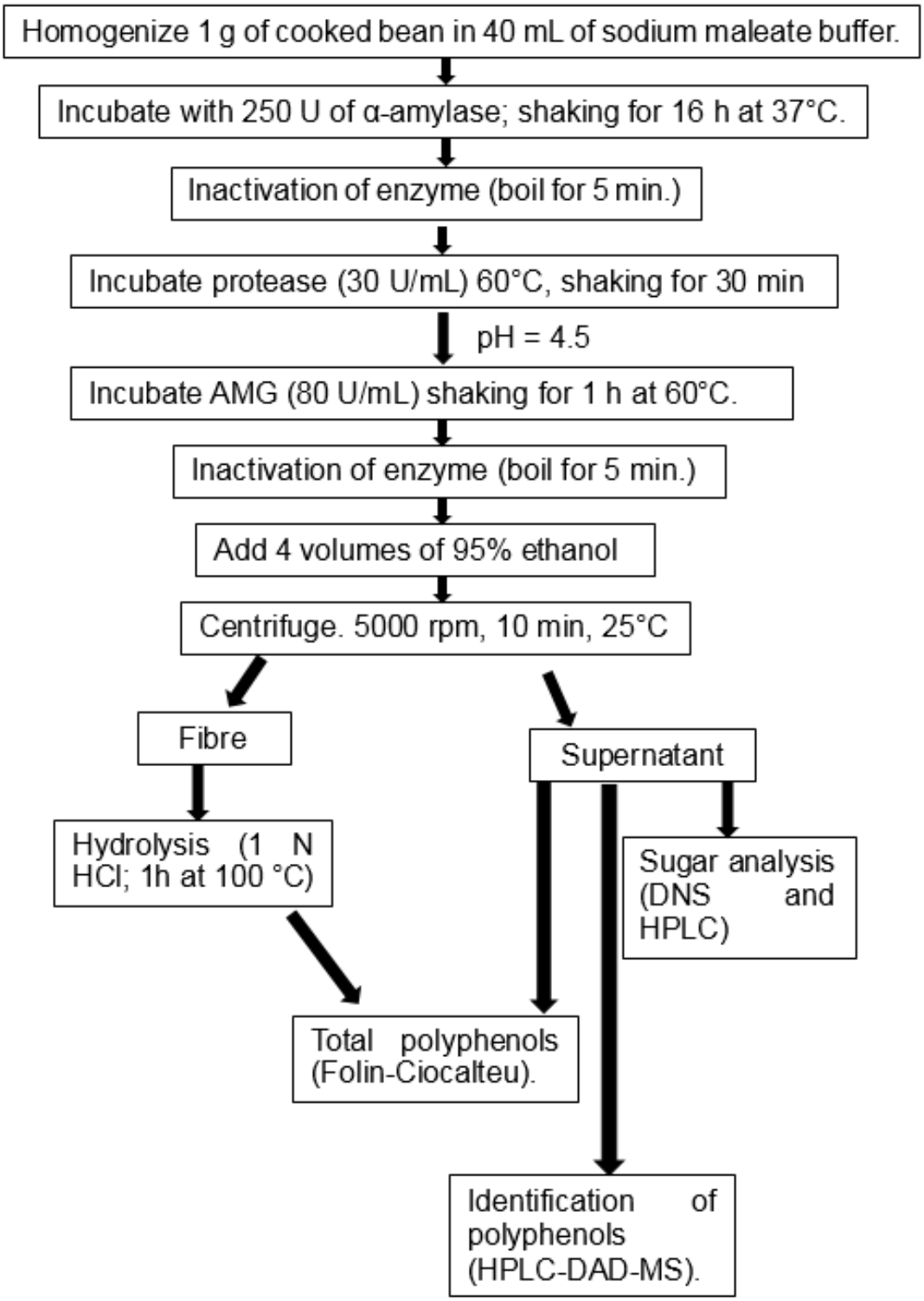


Figure 3.2. Optimized bean digestion method with pancreatic α-amylase, protease and amyloglucosidase (AMG) and further polyphenol analysis performed.

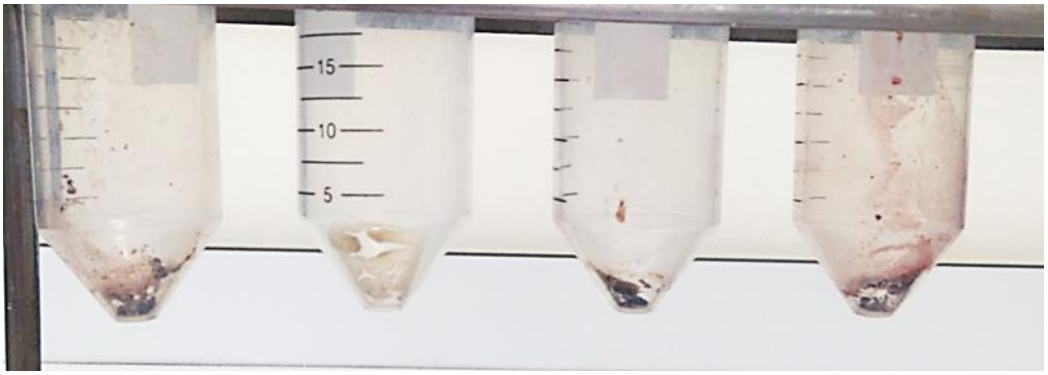


Figure 3.3. Borlotti bean fibre recovered after starch and protein digestion.

Table 3.2. Moisture content, starch content (total, digestible and resistant) and fibre content of cooked *P. vulgaris* and *V. faba* samples.

Sample	Moisture (%)	Total starch (g/100 g dry sample)	Digestible Starch (g/100 g dry sample)	Resistant Starch (g/100 g dry sample)	Dietary fibre (g/100 g dry sample)
Bor	55.6 ± 2.6^{bc}	44.7 ± 1.9^{a}	30.2 ± 2.4^{a}	14.5 ± 0.5^{d}	22.5 ± 1.5^{b}
Nej	52.1 ± 3.0^{bc}	37.9 ± 1.0 ^{b}	27.4 ± 2.2^{ab}	10.5 ± 0.6^{ef}	26.8 ± 3.6^{a}
Azh	50.5 ± 3.67^{c}	42.3 ± 1.2^{a}	23.6 ± 3.8^{ab}	18.7 ± 0.8^{a}	19.5 ± 2.7^{bc}
Pis	59.9 ± 1.2^{a}	35.0 ± 1.7^{e}	22.0 ± 4.0^{bc}	13.0± 1.0^{de}	30.5 ± 1.4^{a}
Mar	59.1 ± 2.9^{ab}	39.8 ± 1.8^{cd}	22.2 ± 3.8^{bc}	17.6± 1.0^{b}	28.8 ± 2.0^{a}
Azu	54.6 ± 2.4^{bc}	37.4 ± 1.9^{bc}	23.4 ± 3.50^{b}	14.0± 1.1^{a}	20.2 ± 2.6^{bc}
Peb	58.2 ± 4.0^{ab}	41.9 ± 0.8^{b}	26.9± 4.6^{ab}	15.0± 0.8^{c}	22.7 ± 1.8^{bc}
Mean	55.7 ± 3.6	39.9 ± 3.3	25.1 ± 3.4	14.8 ± 3.4	24.4 ± 4.3
Div	60.2 ± 4.1^{ab}	39.9 ± 0.6^{d}	27.9 ± 3.2^{b}	11.4 ± 0.3^{f}	20.2 ± 1.3^{c}
Tat	62.9 ± 4.0^{a}	43.4 ± 0.5^{cd}	30.8 ± 2.7^{b}	12.73 ± 0.5^{f}	18.0 ± 2.3^{d}
Wiz	60.2 ± 4.2^{ab}	40.1 ± 0.2^{d}	28.4± 4.4^{ab}	11.6± 0.2^{f}	20.1 ± 2.0^{cd}
Buz	60.8 ± 2.9^{a}	42.1 ± 0.8^{c}	27.4 ± 1.8^{b}	14.6± 0.8^{d}	21.6 ± 1.2^{c}
Mean	61.0 ± 1.3	41.4 ± 1.7	28.7 ± 1.7	12.7 ± 1.5	20.0 ± 1.5

Samples: Bor: Borlotti; Nej: Negro Jamapa; Azh: Azufrado Higuera; Pis: Pinto Saltillo; Mar: Marcela; Azu: Azufrado; Peb: Peruano Bola; Div: Divine; Tat: Tattoo; Wiz: Wizard: Buz: Buzz. Results, apart from moisture, are expressed as g / 100 g cooked sample (dry weight basis). Values show mean ± standard deviation (n = 3 determinations). Different letters indicate significant differences between values in the same column ($p < 0.05$).

ranged from 39.90 ± 0.55 to 43.40 ± 0.46 g / 100 g of dry sample. Significant variations in the starch digestibility of different bean samples were also observed. For *P. vulgaris* beans, the values ranged 55.8% for Azufrado Higuera to 72.3% for Negro Jamapa. In the case of the *V. faba* beans, starch digestibility values ranged from 65.1% for Buzz to 73.2% for Tattoo. *P. vulgaris* beans presented higher amounts of fibre than the *V. faba* samples (table 3.2). For the *P. vulgaris* beans, the sample that presented the highest amount of fibre was Pinto Saltillo (30.50 ± 1.37 g / 100 g of dry sample). In the case of the *V. faba* beans, the cultivar that showed the highest amount of fibre was Buzz (21.6 ± 1.22 g / 100 g of dry sample). The cultivars that presented the lowest fibre content were Azufrado Higuera (19.5 ± 2.71 g / 100 g of dry sample) for the *P. vulgairs* beans and Tattoo (18.00 ± 2.27 g / 100 g of dry sample) for the *V. faba* beans.

3.4.4 Starch and fibre content in *P. vulgaris* and *V. faba*

Starch, starch digestibility and fibre content varied between bean samples (table 3.2). Figure 3.4 shows the scanning electron microscopy (SEM) micrographs of native Borlotti bean starch granules. The size of most of the bean starch granules ranged between 5-10 μm. The total starch content in *P. vulgaris* samples ranged from 35.00 ± 1.72 to 42.30 ± 1.19 g / 100 g of dry sample. The *V. faba* samples presented starch contents that ranged from 39.90 ± 0.55 to 43.40 ± 0.46 g / 100 g of dry sample for the Divine and Tattoo cultivars, respectively. The digestibility of the starches also varied significantly. For the *P. vulgaris* samples, values ranged 55.8% in Azufrado Higuera cultivar to 72.3% in the Negro Jamapa cultivar. For *V. faba* samples, starch digestibility values ranged from 65.1% for the Buzz cultivar to 73.2% for the Tattoo cultivar.

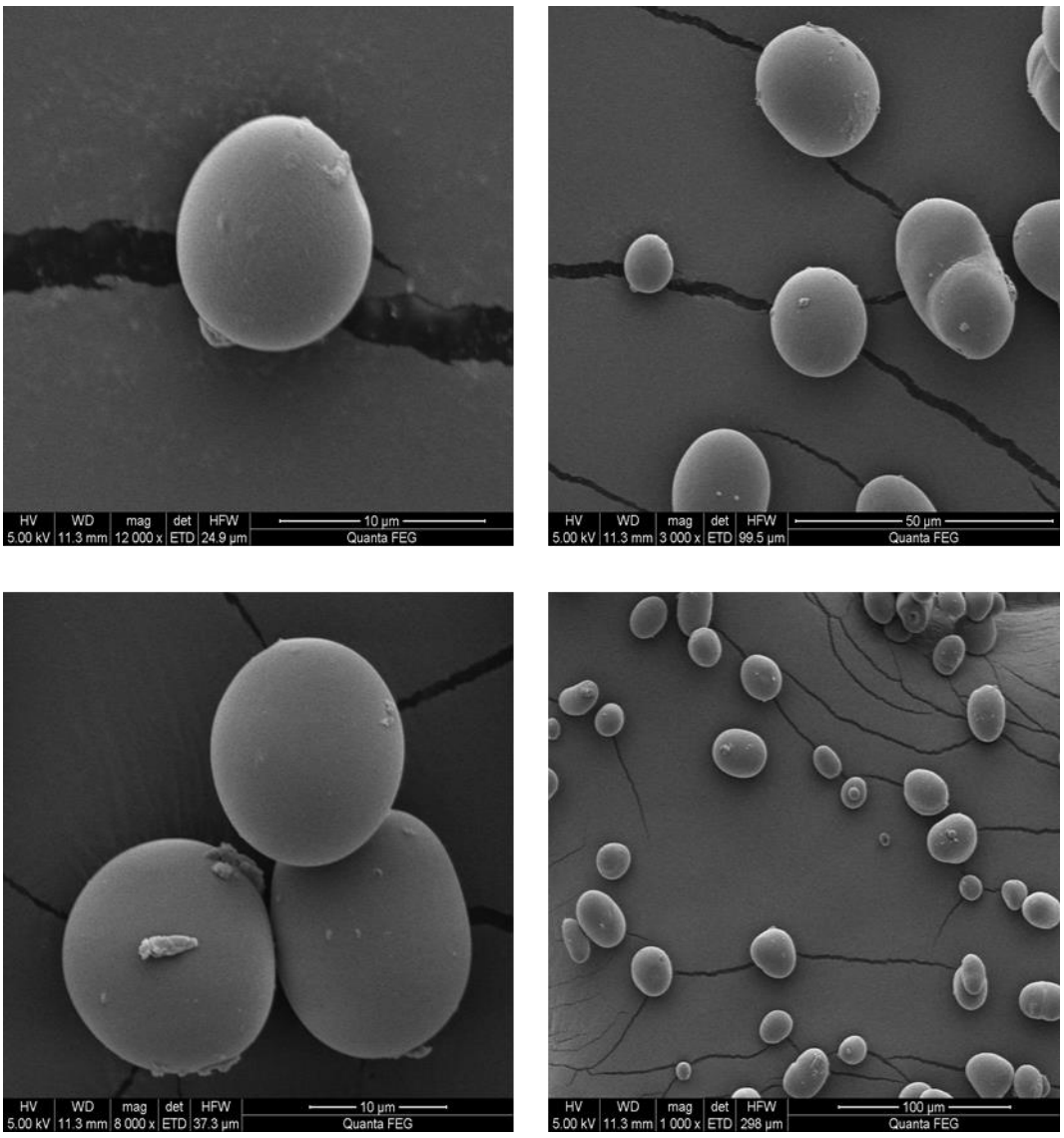


Figure 3.4. Scanning electron microscopy (SEM) micrographs of native Borlotti bean starch granules. As can be observed, the starch granules of this common bean (*P. vulgaris*) vary in size.

In the case of the fibre content, *P. vulgaris* beans presented higher amounts of fibre than the *V. faba* samples. The bean cultivar that showed the highest amount of fibre was Pinto Saltillo (30.50 ± 1.37 g / 100 g of dry sample).

3.4.5 Effect of domestic processing on polyphenol release

Both, soaking and cooking processing, resulted in the release of polyphenols. The amount of total polyphenols found in the soaking and cooking water was lower than 1 mg/gram of dry sample (table 3.3).

For most of the samples, around four times the amount of polyphenols was released by cooking compared to soaking.

3.4.6 Effect of starch and protein digestion on polyphenol release

Results showing the effect of individual enzymes on polyphenol release from Borlotti beans are shown in Figure 3.5. When the cooked bean sample was subjected to the digestion protocol without enzymes, the TPC value was 2.25 ± 0.16 mg GAE / g sample, which is about the same amount of TPC released during cooking. When starch and protein were digested separately, the TPC content detected increased to 30.78 ± 0.75 and 6.53 ± 0.46 (mg GAE / g sample) respectively. The largest release was obtained when amylase, amyloglucosidase and protease digestion were carried out (40.42 ± 2.49 mg GAE / g sample). However, the effects appear to be additive rather than providing significant synergistic effects.

Table 3.3 shows the amount of polyphenols released by combined enzymatic digestion (amylase, amyloglucosidase and protease) of cooked

Table 3.3. Total polyphenol content (TPC) of soaking and cooking water, enzymatic digestion supernatants of cooked beans and acid hydrolysis of fibre of *P. vulgaris* bean and *V. faba* bean samples.

Sample	Soaking (g/100 g dry sample)	Cooking (g/100 g dry sample)	Enzyme Digestion (g/100 g dry sample)	Acid Hydrolysis (g/100 g dry sample)
Bor	0.91 ± 0.04^{a}	2.22 ± 0.09^{a}	40.42 ± 2.49^{ab}	4.15 ± 0.45^{g}
Nej	0.50 ± 0.03^{ab}	1.97 ± 0.09^{b}	28.17 ± 1.90^{c}	14.18 ± 0.76^{b}
Azh	0.32 ± 0.01^{a}	0.58 ± 0.01^{h}	35.52 ± 3.68^{b}	6.51 ± 0.61^{f}
Pis	0.23 ± 0.01^{e}	1.32 ± 0.07^{d}	34.01 ± 2.78^{b}	9.79 ± 0.46^{d}
Mar	0.48 ± 0.01^{d}	1.75 ± 0.04^{c}	34.93 ± 1.40^{b}	15.13 ± 0.69^{b}
Azu	0.41 ± 0.01^{de}	1.06 ± 0.04^{g}	37.98 ± 1.01^{b}	8.42 ± 0.48^{e}
Peb	0.27 ± 0.00^{a}	1.13 ± 0.03^{ef}	28.63 ± 1.21^{c}	12.36 ± 0.31^{c}
Mean	0.45 ± 0.23	1.43 ± 0.57	34.24 ± 4.52	10.1 ± 4.05
Div	0.40 ± 0.01^{de}	1.83 ± 0.06^{bc}	41.71 ± 2.37^{ab}	8.58 ± 0.78^{de}
Tat	0.18 ± 0.00^{c}	1.12 ± 0.01^{f}	44.50 ± 4.40^{ab}	16.87 ± 0.57^{a}
Wiz	0.34 ± 0.02^{b}	2.16 ± 0.05^{a}	47.13 ± 3.30^{a}	15.90 ± 1.00^{ab}
Buz	0.21 ± 0.00^{f}	1.18 ± 0.03^{e}	43.15 ± 2.41^{a}	16.74 ± 0.34^{a}
Mean	0.28 ± 0.10	1.57 ± 0.51	44.12 ± 2.31	14.5 ± 3.98

Samples: bor: Borlotti; nej: Negro Jamapa; azh: Azufrado Higuera; pis: Pinto Saltillo; mar: Marcela; azu: Azufrado; peb: Peruano Bola; div: Divine; tat: Tattoo; wiz: Wizard: buz: Buzz. Results are expressed as mg GAE / g cooked sample (dry weight basis). Values show mean ± standard deviation (n =3 extractions). Different letters indicate significant differences between values in the same column ($p < 0.05$).

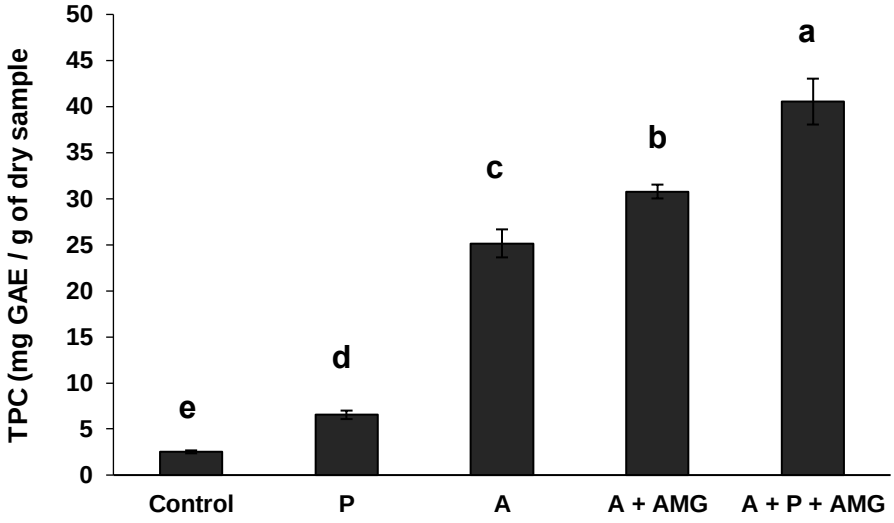


Figure 3.5. Total polyphenol content of digestion supernatants obtained by digestion of cooked Borlotti beans after digestion with individual enzymes. Protease (P), pancreatic α-amylase (A), pancreatic α-amylase and amyloglucosidase (A + AMG) and pancreatic α-amylase, amyloglucosidase and protease (A+ AMG + P). The control sample consisted of the pancreatic α-amylase mixed in the sodium maleate buffer. Data are mean of three separate digestions. Error bars show standard deviation of the mean. Different letters indicate significant differences ($p<0.05$).

beans. The amount of polyphenols released after digestion of the different beans was consistently high, with a mean release of 34.24 ± 4.52 and 44.12 ± 2.31 mg GAE / g sample for *P. vulgaris* and *V. faba* respectively.

Figure 3.6 shows Microscopy of native Borlotti bean starch granules using two different types of emission lights white light and UV light (280 nm). The granules fluoresce with the UV light; this may indicate the presence of polyphenols, since these conjugated compounds absorb UV light.

3.4.7 Effect of acid hydrolysis of undigested residue on polyphenol release.

Acid hydrolysis of the *P. vulgaris* beans undigested residue resulted in an additional release of polyphenols that ranged from 4.15 ± 0.45 to 16.87 ± 0.57 mg GAE / g of sample (table 3.3). *V. faba* presented a higher amount of polyphenols in comparison to *P. vulgaris* upon digestion and acid hydrolysis (Table 3.3).

3.4.8 Total polyphenols in raw and cooked bean methanolic extracts

Figure 3.7 shows the values of total polyphenols determined by the Folin-Ciocalteu method of raw and cooked bean methanolic extracts. For most samples, the cooking process decreased the amount of polyphenols. Wizard and Buzz preserved their total polyphenol values after cooking. The cooking process increased the total polyphenol content of negro jamapa beans.

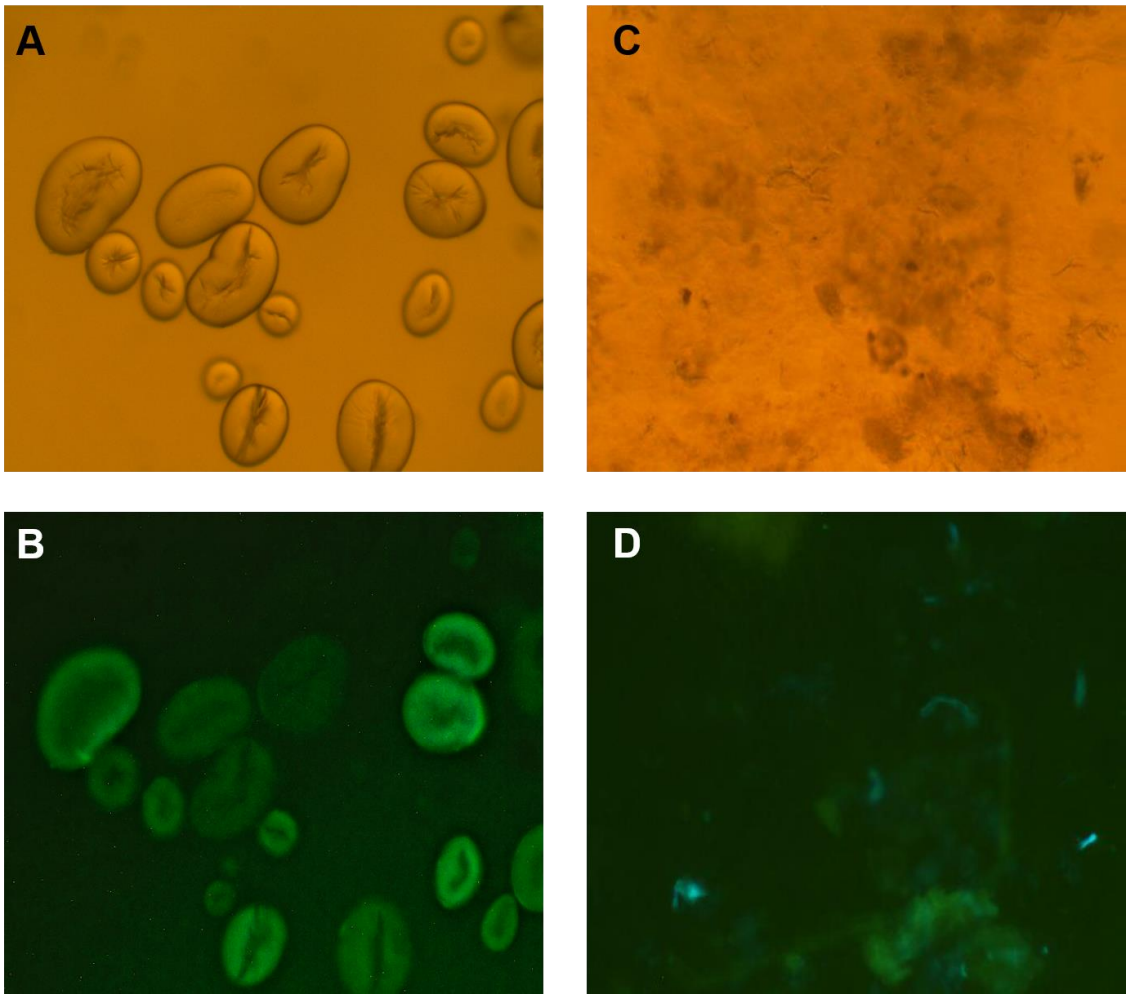


Figure 3.6. Microscopy of native Borlotti bean starch granules using two different types of emission lights: A) white light microscopy of native starch; B) UV light (280 nm) microscopy of native starch. C) white light microscopy of gelatinized starch; D) UV light (280 nm) microscopy of gelatinized starch.

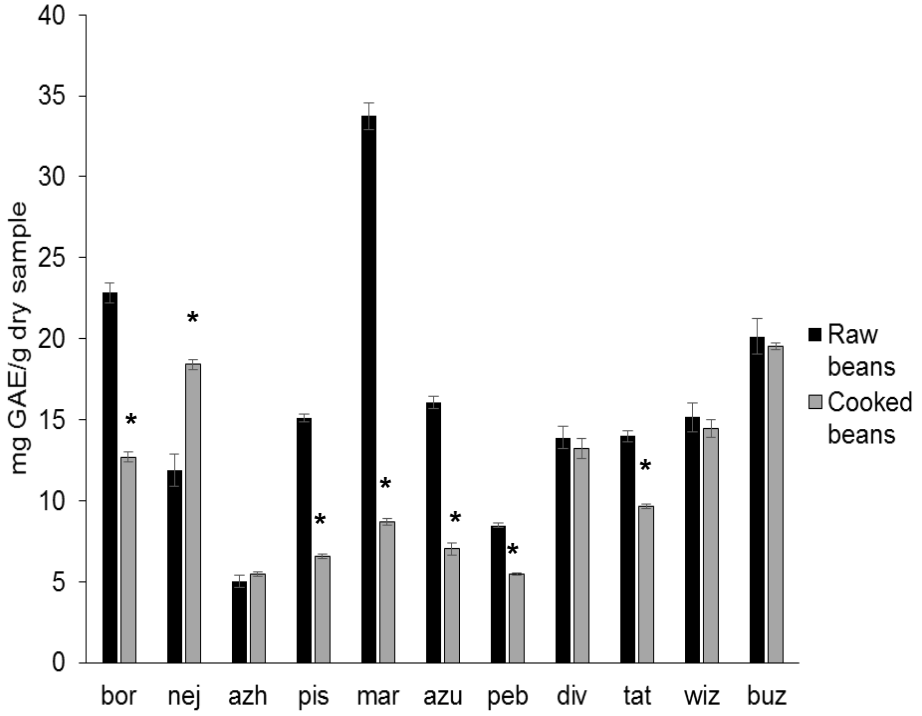


Figure 3.7. Total polyphenols determined by the Folin-Ciocalteu method of raw and cooked beans. Samples: bor: Borlotti; nej: Negro Jamapa; azh: Azufrado Higuera; pis: Pinto Saltillo; mar: Marcela; azu: Azufrado; peb: Peruano Bola; div: Divine; tat: Tattoo; wiz: Wizard: buz: Buzz. Results are expressed as mg GAE / g cooked sample (dry weight basis). Values show mean ± standard deviation (n =3 extractions). * indicates significant differences between raw and cooked sample ($p < 0.05$).

3.5 Discussion

The Folin-Ciocalteu assay corroborated the presence of polyphenols in the fractions analysed. Since phenolic molecules have a polar (hydroxyl groups) and a non-polar (aromatic ring) part, they are soluble in different solvents, including water (Dent et al., 2013). The polyphenolic profile of the soaking and cooking water, as well as the enzymatic digestion supernatant will be discussed in the next chapter.

For most of the samples, around four times the amount of polyphenols was released by cooking compared to soaking and solvent extraction. Other studies have shown similar effects of soaking and cooking (Xu et al., 2008; Chen et al., 2014). In this study, the amount of polyphenols extracted with acidified methanol ranged from 5 ± 0.22 to 34 ± 2.3 mg GAE / g sample. Previous studies have shown similar polyphenol values for *P. vulgaris* and *V. faba* (Akillioglu et al., 2010; Baginsky et al., 2013).

It was expected that the cooking process would decrease the total polyphenols in all of the samples. Yet, this was not observed in Wizard, Buzz and Negro Jamapa. This could be due to a possible hydrolysis during cooking of polyphenols attached to the food matrix in these samples. In the case of Negro Jamapa, for example, the undigested residue still maintained a dark colour even after acid hydrolysis of fibre. This might indicate that a large proportion of polyphenols could be mainly found bound to macronutrients in this sample.

The presence of calcium in the buffer enhanced the activity of pancreatic α-amylase. This enhancement has been observed on thermostable and physiological α-amylase. It has been reported that the presence of calcium

protects α-amylase from thermal degradation and prevents the spontaneous loss of biological activity (Saboury, 2002). The performance of α-amylase is enhanced by the presence of calcium ions, which bind to negatively charged amino acid residues of the enzyme, which helps maintain the 3-D structure (Muralikrishna and Nirmala, 2005).

It was found that the colorimetric DNS assay may not be a good method to measure products of α-amylase digestion in beans since other reducing molecules present in the sample or enzyme preparation (lactose) react with DNS and produce false positive results. The DNS assay is a useful method for the evaluation of reducing sugars; yet more specific methods like HPLC should be used for accurate quantification of sugars due to the lack of specificity and the susceptibility of the DNS assay to detect interferences (Rivers et al., 1984).

The amount of Borlotti bean fibre recovered (13.72 g/ 100 g) with the optimized method was higher than the one reported by the Borlotti bean producer (6.3 g/ 100 g). The fibre content obtained in this study is also higher than the mean NSP values for boiled legumes (4.23 g/ 100 g). This difference may be partly due to the presence of indigestible materials such as resistance starch and lignin (Wolters et al., 1992). The fibre values obtained for all the beans are similar to those obtained for cooked kidney beans (Aldwairji et al., 2014).

The results suggest that most polyphenols in Borlotti beans are bound to the bean matrix, with starch being the main limiting factor of polyphenol bioaccessibility. Studies have shown that the interaction between polyphenols and starch is possible. Takahama and Hirota (2013) studied the effect of the molecular structure of polyphenols (epicatechin-dimetilgallate, rutin and

vignacyanidin) after adding starch solutions; a shift in the UV-spectrum of the native phenolics was observed after adding the starch solutions (Takahama and Hirota, 2013). This could indicate a change in the molecular structure of the polyphenol after adding the starch solution that could be due to an interaction between the polyphenol and starch. Barros et al. (2012) observed that proanthocyanidins interacted better with amylose than with amylopectin, while monomeric polyphenols interacted equally with both amylose and amylopectin (Barros et al, 2012). This shows that the type of interaction (polyphenol with amylose or polyphenol with amylopectin) might depend on the type of polyphenol.

Non-covalent interactions between flavonoids and starch have been reported through observations of textural changes of starch (Bordenave et al., 2014). Wu et al. (2009) observed that the addition of flavan-3-ols from tea to rice starch retards retrogradation, although the mechanism of the interaction between polyphenols and starch is not yet understood (Wu et al., 2009). Amylose may form single left handed helices with smaller molecules which interact hydrophobically within the cavity of the helix (Obiro et al., 2012). Cohen et al. (2008) found that the isoflavone genistein, formed this type of interaction with high-amylose corn starch and pure amylose (Cohen et al., 2008). Van Hung et al. (2013) showed that ferulic acid interacts with the hydrophobic core of the amylose helix in cassava starch (Van Hung et al., 2013).

While it is relatively well established that polyphenols are weak inhibitors of α-amylase (Rohn et al., 2002), it is not clear whether some of the inhibition may actually be through interactions of the polyphenol with the starch substrate, as well as interaction with the enzyme itself. There is not much known about the nature of the interactions between polyphenols and starch. It is believed that

they might interact by hydrogen bonds or form inclusion complexes with amylose (Zhu, 2015). It is well known that amylose can interact with small molecules by forming inclusion complexes (single, left handed helices) that are known as V-type amylose inclusion complex in which the small molecules interact hydrophobically within the cavity of the amylose helix (Obiro et al., 2012). In a study performed by Cohen et al. (2008; 2011), genistein was mixed with an amylose solution in an alkaline solution. X-ray diffraction was used in order to understand the nature of the complex formed. Their results showed that the volume of the amylose increased in some parts of the cavity, which could indicate the formation a V-type inclusion complex with the genistein molecule.

In a similar study, Zhang et al. (2011) studied the starch/polyphenol interaction by mixing quercetin with maize starch. X-ray diffraction results of the quercetin/starch complexes showed that the crystal structure of native maize starch disappeared and new crystalline regions formed. This could indicate that the interaction of starch with polyphenols might alter the original starch structure.

Chai et al. (2013) performed a similar study by mixing high-amylose maize starch with tea catechins. Fourier transform infrared spectral analysis and X-ray diffraction results showed tea catechins might bridge amylose helixes together, indicating that the tea catechins did not form a V-type complex as mentioned previously. In the same study, results of iodine binding and X-ray powder diffraction analysis revealed that the interactions between the tea catechins and high-amylose maize starch might take place by hydrogen bonds. Wu et al. (2011) studied the interaction of tea catechins with rice starch with fourier transform infrared spectral analysis and proton nuclear magnetic resonance; their results indicated that the interactions between the polyphenols

and the starch were non-inclusive and molecular interactions might take place by hydrogen bonding.

In summary, the studies performed lead to different conclusions. Hence, the nature of the interactions between starch and polyphenols is not known and further studies must be performed in order to have more information about this type of molecular interaction. Some polyphenols may lack hydrophobicity and might be too large to form a V-type complex (Zhu et al, 2015). Thus, it seems that the type of interaction might depend on the size and hydrophobicity of the polyphenols.

Microscopy results obtained in the present study indicate that large starch granules isolated from Borlotti beans fluoresce under UV light (Figure 3), indicating localization of polyphenols within the granules. The fluorescence is not lost upon washing with water or acetone showing a strong interaction that is not disrupted by aqueous solvents. Only 16% of TPC was released upon protease digestion of Borlotti beans. Polyphenols may bind with proteins mainly by non-covalent interactions (hydrophobic, van der Waals, hydrogen bridge binding and ionic interactions) (Jakobek, 2015). Our data suggests that starch rather than protein is the main limiting factor in the bioaccessibility of polyphenols from beans. The fluorescence microscopy image suggests that polyphenols bind to starch. This behaviour has recently been observed by Gomez-Mascarque et al. (2017) in potato. This is the first time this has been observed in beans.

Polyphenols bind with components such as cellulose, hemicellulose (e.g. arabinoxylans), lignin and pectin and are normally present as glycosides, linked through an hydroxyl group with a single or multiple sugars (O-glycosides) or

through carbon–carbon bonds (C-glycosides) (Acosta-Estrada et al., 2014). In addition, these molecules may interact with cell well components by hydrogen bonds or hydrophobic interactions (Jakobek, 2015). As mentioned previously, *V. faba* presented a higher amount of polyphenols in comparison to *P. vulgaris* following digestion and acid hydrolysis.

The differences in TPC may be due to environmental factors such as light, humidity, temperature, minerals and the supply of water and CO_2 during the development of the bean plant that may influence the secondary metabolite production (Ramakrishna and Ravishankar, 2011). *V. faba* were grown in the temperate climate of the UK, compared to the *P. vulgaris* (except Borlotti) beans grown in the warmer and moister climate of Western Mexico. The environmental differences of both regions could of had an influence in the difference of TPC values found in both groups of samples analysed in the present study.

3.6 Conclusion

Most of the polyphenols quantified in this study were released following enzymatic digestion, particularly starch digestion. Hence, the digestion of starch may increase the bioaccessibility of bean polyphenols. The fluorescence observed in starch granules may indicate that some bean polyphenols are within the starch granules structure. The implications of this observation are not known. Digestion could have a positive effect on the bioavailability of polyphenols.

Although Borlotti bean was not contemplated in the initial stage of this study, and was only used while waiting for the Mexican beans to arrive, it was determined that including this sample in the study was quite useful since this

bean is consumed in the UK and little information about its fibre and polyphenolic content is available in the literature.

At this stage, the phenolic composition of the soaking water, cooking water and *in vitro* digestion supernatants is unknown. The next step of the study was to identify the polyphenols that were released during the domestic processing of beans and upon *in vitro* digestion.

Chapter 4

LCMS identification of polyphenols released during domestic processing and *in vitro* digestion of *P. vulgaris* and *V. faba*

Summary

- The polyphenolic profile of the beans was slightly variable between the beans of the same species, which is reflected in their different appearance and colour of the bean's hull.
- Most of the polyphenols are lost during domestic processing. The compounds lost during soaking and cooking of beans are mainly phenolic acids and flavonoids that are likely to come from the bean hull.
- The polyphenols released upon *in vitro* digestion were sinapic acid, *p*-coumaric acid and catechin.
- Most of the polyphenols were glycosylated or derivatives of polyphenol aglycones and could not be identified using the available aglycone standards. Therefore, the masses provided by the MS chromatograms were a useful tool to identify the polyphenols present in the fractions.

4.1 Introduction

Common beans are a rich source of biologically active phytochemicals, such as polyphenols, which may be beneficial for human health (Rochfort and Panozzo, 2007). Polyphenols are secondary plant metabolites that constitute a group of compounds that are widely distributed in foods of plant origin (Amarowicz and Pegg, 2008). These compounds are produced in plants through the shikimic acid pathway; different types of polyphenols include phenolic acids (benzoic and hydroxycinnamic acids) and flavonoids such as flavonols and anthocyanins, stilbenes, and lignans (Manach et al., 2004).

Polyphenols that have been identified in common beans (*P.* vulgaris) are flavonoids (including flavonols and their corresponding glycosylated forms) anthocyanins, proanthocyanidins, isoflavones, coumarins, and phenolic acids (hydroxybenzoic and hydroxycinnamic acids) (Takeoka et al., 1997, Beninger and Hosfield, 1999, Beninger and Hosfield, 2003, Choung et al., 2003, Romani et al., 2004, Aparicio-Fernandez et al., 2005, Diaz-Batalla et al., 2006, Macz-Pop et al., 2006, Lin et al., 2008, Aguilera et al., 2011). The polyphenolic composition of beans varies with their colour. In general, dark coloured beans present a higher amount of anthocyanins (Lin et al., 2008). The polyphenols that have been identified by high performance liquid chromatography, in fava beans (*V. faba*) are condensed tannins, procyanidins, prodelphinidins, flavonols and flavones (Baginsky et al., 2013).

The use of legumes as food is sometimes limited due to the presence of certain anti-nutritional factors. Among these are phytates, polyphenols, enzyme inhibitors (trypsin, chymotrypsin, and α-amylase) and hemagglutinins (Fernández et al., 1997, Alonso et al., 1998).

It is necessary to subject beans to a cooking process before their consumption in order to remove these factors. The most common bean preparation methods are soaking, cooking, germination or fermentation (Duenas et al., 2009; Chandrasekara et al., 2012).

Soaking is normally performed before cooking in order to decrease the cooking time of the legumes and remove anti-nutritional factors such as trypsin inhibitors (Siddiq and Uebersax, 2012). El-Hady and Habiba (2003) found that soaking significantly decreased antinutrients (phytic acid, tannins α-amylase and trypsin inhibitors) and polyphenols; total polyphenol content decreased by 11% in fava beans and by 20% in common beans (El-Hady and Habiba, 2003).

In order to cook legumes, thermal processes like boiling are normally applied to the seeds; this process has been the most common method used since humankind began to consume beans as food. Yet, thermal processes normally also result in a decrease in the polyphenol content (Turkmen et al., 2005, Martinez-Herrera et al., 2006). Hence, the soaking and cooking processes might dramatically affect the final polyphenol content and potential health benefits.

The results discussed in the previous chapter showed that significant amounts of polyphenol are released from beans during digestion. However, the composition of the polyphenols released is not known. Hence, it is important to identify the polyphenols released upon bean digestion in order to have an idea of which polyphenols would be bioaccessible and potentially bioavailable. Therefore the aim of this Chapter was to analyse different fractions obtained during soaking, cooking and digestion of Borlotti, Mexican *P. vulgaris* and British *V. faba* beans.

4.2 Methodology

Details of the method and equipment used are described in Chapter 2. The mass spectrophotometer was set in negative mode to assay for ions as shown in table 4.1. The ions selected were according to the data reported by Lopez et al. (2013) for beans (*P. vulgaris*).

4.3. Aim and objective

4.3.1. Aim

Identify the individual polyphenols present in *P. vulgaris* and *V. faba* beans by LCMS analysis.

4.3.2. Objectives

1. Identify the polyphenols present in the soaking and cooking water to determine which polyphenols are lost during the domestic processing of the beans.
2. Identify the polyphenols released after *in vitro* digestion of beans.

4.4 Results

It was observed that the polyphenolic profiles varied between samples and the different fractions analysed, as shown in figures 4.1-4.3. Results for each fraction are described below.

4.4.1. Soaking water

In the case of the *P. vulgaris* beans, the hydroxycinnamic acids released during soaking were caffeic and sinapic acid (table 4.2). The presence of either one of these compounds varied amongst beans. Only two beans, Pinto Saltillo and Azufrado, presented both of these hydroxycinnamic acids. In general, three flavonols were identified in this fraction, catechin, kaempferol and kaempferol glucoside. Pinto Saltillo and Azufrado were the only beans that presented all of these three flavonols. Azufrado Higuera did not present any flavonol. Hence,

Table 4.1. Molecular ions used for the identification of hydroxycinnamic acids, flavonols and anthocyanins by HPLC-DAD-MS in soaking water, cooking water and enzymatic *in vitro* digestion supernatants.

Compounds (*hydroxycinnamic acids*)	$[M-H]^-$	Identification Code
Gallic acid	169	H1
Ferulyl aldaric acid	285	H2
Ferulic acid	193	H3
p-Coumaryl aldaric acid	355	H4
p-Coumaric acid	163	H5
Caffeic acid	179	H6
Sinapyl aldaric acid	415	H7
Sinapic acid	223	H8
Protocatechuic acid	153	H9

Compound (*flavonols*)	$[M-H]^-$	Identification Code
Catechin/epicatechin	289	F1
Procyanidin dimer	577	F2
Procyanidin trimer	865	F3
Hesperetin derivative	579	F4
Naringenin 7-glucoside	433	F5
Naringenin	271	F6
Quercetin derivative	609	F7
Quercetin Derivative	463	F8
Quercetin 3-glucoside acetate	505	F9
Quercetin	301	F10
Kaempferol derivative	447	F11
Kaempferol	285	F12
Myricetin 3-glucoside	479	F13
Biochanin B 7-glucoside	429	F14
Myricetin derivative	521	F15
Myricetin	317	F16
Apigenin 7-glucoside	431	F17

Biochanin B 7-glucoside	445	F18
Genistein hexose	431	F19
Resveratrol glucoside	389	F20

Compound (*anthocyanins*)	$[M-H]^-$	Identification code
Delphinidin 3-glucoside	465	A1
Cyanidin 3-glucoside	449	A2
Pelargonidin glucoside acylated	447	A3
Pelargonidin 3-malonylglucoside	519	A4
Pelargonidin 3,5-diglucoside	595	A5
Pelargonidin 3-glucoside	433	A6
Delphinidin glucoside acylated	479	A7
Petunidin feruloyl glucose	507	A8

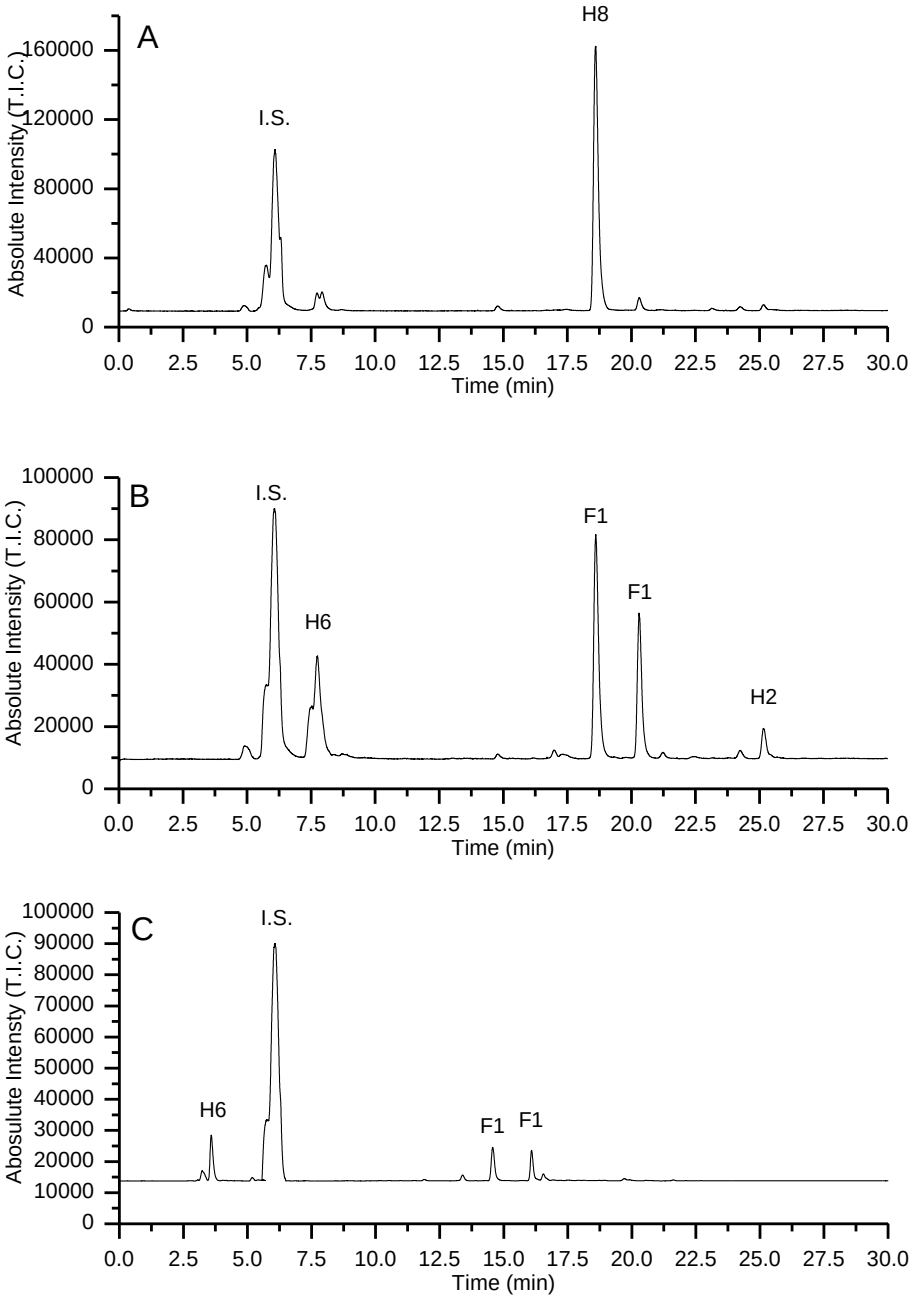


Figure 4.1. Chromatograms obtained from different fractions of Borlotti bean (*P. vulgaris*). A) soaking water; B) cooking water; C) enzymatic digestion I.S.: gallic acid; H2: feruloyl aldaric acid; H6: caffeic acid; H8: sinapic acid; F1: catechin/epicatechin; supernatant.

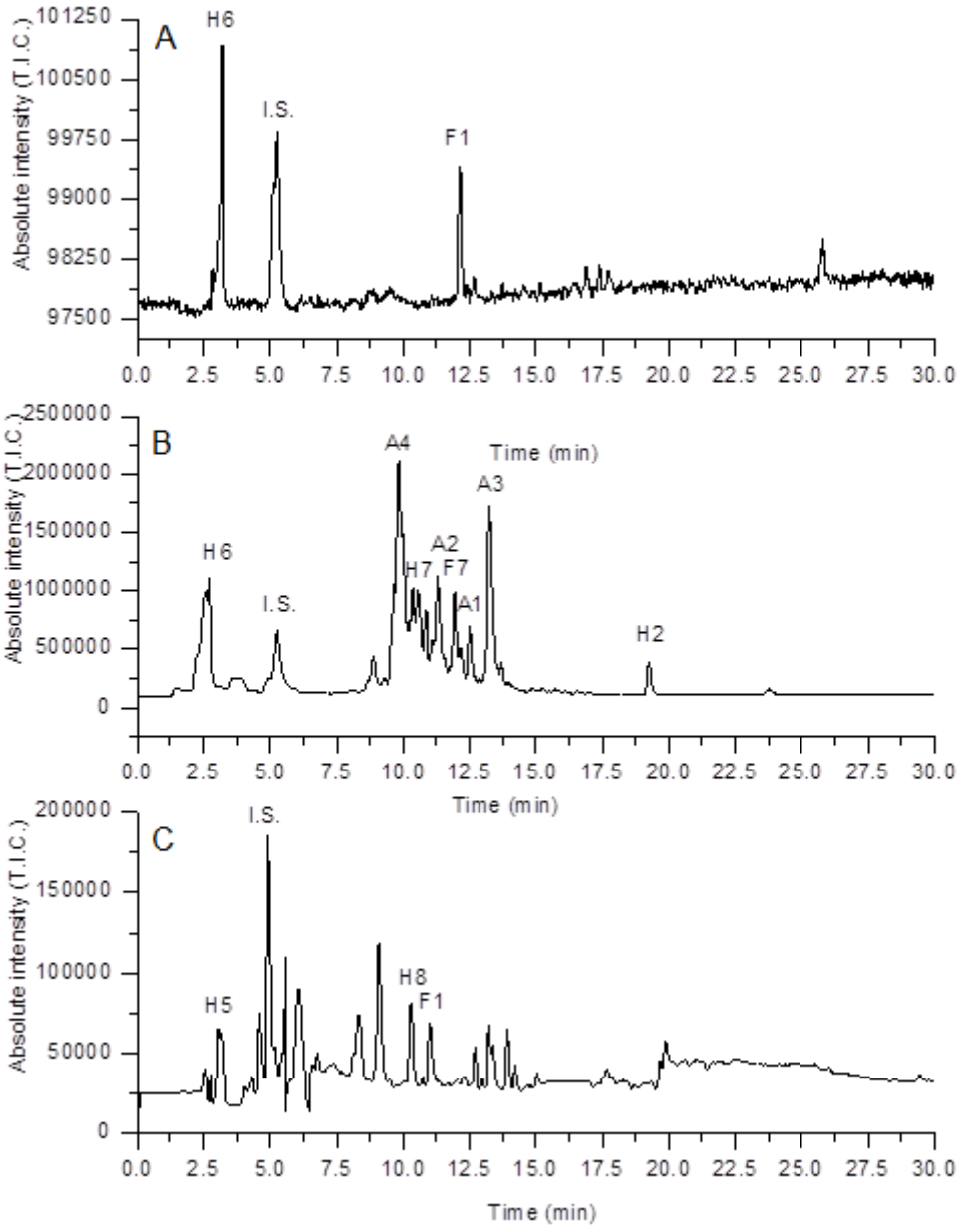


Figure 4.2. Chromatograms obtained from different fractions of Marcela bean (*P. vulgaris*). A) soaking water; B) cooking water; C) enzymatic digestion supernatant. I.S.: gallic acid; H2: feruloyl aldaric acid; H5: *p*-coumaric acid; H6: caffeic acid; H7: sinapyl aldaric acid; H8: sinapic acid; F1: catechin/epicatechin; F7: quercetin derivative; A1: delphinidin 3-glucoside; A2: cyanidin 3-glucoside; A3: pelargonidin glucoside acylated.

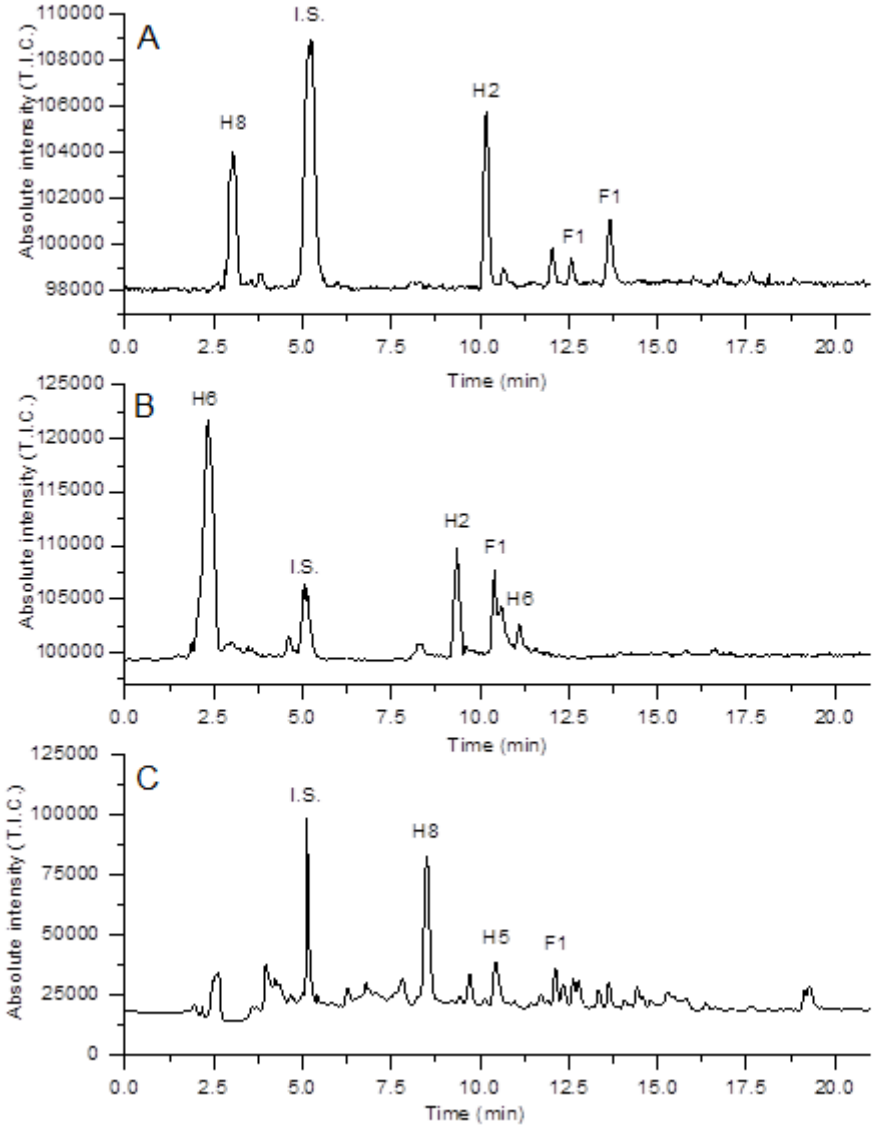


Figure 4.3. Chromatograms obtained from different fractions of Wizard bean (*V. fava*). A) soaking water; B) cooking water; C) enzymatic digestion supernatant. I.S.: gallic acid; H2: feruloyl aldaric acid; H5: *p*-coumaric acid; H8: sinapic acid; F1: catechin/epicatechin.

Table 4.2. Phenolic compounds identified by HPLC-DAD-MS found in soaking water of *P. vulgaris* beans. bor: Borlotti; nej: Negro Jamapa; azh: Azufrado Higuera; pis: Pinto Saltillo; mar: Marcela; azu: Azufrado; peb: Peruano Bola.

Compounds (*hydroxycinnamic acids*)	$[M-H]^-$	bor	nej	Azh	pis	mar	Azu	Peb
Caffeic acid	179	-	-	X	X	X	X	X
Sinapic acid	223	X	-	-	X	-	X	-

Compound (*flavonols*)	$[M-H]^-$	bor	nej	Azh	pis	mar	azu	Peb
Catechin	289	X	-	-	X	X	X	X
Kaempferol derivative	447	-	-	-	X	-	X	
Kaempferol	285	-	X	-	X	-	X	X

caffeic acid was the only polyphenol that was identified in Azufrado bean. The polyphenolic content of the rest of the beans was variable.

V. faba beans presented three hydroxycinnamic acids (feruloyl aldaric acid, caffeic acid and sinapic acid) and one flavonol (catechin) (table 4.3). Feruloyl aldaric acid was identified in all of the *V. faba* beans. Caffeic acid was found in all of the beans except Wizard. Sinapic acid was found only in Wizard and Buzz beans. Catechin, the only flavonol identified, was only found in Divine and Wizard faba beans.

4.4.2. Cooking water

P. vulgaris bean cooking water presented the most complex polyphenolic profiles (table 4.4). Yet, it was observed that Borlotti bean had a less complex polyphenolic profile in comparison to the rest of the Mexican *P. vulgaris* beans.

Sinapoyl aldaric acid was identified in all of the *P. vulgaris* beans, except Borlotti. Sinapic acid was also found in all of the samples, except Azufrado Higuera. Ferulic acid was identified in Azufrado Higuera and Peruano Bola beans. Borlotti and Marcela presented *p*-coumaric acid in their polyphenolic profile. Finally, *p*-coumaroyl aldaric acid was found in all of the beans except Borlotti and Azufrado.

The flavonols identified in the *P. vulgaris* beans are shown in table 4.4. Kaempferol was found in all of the samples except Borlotti beans. Hesperetin 7-glucoside and a hesperetin derivative were identified only in Azufrado beans. Myricetin 3-glucoside was identified only in Negro Jamapa beans. As mentioned previously, this fraction was the only one that presented anthocyanins (table 4.4). Pelargonidin 3-malonylglucoside was found in all of the *P. vulgaris* beans except for Borlotti. Catechin was not identified in any of the cooking waters,

Table 4.3. Phenolic compounds identified by HPLC-DAD-MS found in soaking water of *V. faba* beans. div: Divine; tat: Tattoo; wiz: Wizard; buz: Buzz.

Compounds (*hydroxycinnamic acids*)	$[M-H]^-$	div	tat	wiz	buz
Ferulyl aldaric acid	285	X	X	X	X
Caffeic acid	179	X	X	-	X
Sinapic acid	223	-	-	X	X

Compound (*flavonols*)	$[M-H]^-$	div	tat	wiz	buz
Catechin/epicatechin	289	X	-	X	-

Table 4.4. Phenolic compounds determined HPLC-DAD-MS found in cooking water of *P. vulgaris* beans. bor: Borlotti; nej: Negro Jamapa; azh: Azufrado Higuera; pis: Pinto Saltillo; mar: Marcela; azu: Azufrado; peb: Peruano Bola.

Compound (*hydroxycinnamic acids*)	$[M-H]^-$	Bor	nej	azh	pis	mar	azu	Peb
Ferulic acid	193	-	-	X	-	-	-	X
p- Coumaryl aldaric acid	355	-	X	X	X	X	-	X
p-Coumaric acid	163	X	-	-	-	X	-	-
Sinapyl aldaric acid	415	-	X	X	X	X	X	X
Sinapic acid	223	X	X	-	X	X	X	X
Caffeic acid	179	-	X	X	X	X	X	X

Compound (*Flavonols*)	$[M-H]^-$	Bor	nej	azh	pis	mar	azu	Peb
Hesperetin 7-glucoside	463	-	-	-	-	-	X	-
Hesperetin derivative	579	-	-	-	-	-	X	-
Quercetin derivative	609	-	X	-	X	X	X	-
Quercetin Derivative	463	-	X	-	-	X	-	-
Kaempferol derivative	447	-	-	-	-	X	X	X
Kaempferol	285	-	X	X	X	X	X	X
Myricetin 3-glucoside	479	-	X	-	-	-	-	-

Compound (*anthocyanins*)	$[M-H]^-$	bor	nej	azh	pis	mar	azu	peb
Biochanin B 7-glucoside	429	-	X	-	-	-	-	-
Delphinidin 3-glucoside	465	-	-	-	-	-	-	X
Cyanidin 3-glucoside	449	-	-	X	-	X	X	-
Delphinidin glucoside acylated	479	-	X	-	-	-	-	-
Pelargonidin glucoside acylated	447	-	X	X	-	-	-	-
Pelargonidin 3-malonylglucoside	519	-	X	X	X	X	X	X
Catechin derivative	289	X	-	-	-	-	-	-

except for Borlotti beans. *V. fava* beans had a much less varied profile in comparison to the *P. vulgaris* beans (table 4.5). No anthocyanins were identified for these bean species. The hydroxycinnamic acids identified were ferulic acid, kaempferol and sinapic acid. Catechin was the only flavonol identified and it was present in Divine and Wizard beans.

4.4.3 Enzymatic digestions

For both species, *P. vulgaris* and *V. fava* beans two hydroxycinnamic acids (*p*-coumaric and sinapic acid) and one flavonol (catechin) were identified (table 4.6).

Catechin was released upon digestion in all of the *P. vulgaris* bean samples. Sinapic acid was found in most of the samples (except Azufrado Higuera and Pinto Saltillo). Meanwhile, *p*-coumaric acid was identified in all the beans except Borlotti, Pinto Saltillo and Peruano Bola beans.

The polyphenols present in the *V. fava* digestion supernatant fraction are shown in table 4.7. Catechin was identified in the *V. fava* beans, except Wizard beans. Hydroxycinnamic acids were not identified in the Divine and Buzz beans. Sinapic acid was identified in the Wizard bean, whereas *p*-coumaric acid was found in Tattoo and Wizard beans.

4.5 Discussion

For this part of the study, HPLC-DAD-MS was used as a main tool for the identification of the polyphenol ion derivatives present in the different fractions. Normally, MS is used as an additional tool to confirm HPLC results. The compounds identified were derivatives of polyphenols (such as glycosylated polyphenols), rather than the respective free aglycones. Since polyphenol standards are mostly available for the aglycone rather than their derivatives, it

Table 4.5. Phenolic compounds determined by HPLC-DAD-MS found in cooking water of *V. faba* beans. div: Divine; tat: Tattoo; wiz: Wizard; buz: Buzz.

Compound	$[M-H]^-$	div	tat	wiz	buz
Catechin/epicatechin	289	X	-	X	-
ferulic acid	193	-	-	-	X
Kaempferol	285	X	-	X	X
Sinapic acid	223	-	-	-	X
Caffeic acid	179	X	X	X	X

Table 4.6. Phenolic compounds determined by HPLC-DAD-MS found in digestion supernatants of *P. vulgaris* beans. bor: Borlotti; nej: Negro Jamapa; azh: Azufrado Higuera; pis: Pinto Saltillo; mar: Marcela; azu: Azufrado; peb: Peruano Bola.

Phenolic compound equivalents	$[M-H]^-$	bor	nej	azh	pis	mar	azu	peb
Coumaric acid	163	-	X	X	-	X	-	-
Sinapic acid	193	X	X	-	-	X	X	X
Catechin/epicatechin	289	X	X	X	X	X	X	X

Table 4.7. Phenolic compound derivatives determined by HPLC-DAD-MS found in digestion supernatants of *V. faba* beans. div: Divine; tat: Tattoo; wiz: Wizard; buz: Buzz.

Phenolic compound equivalents	$[M-H]^-$	div	tat	wiz	buz
Coumaric acid	163	-	X	X	-
Sinapic acid	193	-	-	X	-
Catechin/epicatechin	289	X	X	-	X

was not possible to perform an identification of most peaks using retention times of known aglycone standards and UV chromatograms. For this reason, we were only able to identify the compounds by their mass. Different types of individual polyphenols were observed between the soaking and cooking samples. These may primarily originate from the seed coat that is in contact with soaking and cooking water. These fractions presented great variability in polyphenol content, reflective of the samples‘ varied phenotypes. These polyphenols have likely functions in protecting seeds from environmental conditions, and are mainly lost during the cooking process. It was observed that some polyphenols remained bound to the undigested residue obtained after the *in vitro* digestion. After acid hydrolysis, it was seen that the remaining residue still had some colour left, especially for the most colourful samples such as Negro Jamapa and Marcela. This may indicate that bean polyphenols may form strong chemical bonds with the cell wall of beans.

It was observed that some peaks corresponded to the same ion count but had different retention times. Lin et al. (2008) observed that the retention times between glycosylated bean polyphenols and their respective aglycone was different (Lin et al., 2008). Hence, glycosylation affects retention times. The same study reported that glycosylated polyphenols usually appear earlier than their respective aglycone. In nature, most polyphenols in plants are glycosylated with different sugar units bound at different positions of the aglycone (Ramakrishna and Ravishankar, 2011). When starch and protein are digested, it may be possible that the polyphenols released are associated with a sugar or amino acid. Hence, it is possible that different peaks with the same ion count could be glycosylated polyphenols with the same aglycone but bound to different sugars.

For the soaking and the cooking water, Mexican common beans (*P.* vulgaris) presented more polyphenolic compounds in comparison to the Borlotti beans (*P.* vulgaris) and the fava beans (*V. faba*). Borlotti beans are of European origin whereas the fava beans were grown in the UK. It is possible that the different environmental features and growing conditions in the regions of origin of the beans could have an influence in the differences presented in the polyphenolic profile. As mentioned previously, polyphenol compounds are produced through the shikimic acid pathway (Manach et al., 2004). This pathway is induced as a response to environmental stress, like UV-B radiation, drought, chilling and frost, heat, ozone, pollutants (heavy metals, pesticides and aerosols), wind, soil movement, submergence, attack by pathogens, wounding, salinity, drought, flooding, mineral salts, gaseous toxins or nutrient deficiencies (Akula and Ravishankar, 2011, Dixon and Paiva, 1995).

Espinosa-Alonso et al. (2006) determined the polyphenolic profile of Mexican common beans (*P. vulgaris*) (Espinosa-Alonso et al., 2006). Their study revealed that the samples analysed for that study also presented a broad polyphenolic profile, including phenolic acids, flavonols and anthocyanins. Their beans contained caffeic acid, syringic acid, *p*-coumaric acid, ferulic acid, sinapic acid, quercetin and kaempferol, which were also found in the Mexican beans analysed during this study. This might mean that the specific environmental condition of Mexico might stimulate different biochemical pathways that would lead to the production of diverse polyphenolic compounds.

As mentioned previously, the ions used for the MS analysis were selected according to the method performed by Lopez and Denicola (2013). The polyphenolic profile determined in this study is similar to the samples used

in the present study. The samples used in the present study contained caffeic acid and catechin, which were not reported in the published study.

In the case of the digestion supernatants, in general most of the samples presented a similar polyphenolic profile mainly comprised by phenolic acids and one flavonol (catechin). This might indicate that these compounds might interact with the food matrix (cotyledon) in comparison with the polyphenols identified in the soaking and cooking water, which included a more diverse polyphenolic content that included flavonols, anthocyanins and phenolic acids. Ranilla et al. (2007) analysed the total polyphenols of bean hulls and cotyledons separately; their results showed that bean hulls contain flavonoids and hydroxycinnamic acid whereas the cotyledons contained only hydroxycinnamic acids (Ranilla et al., 2007). The phenol explorer databases for *P. vulgaris* and *V. fava* (Table 4.10) show a similar tendency, that is, bean seed coats present a more variable polyphenolic profile (which includes flavonols, anthocyanins and phenolic acids); meanwhile, dehulled beans (cotyledon) contain mainly phenolic acids (http://phenol-explorer.eu/). This indicates that the polyphenols lost during domestic processing might mainly come from the seed coats.

Since the cooked beans were homogenised prior to digestion, the cotyledon components would be exposed. The function of polyphenols in the cotyledon is not clear, but they could act as a protection mechanism against invasive pathogens or herbivores. The observation that bean starch granules are fluorescent under UV light indicates a strong interaction between starch and polyphenols, supported by the digestion experiments in chapter 3. The way the polyphenols are incorporated into the granules and what their function is not clear.

Table 4.10. Polyphenols identified and quantified in raw whole and dehulled fava beans (*V. faba*), white beans and black beans.

Legume	Polyphenol type	Compound	Mean content (mg/ 100 g FW)
Raw fava beans (*V. faba)*	Flavonol	Catechin	12.83
		Epicatechin	22.51
		Epigallocatechin	14.03
Dehulled raw fava beans (*V. faba*)	Hydroxycinnamic acid	Ferulic acid	1.50
		p-coumaric acid	1.60
Raw white beans (*P. vulgaris*)	Flavanols	Catechin	0.01
		Epicatechin	0.09
		Procyanidin dimer B3	0.03
	Flavonol	Kaempferol	0.82
		Kaempferol 3-O-acetyl glucoside	7.15
		Kaempferol 3-O-glucoside	30.85
		Kaempferol 3-O-xylosyl glucoside	11.14
	Isoflavone	Daidzein	0.30
		Genistein	0.20
Dehulled raw white beans (*P. vulgaris*)	Hydroxycinnamic acid	5-O-caffeoylquinic acid	0.06
		Ferulic acid	0.43
		Sinapic acid	0.04
Raw black beans (*P. vulgaris*)	Anthocyanin	Cyanidin	1.63
		Cyanidin 3,5-O-diglucoside	1.98
		Cyanidin 3-O-glucoside	3.99
		Delphinidin 3-O-feruloyl-glucoside	1.10
		Delphinidin 3-O-glucoside	14.50
		Malvidin 3-O-glucoside	0.60
		Pelargonidin	0.95
		Pelargonidin 3,5-O-diglucoside	1.54
		Pelargonidin 3-O-glucoside	12.60
		Peonidin	1.36

		Petunidin 3-O-glucoside	0.80
	Flavonol	Kaempferol	1.80
		Kaempferol 3-O-acetyl glucoside	3.40
		Kaempferol 3-O-glucoside	6.60
	Isoflavonoid	Daidzein	0.80
		Genistein	0.60
Dehulled raw black beans (*P. vulgaris*).	Hydroxycinnamic acid	5-O-caffeoylquinic acid	0.25
		Ferulic acid	0.35
		Sinapic acid	0.04

Source: http://phenol-explorer.eu/

Chen et al. (2015) performed an *in vitro* digestion of cranberry beans (*P. vulgaris*), which are very similar to Borlotti beans (pink spots, but lighter beige colour) (Chen et al., 2015). Upon digestion, it was determined that the main compounds released were *p*-coumaric, ferulic, and sinapic acids. For Borlotti beans, sinapic acid was also detected in addition to catechin. Some of the beans also showed the release of *p*-coumaric acid upon digestion (Negro Jamapa, Azufrado Higuera, Marcela, Tattoo and Wizard). La Parra et al. (2008) found that the main compounds released upon *in vitro* digestion were ferulic acid and kaempferol, which were not identified in the digestion supernatants of the present study (LaParra et al., 2008). For most of the samples, ferulic acid was lost mainly during the soaking and cooking process.

The analysis of polyphenols present in the fibre fraction was initially planned for this study. As mentioned in the previous chapter, to recuperate the polyphenols associated to this fraction, HCl hydrolysis was performed. Due to the technical difficulties encountered during the analysis of samples in strong acid with the equipment available, we were unable to produce results for this fraction.

4.6. Conclusion

Due to the technical limitations presented during this part of the study, the identification of specific polyphenols was not possible. However, the identification of the ions of interest was achieved. Further studies are needed in order to confirm the results obtained for this study. Even though the identification of the compounds was not fully achieved, different peaks corresponding to different ions were found in the different fractions analysed.

This may indicate that the composition differs in the fractions, which could indicate differences in the polyphenolic profiles between the fractions.

In general, the polyphenolic profile of the beans was slightly variable between the beans. This is reflected in their different appearance and colour of the bean's hull. Results indicate that a broad variety of polyphenols are lost during the domestic preparation process (soaking and cooking). This may show that most of the polyphenols in beans are in the bean hull. This is similar to the data published in phenolexplorer.com. More experiments are required to investigate the polyphenolic content present in different parts of the bean. The results of the Folin-Ciocalteu assay mentioned in chapter 3, indicate that more polyphenols are released upon digestion. Since we were unable to quantify polyphenols by the LCMS method, further experiments are required to explain this behaviour. The analysis of the digestion supernatants revealed that the polyphenols released upon digestion are mainly phenolic acids and catechin/epicatechin. It is likely that the remaining undigested material (fibre) still contains polyphenols, which would be metabolized in the colon. Further analysis is required to confirm this premise.

The Mexican beans presented a larger variety of compounds and appearance. Most beans presented sinapic acid and catechin/epicatechin in their composition. In order to evaluate further biological effects, we decided to select the beans with the most unique colours reflecting the different polyphenols in the hull. Since most Mexican beans presented a similar polyphenol profile despite their appearance, we found it interesting to see if their biological activity was similar.

Chapter 5

Evaluation of the anti-inflammatory activity of common bean (*P. vulgaris*) extracts in murine RAW264.7 macrophages

Summary

- Sinapic acid, a phenolic acid identified previously in the *in vitro* digestion supernatants, decreased mRNA levels of IL6 by 27.64% at 10 μg/mL and 22.84% at 50 μg/mL.
- Negro Jamapa extract showed an anti-inflammatory effect by reducing mRNA levels of IL6 (by 18.28% at 50 μg/mL and 20.37% at 100 μg/mL), IL1β (15.55% at 100 μg/mL) and iNOS (15.79% at 100 μg/mL). Borlotti bean extract decreased mRNA levels of IL1β (by 25.28% at 50 μg/mL and 22.84% at 100 μg/mL) and iNOS (39.61% at 100 μg/mL).
- Marcela and Azufrado seemed to induce inflammation in the macrophages by increasing mRNA levels of IL1β and IL6, respectively.
- Results indicate that different samples of beans belonging to the same species induce a different biological response. Hence, the consumption of some types of beans may be more beneficial than others.

5.1 Introduction

Legumes have been associated with anti-inflammatory properties. Oomah et al. (2010) reported that bean hull extracts of some common beans exhibited an inhibitory effect on both cyclooxygenases, COX-1 and COX-2, and of lipoxygenase 15-LOX (Oomah et al., 2010). Zhang et al. (2014) found that cooked navy and black bean diets improve biomarkers of colon health and reduce inflammation during colitis in mice (Zhang et al., 2014). As mentioned previously, polyphenols may exert an anti-inflammatory effect by inhibiting the NF-kB pathway. As mentioned in the previous chapter, the main polyphenols present in beans are hydroxycinnamic acids. Phenolic acids may exert an anti-inflammatory effect. It has been reported that hydroxycinnamic acid derivatives such as curcumin and caffeic acid are inhibitors of NF-κB activation (Nagasaka et al., 2007). It has also been reported that chlorogenic acid inhibits lipopolysaccharide induced cyclooxygenase-2 expression in RAW264.7 macrophages (Shan et al., 2009).

Information about the anti-inflammatory activity of the common beans remains scarce and there is no information about the bioactivity of Mexican beans. When Negro Jamapa and the Marcela beans were digested, the main polyphenols identified were sinapic acid, *p*-coumaric acid and catechin; the digestion supernatans of Azufrado bean contained the same polyphenols except for *p*-coumaric acid (Chapter 4). Sinapic acid was found in the digestion supernatants of most of the beans analysed during this study. For this reason, the anti-inflammatory effect of this individual polyphenol was evaluated.

As mentioned before, macrophages play a major role during inflammatory and immune response and as a result, these cells are commonly used as a model to evaluate the anti-inflammatory activity of compounds and

extracts *in vitro*. Thus, these cells were selected to evaluate the *in vitro* anti-inflammatory activity of for selected beans (Borlotti, Negro Jamapa, Marcela and Azufrado).

5.2 Methodology

The methodology used for this section is described in chapter 2. Figure 5.1 shows a summary of the steps used.

5.3 Aim and objectives

5.3.1 Aim

The aim of this part of the study is to evaluate the anti-inflammatory effect of 4 common beans (*P.* vulgaris) using murine RAW264.7 macrophages as a model system for inflammation.

5.3.2 Objectives

1. Test two different types of lipopolysaccharide (LPS) (from *E. coli* and *Salmonella*) at different concentrations (10-1000 nM) in order to find the optimal conditions to stimulate inflammation in RAW264.7 macrophages.
2. Evaluate the cytotoxic effect of sinapic acid and cooked bean extracts at different concentrations.
3. Evaluate the effect of different concentrations of sinapic acid and bean extracts on the production of inflammation target genes (IL6, IL1β, TNFα, iNOS and HO-1).

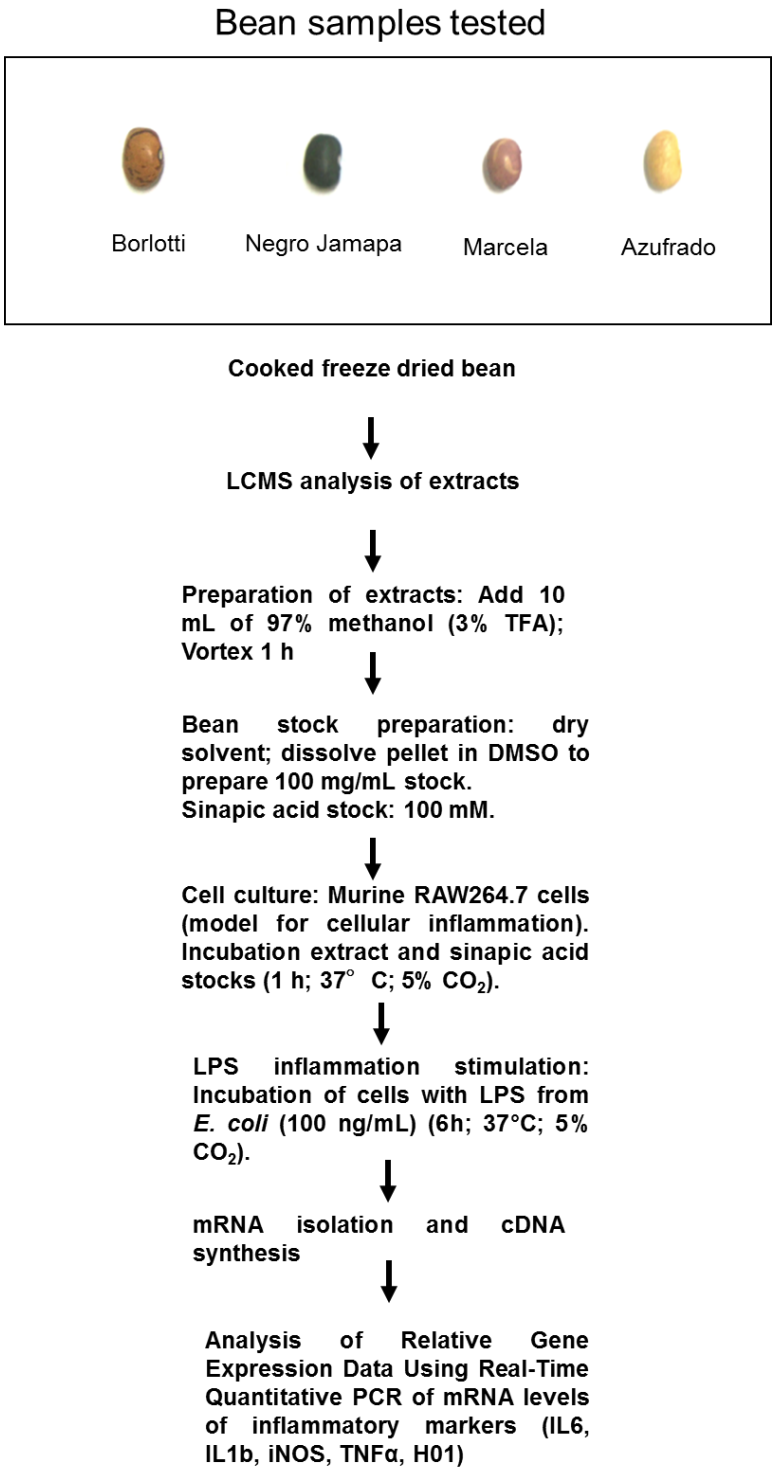


Figure 5.1. General methodology used for the evaluation of the anti-inflammatory activity of common bean (*P. vulgaris*) extracts in murine RAW264.7 macrophages.

5.4 Results

5.4.1 LCMS analysis of cooked bean extracts

The polyphenols identified in the methanolic cooked bean extracts were mainly hydroxycinnamic acids and are shown in table 6.1.

5.4.2 Effect of lipopolysaccharide (LPS) on NO production in RAW264.7 macrophages

Two sources (Salmonella and *E.* coli) and different concentrations (10-1000 nM) of LPS were tested in order to select the optimal stimulation conditions for the RAW 264.7 cells. All of the concentrations tested showed a all of the concentrations evaluated showed a production of NO_2, even the lowest concentrations tested (10 nM). A dose response effect was observed in NO_2 production stimulated by LPS. However, this increase was not significantly different between the increasing concentrations of LPS.

Table 5.1. Polyphenols found in methanolic cooked bean extracts.

Sample	Sinapic acid	Ferulic acid	*p*-coumaric acid	Catechin
bor	X		X	X
nej	X	X	X	X
mar	X	X	X	X
azu	X	X	X	X

bor: Borlotti; nej: Negro Jamapa; mar: Marcela: azu: Azufrado

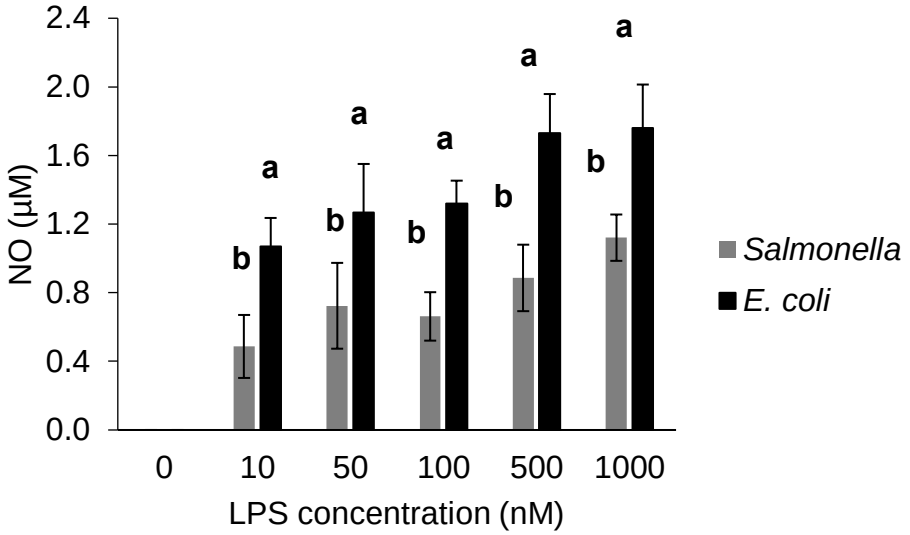


Figure 5.2 . Effect of different concentrations of LPS from two different sources (*Salmonella* and *E. coli*) on NO_2 production in RAW 264.7 cells. Data are mean of triplicate experiments. Error bars show standard error of the mean. Different letters indicate significant differences ($p<0.05$).

Higher values (twofold) of NO were detected when cells were stimulated with LPS from *E. coli*, which indicates a greater inflammatory response (Figure 5.2). Hence, *E. coli* LPS was selected to stimulate inflammation in future experiments. No significant differences in NO production were found when stimulating with different LPS concentrations. This was mainly due to variability of results among experiments. The LPS concentration selected was 100 nM because it has been shown that this amount is enough to promote NF-κB and study the inhibitory effect of this pathway by a polyphenol (chlorogenic acid) in RAW 264.7 cells (San et. Al, 2009).

5.4.3 Evaluation of the cytotoxic effect of sinapic acid and bean extracts

As mentioned previously, the cytotoxic effects of sinapic acid and bean extracts were determined by the neutral red assay. Any cytotoxic effect may affect the final results, since cytotoxicity induces oxidative stress and inflammation. The concentrations tested were 1-100 μM for sinapic acid and 10-100 μg/mL for the three cooked bean extracts tested.

The controls were cells in DMEM control (medium control), DMSO (0.1%) and sulforaphane (5 μM in DMSO). No cytotoxic effect was observed when macrophages were incubated with sinapic acid (at the concentrations evaluated), as there were no significant differences between the different treatments and the DMEM control (figure 5.3). Similar results were seen for the bean extracts, except for the Azufrado bean (figure 5.3). In this case, when cells were incubated at a concentration of 50 μg/mL of these extracts, the percentage of cytotoxicity was significantly lower than the DMEM control. However, even though there was a significant difference with the control, the percentage of

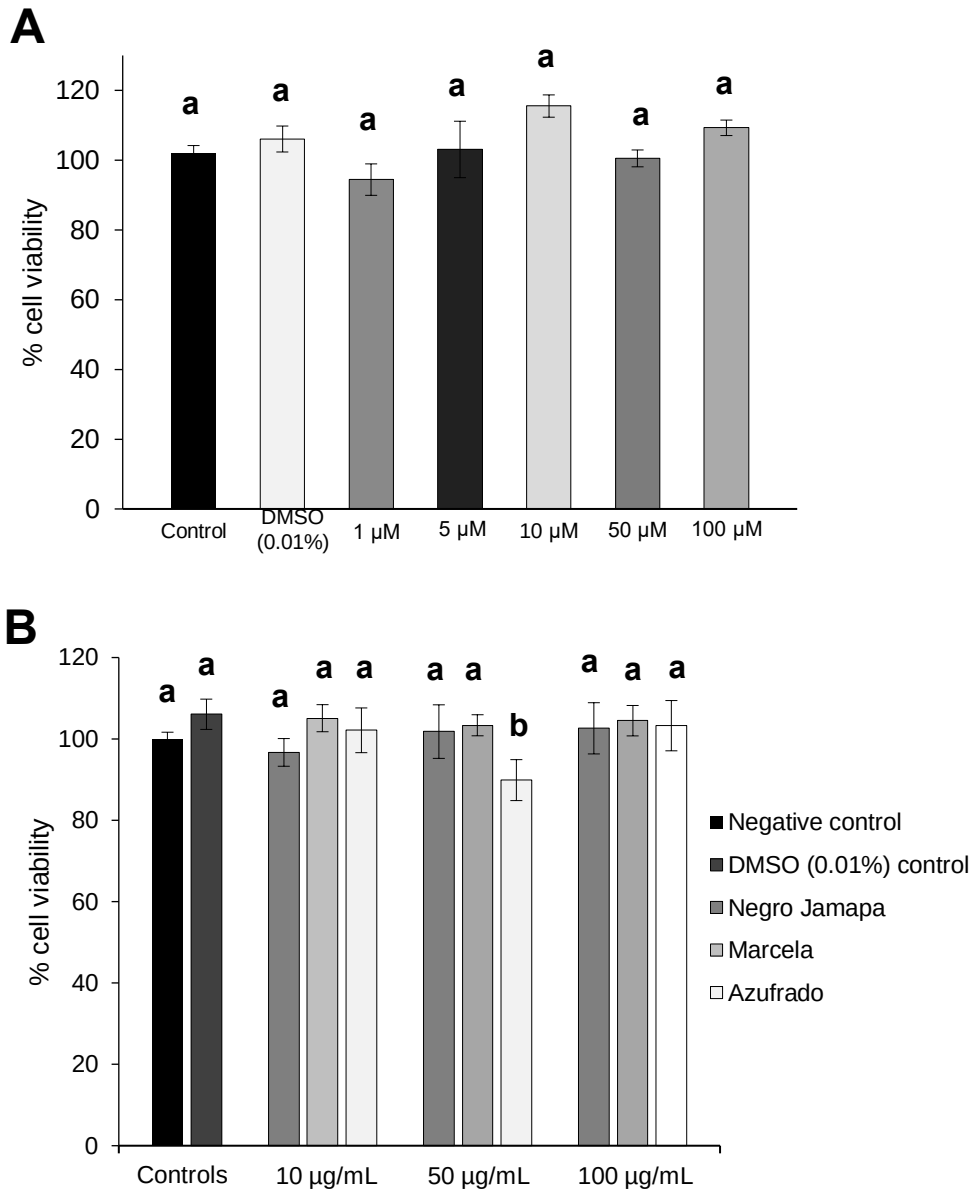


Figure 5.3. Cell viability of RAW 264.7 macrophages after 24 h incubation with different concentrations of (A) sinapic acid and (B) bean extracts determined by the neutral red assay. The results represent the mean with SD of two independent experiments performed in triplicate ($p < 0.05$).

cytotoxicity for this concentration of Azufrado bean extract was still over 90% (93.52%), making it suitable for further experiments. There were no significant differences between the cytotoxicity percentage of the DMEM control and the cells incubated with 0.1% of DMSO or with the sulforaphane (5 μM) control.

5.4.4. Effect of sinapic acid in the production of inflammatory gene expression

While there was no significant effect of sinapic acid (at any concentration evaluated) on the production of IL1β and H01 mRNA levels (Figure 5.4), sinapic acid showed a significant reduction of IL6, iNOS and TNFα mRNA levels (Figure 5.4). The percentage of inhibition of the IL6 target gene was 27.64% and 31.29% when incubating with 10 μM and 50 μM of sinapic acid, respectively. For this same target gene, no significant reduction or increase was observed when incubating with 100 μM of sinapic acid in comparison to the DMEM control.

Results showed that the iNOS target gene was not inhibited by the 10 and 50 μM sinapic acid concentrations. However, when incubating RAW 264.7 cells with 100 μM sinapic acid, the production of this target gene was reduced 30.22%.

Sinapic acid suppressed the production of the TNFα target gene when incubating with the highest concentrations evaluated (50 and 100 μM). This target gene was reduced 33.65% when incubating with 50 μM and 50.22% with 100 μM of sinapic acid. Sinapic acid concentration of 100 μM had a significantly stronger suppressive effect of this target gene in comparison to 50 μM. No significant decrease or increase in the production of the TNFα target gene was observed when incubating with 10 μM of sinapic acid.

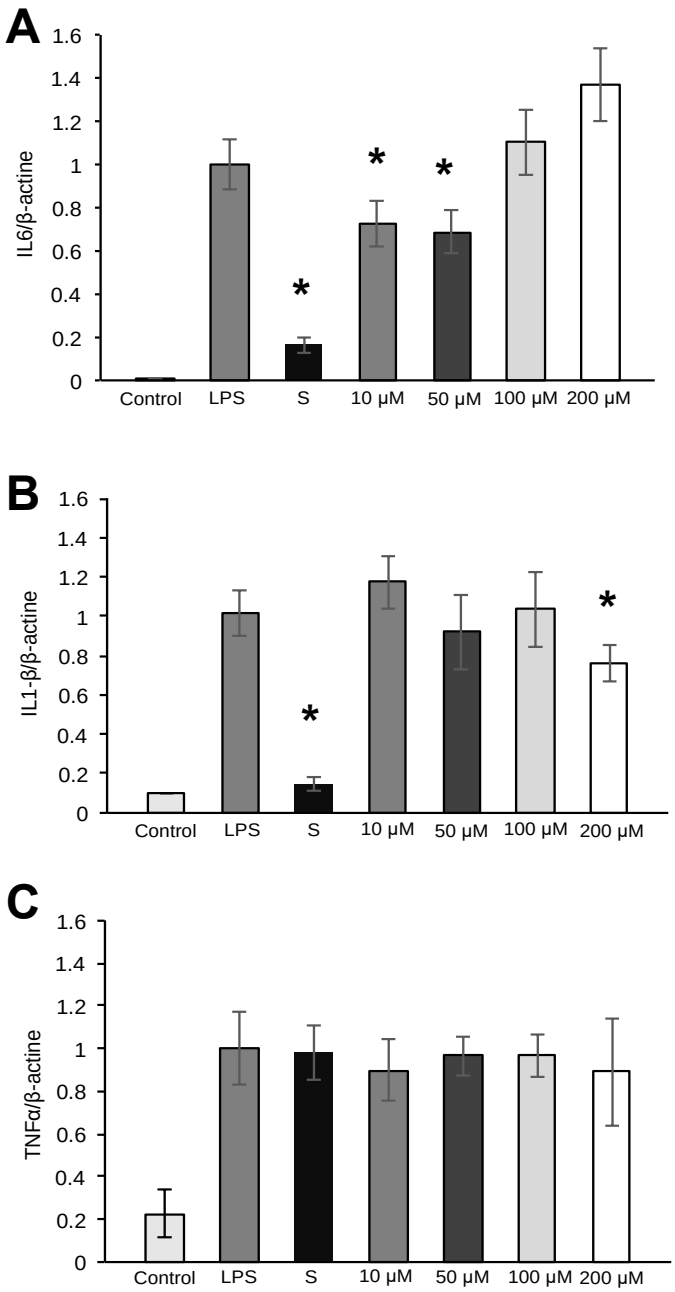


Figure 5.4. Effect of different concentrations of sinapic acid on mRNA levels for inflammatory cytokines. (A) IL6, (B) IL1-β and (C) TNFα. The results represent the mean with SD of three independent experiments performed in triplicate. * indicates significant differences between compound treated cells and LPS control ($p < 0.05$).

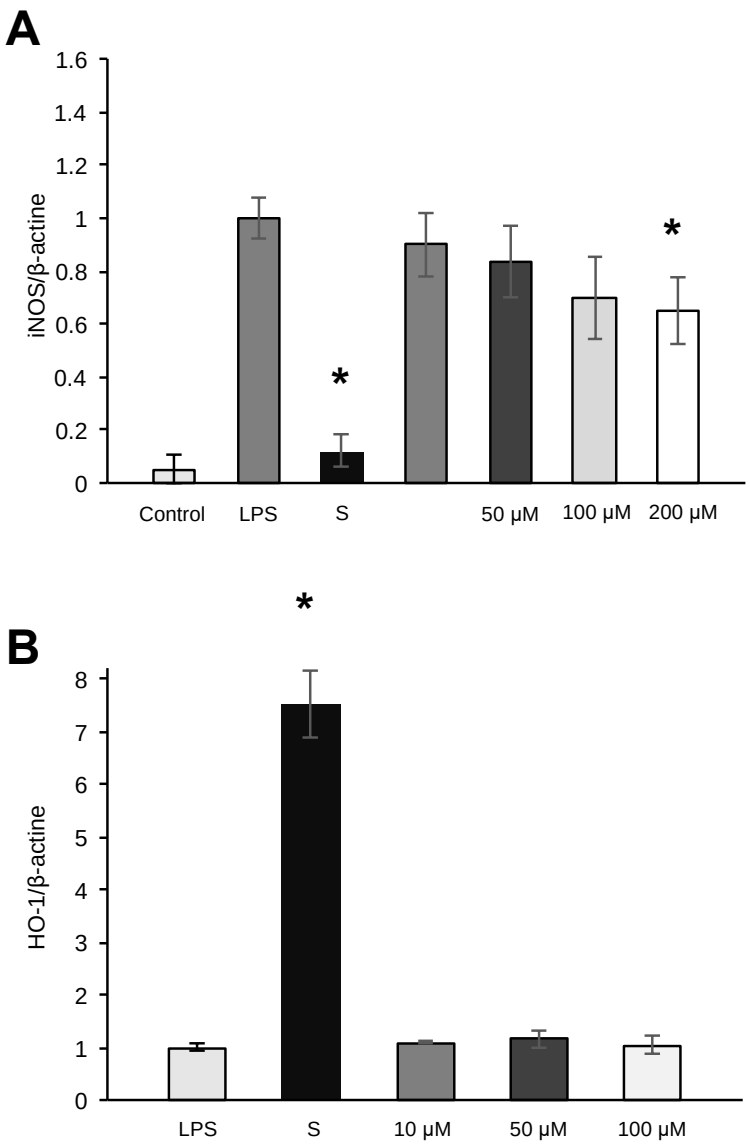


Figure 5.5. Effect of different concentrations of sinapic acid on the mRNA levels of specific for oxidative stress related enzymes. (A) iNOS and (B) HO-1. The results represent the mean with SD of three independent experiments performed in triplicate. * indicates significant differences between compound treated cells and LPS control ($p < 0.05$).

After evaluating sinapic acid concentrations that ranged from 10- 100 μM, it was decided to test an additional concentration of 200 μM in order to determine if there was an additional effect of this polyphenol in the expression of the specific inflammation target genes. In the case of the IL6 target gene it was first assumed that no inhibitory effect would be exerted with a concentration of 200 μM of sinapic acid since this effect was only observed with the lowest concentrations tested.

Yet, it was unknown weather this concentration would stimulate the production of IL6. Results indicate that a concentration of 200 μM of this compound increased the production of IL6 by 20%.

5.4.5 Effect of bean extracts in the reduction of mRNA levels of inflammatory target genes

The Negro Jamapa bean extract decreased the IL6 mRNA levels as shown in figure 5.6. This effect was observed with the two highest concentrations tested, 50 and 100 μg/mL. The IL6 gene target was suppressed by 18.28% with the 50 μg/mL concentration and 20.37% with the 100 μg/mL concentration. No reducing or increasing effect was seen when incubating with the 10 μg/mL concentration for any of the target genes assessed. Marcela bean extract showed no suppressive or stimulant effect for the IL6 target gene. Azufrado bean extract increased in IL6 mRNA levels when incubating the RAW 264.7 cells with 50 μg/mL of the Azufrado bean extract.

For IL1β, the only treatment that showed a decrease in the production of this marker was 100 μg/mL of Negro Jamapa extract. In this case, the IL1β was decreased 15.55%. Azufrado did not show a significant increase or decrease of

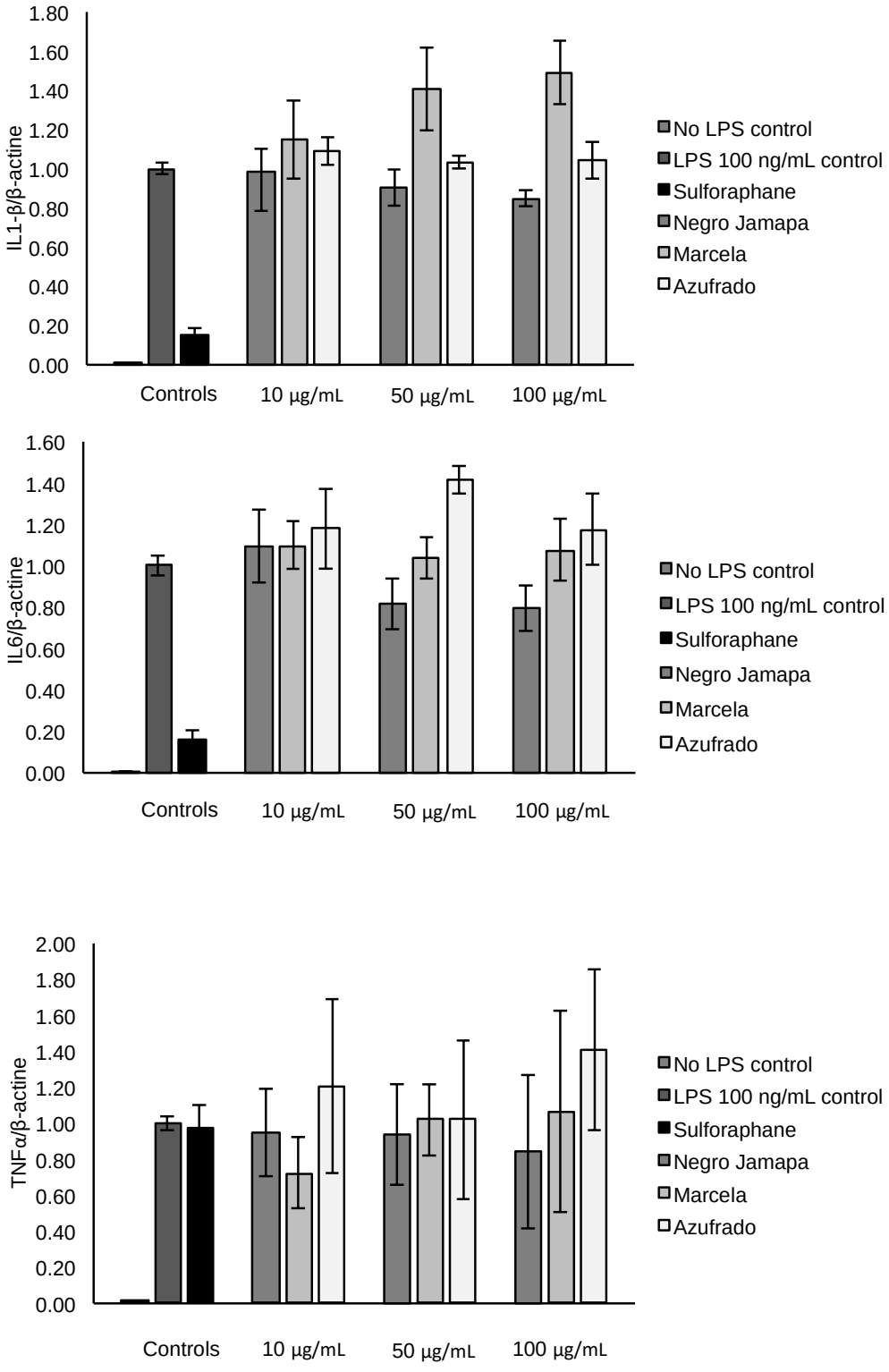


Figure 5.6. Effect of different concentrations of bean extracts on the production of target genes for inflammatory cytokines. (A) IL6, (B) IL1-β and (C) TNFα. The results represent the mean with SD of three independent experiments performed in triplicate. * indicates significant differences between compound treated cells and LPS control ($p < 0.05$).

this marker. However, an increase in the IL1β marker was seen when cells were incubated with 50 and 100 μg/mL of Marcela bean extract.

Results of the effect of bean extracts in the reduction of mRNA levels of iNOS and HO-1 are shown in figure 5.7. The iNOS mRNA levels were reduced by 15.79% when the RAW 264.7 cells were incubated with 100 μg/mL of the Negro Jamapa bean extract. There were no significant differences in the production of the mRNA levels of this target gene when the cells were incubated with lower concentrations of the Negro Jamapa bean extract or with any concentrations of the Marcela and Azufrado bean extracts.

In general, TNFα results were inconsistent and varied between experiments. Due to this behaviour, we were unable to determine if any of the treatments would produce a suppression or a stimulation in the production of the TNFα gene marker. Despite the differences and inconstancies observed in the experiments, Azufrado bean showed an increase of 49.25% in the mRNA levels of this target gene at 10 μg/mL.

Unlike the rest of the target genes tested, the increase in HO-1 production is beneficial for the oxidative balance of the cells. In general, beans did not increase the production of the HO1 target gene. For this target gene, a stimulating effect was observed only with the Marcela bean extract at the highest concentration tested (100 μg/mL). In this case, the increase in the production of H-O1 mRNA levels was of 66.11%.

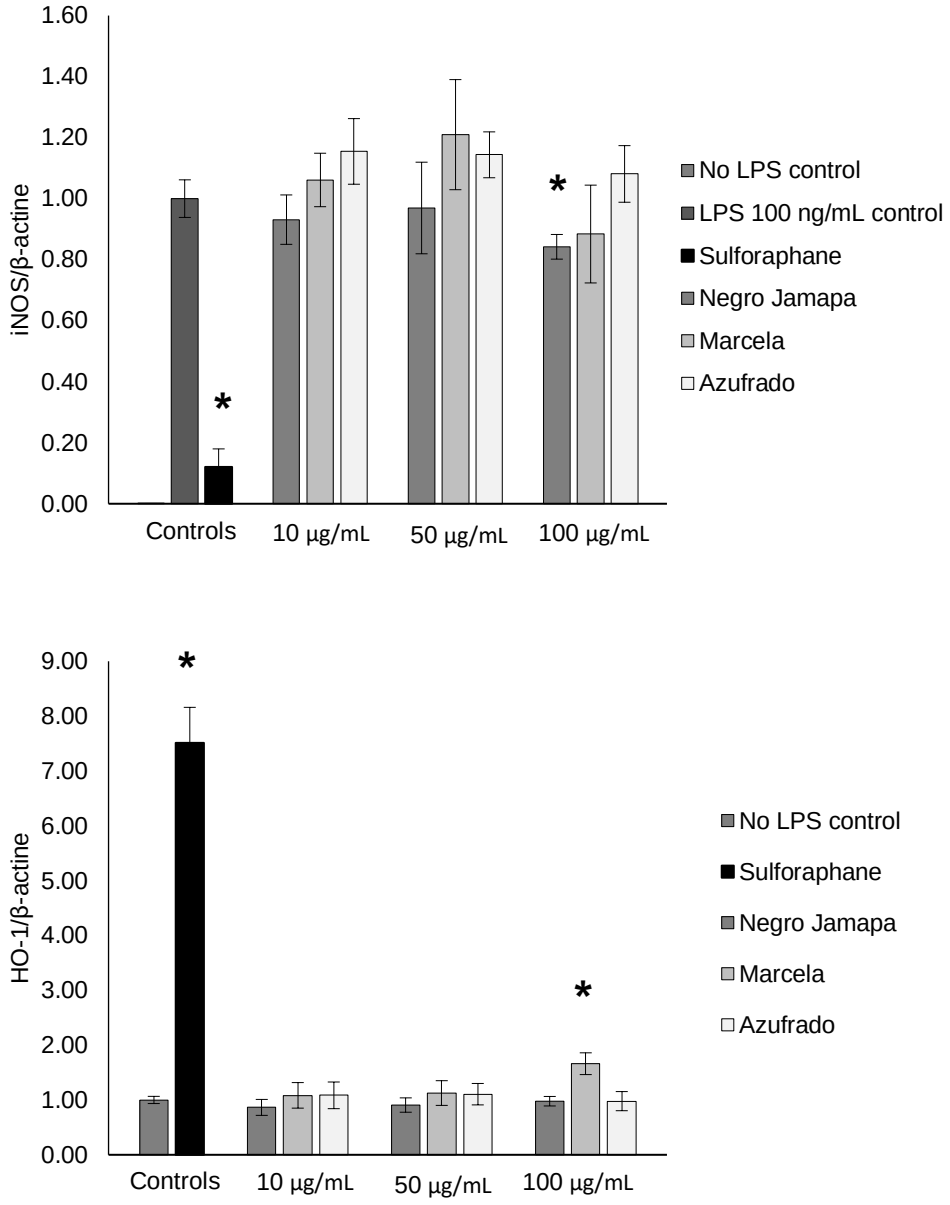


Figure 5.7. Effect of different concentrations of bean extracts on the production of target genes for specific for oxidative stress related enzymes. (A) iNOS and (B) HO-1. The results represent the mean with SD of three independent experiments performed in triplicate. * indicates significant differences between compound treated cells and LPS control ($p < 0.05$).

5.4.6 Anti-inflammatory activity of Borlotti bean extract

Initially, the only samples contemplated for this part of the study were Negro Jamapa, Marcela and Azufrado beans since these are unique cultivars found in a certain area of Mexico and because of their varied polyphenolic profile and appearance. It was decided afterwards to evaluate the anti-inflammatory activity of Borlotti bean. For this sample, the mRNA inflammatory levels evaluated were IL6, IL1β and iNOS, since only the effect of these were reduced by Negro Jamapa (the bean sample that presented a positive effect). The concentrations selected for evaluation were 50, 100 and 200 μg/mL because lower concentrations of the bean extracts did not exert an effect in the previous experiments.

Results showed that the Borlotti bean extract decreased mRNA levels of IL1β by 25.28% at 50 μg/mL and by 22.84% at 100 μg/mL. Additionally, iNOS mRNA levels were decreased by 39.61% at a concentration of 100 μg/mL. No significant decrease was observed for the IL6 mRNA levels (Figure 5.8).

5.4.7 Antioxidant activity of raw and cooked bean samples

The antioxidant activity of raw and cooked bean samples was determined by the TEAC and FRAP assays. As showed in figure 5.9, for most samples, cooking decreased the antioxidant activity. However, in the case of the Wizard fava bean, the antioxidant activity was preserved after cooking, as determined by both methods. Interestingly, results showed that the antioxidant activity of Negro Jamapa bean increased after cooking.

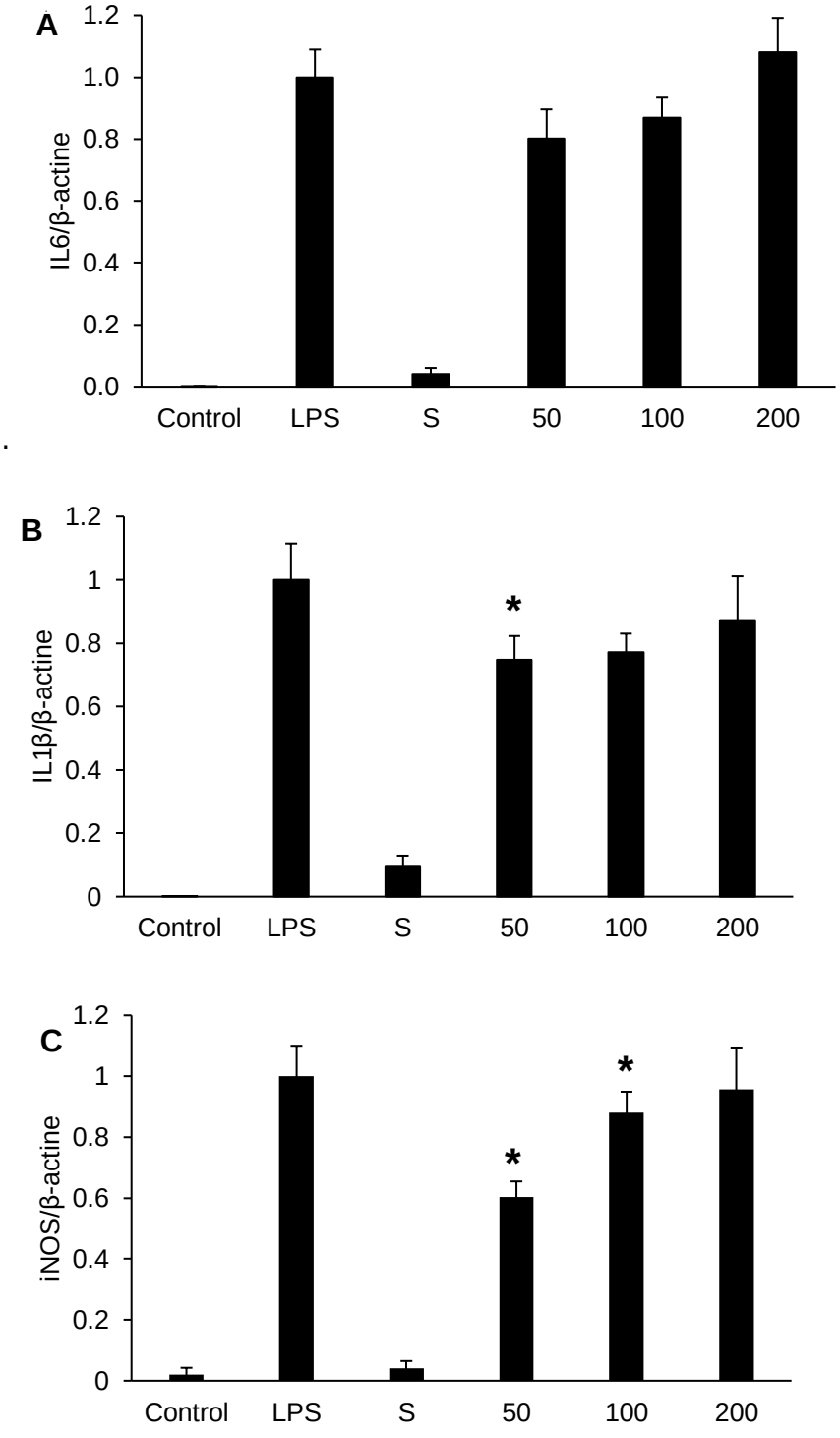


Figure 5.8. Effect of different concentrations of Borlotti bean extracts on the production of target genes for inflammatory related target gene. (A) IL6, (B) IL1-β and (C) iNOS. The results represent the mean with SD of three independent experiments performed in triplicate. * indicates significant differences between compound treated cells and LPS control ($p < 0.05$).

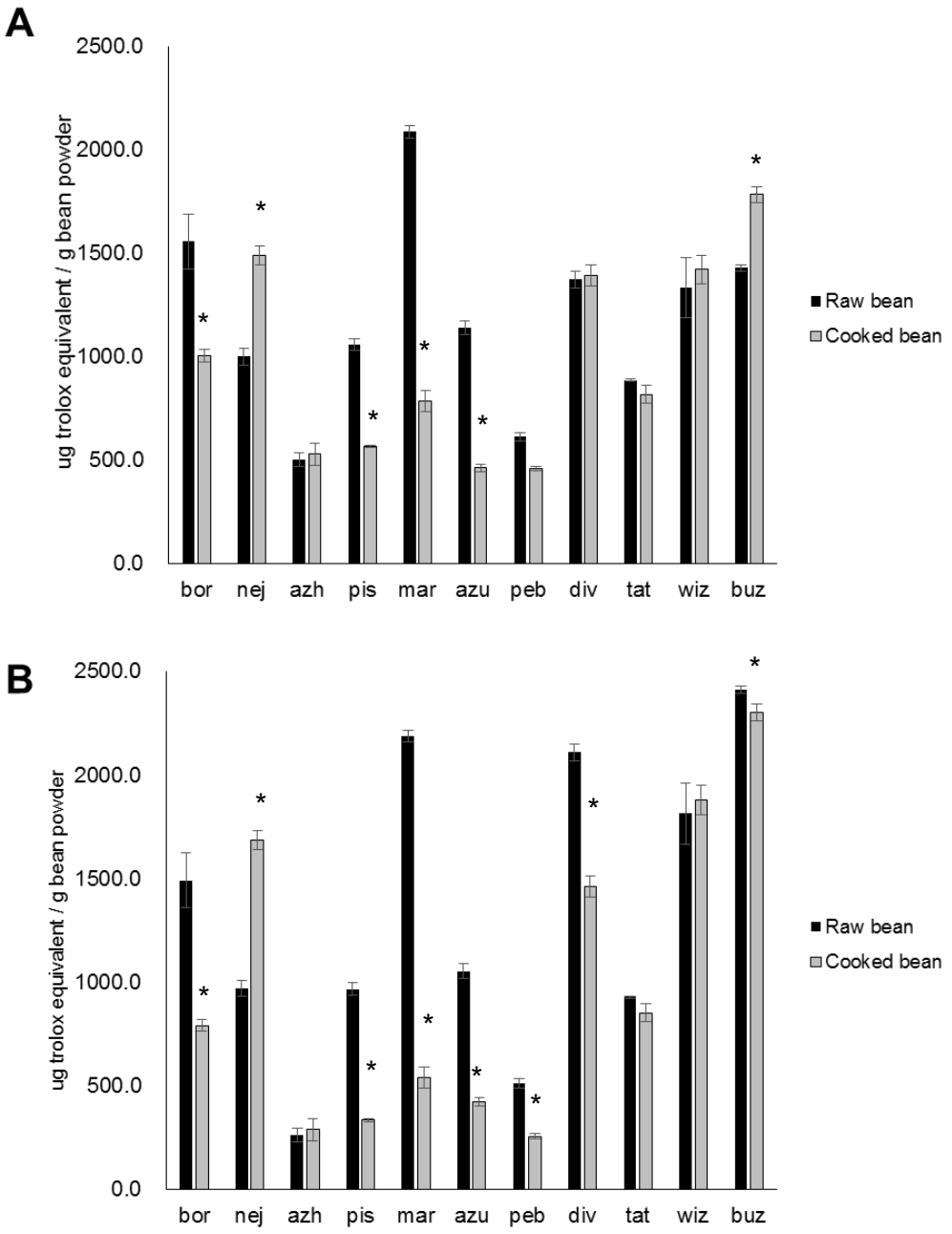


Figure 5.9. Antioxidant activity of methanolic extracts of cooked and dried beans measured by two different methods. A) TEAC; B) FRAP. The results represent the mean with SD of three independent experiments. * indicates significant differences between raw and cooked samples ($p < 0.05$).

Of the samples tested in this chapter, Negro Jamapa exhibited the highest antioxidant activity, followed by Borlotti. Marcela and Azufrado beans presented the lowest antioxidant activity. There were no significant differences in the antioxidant activity values of Marcela and Azufrado beans.

Figure 5.10 shows the correlation between TEAC assay vs Folin-Ciocalteu assay, FRAP assay vs Folin-Ciocalteu assay and TEAC assay vs FRAP assay. The TEAC and FRAP assay correlated positively with the Folin-Ciocalteu total polyphenol assay, which indicated that the antioxidant activity of the extracts is likely due to the polyphenols. Both antioxidant activity assay correlated positively, which explains that both assays showed a similar tendency in the results obtained.

5.5 Discussion

Results showed that the behaviour exerted by the beans in the macrophages was variable, in spite of the fact that the polyphenolic profile of the extracts was the similar. The polyphenolic profiles of the extracts resemble to the profile of the digestion supernatants described in chapter 4. Thus, the anti-inflammatory activity should be similar if the digestion supernatants would have been tested. The reason the digestion supernatants were not used for this part of the study was that this fraction contains buffers and enzyme residues that could alter the final results.

The antioxidant activity results showed that domestic processing decreased the antioxidant activity in most cases, except for Negro Jamapa bean, in which cooking had a positive effect in this parameter. This could be due to the fact that polyphenols in the Negro Jamapa bean could be strongly bound to the food matrix components. Thus, cooking might hydrolyse these

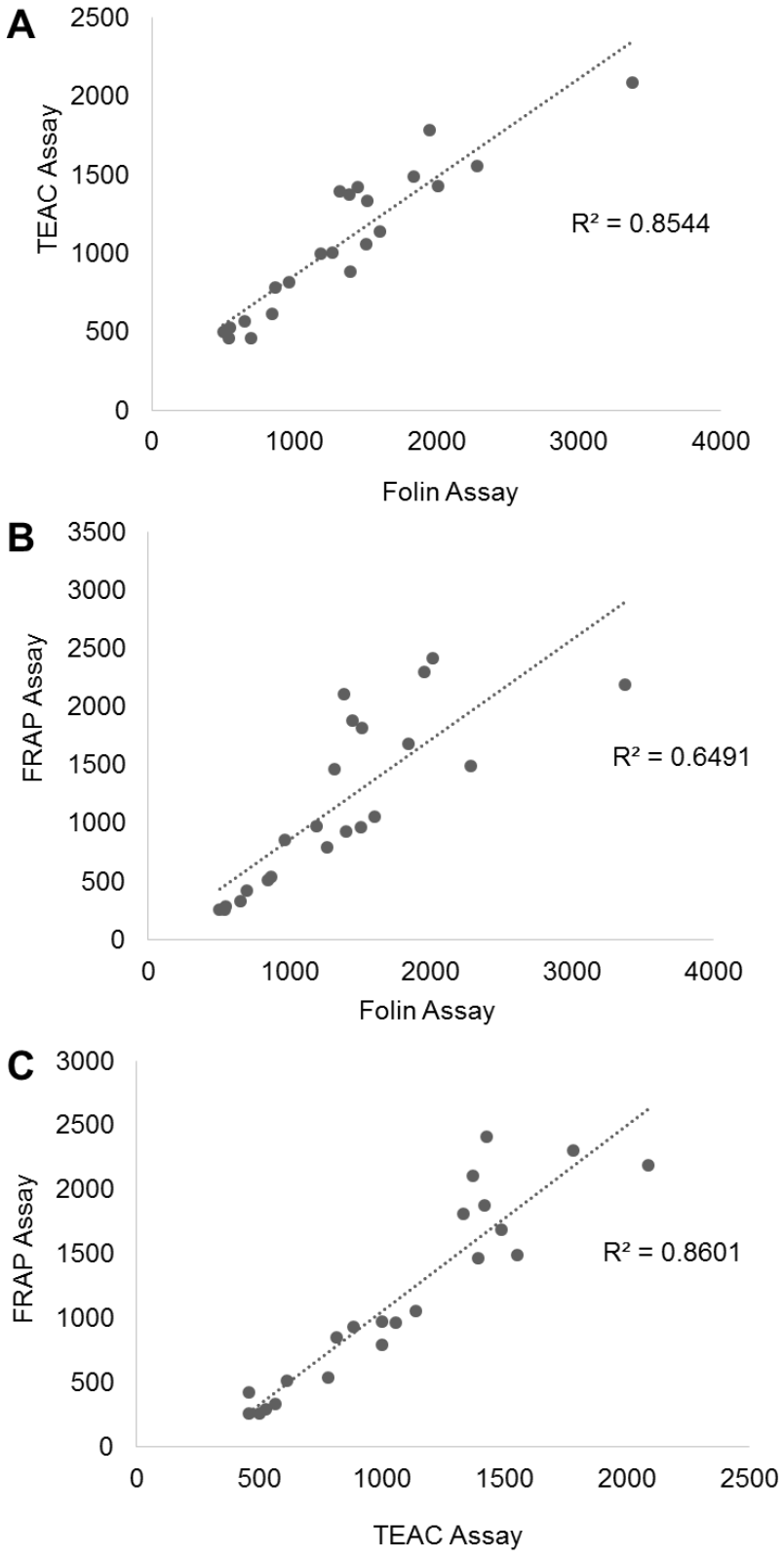


Figure 5.10. Correlation of A) TEAC assay vs Folin-Ciocalteu assay; B) FRAP assay vs Folin-Ciocalteu assay; C) TEAC assay vs FRAP assay.

bonds, liberating the polyphenols. In addition, it was observed that after Negro Jamapa (black bean) digestion, the remaining residue still presented a dark colour, which might indicate the presence of polyphenols bound to the fibre. This could explain why the polyphenolic profile of this bean was similar to the rest of the samples analysed in this part of the study, even though the dark colour of the sample indicates the presence of a more variable polyphenolic profile.

Anti-inflammatory effect results showed that sinapic acid and Negro Jamapa had a moderate positive effect. Yet, this was not seen with Marcela or Azufrado beans. When the macrophages were incubated with the Negro Jamapa bean extract, a reduction of IL6, iNOS and IL1β mRNA levels was observed. As mentioned previously in the results section, Negro Jamapa decreased the IL6 mRNA levels by about 20%, iNOS mRNA levels by 15.79% and IL1β mRNA levels by 15.55%. The reduction was only found with the highest concentrations evaluated. The reduction could be considered mild in comparison to the effect obtained with sulforaphane, which is a powerful anti-inflammatory used as a positive control. Yet, these results show the same tendencies as those reported by the *in vivo* study performed by Zhang et al. (2014) in which navy bean also reduced IL6 and IL1β cytokines in mice.

Marcela bean extract increased IL1β mRNA levels in more than 40%. This indicates that this extract stimulated inflammation at the concentrations tested. This same sample increased the mRNA levels of HO-1, which could be due to a possible stimulation of the NrF2 biochemical pathway. HO-1 is a target gene for heme oxygenase production. This enzyme is involved in catalization of the cleavage of the porphyrin ring of haeme into Fe2+, carbon monoxide and biliverdin, which is later transformed to bilirubin, an antioxidant. The production

of bilirubin decreases the oxidative status of the cell, which may inhibit the NF-κB pathway (Wardyn et al., 2015). The Nrf2 and the NF-κB pathways are both regulated by redox factors. Cytokine production by the NF-κB pathway increases the activity of Nrf2. The NF-κB creates an oxidative environment in the cell, which activates the antioxidant NrF2 pathway in order to restore cell homeostasis (Yerra et al, 2013). As a result, it is likely that Marcela does not exert an anti-inflammatory effect in the macrophages and the HO-1 mRNA increase is the effect of the production of an inflammatory response. Azufrado bean did not show an anti-inflammatory effect either since it increased the IL6 mRNA levels by almost 50%. It is probable that, like the Marcela bean extract might produce an inflammatory effect. No cytotoxic effects were found when incubating the cells with this extract, hence it is unlikely that a pro-oxidant effect is producing the inflammatory response produced by these two bean samples. It is likely that the bean extracts could contain an unidentified compound that might induce inflammation. Further analysis of the sample and other anti-inflammatory assays are required in order to understand this response.

According to the results obtained in this study, Azufrado bean increased the TNFα mRNA levels in almost 50%. Yet, in general, it was seen that the results for the mRNA levels of this cytokine varied between different experiments. In the experiments performed in the present study, macrophages are stimulated with LPS for 7 h. It has been reported that macrophages start producing TNFα mRNAs after 0.5-1 h of LPS stimulation (Dumitru et al., 2000). This could explain the variability of the results obtained for this target gene.

Information about the anti-inflammatory effect of beans is scarce. Zhang et al. (2014) reported that cooked navy and black bean diets improve biomarkers of colon health and reduce inflammation during colitis in mice

(Zhang et al., 2014). The possible mechanism was not reported but this study suggests that the reduction of inflammation may be due to an inhibition of the IL6 and IL1β. Negro Jamapa beans also showed an reduction on iNOS mRNA levels.

In our study, results showed that a sinapic acid suppressed IL6 (approximate 30% decrease) and TNFα expression mRNA levels (34%). Additionally, Negro Jamapa decreased lL1β mRNA levels. This behaviour has not been reported for catechin of sinapic acid when tested individually. The decrease in lL1β mRNA levels by the Negro Jamapa extract could be due to a synergistic action of these two polyphenols plus the other polyphenols that are present in a smaller amount. As mentioned previously, it is assumed that the polyphenols present in the digestion supernatants are also found in the bean extracts used for this part of the study. A further analysis of individual polyphenols found in the bean extracts is required have a better understanding of the behaviour observed for the different bean samples.

As mentioned before, the antioxidant capacity of cooked Negro Jamapa and Borlotti bean was significantly higher than Marcela and Azufrado cooked beans. Also, as mentioned in chapter 3 (figure 3.11) the polyphenolic content was also lower in Marcela and Azufrado cooked beans in comparison to the Negro Jamapa and Borlotti cooked beans. Since the antioxidant and anti-inflammatory activity are related, it is likely that the relatively low antioxidant activity and low total polyphenol content might be responsible for the incapacity of this samples to reduce the production of inflammatory related mRNA levels. This, however, does not explain why Marcela and Azufrado bean induced inflammation rather than reducing it. This behaviour might be due to the

presence of an unidentified compound (not a polyphenol) that might induce inflammation.

Even though studies regarding the anti-inflammatory effect of beans are scarce, the anti-inflammatory activity of polyphenols has been reported. Polyphenols that exert a strong anti-inflammatory activity are resveratrol, catechins (like the ones present in the beans used in this study) and curcumin (Rahman et al., 2006). The anti-inflammatory activity of resveratrol has been observed in monocytic U937 cells and alveolar epithelial A549 cells, in which resveratrol has shown to inhibit the NF-kB pathway (Manna et al., 2000, Donnelly et al., 2004). The same compound has also exhibited an anti-inflammatory effect *in vivo*, by inhibiting the expression of inflammatory cytokines after LPS stimulation in rats (lungs) (Birrell et al., 2005). It has been reported that catechin derivatives (epigallocatechin gallate, epicatechin gallate and theaflavin-3'3-digalate) inhibit the IL6 cytokine and iNOS in fibroblasts (Chen *et al.*, 2003; Hosokawa et al., 2010). Ichikawa et al. (2004) studied the effect of epicatechin gallate in myocardial injury after ischemia and reperfusion in rats. Their results indicate that this compound decreased the IL6 cytokine and the activation of IKK and degradation of IκB-α (supressing the NFκB pathway) (Ichikawa et al., 2004). It has been reported that curcumin inhibits the expression of TNF-α, IL-12, COX-2, and iNOS in Kupffer cells (liver macrophages) (Nanji et al., 2003). The possible mechanism by which the polyphenols may inhibit the NFκB was described by Karunaweera et al. (2015) with curcumin. This compound inhibits NF-κB by avoiding the phosphorylation and degradation of IκBα and the subsequent translocation of the P65 and P50 subunits to the nucleus (Karunaweera et al., 2015). Further studies are needed

in order to understand the mechanism in which polyphenols inhibit the NF-κB pathway.

5.6 Conclusion

Negro Jamapa and Borlotti beans showed a moderate anti-inflammatory effect. A similar tendency has been reported in an *in vivo* model with beans. Further studies are required in order to determine if these beans may prevent inflammation related diseases (such as cancer, diabetes or Alzheimer). Marcela and Azufrado seemed to induce inflammation rather than preventing it. These beans have a different pigmentation in their seed coat, yet, the polyphenolic profiles analysed for these bean extracts are similar. The reason why these beans exerted an inflammatory effect is unknown. During the extraction method, compounds other than polyphenols may be isolated. Since the extracts consist not only of polyphenols, it is likely that an unknown compound present in the extracts may induce the NF-κB pathway. Results lead to believe that different beans may exert positive biological effect, while others could produce the opposite. Further experiments are required to understand the biological behaviour of these samples.

Chapter 6. Discussion and future work

Several studies that evaluate the nutritional aspects of beans focus on their protein content and the presence of anti-nutrients in these legumes. Yet, recently, the consumption of beans has been generally associated with health benefits because they are an important part of vegetarian and Mediterranean-style diets; the possible health benefits of bean consumption may be due to their low saturated fat, their high fibre content and the presence of essential nutrients and phytochemicals (Messina, 2014). Human and epidemiological studies have concluded that beans contribute to positive health benefits. Some of these studies are described in table 6.1.

Studies regarding the effect of bean polyphenols in health are scarce. As a result, it is not known if the polyphenols present in these legumes are responsible for the health benefits reported in the literature. As shown in table 6.1, epidemiological studies suggest that a diet that incudes beans exerts a protective effect against cancer (Mills *et al*., 1989; Hernandez-Ramirez *et al.*, 2009) and cardiovascular disease (Mattei et al., 2017). It has been reported that polyphenols act on multiple key steps in carcinogenesis, inflammation, angiogenesis, cellular proliferation, differentiation, apoptosis and metastasis (Heim et al., 2012). It is believed that polyphenols may exert an anticancer effect by inhibiting COX-2, LOX, and iNOS (Nakagawa and Yokozawa, 2002). Yu et al. (2011) evaluated the anti-inflammatory activity of *Phaseolus angularis* beans in macrophages; their results showed that this bean reduced iNOS mRNA levels. Results of the present study showed that Negro Jamapa and Borlotti bean extract reduced mRNA levels of iNOS in RAW 264.7 macrophages. This could indicate that bean polyphenols

may be responsible for the protective effect against cancer reported by Mills et al. (1989) and Hernandez-Ramirez et al. (2009). Polyphenols may prevent cardiovascular disease by inhibiting TNFα and IL6 (Tagney and Rasmussen, 2013). This study showed that Negro Jamapa bean reduced IL6 mRNA levels. Zhang et al., (2014) also observed a decrease in IL6 in mice colon tissue after following a diet rich in navy and black beans. Hence, beans could have a protective effect against inflammation and cancer.

According to the literature, beans contain hydroxycinnamic acids and flavonoids. Hydroxycinnamic acids (curcumin, caffeic acid and chlorogenic acid) exert an anti-inflammatory effect by inhibiting the NFκB pathway (Nagasaka et al., 2007; Shan et al., 2009). Flavonoids (such as quercetin, kaempferol, luteolin, apigenin, morin, EGCG, resveratrol, indole-3-carbinol and oroxylin) also exert an anti-inflammatory effect by reducing COX2, LOX, TNFα and iNOS *in vitro* and *in vivo* (Reddy et al., 1991; Soliman et al., 1998; Williams et al., 1999;Wadsworth et al., 1999; Tsai et al., 1999; Chen et al., 2000; Chen et al., 2003; Ippoushi et al., 2003; Comalada et al., 2005). Since the identification of individual polyphenols in the beans used in this study was not validated, further analytical studies regarding the polyphenolic profile of the samples are needed in order to know which polyphenols might be responsible for the anti-inflammatory effect observed with the Negro Jamapa and Borlotti bean extracts.

Besides cancer and cardiovascular disease, polyphenol consumption has also been linked to the prevention of other chronic inflammatory diseases such as diabetes (Bahadoran et al., 2013) and Parkinson disease (Aquilano et al., 2008). This could lead to believe that populations that consume beans as a staple should have a low incidence of the mentioned diseases. Yet, in Mexico, a country that includes beans as part of a traditional diet, the main causes of

death by disease are cardiovascular disease (17% of the total deaths) and cancer (14% of the total deaths) (WHO, 2016). From 2000 to 2016, the cases of diabetes in Mexico increased from 287,180 to 368,069 (80,908 new cases) (www.epidemiologia.salud.gob.mx). According to Velázquez-Monroy et al. (2007) the rate of death by cardiovascular disease in Mexico increased by 288% from 1970 to 1990. One of the most prevalent types of cancer in Mexico is breast cancer and it is the second cause of death in Mexican women. In 1955, 4 out of 100,000 women in Mexico died due to breast cancer. In 1990, this incidence increased to 9 out of 100,000 women (Knaul et al., 2009).

The increase in the mentioned diseases in Mexico could be due, in part, to changes in diet patterns. For instance, according to the Mexican Secretariat of Agriculture, Livestock, Rural Development, Fisheries and Food (SAGARPA), the consumption of beans in Mexico decreased from 16 kg per person per year in 1980, to 8.4 kg per person per year in 2016 (SAGARPA, 2016). This reduction in bean consumption in Mexico may have an influence in the increase of the incidence of chronic inflammatory diseases. In order to confirm this, more studies about the anti-inflammatory effect of beans, together with epidemiological studies regarding bean consumption, should be performed.

Epidemiological studies are a useful tool to correlate the putative health effects of certain types of diets since they study the health tendencies of a high number of participants over a considerable amount of time (sometimes several years). However, epidemiological studies may not take into account the additional factors that may be associated with the development of a condition (such as genetic or environmental factors) and may attribute their outcomes solely on diet. The evaluations used normally rely on questionnaires that could

be misunderstood by the participants. It is also likely that participants might forget/omit important information when completing the questionnaires, affecting the overall result. Epidemiological studies often use composition tables that do not have enough detail about individual varieties of beans. Sometimes the term “legume” is used, which could be broad and unspecific (normally the species or bean cultivar studied is not mentioned). For example, the European Prospective Investigation into Cancer and Nutrition (EPIC), which is one of the largest cohort studies in the world, include legumes in their evaluation, but they don’t specify the bean cultivars taken into account for their study (http://epic.iarc.fr, 2017).

It is important for studies to specify the type of legume used, since there are a wide variety of these pulses that may exert different health benefits. According to the Food and Agriculture Organization of the United Nations (FAO), there are more than 40,000 varieties of beans in the world. Beans present a wide scope of colours in their hulls, which indicates variability in their polyphenolic content. As mentioned in previous chapters, the phenolic profile of beans depends on genetic factors and abiotic stress, which varies from region to region. Beans are cultivated in many countries around the world, each area with specific environmental conditions that could influence the polyphenol content of the legumes; this could contribute even more to the differences in bean phenolic profiles.

In consequence, an epidemiological study of the effect of bean polyphenols on health would be difficult to achieve since there is a huge number of bean varieties available, each with different polyphenol compositions. This would not be an issue if the population studied consumed only a few types of beans (like the UK for example).

The advantage of human studies is that a specific bean sample of interest is defined. In addition, the diets are normally controlled (including the amount of sample consumed) and are performed for relatively short periods of time. Hence, the effects observed are more likely due to the sample studied. The disadvantage is that the number of subjects used are much lower than the epidemiological studies, therefore, the standard error is likely to be larger and/or

Table 6.1. Epidemiological studies focused on the effect of bean consumption on different health conditions, characteristics of each study and final outcome.

Study	Condition evaluated	No. subjects	Age (years)	Duration of follow up (years)	Country	Outcome
Mills et al., 1989	Prostate cancer	35,000	> 25	6	USA	Increasing consumption of beans was associated with a significant reduction of prostate cancer.
Mattei et al., 2017	Cardiovascular disease	1194	45-75	1	USA (Puerto Rican subjects)	A diet that includes beans (Mediterranean diet) is associated with maintaining a low BMI and waist circumference.
Hernández-Ramírez et al., 2009	Gastric cancer	735 (257 confirmed gastric cancer patients and 478 controls)	> 25	1	Mexico	A diet rich in polyphenols (beans were a source in the diet evaluated) exert a protective effect against gastric cancer by inhibiting endogenous nitrosation of *N*-Nitroso compounds.
Chang et al., 2012	All-cause mortality	5752	> 15	6.5	Taiwan	Beans were beneficial for women, as they may be an important predictor of overall mortality, reduce waist circumference and decrease the levels of glycated haemoglobin (HbA1c). A bean-free diet may be associated with the development of the metabolic syndrome in men and women.

Study	Condition evaluated	No. subjects	Age (years)	Duration of follow up (years)	Country	Outcome
Darmadi-Blackberry et al., 2004	Survival rate in older people (different ethnicities)	785	>70	5	Japan, Sweden, Greece and Australia	For every 20 g increase in legume consumption, the mortality hazard ratio decreases 7-8%. This increase was independent of the ethnicity of the participants. Legumes consumed in this study varied with the ethnicity of the participants. Japanese: ate soy, tofu, natto and miso. Swedes: brown beans (similar to kidney beans) and peas. Mediterraneans: lentils, chickpeas and white beans.
Trichopoulou et al., 2009	All-cause mortality	23,349, healthy	45 – 65	8.5	Greece	The practice of a Mediterranean diet is related to a significant reduction in overall mortality of the subjects. A high legume consumption contributes to 9.7% of the mortality reduction. Other important factors of this diet and their contribution on overall mortality are: moderate ethanol consumption (23.5%), low consumption of meat products (16.6%), high vegetable consumption (16.2%), high fruit and nut consumption (11.2%) and high monounsaturated to saturated lipid ratio (10.6%).

the results obtained may not be representative of a larger population. These types of studies cannot normally determine a possible mechanism of action exerted by the sample. Thus, an experiment using a cellular model can give complementary information about the biochemical pathways inhibited or stimulated by the compounds found in the sample.

6.1 Advantages of the *in vitro* digestion method used in this study

After the optimization for the present method was performed, a general consensus *in vitro* digestion method was published by INFOGEST (Minekus et al., 2014). The INFOGEST method has the advantage that it emulates all the required physiological digestion conditions (temperature, digestion time, enzyme source and pH). It is suitable for the digestion of any food sample. However, the main disadvantage of this method is that it requires training and different buffers and enzymes, making it more expensive to perform than the method optimized in the present work.

The method optimized in this study uses similar amounts of pancreatic α-amylase as the INFOGEST protocol. The present method is relatively easy to perform, making it a practical tool for bioaccessibility analysis. Also, as it was standardized especially for beans, it digests a maximum amount of starch present in the sample (further increase in enzyme or incubation time did not increase digestion). Legume starch is known to be resistant to amylase digestion. However, resistant starch content appears to be dependent on the amount of amylase added to the assay. It may be that resistant starch content of beans has been previously over-estimated. Digestion of the starch appeared to release a considerable amount of polyphenols. Information about the starch/polyphenol interaction is scarce as most of the information available in the literature focuses on phenolic interaction with proteins or the carbohydrates

in cell walls. With the present *in vitro* digestion method, it was determined for the first time that an important amount of bean polyphenols interacts with starch. As a result, even though the INFOGEST method may be more related to what would happen during the digestion processes of an organism, a method optimized for a specific food sample (like the one t used in this work) can give additional information about the way in which polyphenols are distributed in the food matrix.

6.2 Effect of domestic processing in polyphenolic content and anti-nutrients

The present work showed that during soaking, most of the flavonoids were lost because they were solubilized in the water. Soaking is mainly performed to facilitate cooking and to remove antinutrients. The main antinutrients present in beans are protease inhibitors (PIs), lectins, phytates and oxalates (Campos-Vega et al., 2010, Bouchenak and Lamri-Senhadji, 2013). Hence, soaking can have both, positive and negative effects. Some authors consider that soaking is not always necessary to remove antinutrients. Several studies have shown that lectins can be inactivated with aqueous heat treatment. Lectins in soya bean can be inhibited by heating at 100°C for only 10 minutes (Pusztai and Grant, 1998). For both *P. vulgaris* and *V. fava* beans, lectins can be inactivated when the legumes are heated at 90°C for 1 h or, like in the case of soy beans, at 100°C for only 10 minutes (Grant et al., 1982, Grant and Van Driessche, 1993). Poel and Blonk (1990) found that steam treatment at 119°C for 5 or 10 min inhibited trypsin inhibitor in *P. vulgaris* beans (Van Der Poel et al., 1990). Rayas-Duarte et al. (1992) found that boiling for 30 min reduced the trypsin inhibitor activity by 80-90% in *P. vulgaris* beans (Rayas-Duarte et al., 1992).Therefore, boiling seems enough to inactivate lectins and protease

inhibitors without soaking the beans and consequently, avoiding the loss of polyphenols by this preparation process.

Unlike protease inhibitors and lectins, phytates and oxalates are not heat-labile. Oxalates affect calcium absorption; in beans, only around 22% to 27% of calcium is absorbed and it is believed this is due to the presence of oxalates in this legume (Weaver et al., 1999). Yet, beans are relatively low in calcium (45-55 mg/100 g of cooked bean in comparison to soybean, which contains 100 mg/100g of cooked soybean); hence, beans would likely make a minor contribution of calcium intake if other calcium-rich foods are included in the whole diet (Messina, 2014). The oxalate content in beans varies from 4-80 mg/100 g of beans, which is lower than other foods such as almonds (469 mg/ 100 g) cashews (262 mg / 100 g) and hazelnuts (222 mg/ 100 g); yet, beans high in oxalates should be avoided by people prone to kidney stones, since these are mainly composed of calcium oxalates (Chai and Liebman, 2005). Phytates affect mineral absorption, including iron; beans contain around 0.1-2% of this antinutrient (Oberleas and Harland, 1981, Campos-Vega et al., 2010). Phytates can be eliminated by soaking and fermentation, which can be beneficial to stimulate mineral absorption when beans are consumed (Gibson et al., 1998). However, the form in which iron is stored in the legume is an important factor that determines if the absorption of this mineral is affected or not by phytates. According to Murray et al. (2003), when iron is stored in the form of ferritin, iron absorption may be enhanced since this chemical form of iron is resistant to phytate action (Murray-Kolb et al., 2003). Lighter beans usually have a higher amount of ferritin; in white beans, ferritin comprises 90% of the total iron content (Hoppler et al., 2008). As in the case of oxalates, the possible negative effect of phytates could be reduced with a balanced diet. It is

important to have a diet that includes food rich in iron to cover the daily requirements of this mineral.

6.3 Outcomes of the LCMS polyphenol analyses

The LCMS analysis performed during this study presented diverse difficulties. In this case, due to the complexity of the samples, HPLC was not very useful since the mass as determined by LCMS equipment was the only parameter used for the identification of polyphenols in the samples. The methods that could be used in addition to MS analysis are UV spectrometry to identify the number of conjugated dienes, and infrared spectrometry to determine the functional groups of the molecules. In this way, with the information generated by these two methods mentioned, plus the mass of the compound, it is possible to predict the molecular structure of the compounds present. An alternative method could be to hydrolyse the fractions in order to separate the sugars from the aglycones and then be able to identify the polyphenols by HPLC.

As mentioned in the Introduction, the microflora may exert positive or negative effects on bean polyphenols. Some bacteria may hydrolyse the covalent bonds formed between the polyphenol and the cell wall components or sugars, making the phenols more available for absorption. Comalada et al. (2005) tested the anti-inflammatory effect of quercetin and quercitrine (a glycosylated form of quercetin) *in vitro* and *in vivo*. The *in vitro* anti-inflammatory activity was tested using macrophages; the *in vivo* assay consisted of the effect of the compounds on the reduction of induced colon inflammation in rats. Their results showed that quercetin reduced inflammation in the *in vitro* assay but not in the *in vivo* experiment. However, the glycosylated form had a minimum effect

in vitro and a higher effect *in vivo* in comparison to the aglycone. Furthermore, faecal fermentation of the quercitirin tested was performed. Their outcome was that the glycoside was hydrolysed during the faecal fermentation to their respective aglycone and was absorbed in the colon, reducing inflammation in the colon. Therefore, it is likely that these polyphenols may be metabolized by the microflora in the colon, hydrolysing them and making them more available for absorption in this part of the intestine. Some bacteria may alter the chemical structure of the polyphenols, which in consequence lead to the loss of biological activity of these phytonutrients. More experiments are required to investigate the role of the microflora on the bioavailability and biological activity of bean polyphenols.

6.4 Outcomes of the studies on the anti-inflammatory effect of Mexican *P. vulgaris* beans

The cell culture experiments showed that two of the bean samples (Borlotti and Negro Jamapa) possessed anti-inflammatory activity, whereas the other two beans analysed (Marcela and Azufrado) stimulated the inflammation process in the cells. As mentioned in Chapter 5, it is likely that an unknown compound present in the Marcela and the Azufrado beans could be stimulating oxidative stress in the cells, resulting in the increase of inflammation target genes. These results indicate that these two beans might not be suitable to prevent inflammatory-related diseases and that the consumption specific beans may have positive health effects. On the other hand, other beans could induce negative biological responses.

6.5 Further work

Studies such as those performed in the present work are scarce and have been carried out for less than a decade. As a result, modifications should be done in order to improve these types of studies. Based on the outcomes of this study, the following complementary work could be done in the future:

- Compare the INFOGEST *in vitro* digestion method in order to identify differences between this method and the one optimized in the present study.
- Use additional identification methods (besides LCMS) to identify the polyphenols in the samples.
- Evaluate the anti-inflammatory activity of more bean samples using the same methods used in the present study. In addition, identify P65 in macrophages after incubation of Negro Jamapa beans to confirm that these beans inhibit the NFκB pathway. This can be performed by isolating the p65 protein and analysing it by Western Blotting.
- Evaluate the effect that microflora has in the bioavailability of polyphenols This effect could give valuable information that could help us predict if beans can reduce colon inflammation.
- Perform a human study in order to evaluate the bioavailability and biological activity of the beans used in this study.

6.6 Conclusion

The health benefits of beans are yet inconclusive and further studies should be performed in order to understand in depth if beans have positive or negative attributes. In this study, it was found that even though some beans may belong to the same species, cultivars may have a diverse composition and

contrasting biological activities. This behaviour could also be found in other foods. Hence, it is very important to avoid generalization of possible activities of different samples, even though they might be similar or closely related (taxonomically). As a result, it would be ideal to analyse as many samples as possible to know which would be more recommendable for people to consume.

When evaluating the possible health benefits of foods, several factors should be taken into account. It is important to establish optimal methods for each sample studied since these are crucial for the final outcomes. Further work should include the optimization of analytical methods that can provide clearer information regarding their composition. Digestibility is an important factor that should be taken into account when evaluating the health benefits of food. Some food components might not be liberated during digestion and could be strongly associated with the food matrix. Therefore, extraction of food components with solvents might underestimate the amount of compounds isolated.

The methods used for the preparation of different dishes follows ancient recipes and traditions that are passed from generation to generation. As mentioned before, it is a popular belief that soaking beans will get rid of antinutrients that could be detrimental for human health. People normally follow preparation protocols and believes from their ancestors without questioning them. Bean soaking is an example of this. However, in this study it was shown that polyphenols are lost during this process. Cooking also contributes to the loss of these compounds. Yet, if soaking is avoided, the overall loss could be reduced. In addition, there is evidence in the literature that antinutrients are destroyed during cooking and the thermostable ones do not have an impact in the overall health of individuals, as long as bean consumption is complemented with a balanced diet. Thus, it is important to study traditional cooking methods in

order to know if these are the most appropriate preparation methods for the food in question or if some steps should be added or avoided to obtain a healthier meal.

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ANNEX

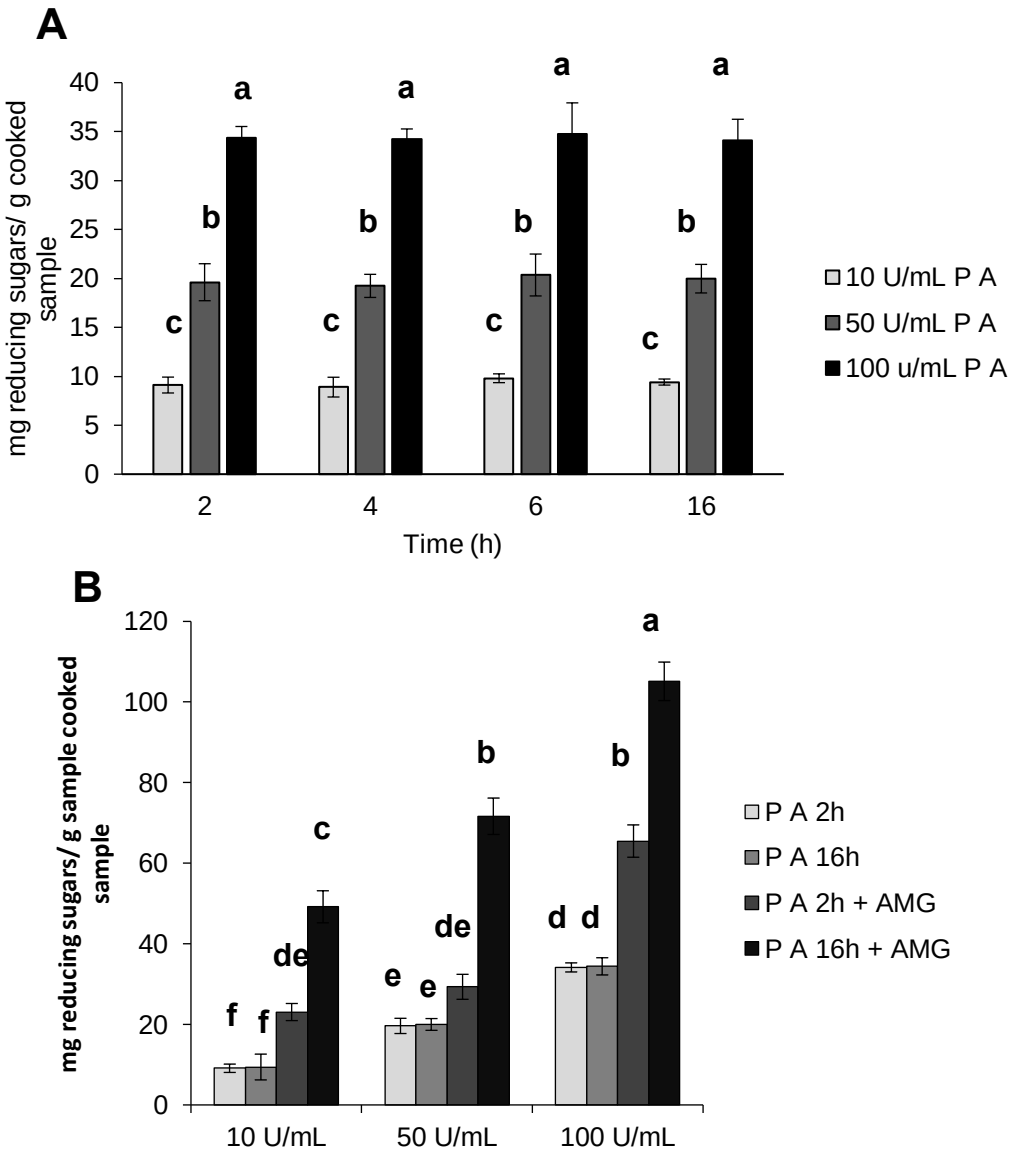

Figure A1. Reducing sugars (determined by the DNS assay) released upon Borlotti bean digestion. A) effect of different concentrations of pancreatic α-amylase (PA, 10, 50 and 100 U/mL) at different incubation times (2, 4, 6 and 16 h) (B) effect of PA digestion with or without subsequential digestion with amyloglucosidase (AMG) digestion at 2 h and 16 h.

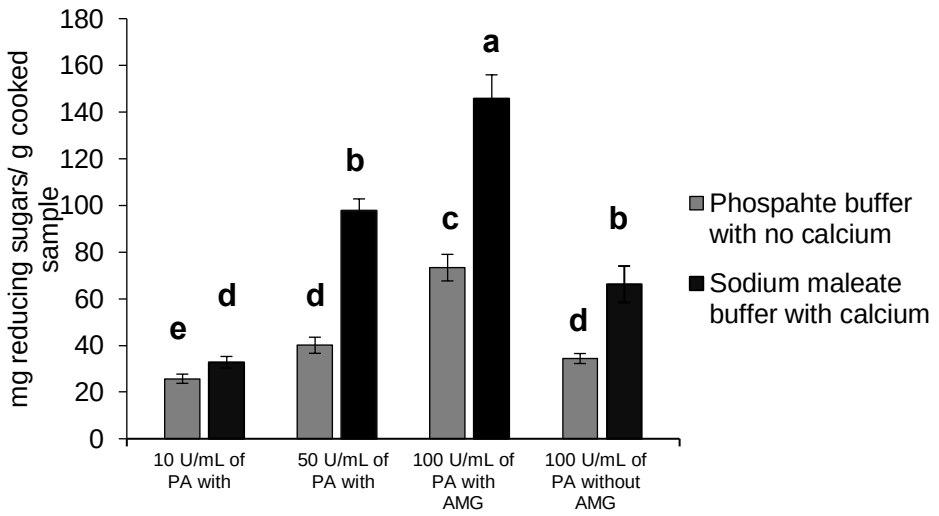

Figure A2. Reducing sugars determined by the DNS assay released upon Borlotti bean digestion in phosphate buffer (with no calcium) and sodium maleate buffer (with calcium) with different enzymatic treatments.

Table A1. DNS results in digestion supernatant after incubating sample with 100 U of pancreatic α-amylase (PA) (16h) and 30 U amyloglucosidase (AMG) using different incubation times.

Only PA	PA and AMG (30 min)	PA and AMG (60 min)	PA and AMG (120 min)
34.12 ± 4.10	43.14 ± 4.10	78.02 ± 7.08	77.03 ± 4.38

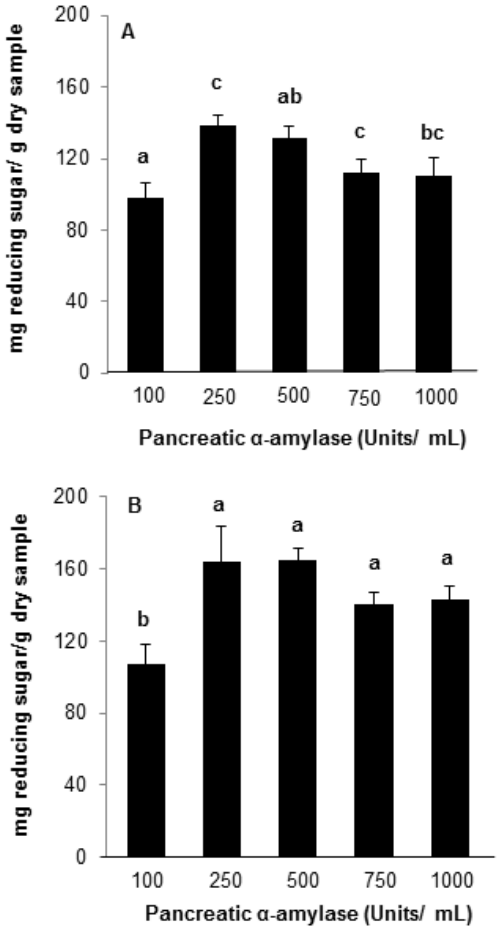

Figure A3. Results of supernatants obtained by Borlotti bean digestion with different amounts of pancreatic α-amylase and a posterior hydrolysis with 80 U/g of AMG. (A) DNS results; (B) HPAEC-PAD results.