Universidade de Aveiro Departamento de Química
Ano 2016

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Extração assistida por alta pressão de compostos bioativos a partir da casca de figo da Índia amarelo

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, ramo Alimentar, realizada sob a orientação científica do Doutor Jorge Manuel Alexandre Saraiva, Professor Investigador Auxiliar do Departamento de Química da Universidade de Aveiro e da Doutora Elisabete Maria da Cruz Alexandre, Investigadora da Universidade de Aveiro e da Escola Superior de Biotecnologia da Universidade Católica Portuguesa.

Aos meus pais com muito amor e carinho

o júri

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palavras-chave

Extração assistida por alta pressão, casca de figo da Índia, compostos bioativos, atividade antioxidante, atividade antimicrobiana.

resumo

O principal objetivo deste trabalho foi estudar o efeito da pressão (0.1, 300 e 600 MPa), tempo de extração (5, 17.5 e 30 min) e concentração de etanol (0, 40 e 80%) na extração de compostos fenólicos, flavonóides, antocianinas e betalainas a partir de casca de figo da Índia resultante do processo de produção de vinagre. A atividade antioxidante foi determinada pelos métodos de ABTS, DPPH e FRAP em todos os extratos assim como os rendimentos totais. Foi efetuado um desenho experimental fatorial e os resultados foram analisados pela metodologia de resposta de superfície, tendo-se determinado depois as condições óptimas de extração previstas pelo modelo. Todos os modelos foram validados e a atividade antimicrobiana dos extratos obtidos nas condições optimas foi analisada usando a *Escherichia coli* e *Listeria innocua*.

A concentração de etanol foi a variável que apresentou o maior efeito sobre o rendimento das extrações, seguindo-se a pressão e depois o tempo de extração. No geral, a alta pressão aumentou os rendimentos entre 6 e 17%, quando comparados com extrações realizadas sob as mesmas condições, mas a 0.1 MPa. Os modelos mostraram um ajuste satisfatório e adequado aos dados experimentais e as correlações dos modelos matemáticos obtidas indicaram que os modelos polinomiais quadráticos podem ser utilizados para prever os resultados. As condições ótimas de extração foram diferentes de acordo com o tipo de composto analisado. Os resultados experimentais e previstos diferiram menos do que 10%. Os extratos selecionados inibiram o crescimento de *Escherichia coli* e *Listeria innocua*, que se mostrou mais sensível. A extração por alta pressão apresentou vantagens em relação à extração por Soxhlet, aumentando a atividade antioxidante em média 27%. A extração de compostos fenólicos totais e betaxantinas aumentou 19% e 117%, respetivamente. Para além disto, os tempos de extração foram 8 a 48 vezes menores quando alta pressão foi usada.

Os resíduos de figo da Índia são ricos em compostos bioativos que, quando convenientemente recuperados, pode ter inúmeras aplicações em diversos sectores, e ao mesmo tempo valorizam-se os resíduos de fruta. As otimizações obtidas neste trabalho tornam a tecnologia de alta pressão num processo de extração promissor para *scale-up*.

keywords

High pressure assisted extraction; prickly pear peel; bioactive compounds; antioxidant activity; antibacterial activity.

abstract

The main objective of this research was to study the effect of pressure (0.1, 300 and 600 MPa), extraction time (5, 17.5 and 30 min) and ethanol concentration (0, 40 and 80%) on total phenolics, flavonoids, anthocyanins and betalains from yellow prickly pear peels, a sub product of vinegar production. Antioxidant activity (ABTS, DPPH and FRAP methods) and total extraction yields were also determined for all extracts. A Box–Behnken design and Response Surface Methodology (RSM) were used to evaluate the effects and to estimate the optimum extraction conditions. Antimicrobial activity was evaluated against *Escherichia coli* and *Listeria innocua* in extracts performed under optimized conditions.

Ethanol concentration was the variable that showed the greatest effect on extraction yields, followed by pressure and then the extraction time. In general, high pressure increased extraction yields between 6 and 17%, when compared with extractions performed under same conditions but at 0.1 MPa. The models showed satisfactory fitting and adequacy to the experimental data and the high correlation of mathematical models indicated that the quadratic polynomial models could be employed to predict the results and optimize the extraction conditions. The optimum extraction conditions were dependent on the class of compounds analyzed. Experimental and predicted results differ less than 10%. The selected extracts inhibited *Escherichia coli* and *Listeria innocua* growth. High pressure assisted extraction showed advantages in relation to Soxhlet increasing the antioxidant activity in average 27% and presenting similar total extraction yields. Total phenolic compounds and betaxanthins increased 19% and 117%, respectively. Moreover, the extraction times were between 8 to 48 times smaller when high pressure extraction was used.

The prickly pear residues are rich in bioactive compounds that, when conveniently recovered, can have numerous applications in different sectors and at same time valorising the fruit residue. The optimizations obtained in this work make the high pressure technology applied to extraction process a promising process for scale up.

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List of Abbreviations

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AIS	Ammonium iron sulfate (II)
ANOVA	Analysis of variance
BC	Betacyanins
BT	Betanin
BX	Betaxanthins
CD	Cyanidin-3-glucoside
CE	Conventional extraction
CT	Catechin
CV	Coefficient of variation
DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	Dried weight
EOV	Experimental optimum values
FL	Flavonoids
FRAP	Ferric reducing ability of plasma
FW	Fresh weight
GA	Gallic acid
HPE	High pressure extraction
IN	Indicaxanthin
INE	Instituto Nacional de Estatísca
MW	Molecular weight
OEC	Optimum extraction condition
PC	Phenolic compounds
POV	Predicted optimum values
QR	Quercetin
RSM	Response surface methodology
SE	Soxhlet extraction
t	Time
T	Temperature
TPTZ	2,4,6-Tripyridyl-s-triazine
TROLOX	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
TY	Extraction yields

1. Literature Revision

1.1. Fruit production, consumption and residues

Every year are produced large amounts of fruits in which, a huge part is processed increasing also fruit residues production by industrial sector. Between 2011 and 2013, the global production of fruits increased about 9% (Statista, 2016). In 2013, Europe produced about 7320000 tons of fruits, which corresponded to 11% of global world production (Statista, 2015). Between 2012 and 2015, the Portuguese fruit production increased 44%. The most produced fruits where apples, pears, oranges and olives for olive oil with a production increase of 47%, 21%, 18%, and 68%, respectively between 2012 and 2015 (INE, 2015c; INE, 2016). In the last four years, between 2012/2013 and 2014/2015, the fruit consumption per capita in Portugal increased 6%, which represent a consumption of 31 tons. In Portugal, 630 tons of fruit were consumed in 2014/2015 (INE, 2015a; INE, 2015b). Recent studies were not found concerning European fruit residues production. However, according to Baiano (2014), although the production and processing of fruits and vegetables sector did not generate the highest amount of residues when compared with other sectors, such as the dairy products and ice cream industry sector, it was this sector that produced more residues generated per amount of production of each industrial sector (Table 1).

Table 1. Amount of residues produced and ratio between amount of residues generated and amount of production by the food industry (Baiano, 2014).

INDUSTRIAL SECTOR	RESIDUE (tons)	RESIDUE (%)
Production and processing of fruits and vegetables	279000	4.5
Production and processing of fish and fish products	8000	3.5
Dairy products and ice cream industry	404000	3
Production, processing and preserving of meat	150000	2.5
Manufacture of other food products	239000	2
Drinks industry	492000	2
Production of grain and starch products	245000	1.5
Manufacture of vegetable and animal oils and fats	73000	1.5
TOTAL	**1890000**	**2.6**

Until a few decades ago, these industrial residues were considered neither a benefit nor a cost, ending up brought to landfills, being used as animal feed, or sent for composting causing a significant negative impact on the environment, leading to environmental pollution. In the last years, several efforts have been done to change this attitude due to

the growing environmental concerns in the European Union and worldwide; the demand for controls to minimize the impact of residues on human health (that is bringing more stringent regulations); the high disposal costs (that are eroding the already low profits of the food industry); and the growing awareness of the benefits deriving from potentially marketable components present in foods residues are good examples of it (Baiano, 2014). These residues can be perceived as a source of valuable compounds such as carotenoids, fibers (Hernández-Santos et al., 2015) and phenolic compounds such as flavanols, flavonols and phenolic acids with important biological activities (Deng et al., 2012; Ma et al., 2012). Several research works have been developed to valorize the fruit residues, reporting that they can be used to obtain higher yields of cellulose produced by bacteria, resulting in cellulose with high quality (Kumbhar et al., 2015) and can be novel substrates for enzyme production (Almeida et al., 2015). Fruit extracts showed the capacity to inhibit human fungi, gram-positive and negative bacteria pathogens, making these residues useful to control the spread of human food-borne pathogens (Rakholiya et al., 2014; Mahadwar et al., 2015). Fruit residues can be transformed in flours, suggesting that they may be used to improve bowel health, as reported by Silva et al. (2014) in rats. Choi et al. (2015) used citrus peel residues in combination with other fruit residues/pomaces to produce bioethanol with high yields. Another possible application of fruit residues is related with the use of fruit residues as low-cost biosorbents to remove dyes and toxic metal ions (Mallampati et al., 2015; Pathak Pranav et al., 2015; Sahetya et al., 2015). Naseer et al. (2014) reported that fruit residues were also used to inhibit the synthesis of mycotoxins, showing that peels can replace chemical treatments to detoxify aflatoxins, being safer for long-term food storage.

1.2. Prickly pear fruit

With a production of 345000 tons/year, Mexico is the main producer of prickly pear fruit (*Opuntia* spp.). In the Mediterranean, Italy is the biggest producer with intensive plantations yielding about 70000 tons. Sicily is the responsible for nearly all of the Italian production and San Cono Hills, Santa Margherita Belice district, and the southwestern foothills of the Etna volcano are the three major production zones representing 90% of intensive cultivation (Inglese et al., 2002). The *Opuntia ficus-indica*, *O. megacantha*, *O. streptacantha* and *O. amyclaea* are the most common cultivated species (Cardador-Martinez et al., 2011). Nevertheless, this fruit is cultivated worldwide being eaten raw or

processed into fruit based products. This fruit contributes substantially to the food diet in rural populations from Mexico, Peru and North Africa, while in Europe and the USA is still considered an exotic fruit (Dubeux et al., 2012). Several important biological proprieties have been attributed to this fruit such as antioxidant (Koubaa et al., 2015), anti-diabetic (Berraaouan et al., 2014), antibacterial (Ali and El-Mohamedy, 2011), anti-inflammatory (Allegra et al., 2013; Tesoriere et al., 2014), anticancer (Zou et al., 2005), hepatoprotective (Galati et al., 2005), antiulcerogenic (Galati et al., 2003), neuroprotective (Dok-Go et al., 2003), hypolipidemic (Palumbo B. et al., 2003), and antiproliferative activities (Sreekanth et al., 2007). Because of all of these activities, the prickly pear fruit is important from a health and medical point of view since it can be used to control/prevent diseases. The prickly pear can be used to prepare products such as fruit juices, alcoholic beverages, jams, natural liquid sweetener, wine, flours for food supplements, squash, pickle, body lotions, shampoo, creams and flavouring agents (Moßhammer et al., 2006; Kaur et al., 2012; Yeddes et al., 2013).

The pulp represents 45-67% of total fruit, while the pericarp represents 33-55% and the seeds 2-10% (Gurrieri et al., 2000; Piga, 2004). Thus, the processing process generates high amount of fruit residues, mainly peels that may create value in the entire chain-production. The valorization is not only a market need but also an opportunity to recover basic nutrients and/or high value compounds (such as vitamins, carotenoids and phenolic compounds) and to produce relevant metabolites by chemical or biotechnological assays having a significant impact on industries economy (Pintado and Teixeira, 2015). Thus, the reuse and recycling of food residues has been highly encouraged using new technologies environmentally clean. Prickly pear peels are excellent sources of bioactive compounds, such as betalains, phenolic compounds, flavonoids, and tannins (Cardador-Martinez et al., 2011; Chougui et al., 2015). Some prickly pear cultivars are very attractive due to the intense color, and depending on the cultivar, the color may change from a blood-red to orange-yellow due the presence of different betalains (Gurrieri et al., 2000). These vacuolar alkaloid pigments result from the condensation between cyclo-DOPA (L-3,4-dihydroxy-phenylalanine)/its glucosyl derivatives and betalamic acid with amino acids/derivatives leading to the formation of two categories of betalains: red-violet betacyanins and yellow-orange betaxanthins, respectively (Jain and Gould, 2015; Khan and Giridhar, 2015). Betalains can be used as natural food colorant (E162), in pharmaceutical and cosmetics industries (Esatbeyoglu et al., 2015), having also shown

antioxidant activity in model rats (Allegra et al., 2014), among others biological activities, such as anti-inflammatory, anti-microbiological, anti-cancer, and anti-lipidemic properties (Gengatharan et al., 2015). Phenolic compounds are characterized by having one or more aromatic rings and one or more hydroxyl group and can be divided into phenolic acids, tannins, stilbenes, coumarins and flavonoids (Huang et al., 2009), presenting antioxidant and antimicrobial activities that play an important role in prevention of some diseases (Cushnie and Lamb, 2005; Treml and Šmejkal, 2016). The extraction of bioactive compounds can be done using different methods that play an important role in sample preparation, experimental research and qualitative and quantitative analysis of bioactive compounds (Bernhoft, 2010). Soxhlet, maceration and hydrodistillation are the widely and most used traditional extraction techniques (Ngamwonglumlert et al., 2015).

1.3. Traditional extraction techniques

These techniques are based on the extracting power of different solvents and the application of heat and/or mixing to increase the rate of mass transfer increasing the solubility of compounds (Wu et al., 2001). The extraction efficiency depends on the choice of solvents, where the polarity of the targeted compound is the most important factor (Shahid et al., 2013). Questions concerned with environmental safety, toxicity and financial feasibility should also be considered in selection of solvent.

1.3.1. Extraction by Soxhlet

Franz Ritter von Soxhlet proposed Soxhlet extraction firstly for the determination of fat milk (Soxhlet, 1879). Nowadays, its application was extended and is the most used traditional extraction technique for a number of decades, as an efficiency reference for the comparison of its conventional and new counterparts, surpassing the performance of other extraction methods (Luque de Castro and García-Ayuso, 1998). A small amount of dry sample is placed in a thimble that is placed in distillation flask containing the solvent of interest. After reaching to an overflow level, the solution of the thimble-holder is aspirated by a siphon that unloads the solution back into the distillation flask. This solution carries extracted solutes into the bulk liquid. The solute remains in the distillation flask and solvent passes back to the solid bed of plant. The process runs repeatedly until the extraction is completed (Azmir et al., 2013). Some disadvantages of the Soxhlet

extraction are the extraction of thermolabile compounds, which undergo thermal degradation due to high temperature (Kanasawud and Crouzet, 1990); the very long extraction times, increasing the extraction cost; and the used of potential harmful organic solvents to the environment and health such as petroleum ether and hexane (Table 2). Beside peels, Soxhlet is also used to extract essential oils from seeds (Gurrieri et al., 2000; Ennouri et al., 2005; Xhaxhiu et al., 2013; Chemat et al., 2014; Kukeera et al., 2015).

Table 2. Compounds extracted from fruit peels by Soxhlet.

Source	Compounds	Solvent(s)	OEC T(°C)/t(h)	Max. yield (%)	Reference
Citrus	Nobiletin and tangeretin	ethanol	-/4	14.34[a)]	Lee et al. (2010)
Citrus	Limonene (L) β-myreene (Bm) Linalool (Ln)	methylene, chloride, hexane, acetone, methanol	-/6	0.35 (L) 0.085 (Bm) 0.055 (Ln)	Xhaxhiu et al. (2013)
Citrus	D-limonene	hexane	68/4	0.95	Lopresto et al. (2014)
Mango	Phenolic compounds	ethanol	-/4	25.13[a)]	Tunchaiyaphum et al. (2013)
Mango	Mangiferin (M) Lupeol (Lu)	ethanol hexane	-/8	9-11[a)] (M) 0.2-0.3[a)] (Lu)	Ruiz-Montañez et al. (2014)
Pomegranate	Phenolic compounds (PC) Flavonoids (FL) Anthocyanins (A) Punicalagins (P) Ellagic acid (EA)	ethyl acetate	-/3-15	2.462[a)] (PC) 0.676[a)] (FL) 0.421[a)] (A) 7.39[a)] (P) 63.61[a)](EA)	Masci et al. (2016)
Pomegranate	Phenolic compounds	ethyl acetate, methanol, water, acetone	-/4	52	Negi et al. (2003)
Banana	Essential oil	hexane	68/7	62.42	Hamid et al. (2016)
Garcinia cowa	Organic acids	acetone, methanol	60/8	25.4	Jena et al. (2002)

OEC: optimum extraction conditions; T: Temperature; t: time; a) mg equivalent standard/g DW; DW: dried weight.

1.3.2. Extraction by hydrodistillation

Hydrodistillation is a traditional method very used to extract essential oils from peels (Table 3), but also from seeds (Zito et al., 2013; Du et al., 2014; Lee et al., 2014; Papa et al., 2014; Liu et al., 2015). As similar to the Soxhlet extraction, at high extraction

temperatures some volatile components may be lost, which limits their application for thermolabile compound extraction. The long extraction times is also a disadvantage, but the extraction by hydrodistillation is more environmental friendly since water is the main extraction solvent used (Azmir et al., 2013).

Table 3. Essential oils extracted from fruit peels by hydrodistillation.

Source	Solvent(s)	OEC t (h)	Max. yield (%)	Reference
Bergamot	-	3.0	0.60	Djenane (2015)
Grapefruit	water	3.0	0.42	Uysal et al. (2011)
Lemon	-	3.0	0.70	Djenane (2015)
Lemon	water	3.0	4.12	Gök et al. (2015)
Mandarin	-	3.0	3.5-5.5	Hosni et al. (2010)
Orange	-	3.0	1-3	Hosni et al. (2010)
Orange	water	4.0	1.63	Allaf et al. (2013a)
Orange	water	4.0	1.97[a)]	Allaf et al. (2013b)
Orange	-	1.7	7.01	Pingret et al. (2014)
Orange	-	3.0	0.58	Djenane (2015)
Pomelo	-	3.0	1	Hosni et al. (2010)
Pomelo	water	2.0	756[b)]	Sun et al. (2014)
Yellow prickly pear	-	3.0	0.053	(Zito et al., 2013)
Red prickly pear	-	3.0	0.071	(Zito et al., 2013)

OEC: optimum extraction conditions; t: time; a) mg/g DW; b) µg/mL; DW: dried weight.

According to Vankar (2004), there are three types of hydrodistillation: water distillation, direct steam distillation and water and steam distillation. Water distillation consists in the mixture of plant materials with water in sufficient amount and then boiled. Direct steam distillation consists in the injection of steam into the sample and water and steam distillation technique consists in a mix of both principals. Hot water and steam act as the main influential factors to release bioactive compounds from plant tissues. During hydrodistillation, the indirect cooling by water condensates the vapor mixture of water and oil, which flows from condenser to a separator, where oil and bioactive compounds are separated automatically from the water (Silva et al., 2005).

1.3.3. Extraction by maceration

Maceration is a traditional technique frequently used for a small-scale extraction. The plant materials are grounded to increase the surface area for mixing with solvent and appropriate solvent is added in a vessel. Finally, the liquid is strained off but the solid residue of the extraction is pressed to recover large occluded solutions. The obtained

strained and press liquids are mixed and filtered (Azmir et al., 2013). The main disadvantage of this technique is that can be very time-consuming and uses potential harmful organic solvents to the environment and health, but more friendly solvents are used such as water and ethanol (Table 4). Like Soxhlet, maceration is also used to extract essential oils from seeds (Yoswathana and Eshtiaghi, 2014; Jadhav et al., 2016; Lourith et al., 2016; Oliveira et al., 2016).

Table 4. Compounds extracted from fruit peels by maceration.

Source	Compounds	Solvent(s)	OEC	Max. yield (mg/g DW)	Reference
			T(ºC)/t(h)		
Citrus	Pectin	water	80-82/1	15[a)]	Guo et al. (2012)
Citrus	Phenolic compounds (PC) Flavonoids (FL) Vitamin C (VC) Carotenoids (C)	methanol, hexane, dichloromethane, ethyl acetate, butanol	-/72	9.2 (PC) 7.1 (FL) 0.35 (VC) 0.0062 (C)	Singh et al. (2014)
Grape	Phenolic compounds (PC) Proanthocyanidins (Pat)	methanol, acetone	25/3	3-41 (PC) 1-60 (Pat)	Sá et al. (2014)
Mango	Phenolic compounds (PC) Flavonoids (FL)	methanol and ethanol	25/240	127 (PC) 78 (FL)	Vega-Vega et al. (2013)
Mango	Mangiferin (M) Lupeol (Lu)	ethanol hexane	25/24	4-6 (M) 0.15-0.25 (Lu)	Ruiz-Montañez et al. (2014)
Pomegranate	Antioxidant compounds	water	25/4	10[a)]	Qu et al. (2010)
Persimmon	Phenolic compounds	acetone, ethanol, methanol, water	25/16	18[a)]	Jang et al. (2010)
Hazelnuts	Phenolic compounds (PC) Tannins (Tn)	ethanol	20-22/20	502 (PC) 358 (Tn)	Contini et al. (2008)

OEC: optimum extraction conditions; T: Temperature; t: time; DW: dried weight; a) %.

Exhaustive maceration can consume large volumes of solvent and lead to the loss of material and/or metabolites. Furthermore, some compounds may not be efficiently extracted if they are poorly soluble at room temperature but since extraction is performed at room temperature, is less likely to occur degradation of compounds sensitive to high temperatures (Seidel, 2012).

1.4. High pressure assisted extraction

To overcome the limitations of the traditional extraction techniques and due to the fact that betalains and phenolic compounds are sensible compounds to high temperatures, light, oxygen, and other extraction conditions (Delgado-Vargas et al., 2000; Palma et al., 2001), new and promising extraction techniques were developed such as high-pressure extraction, enhancing the overall yield and selectivity of bioactive components from plant materials.

High pressure assisted extraction (HPE), also known as ultra-high pressure extraction or high hydrostatic pressure extraction, was firstly studied in 2004 by Shouqin et al. (2004). These authors performed some pilot studies to demonstrate the applicability of HPE of compounds from herbs. These authors concluded that HPE was able to shorten effectively the extraction time and increase the efficiency of the process. This technique has been applied to extract several compounds from various natural plant materials such as catechins, phenolic compounds and caffeine from green tea leafs (Jun, 2009; Xi et al., 2009; Jun et al., 2010; Xi et al., 2011), lycopene from tomato paste (Xi, 2006), ginsenosides from ginseng power (Shin et al., 2010), flavonoids from propolis (Shouqin et al., 2005), phenolic compounds, flavonoids and sulforaphane from seeds (Briones-Labarca et al., 2015). Different bioactive compounds have been also extracted frequently from fruit peels (Table 5). HPE consists in the application of high pressures between 100 and 600 MPa, low volumes of organic solvents and low-to-mild temperatures, which results in a conservation of the compounds structure, since it does not affect covalent bonds (Pereira and Vicente, 2010; Jun, 2013). The extraction times are reduced, when compared with the traditional technologies. HPE also allows to use any type of solvent without solvent volatilization to occur. This extraction technique is environmental friendly being considered as ‘‘green technique’’ since complies with standards set by the Environmental Protection Agency, USA due the reduction of synthetic and organic chemicals used, the reduction in operational time and due the achievement of better yields and extracts with a high quality (Azmir et al., 2013).

Table 5. Compounds extracted from fruit peels by HPE and increase in extraction yields comparing with traditional methods.

Source	Compounds	Solvent	OEC	Max. yield HPE / CE (mg/g DW)	Reference
			P(MPa)/T(ºC)/t(min)		
Honey pomelo	Pectin	water	500/55/10	Similar amount	Guo et al. (2014)
Lemon	Phenolic compounds	ethanol	500/25/3	3.75-4.00[a)]/3.40-3.60[a)]	Casquete et al. (2014)
Lemon	Phenolic compounds	ethanol	300/-/3	2.66[a)]/2.23[a)]	Casquete et al. (2015)
Lime	Phenolic compounds	ethanol	300/-/3	3.97[a)]/3.62[a)]	Casquete et al. (2015)
Longan fruit	Phenolic compounds	ethanol	500/50/2.5	23/21	Prasad et al. (2009b)
Longan fruit	Corilagin	ethyl acetate, ethanol, methanol and water	500/30/2.5	9.65/2.35	Prasad et al. (2009a)
Longan fruit	Phenolic compounds	ethanol	500/30/30	10.59/5.597	Prasad et al. (2010)
Orange	Pectin	water	500/55/10	20.44[a)]/15.47[a)]	Guo et al. (2012)
Orange	Phenolic compounds	ethanol	300/25/10	4.0[b)]/-	Casquete et al. (2014)
Orange	Phenolic compounds	ethanol	300/-/3	2.88[b)]/2.84[b)]	Casquete et al. (2015)
Orange	Phenolic compounds (PC) Flavonoids (F)	ethanol	50-100/35/30	12-14/15-17 (PC) 7-9/6-8 (F)	M'hiri et al. (2015)
Mandarin	Anthocyanins	ethanol	600/50/30-90	More 23[b)] than control	Corrales et al. (2009)
Mandarin	Phenolic compounds	ethanol	300/-/3	5.87[a)]/5.30[a)]	Casquete et al. (2015)

OEC: optimum extraction conditions; P: Pressure (MPa); T: Temperature (ºC); t: time (min); CE: conventional extraction; DW: dried weight; a) mg equivalent standard/g FW; b) %.

Table 5. Compounds extracted from fruit peels by HPE and increase in extraction yields comparing with traditional methods (continued).

Source	Compounds	Solvent	OEC P(MPa)/T(ºC)/t(min)	Max. yield HPE / CE (mg/g DW)	Reference
Mandarin	Phenolic compounds (PC) Anthocyanins (A)	ethanol	600/70/60	0.25-0.38/0.18-0.25 (PC) 0.0061-0.011/0.0075-0.0079 (A)	Corrales et al. (2008)
Mango	Mangiferin (M) Lupeol (Lu)	ethanol and hexane	150/25/20	9.6-11.8/8.4-10.8 (M) 0.45-0.55/0.2-0.28 (Lu)	Ruiz-Montañez et al. (2014)

OEC: optimum extraction conditions; P: Pressure (MPa); T: Temperature (ºC); t: time (min); CE: conventional extraction; DW: dried weight; a) mg equivalent standard/g FW; b) %.

The extraction mechanism by HPE was elucidated and reported by (Huang et al., 2013). The pressure increases from atmospheric to the set pressure in a short time, leading to cell deformation due to the large differential pressure that was created between the interior and the exterior of the cell, because the pressure inside the cells is very low. With the membranes altered and under the differential pressure, the solvent permeates very fast into the cells with a large rate of dissolution. The second stage occurs when the extraction pressure equals the set pressure and is maintained for a certain period of time (the extraction time) to balance the pressure inside and outside the cells. The final stage is finished in a matter of seconds, were occurs a suddenly pressure drop to atmospheric pressure. Jun et al. (2011) studied the microstructure and ultrastructure of the untreated and pressurized green tea leaves samples with 50% ethanol as extraction solvent by scanning and transmission electron microscopy. The authors concluded that untreated samples were in an orderly intact state and stand relatively closely in an orderly manner with intact organelles inside the cells, while pressurized samples were found remarkably cracked, distorted and loosened shape. The cell organelles were broken into pieces, the vacuoles were destroyed and cellular membrane was totally collapsed and disrupted, which resulted in broken organelles being remixed and redistributed in the cells.

1.5. Objectives

No literature has reported the high pressure assisted extraction of bioactive compounds from yellow prickly pear peels and so, based on the present review, the objectives of this work were:

1. Study the impact of pressure (0.1, 300 and 600 MPa), extraction time (5, 17.5, and 30 min) and ethanol concentration (0, 40, and 80%) on total compounds such as phenolics, flavonoids, betalains and anthocyanins from yellow prickly pear peel. Evaluate the antioxidant activity by DPPH, ABTS and FRAP methods and determine the total yields also in the extracts;

2. Analyze each parameter by response surface methodology, determine the optimum extraction conditions and validate the models;

3. Compare the experimental optimum values with Soxhlet extraction method;

4. Evaluate the antibacterial activity of extracts obtained under optimum conditions against *Escherichia coli* and *Listeria innocua*.

2. Materials and Methods

2.1. Chemicals

All chemicals used were of analytical grade. DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and quercetin were purchased form Sigma-Aldrich (Missouri, EUA). TPTZ (2,4,6-Tripyridyl-s-triazine), potassium persulfate and aluminium chloride were obtained from Acrōs Organics (New Jersey, USA). Methanol, citric acid, glacial acetic acid and monopotassium phosphate were supplied by Chem-Lab (Zedelgem, Belgium). Folin-Ciocalteu reagent, ampicillin sodium, sodium carbonate and dipotassium phosphate were acquired form AppliChem Panreac (Darmstadt, Germany). Gallic acid, iron (III) chloride hexahydrate, potassium chloride and sodium acetate were purchased form Panreac (Barcelona, Spain). Ammonium iron sulfate (II) was supply by Fisher Chemical (Leicestershire, UK). Hydrochloric acid was obtained from Scharlau (Barcelona, Spain). Ringer was acquired form Merck KGaA (Darmstadt, Germany) and Müller-Hinton agar form Oxoid LTD (Hanupshire, England).

2.2. Peel residue

Yellow prickly pear peels were kindly provided by the processing industry "Cactus Extractus Lda" from Beja. The fermented peels were dried at 40ºC in a laboratory incubator equipped with an internal fan, promoting air circulation until moisture content around 13±2% (dry basis). Dried samples were then frozen in liquid nitrogen, grounded in a mill (Moulinex, Indonesia), vacuum packaged and stored at -20ºC until used for the extractions.

2.3. Extraction procedure

High pressure extracts were performed using an industrial hydrostatic press equipment (Hiperbaric 55, Hiperbaric, Burgos, Spain) with a pressure vessel of 200 mm inner diameter and 2000 mm length and a maximum operation pressure of 600 MPa. The equipment was connected to a refrigeration unit (RMA KH 40 LT, Ferroli, San Bonifacio, Italy) that allowed to control the temperature of the input water used as a pressurizing fluid. An amount of 0.5 g of grounded residue was mixed with 20 mL of ethanol (0, 40, and 80%) in plastic bags, which were pressurized at 300 and 600 MPa during 5, 17.5, and 30 min, at room temperature. Control experiments at 0.1 MPa were preformed alike but

without the application of pressure. All the variable combinations were tested according to a full 3^3 factorial design, which are presented Table 6.

Table 6. Full 3^3 factorial experiment design with repetition of central point 3 times.

Pressure (MPa)	Time (min)	Ethanol concentration (%)	Nomenclature
0.1	5	0	P0.1/t5/E0
		40	P0.1/t5/E40
		80	P0.1/t5/E80
	17.5	0	P0.1/t17.5/E0
		40	P0.1/t17.5/E40
		80	P0.1/t17.5/E80
	30	0	P0.1/t30/E0
		40	P0.1/t30/E40
		80	P0.1/t30/E80
300	5	0	P300/t5/E0
		40	P300/t5/E40
		80	P300/t5/E80
	17.5	0	P300/t17.5/E0
		40	P300/t17.5/E40
		80	P300/t17.5/E80
	30	0	P300/t30/E0
		40	P300/t30/E40
		80	P300/t30/E80
600	5	0	P600/t5/E0
		40	P600/t5/E40
		80	P600/t5/E80
	17.5	0	P600/t17.5/E0
		40	P600/t17.5/E40
		80	P600/t17.5/E80
	30	0	P600/t30/E0
		40	P600/t30/E40
		80	P600/t30/E80
300	17.5	40	P600/t17.5/E40
		40	P600/t17.5/E40
		40	P600/t17.5/E40

Soxhlet extraction was performed using the method reported by Manasathien et al. (2012). For the extraction, 3.75 g of residue were weighted and placed inside the thimble of the Soxhlet extraction apparatus with 150 mL of 40% ethanol. The extraction was carried out in a bath of silicone oil at 115ºC for 4 h, until the extraction was complete.

All the extracts were centrifuged at 15.000 rpm for 10 min at 4ºC (Thermo Fisher Scientific, Massachusetts, USA), filtered (Whatman Nº 1) and frozen at -80ºC until used for compounds analyses which were performed in 96-well microplates (0.77 cm length path) and read in a microplate reader (Microplate Spectrophotometric Multiscan Go, Thermo Scientific, USA). All analyses were performed in triplicate.

2.4. Total phenolic content

The Folin-Ciocalteu assay was used to determine the total phenolic content (Singleton et al., 1999). Folin reagent is a mixture of phosphomolybdic and phosphotungstic acids and when is in the presence of phenolic compounds (reducing agents) on a alkaline medium, forms a blue chromophore due the phosphotungstic-phosphomolybdenum complex (Blainski et al., 2013).

For the analysis, 20 µL of each extract were mixed with 100 µL of 1:4 diluted Folin-Ciocalteu reagent. After 4 min, 75 µL of sodium carbonate solution prepared in water (100 g/L) was added and allowed to react in the dark, at room temperature for 2 h, after which the reads were performed at 750 nm. For blanks, the 20 µL of each extract were replaced by 20 µL of water. A gallic acid (GA) stock solution (0.2 mg/mL) was used as a standard and a series of gallic acid solutions (0 - 0.2 mg/mL) were prepared to establish the standard curve ($y=0.0062x+0.0130$; $R^2=0.9996$) (Figure A1 from Appendix A). Results were expressed as milligrams of gallic acid equivalent per gram of dried weight (mg GA Eq./g DW).

2.5. Total flavonoids

Total flavonoid content was determined using the Dowd method described by Cruz et al. (2014). According to Magalhães et al. (2012), this spectrophotometric method relies on the detection at 415 nm, yellow colored complexes formed between aluminum (III) (aluminum trichloride) and the carbonyl and hydroxyl groups of flavonoids, in alkaline medium.

For the analysis, 150 µL of each extract were mixed separately with 150 µL of an aluminum trichloride ($AlCl_3$) solution prepared in methanol (0.2 mg/mL). For blanks, the 150 µL of aluminum trichloride solution were replaced by 150 µL of methanol. All reactions were kept in the dark for 10 min and the absorbance was read at 415 nm. A quercetin (QR) stock solution (0.025 mg/mL) prepared in absolute ethanol was used as a

standard and several quercetin solutions (0 – 0.025 mg/mL) were prepared to establish the standard curve (y=0.0245x-0.0160; R^2 =0.9842) (Figure A2 from Appendix A). Results were expressed as milligrams of quercetin equivalent per gram of dried weight (mg QR Eq./g DW).

2.6. Total condensed tannin

The condensed tannin content of extracts was determined by the vanillin method reported by Naczk et al. (2000). Price et al. (1978) reported the vanillin assay involves the reaction between vanillin, an aromatic aldehyde, and flavanols, the monomeric units of tannins, forming a red adduct that has a maximum absorbance at 500 nm.

For the analysis, 50 µL of each extract was mixed with 150 µL of vanillin (1% in 7 M H_2SO_4) in an ice bath. The reaction was maintained in the dark, at room temperature, for 15 min and the absorbance was read at 500 nm. Absolute ethanol (200 µL) was used as blank. A catechin (CT) stock solution (0.08 mg/mL) prepared in absolute ethanol was used as a standard and a series of catechin solutions (0 - 0.08 mg/mL) were prepared to establish the standard curve (y=0.0046x+0.0388; R^2 = 0.9785) (Figure A3 from Appendix A). Results were expressed as milligrams of catechin equivalent per gram of dried weight (mg CT Eq./g DW).

2.7. Total betacyanins and betaxanthins

Total betacyanins (BC) and betaxanthins (BX) content were determined using the method reported by Stintzing et al. (2005) with some minor adaptations. For this analysis, 275 µL of extract were mixed with 25 µL of McIlvaine buffer (pH 6.5, citrate-phosphate) to obtain absorption values of $0.9 \leq A \leq 1.1$ at their maximum absorption, mixed for 10s and read at 480 and 538 nm for betaxanthins and betacyanins, respectively. For blanks, the 275 µL of extracted were replaced by water. Concentrations were calculated using the Equation 1 reported by Castellanos-Santiago and Yahia (2008).

$$C = \frac{A \times MW \times df \times 1000 \times V}{0.77 \times m \times \varepsilon} \quad (1)$$

Where A is the observance difference between samples and blank; MW and ε is the molecular weights and molar extinction coefficients of betanin (BT) (MW=550g/mol; ε=60000 L/mol.cm in water; 538 nm) and indicaxanthin (IN) (MW=308g/mol; ε=48000

L/mol.cm in water; 480 nm) for quantification of betacyanins and betaxanthins, respectively; *m* and *V* is the mass of residue (0.5 g) and the volume of extract in liters, respectively. The parameter *df* is the dilution factor and 0.77 is the path length in centimeters for microplate reader. The results were expressed as milligrams of betanin equivalent per gram of dried weight (mg BT Eq./g DW) for betacyanins and as milligrams of indicaxanthin equivalent per gram of dried weight (mg IN Eq./g DW) for betaxanthins.

2.8. Total monomeric anthocyanin

Monomeric anthocyanin content was determined using a pH differential method (Lee et al., 2005). The pH differential method is based on the reversible structural change of the anthocyanin chromophore between pH 1.0 and 4.5, having a colored pink oxonium form and a colorless hemiketal form, respectively for each pH. These difference in absorbance (at the maximum absorbance) is proportional to the concentration of monomeric anthocyanin (Lee et al., 2005).

For the analysis, 60 µL of each extract was mixed with 240 µL of a potassium chloride solution (0.025 M, pH 1.0) and 240 µL of a sodium acetate solution (0.4 M, pH 4.5) separately. Solutions were prepared with water and pH was adjusted with concentrated HCl. After 30 min, the absorbance was measured at 520 and 700 nm. For blanks, the 60 µL of extract were replaced by 60 µL of water. Pigment content was calculated using the Equations 2 and 3.

$$A = (A_{520} - A_{700})_{pH\ 1.0} - (A_{520} - A_{700})_{pH\ 4.5} \qquad (2)$$

$$C = \frac{A \times MW \times df \times 1000}{0.77 \times \varepsilon} \qquad (3)$$

Where, *A* is total difference of observances measured at 520 and 700 nm at pH 1.0 and 4.5 without blanks; *MW* and ε is the molecular weights and molar extinction coefficients of cyanidin-3-glucoside (*MW*=449.2 g/mol and ε= 26.900 L/mol.cm) for quantification of monomeric anthocyanin. The parameter *df* is the dilution factor and 0.77 is the path length in centimeters for microplate reader. Results were expressed as milligrams of cyanidin-3-glucoside (CD) equivalent per gram of dried weight (mg CD Eq./g DW).

2.9. Total antioxidant activity

Antioxidant activity was performed using free radical DPPH scavenging capacity, radical cation $ABTS^{\bullet+}$ scavenging activity and ferric reducing antioxidant power (FRAP) assays according to the methods described by Bobo-García et al. (2015), Cardador-Martinez et al. (2011) and Benzie and Strain (1996), respectively.

2.9.1. DPPH assay

The DPPH radical is stable and has a dark purple color with a maximum absorbance at 515 nm. When reduced, the radical originates its corresponding hydrazine (pale yellow color) in the presence of a substance capable of donating a hydrogen atom, losing color and resulting in a decreased in absorbance (Huang et al., 2005).

For this analysis, 20 µL of extract were mixed with 180 µL of DPPH reagent (150 µM) in the dark at room temperature. The absorbance of mixture was read at 515 nm after 40 min. Blanks and controls were performed with 20 µL of water more 180 µL of solvent or 180 µL of DPPH reagent, respectively. A Trolox stock solution (0.175 mg/mL) was prepared in ethanol 80% was used as a standard and a series of Trolox solutions (0 - 0.122 mg/mL) were prepared to establish the standard curve ($y=0.5317x+4.4419$; $R^2=0.9901$) (Figure A4 from Appendix A) of the percentage of DPPH inhibition *versus* the concentration of the Trolox solutions. The percentage of DPPH inhibition was determined using the Equation 4.

$$\%\ \text{DPPH inhibition} = \left[1 - \left(\frac{A - A_{blank}}{A_{control} - A_{blank}}\right)\right] \times 100 \quad (4)$$

Where A is the absorbance of sample or Trolox solution and A_{blank} and $A_{control}$ are the absorbance of the blanks and controls. Results were expressed as milligrams of Trolox equivalent per gram of dried weight (mg Trolox Eq./g DW).

2.9.2. ABTS assay

In this method, the monocationic radical of $ABTS^{\bullet+}$ is generated by oxidation of ABTS by potassium persulfate, resulting in a blue-green chromophore, which is then reduced in the presence of hydrogen or electron donor antioxidants. This reduction causes a discoloration that can be converted into a $ABTS^{+}$ inhibition that is proportional to the antioxidant concentration (Re et al., 1999). For this analysis, ABTS reagent was obtained by the reaction of 50 mL of 7 mM ABTS with 25 mL of 2.45 mM potassium persulfate for 24h under constant mixing in the dark, being then diluted to an absorbance of

0.80±0.02 at 734 nm. A volume of 200 µL of diluted $ABTS^{\bullet+}$ solution was mixed with 20 µL of extract for 6 min in the dark, at room temperature, and the absorbance was read at 734 nm. Blanks and controls were performed with 20 µL of water and 200 µL of solvent or 200 µL of DPPH reagent, respectively. A Trolox stock solution (0.175 mg/mL) was prepared in ethanol 80% was used as a standard and a series of Trolox solutions (0 - 0.175 mg/mL) were prepared to establish the standard curve (y=0.7767x+0.3057; R^2=0.9904) (Figure A5 from Appendix A) of the percentage of ABTS inhibition *versus* the concentration of the Trolox solutions. The percentage of ABTS inhibition was determined using the Equation 5.

$$\%\ \text{ABTS inhibition} = \left[1 - \left(\frac{A - A_{blank}}{A_{control} - A_{blank}}\right)\right] \times 100 \quad (5)$$

Where A is the absorbance of sample or Trolox solution and A_{blank} and $A_{control}$ are the absorbance of the blanks and controls. Results were expressed as milligrams of Trolox equivalent per gram of dried weight (mg Trolox Eq./g DW).

2.9.3. FRAP assay

The ferric salt is used as the oxidant and is prepared by mixing TPTZ and iron (III) chloride hexahydrate in acetate buffer. A FRAP unit is defined as the reduction of 1 mole of Fe (III) to Fe (II), leading to the formation of a dark blue complex (Huang et al., 2005). FRAP reagent was prepared daily mixing 50 mL of acetate buffer (300 mM, pH 3.6), 2.5 mL of TPTZ 10 mM (0.031g of TPTZ dissolved with 10 mL of 40 mM HCl at 50ºC) and 2.5 mL of ferric chloride 20 mM (0.054g of iron (III) chloride hexahydrate dissolved with 10 mL of water), which was warmed at 37ºC for 10 min before used. Acetate buffer was prepared mixing 3.1 g of sodium acetate trihydrate with 16 mL of glacial acetic acid and completed for 1 L with water.

For the analysis, 280 µL of this solution were mixed with 20 µL of extract, incubated at 37ºC for 30 min and absorbance was read at 595 nm. For blank, the 20 µL of each extract were replaced by 20 µL of water. An ammonium iron (II) sulfate (AIS) stock solution (0.39 mg/mL) was used as a standard and a series of solutions (0 - 0.392 mg/mL) were prepared to establish the standard curve (y=0.0031x-0.0200; R^2=0.9993) (Figure A6 from Appendix A). Results were expressed as milligrams of ammonium iron (II) sulfate equivalent per gram of dried weight (mg AIS Eq./g DW).

2.10. Total extraction yields

Total extraction yields were determined according to the method described by Zhang et al. (2007) through Equation 6.

$$\text{TY}\ (\%) = \frac{mass\ of\ peel\ extract}{mass\ of\ residue} \times 100 \quad (6)$$

One milliliter of each extract was evaporated to remove the ethanol using a rotary evaporator (Büchi, Flawil, Switzerland), frozen at -80°C during 24 h and then freeze-dried for 60 h (Vitris Benchtop K, Pennsylvania, USA) to determine the mass of residue.

2.11. Antibacterial activity

Antibacterial activity was tested against *Escherichia coli ATCC 25922* and *Listeria innocua ATCC* 33090 (Liofilchem, Roseto degli Abruzzi (TE), Italy) using the Kirby-Bauer well-diffusion method reported by Biswas et al. (2013) with some adaptations to use a biosafety cabinet (Telstar Bio II Advance, Terrassa, Spain) (Al-Zoreky, 2009). Each bacteria strains was cultivated onto nutrient agar plates and incubated at 37 ºC for 24 and 72h for *Escherichia coli* and *Listeria innocua,* respectively, to obtain the colonies. After incubation, colonies were selected with a sterile disposable inoculating loop and transferred to a glass tube of sterile physiological saline and vortex thoroughly. Each bacterial suspension turbidity is then compared to that of the 0.5 McFarland standard solution (containing about 1.5×10^8 CFU/mL). After incubation, the bacterial suspensions were standardized by adjusting to the scale of 0.5 MacFarland (1×10^8 CFU/mL), which corresponds to an absorbance between 0.08-0.10 at 625 nm. Plates with 13.5 cm of diameter were filled 30 mL of Müller-Hinton medium prepared according to the instruction on the fabricant. After 1 day, all plates were inoculated with the adjusted test bacterium with a sterile cotton swab streaking over the entire sterile agar surface and rotating the plate to ensure even distribution of the bacteria with a final swab around the rim. Then, 6 mm diameter wells have been perforated using a sterile cork borer, after witch 50 µL aliquots of each extract (1 mg/µL) was dispensed into each well and petri plates were incubated for 24 h at 37ºC. Ampicillin (10µg/100µL) prepared in 0.1 M of potassium phosphate buffer, pH 8.0 and water were used as positive and negative controls, respectively. The halos formed by inhibition zones surrounding wells were considered as a measurement of the antimicrobial activity and the inhibition zones

diameters (with wells) were measured with a ruler (error ± 0.5 mm) and results reported in millimeters.

2.12. Experimental design, statistical analysis and models validation

The experimental extraction methodology was developed following a Box–Behnken design formed by a full 3^3 design, where experiments were conducted in a randomized order and data were analyzed by response surface methodology (RSM). The effect of 0.1, 300 and 600 MPa, applied during 5, 17.5 and 30 min using as solvent 0, 40 and 80% of ethanol were analyzed on the total phenolic compounds, flavonoids, betalains, anthocyanins, antioxidant activity (DPPH, ABTS and FRAP methods) and total yields. Error assessment was based on replication of the central point (300 MPa, 17.5 min, ethanol 40%). The output results were fitted to a second-order polynomial equation (quadratic model), according to the model in Equation 7.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \beta_{ii} x_i^2 + \sum_{i \neq j=1}^{3} \beta_{ij} x_i x_j \qquad (7)$$

Where Y is the predicted response, β_0 is the model intercept coefficient, β_i, β_{ii} and β_{ij} are the linear, quadratic and linear interactive coefficients, respectively and x_i and x_j represent the independent variables. The regression coefficients (individual linear, quadratic and interaction terms) were determined using analysis of variance. The regression coefficients were used to generate 3-D surface plots from the fitted polynomial equation to obtain the relationship between the response and experimental levels of each factor and to achieve the optimum conditions for each parameter analyzed. Analysis of variance (ANOVA) was performed for response variable using the full models where p-values indicated whether the terms were significant ($p<0.05$) or not ($p>0.05$). To validate the models, additional extraction trials performed in triplicate were carried out at the predicted optimal conditions and the experimental data were compared to the values predicted by the regression model. Antibacterial activity was performed in triplicate only for these extraction conditions and were analyzed by ANOVA. Antibacterial activity and comparison between HPE and Soxhlet extractions was performed using one-way ANOVA followed by Tukey's HSD test at a 5% level of significance.

3. Results and Discussion

3.1. Total phenolic compounds, flavonoids and tannins

The highest extraction yield obtained for total phenolics was 26.30±1.06 mg GA Eq./g DW, for the combination P600/t17.5/E0 (see Appendix B, and some representative figures are in Appendix C). This represents an increase of 14% in relation to extractions performed in same conditions but at atmospheric pressure (P0.1/t17.5/E0). High pressure impact was significant (F value of 24). In general, the use of high pressure (300 MPa and 600 MPa) conduced to an increase in the same extraction of total phenolics of 11% when compared to extraction performed at 0.1 MPa.

When the highest extraction yield is compared to the extraction performed in the same conditions but during 5 min (P600/t5/E0) the extraction of total phenolic compounds increase 22%. Extraction time had not a significant effect in the extraction ($p<0.05$), but phenolic compounds extraction increased 9% and 4% for extractions performed during 17.5 min and 30 min when compared to extraction performed for 5 min. The ethanol concentration was the independent variable with the highest impact (F values of 326 and 173 for linear and quadratic effects), following the pressure (Table 7).

Table 7. Analyses of variance for linear, quadratic and crossed effects of pressure, extraction time and ethanol concentration at a significance level of 95% confidence for total phenolic compounds and flavonoids models. The significant coefficients in each case are written in bold.

ANOVA	Phenolic compounds		Flavonoids	
	F	***p***	***F***	***p***
P (L)	24.07	**0.00**	0.57	0.45
P (Q)	0.31	0.58	0.58	0.45
t (L)	3.21	0.08	2.01	0.16
t (Q)	2.10	0.15	1.57	0.21
E (L)	325.61	**0.00**	170.60	**0.00**
E (Q)	173.95	**0.00**	67.76	**0.00**
P (L) x t (L)	1.91	0.17	0.04	0.84
P (L) x E (L)	9.08	**0.00**	3.41	0.07
t (L) x E (L)	1.36	0.25	0.76	0.38
R^2	0.875		0.757	
R^2 adjst	0.861		0.730	

L: linear; Q: quadratic; P: Pressure (MPa); t: time (min); E: Ethanol percentage (%).

When ethanol (40%) was used as extraction solvent, the extraction yields increased 4%, when compared to the extractions performed in the same conditions but with water, while

ethanol 80% lead to a decreased of extraction yield of 35%. However, at this ethanol concentration (80%), the increase of high pressure as well as the extraction time lead to higher extractions (Figure 1).

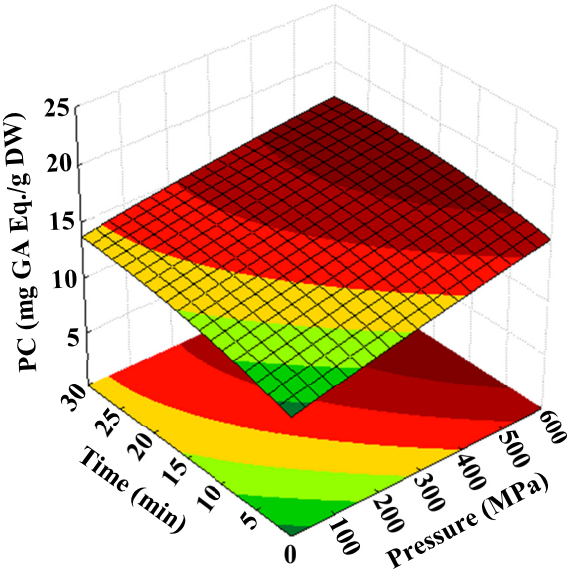

Figure 1. Response surfaces of phenolic compounds extraction for ethanol 80%.

For total flavonoids the highest extraction yield obtained was 2.19±0.04 mg QR Eq./g DW, for the combination P300/t5/E40 (see Appendix B, and some representative figures are in Appendix D), which means an increase of 16% in relation to extractions performed at 0.1 MPa. High pressure impact was not significant ($p<0.05$), but in general, 300 and 600 MPa conduced to an increase of the extraction of total flavonoids of 7% and 3%, respectively, when compared to extraction performed at 0.1 MPa. When results from the highest extraction yield were compared to the extraction performed in the same conditions but during 5 min, the total flavonoids extraction increase 110%. Similar to phenolic compounds, the ethanol concentration was the independent variable with the highest impact on extraction of flavonoids (F values of 171 and 68 for linear and quadratic effects) (Table 7). In general, when ethanol 40% and 80% were used as extraction solvent, the extraction yields increased 70%, when compared to the extractions performed in the same conditions with water. Figure 2 shows that when ethanol 80% was used, the highest extraction yields of flavonoids were obtained for higher high pressure, being necessary only necessary 5 min to reach the maximum extraction, meaning that extraction time had not a significant effect.

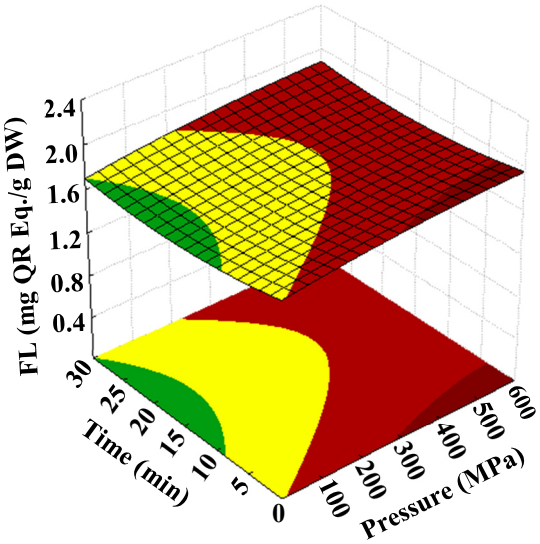

Figure 2. Response surfaces of flavonoids extraction for ethanol 80%.

Therefore, to assure high yields of phenolic compounds and flavonoids low ethanol concentration and intermedium pressures should be used. In relation to time variable, intermedium extraction times will be preferred for extraction of phenolic compounds, and short extraction times are sufficient to reach maximum extraction values for flavonoids. Jayaprakasha et al. (2008) reported that 40 to 80% of ethanol in water were more efficient on the extraction of phenolic compounds from grape fruit and orange that water and pure ethanol. Shouqin et al. (2005) reported that flavonoids from propolis were more soluble in ethanol than in water, due to the weaker and stronger polarities of ethanol and water, respectively. Also verified that when pressure was increased from 100 to 600MPa, the extraction yield of flavonoids also increased from 4.19% to 4.73% using ethanol 75% for a extraction time of 5 min. Extraction time did not had an impact on the extraction, which means duration of extraction had no close relationship to the increase in the extraction. Prasad et al. (2009b) verified that when the ethanol concentration increased between 25 to 50%, the phenolic compounds extracted from longan fruit pericarp also increased, but with ethanol 75% the concentration decreased. Extraction time also did not had a significant impact on the extraction. These authors also studied high pressure effect on total phenolics extracted from longan fruit pericarp and obtained an increase of 44% when 500 MPa were used. The main reason for the extraction of phenolics and flavonoids increase with pressure increase can be related with the cell membrane breakdown. The influx of greater amounts of solvent to the inner membranes can facilitate the extraction of compounds (Casquete et al., 2015). According to the phase behavior theory, the

solubility of phenolic compounds and flavonoids increase as the pressure increases and accordingly to the mass transfer theory, pressurized cells have an increased permeability. Therefore, the higher the extraction pressure is more solvent can enter into the cells and the more compounds can permeate out the cell membrane (Shouqin et al., 2005). Phenolic compounds exhibit extensive free radical scavenging activities through their reactivity as hydrogen or electron-donating agents, and metal ion chelating properties. High pressure assisted extraction can also cause enhancement of chemical reactions in the cells, deprotonating charged groups, and break of salt bridges and hydrophobic bonds, resulting in conformational changes and denaturation of proteins and then rendering phenolic compounds (many times associated with proteins) more available to extraction (Oey et al., 2008; Prasad et al., 2009b). Moreover, may provide the possibility of inactivating degrading enzymes which may account for higher extraction yield and antioxidant activity compared to other methods (Oey et al., 2008; Prasad et al., 2009b).

Total condensed tannins were determined for all extraction conditions, but no quantifiable amounts were detected in prickly pear peels extracts. According to Cardador-Martinez et al. (2011) this fruit is typically rich in tannins (0.3-1.4 mg CT Eq./g DW), however the sample used in this work was a fermented by-product. The fermentation, possibly led to the degradation or transformation of initial tannins present in the fresh fruit.

3.2. Total betalains and monomeric anthocyanins

Betalains were quantified in terms of red-violet betacyanins and yellow-orange betaxanthins. Red-violet betacyanins were not detected when compared to yellow-orange betaxanthins, which was expected since the prickly pear peels analyzed were yellow. The highest extraction yield of betaxanthins (0.25±0.01 mg IN Eq./g DW) was obtained for the combination at P300/t5/E40 (see Appendix B, and some representative figures are in Appendix E), which represented an increase of 7% and 27% in relation to extractions performed in same conditions at atmospheric pressure (P0.1/t5/E40) or in the same conditions but using water as solvent (P300/t5/E0), respectively. Concerning betaxanthins extraction, according to the results presented in Table 8, the ethanol concentration had the highest effect (F values of 1452 and 1234). High pressure and extraction time effects were significant presenting F values of 18 and 7, respectively.

Table 8. Analyses of variance for linear, quadratic and crossed effects of pressure, extraction time and ethanol concentration at a significance level of 95% confidence for total betaxanthins model. The significant coefficients in each case are written in bold.

ANOVA	Betaxanthins	
	F	***p***
P (L)	0.68	0.41
P (Q)	18.49	**0.00**
t (L)	7.24	**0.01**
t (Q)	0.75	0.39
E (L)	1452.25	**0.00**
E (Q)	1234.21	**0.00**
P (L) x t (L)	0.77	0.38
P (L) x E (L)	86.10	**0.00**
t (L) x E (L)	0.98	0.33
R^2	0.973	
R^2 adjst	0.970	

L: linear; Q: quadratic; P: Pressure (MPa); t: time (min); E: Ethanol percentage (%).

Visually, the aqueous and 40% ethanolic extracts were orange and 80% ethanolic extracts were yellow (Figure 3).

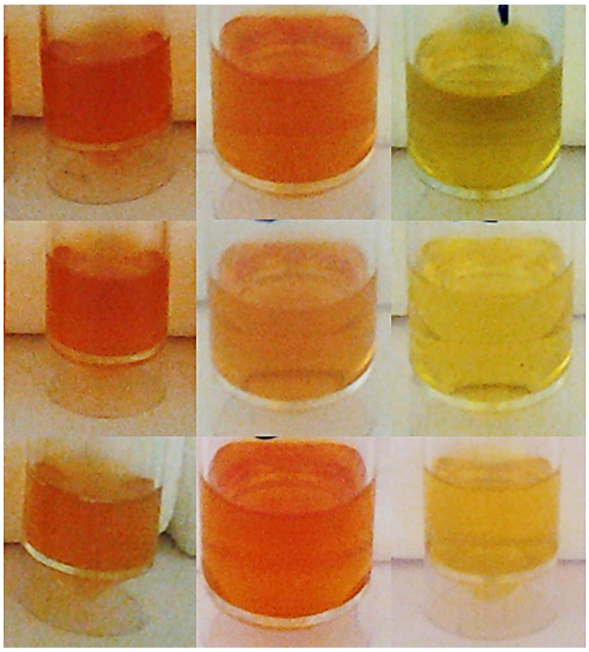

Figure 3. Extracts color performed during 30 min using water (left column), ethanol 40 (middle) and 80% ethanol (right) at 0.1 (top), 300 and 600 MPa (bottom).

These color differences were due to higher concentration of betaxanthins on the aqueous and 40% ethanolic extracts than on 80% ethanolic extracts. The 40% ethanol

concentration increased the betaxanthins extraction in 14% when compared with extractions performed using water, but in relation to 80% ethanol concentration, the extraction of betaxanthins decreased, being preferable low concentrations to ensure higher yields of betaxanthins. Azeredo (2009) reported that betalains are almost exclusively water soluble, but they are slightly soluble in ethanol (Stintzing and Carle, 2008). Fernandez-Lopez et al. (2012) reported that a 60% ethanol concentration increased betaxanthin extraction in relation to water or ethanol. This information might provide a possible explain for the impact of the ethanol concentration on the extraction of betaxanthins and the concentrations obtained are in accordance with those reported by Castellanos-Santiago and Yahia (2008), which used water (0.12±0.01 mg IN Eq./g DW to 0.44±0.03 mg IN Eq./g DW) and citrate-phosphate buffer (0.09±0.01 mg IN Eq./g DW to 0.84±0.12 mg IN Eq./g DW). In relation to high pressure, 300 MPa conduced to an increase of betaxanthins content of 13%, when compared to 0.1 MPa, however 600 MPa decreased slightly the total extraction yields. No literature was found about the extraction of betalains by high pressure. For the extraction time, extractions performed for 17.5 min increase the extractions in 5%, but extraction performed for 30 min decrease the extractions. However, it is worthy to mention that extractions performed with ethanol 80%, the increase in pressure lead to higher extraction (Figure 4).

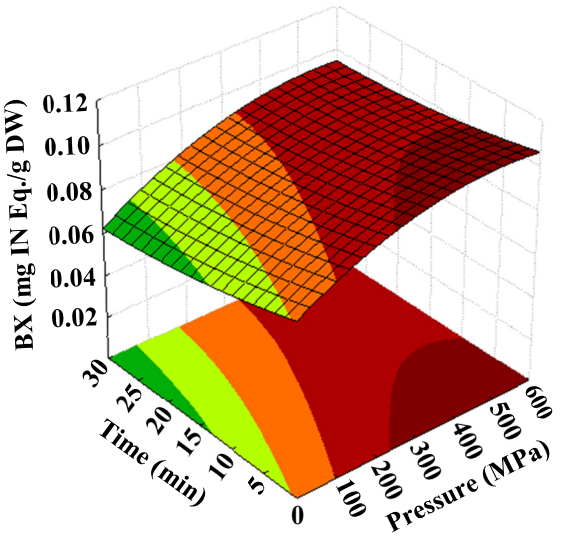

Figure 4. Response surfaces of betaxanthins extraction for ethanol 80%.

During 5, 17.5 min and 30 min, at ethanol 80%, the extractions increase 47% and 52%, 69% and 22%, and 74% and 81%, respectively for 300 MPa and 600 MPa, when compared to those performed in the same conditions but with water.

Total anthocyanins were not detected in any cactus pears samples but betalains were quantified. Lee et al. (2009) analyzed and quantified anthocyanins in cactus pears (4.7 mg cyanidin/g DW), but anthocyanins and betalains are mutually exclusively compounds, i.e., when one is present the other is not (Stafford, 1994; Khan and Giridhar, 2015).

3.3. Total antioxidant activity

The highest extraction yields of 15.34±0.45 mg Trolox Eq./g DW, 16.12±0.74 mg Trolox Eq./g DW and 52.58±0.54 mg AIS Eq./g DW were obtained using P300/t30/E40, P300/t17.5/E40 and P300/t30/E40, respectively for ABTS, DPPH and FRAP methods (see Appendix B, and some representative figures are in Appendices F to H). These values were 21%, 19% and 13% higher than the ones obtained in the same ethanol concentration and extraction times but at atmospheric pressure, respectively for ABTS, DPPH and FRAP methods. High pressure also had a significant impact when ABTS and FRAP methods were used (F values of 18 and 5 for ABTS, and 22 and 38 for FRAP on linear and quadratic effects, respectively) (Table 9).

Table 9. Analyses of variance for linear, quadratic and crossed effects of pressure, extraction time and ethanol concentration at a significance level of 95% confidence for ABTS, DPPH and FRAP total antioxidant models. The significant coefficients in each case are written in bold.

ANOVA	ABTS		DPPH		FRAP	
	F	***p***	***F***	***p***	***F***	***p***
P (L)	18.42	**0.00**	0.01	0.92	22.34	**0.00**
P (Q)	5.96	**0.02**	1.89	0.17	37.79	**0.00**
t (L)	12.40	**0.00**	4.80	**0.03**	10.72	**0.00**
t (Q)	1.72	0.19	0.23	0.63	30.67	**0.00**
E (L)	166.05	**0.00**	242.35	**0.00**	209.46	**0.00**
E (Q)	302.43	**0.00**	128.17	**0.00**	280.84	**0.00**
P (L) x t (L)	4.33	**0.04**	10.17	**0.00**	12.54	**0.00**
P (L) x E (L)	0.81	0.37	9.72	**0.00**	0.33	0.56
t (L) x E (L)	0.17	0.69	0.16	0.69	0.37	0.55
R^2	0.863		0.836		0.885	
R^2 adjst	0.848		0.817		0.872	

L: linear; Q: quadratic; P: Pressure (MPa); t: time (min); E: Ethanol percentage (%); ABTS, DPPH and FRAP: antioxidant activity by the ABTS, DPPH and FRAP methods.

In general, the pressure of 300 MPa conduced to an increase of the total antioxidant activity between 6-17% when compared to extractions performed at 0.1 MPa. Using a pressure of 600 MPa the total antioxidant activity increased between 9-11%.

When the highest extraction yields were compared to the corresponding extractions performed at the same conditions but during an extraction time of 5 min, antioxidant activity increased 34%, 30% and 3% for ABTS, DPPH and FRAP methods, respectively. Extraction time had the lowest effect but still significant for all methods presenting F values between 5 and 31. Globally, the extracts performed during 17.5 min and 30 min increased the antioxidant activity in 7% and 9% (ABTS), 0.4% and 9% (DPPH), and 9% and 6% (FRAP) respectively, when compared to extractions performed during 5 min.

Also, when the highest yields were compared to the ones obtained in the same pressure and extraction times but using water as extraction solvent, the antioxidant activity increased 38%, 74% and 16% for ABTS, DPPH and FRAP methods, respectively. Antioxidant activity was significantly affected mainly by the ethanol concentration since F values were between 166 and 302 for ABTS, 242 and 128 for DPPH, 209 and 280 for FRAP assays for the linear and quadratic effects, respectively (Table 9). In general, when ethanol 40% was used, the antioxidant activity increased 17%, 5% and 11% comparatively to extractions performed with water, but when ethanol 80% was used, antioxidant activity decreased 31%, 30% and 25 % for ABTS, DPPH and FRAP methods, respectively. Therefore, high extraction time and intermediate ethanol concentration allowed higher antioxidant activity quantifications. In relation to high pressure variable, higher pressures will be preferred according ABTS method but only intermediate pressures will be necessary to improve antioxidant activity according DPPH and FRAP methods (Figure 5a-c).

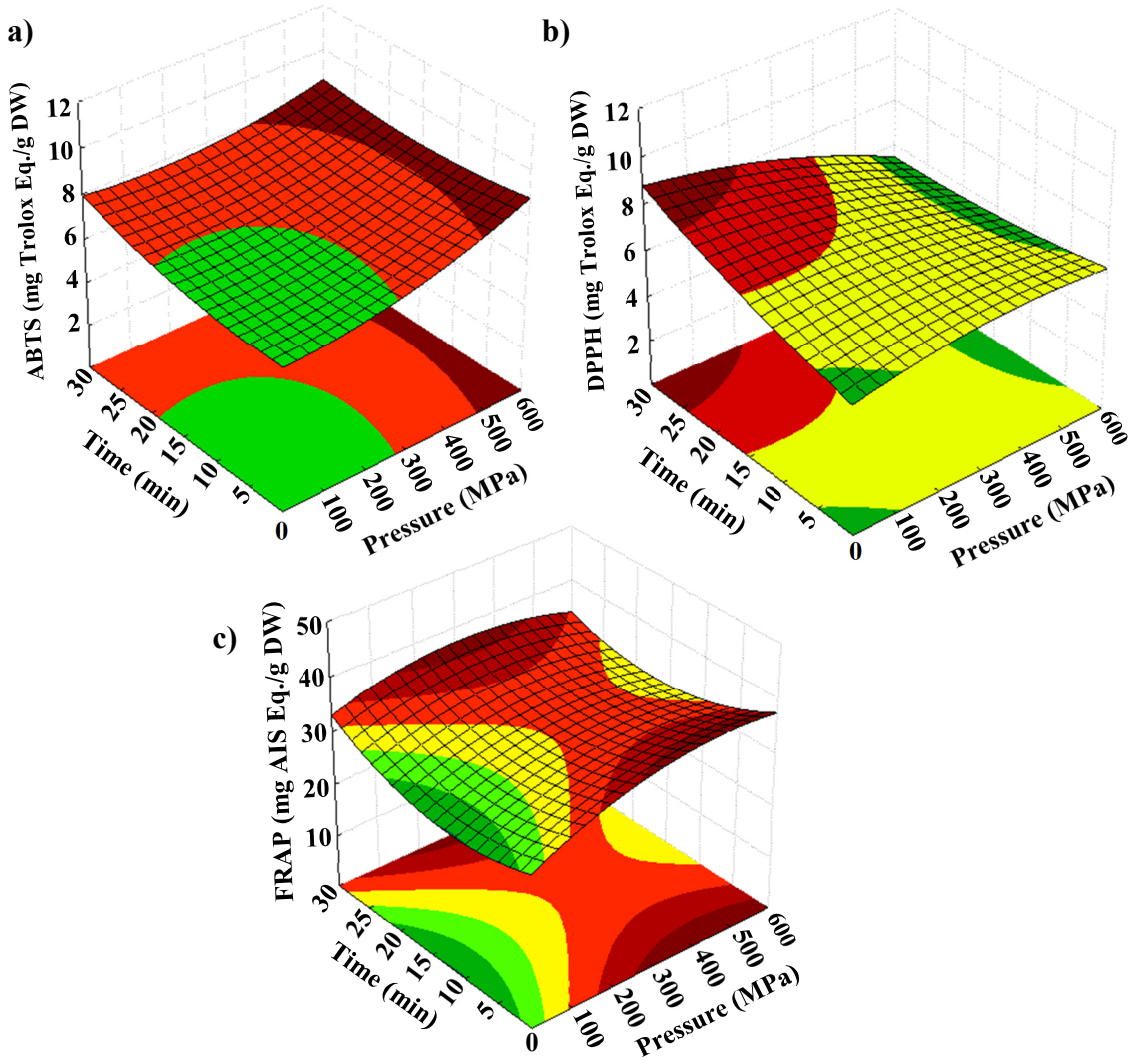

Figure 5. Response surfaces of a) ABTS antioxidant activity, b) DPPH antioxidant activity, c) FRAP antioxidant activity for ethanol 80%.

The amount of phenolic compounds increased with pressure increase, which in turn lead to an increase of antioxidant effects. The reduction of DPPH radical by the peel extracts has been reported by the presence of phenolic compounds, which easily reduce protons. Their capacity varies from one compound to another and but there is a synergy between them and/or other constituents that might be present in the extracts (Casquete et al., 2015). ABTS and DPPH antioxidant values were relatively similar, since both assays are based in the same principal: stabilization of the radical oxidizing reagent. Comparing the ABTS and DPPH values with the individual FRAP counterparts, the last ones were higher, due to the fact of FRAP assay relies on a different principal than the ABTS and DPPH assays: the reduction of Fe (III) of the FRAP reagent to Fe (II). Casquete et al. (2015) reported that 300 MPa, 10 min and 500 MPa, 3 min conduced to higher quantifications of

antioxidant activity (DPPH method) in orange and lemon peels, respectively. These authors obtained similar increases to ours, of 14 and 25% in the antioxidant activity of orange and lemon peels, respectively.

3.3. Total extraction yields

Total extraction yields were main significantly affected by the ethanol concentration since F values were 369 and 243 for linear and quadratic effects, respectively (Table 10).

Table 10. Analyses of variance for linear, quadratic and crossed effects of pressure, extraction time and ethanol concentration at a significance level of 95% confidence for total yields model. The significant coefficients in each case are written in bold.

ANOVA	Total Yields	
	F	***p***
P (L)	9.86	**0.00**
P (Q)	13.39	**0.00**
t (L)	0.00	0.98
t (Q)	0.30	0.59
E (L)	369.22	**0.00**
E (Q)	243.25	**0.00**
P (L) x t (L)	2.56	0.11
P (L) x E (L)	30.90	**0.00**
t (L) x E (L)	0.96	0.33
R^2	0.897	
R^2 adjst	0.886	

L: linear; Q: quadratic; P: Pressure (MPa); t: time (min); E: Ethanol percentage (%).

However, high pressure effects also were significant presenting F values of 9.86 and 13.39 for linear and quadratic effects, while extraction time effects were not significant, meaning that an extraction performed during 5 min is sufficient to reach the maximum extraction yield. The highest total extraction yield (48.11±2.53%) was obtained for the combination at P300/t5/E40 (see Appendix B, and some representative figures are in Appendix I), which represented an increase of 12% and 17% in relation to extractions performed in same conditions at atmospheric pressure (P0.1/t5/E40) and in the same conditions with water (P300/t5/E0), respectively. In general, when ethanol 40% was used the extraction yields increased 6% comparatively to extractions performed with water, but when an 80% ethanol concentration was used the extracting yields decreased 32%. This might be related to the low solubility of the several compounds on the extraction

solvent or ethanol concentrations higher than 40% may affect the composition or quality of the compounds. Similar results were found by Jun (2009) on the total extraction yields of caffeine from tea leafs, where the maximum extraction yield was obtained for intermedium ethanol concentrations (50%). From this ethanol concentration, authors verified that increasing the ethanol concentration, the extraction yields decreased. However, when 80% ethanol was used as solvent (Figure 6), the total extraction yields increased when the pressure increased up to intermedium values regardless the extraction time used.

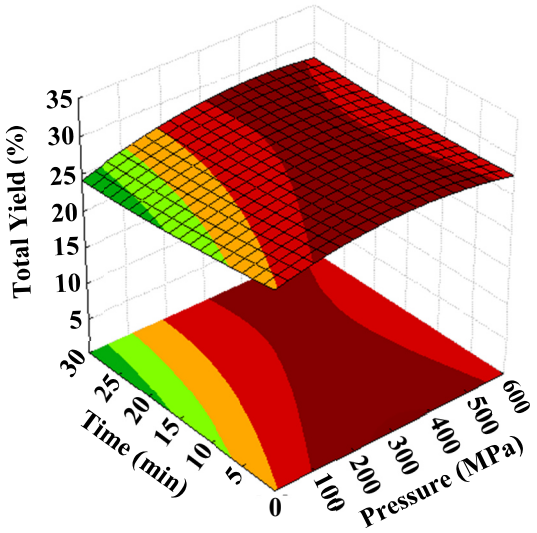

Figure 6. Response surfaces of total extraction yields for ethanol 80%.

Concerning the pressure, the 300 MPa pressure conduced to an increase in the extraction of total extraction yields of 6%. Same increase of 6% was obtained when an ethanol concentration of 40% was used, compared with extractions performed using water. Prasad et al. (2009b) studied the effect of high pressure extraction upon extraction yield, total phenolic content and antioxidant activity of longan fruit pericarp. These authors also observed that extraction yield was influenced by high pressure treatment, increasing more 3% when compared to conventional extraction, and required shorter extraction time. As mention above, the higher pressure is, the more solvent can enter into the cell and the more compounds can permeate the cell membrane increasing also total extraction yields. The differential pressure between the cell interior and the exterior of cell membranes is so large that it will lead to rapid permeation. Moreover, the disruption of cellular walls and hydrophobic bonds in the cell membrane can increase the rate of mass transfer and

enhance solvent penetration into the cells (Oey et al., 2008). Therefore, lower ethanol concentrations and intermediate pressures increased total extraction yields.

3.4. Models fit and adequacy

In general, the predict values were in good agreement with the experimental results. Experimental and predicted values differed in average less than 6%, except for the models developed for flavonoids and antioxidant activity performed by DPPH method, where values differ in average 9 and 10% (see Appendix B), indicating that RSM is satisfactory and accurate.

The coefficient of determination (R^2) and adjusted coefficient of determination (R^2_{ajust}) was calculated for all models by analysis of variance and are reported in the (Table 7, Table 8, Table 9 and Table 10). The coefficient of determination gives the total variation proportion of the model-predicted response, i.e., the higher the R^2 is, the higher will be the satisfactory adjustment of the predicted values of the quadratic model to the experimental data (Prakash Maran and Manikandan, 2012). Betaxanthins model presented a very strong correlation (R^2=0.973), meaning that only 2.7% of the experimental values were not described by the model. The total phenolic compounds, antioxidant activity methods (ABTS, DPPH and FRAP) and extraction yields models presented a modest correlation (R^2 of 0.875, 0.863, 0.836, 0.885 and 0.897, respectively), indicating that only 12.5%, 13.7%, 16.4%, 11.5% and 10.3% of experimental values were not described by the model, respectively. Flavonoid model presented the lowest correlation (R^2=0.757), meaning that the model only explain 75.7% of the obtained results. Despite being modest, these are considered strong correlations (Mukaka, 2012).

The second parameter (R^2_{ajust}) evaluate the model adequacy and fit and corrects the R^2 values relatively to the size of the sample and to the number of terms in the model (Prakash Maran and Manikandan, 2012). In all models the R^2_{ajust} parameter was very close to the corresponding R^2 values and models showed a high fit and adequacy to experimental data since all the developed models could explain more that 73% of the experimental values. Particularly for betaxanthins model, it explains 97% of total variation. Consequently, the regression models defined well the true behavior of the system representing efficiently the experimental data.

The coefficient of variation (CV) measures the relative dispersion of the experimental results from the one's predictions and also were calculated (Table 11). The CV-values

were lower than 10% with few exceptions and according to Koocheki et al. (2009), the CV should not surpass 10% to express a good precision and repeatability of the conducted experiments. Thus, the conducted experiments presented a good precision and repeatability. The best method to evaluate the antioxidant activity of the prickly pear peels was the FRAP assay, because model presented higher R^2 and R^2_{ajust} values and the lowest CV values.

Table 11. Coefficient of variance for all experimental results (%).

Nomenclature	PC	FL	BX	ABTS	DPPH	FRAP	TY
P0.1/t5/E0	1.9	5.0	0.8	3.6	12.1	0.0	1.4
P0.1/t5/E40	2.7	2.4	0.9	4.6	8.0	1.7	2.3
P0.1/t5/E80	4.3	2.3	2.0	5.2	4.1	1.5	2.8
P0.1/t17.5/E0	1.0	4.3	1.2	0.6	5.6	0.3	1.1
P0.1/t17.5/E40	7.1	0.9	2.6	7.0	4.6	0.5	1.1
P0.1/t17.5/E80	6.4	1.6	1.9	6.6	9.8	2.9	2.7
P0.1/t30/E0	0.9	5.4	0.2	2.9	6.3	1.1	8.4
P0.1/t30/E40	3.4	3.3	2.0	7.0	2.7	2.5	1.2
P0.1/t30/E80	3.4	3.4	7.3	5.4	1.4	1.3	14.2
P300/t5/E0	0.5	4.4	0.8	7.3	2.1	3.0	1.2
P300/t5/E40	3.1	5.0	4.2	0.3	4.0	4.6	5.3
P300/t5/E80	0.5	3.7	0.9	9.3	1.7	1.6	3.1
P300/t17.5/E0	1.2	7.7	1.8	1.9	6.1	1.8	0.6
P300/t17.5/E40	5.6	4.8	0.6	5.1	4.6	4.0	4.4
P300/t17.5/E80	6.0	2.1	0.4	6.4	5.7	5.8	3.2
P300/t30/E0	7.0	1.8	2.4	2.1	4.2	1.2	2.5
P300/t30/E40	5.1	1.6	1.6	2.9	1.3	1.0	3.5
P300/t30/E80	10.9	1.2	1.0	2.3	7.5	2.9	1.3
P600/t5/E0	6.6	8.7	0.6	1.8	1.5	2.4	7.7
P600/t5/E40	3.7	4.8	0.7	3.9	7.0	1.1	1.8
P600/t5/E80	13.0	2.2	1.8	2.3	2.6	1.3	2.3
P600/t17.5/E0	3.8	9.8	3.7	5.0	2.0	0.7	0.8
P600/t17.5/E40	1.6	1.9	0.8	1.0	2.6	1.6	1.0
P600/t17.5/E80	9.8	2.1	4.3	0.5	6.6	3.2	1.8
P600/t30/E0	1.3	5.3	3.8	1.7	2.6	0.5	5.0
P600/t30/E40	6.9	1.7	0.5	6.5	3.1	1.7	4.8
P600/t30/E80	11.8	2.2	0.7	4.1	0.8	1.4	3.0
P600/t17.5/E40	7.6	2.7	0.9	4.9	8.3	4.7	5.0
P600/t17.5/E40	2.4	5.0	0.3	7.7	9.0	1.8	1.2
P600/t17.5/E40	4.1	4.4	0.7	2.3	3.9	5.0	1.1

P: Pressure; t: time; E: Ethanol percentage; TY: extraction yields; ABTS, DPPH and FRAP: antioxidant activity by the ABTS, DPPH and FRAP methods; PC: total phenolic compounds; FL: Flavonoids; BX: betaxanthins.

3.5. Correlation matrix

The correlations coefficients (r) between the total phenolic compounds, flavonoids, betaxanthins and antioxidant activity were determined and are presented in Table 12.

Table 12. Coefficient correlation matrix between the responses of all dependent variables. Bold correlations were significant at $p<0.05$.

r	PC	FL	BX	ABTS	DPPH	FRAP	TY
PC	1	-	-	-	-	-	-
FL	**0.078**	1	-	-	-	-	-
BX	**0.873**	-0.142	1	-	-	-	-
ABTS	**0.738**	0.013	**0.828**	1	-	-	-
DPPH	**0.611**	**0.092**	**0.793**	**0.590**	1	-	-
FRAP	**0.634**	0.000	**0.843**	**0.688**	**0.640**	1	-
TY	**0.680**	0.033	**0.952**	**0.545**	**0.529**	**0.574**	1

TY: extraction yields; ABTS, DPPH and FRAP: antioxidant activity by the ABTS, DPPH and FRAP methods; PC: total phenolic compounds; FL: Flavonoids; BX: betaxanthins.

All correlations were significant ($p<0.05$) and presented correlation values between 0.53 and 0.95, with the exception of total extraction yield and total flavonoids content correlations. The strongest correlation was found between the total extraction yields and betaxanthins (r=0.95), however betaxanthins also presented high correlations with antioxidant activity assays (r=0.79-0.84) and with total phenolic compounds (r=0.87). The correlation values between phenolic compounds and the total extraction (r=0.68) and phenolic compounds with the antioxidant activity assays (r=0.73-0.63) were smaller when compared to phenolic compounds and betaxanthins correlations. In the case of flavonoids, the correlations with other parameters were not significant and very low but still were significant for phenolic compounds and antioxidant activity measured by DPPH method. This may mean that the most part of antioxidant activity maybe related with betaxanthins and not so much with phenolic compounds and flavonoids. Cardador-Martinez et al. (2011) also studied the correlation between total phenolics and antioxidant activity (DPPH and ABTS methods) from prickly pear peels. The authors reported similar significant correlation ($p<0.05$) between the phenolic compounds and DPPH antioxidant activity (0.52), and between DPPH and ABTS antioxidant activities (0.64). In the case of flavonoids, the authors found a significant correlation with the antioxidant activity measured by DPPH assay but not with ABTS, but r values founded by authors were higher (0.50 and 0.13, respectively). However, that authors did not study betaxanthins pigments.

3.6. Regression coefficients

Each model could be expressed by the quadratic polynomial equation (Equation 7) as a function of the independent variables within the region under investigation by applying multiple regression analysis on the experimental data. The regression coefficients were determined and presented in Table 13.

Table 13. Regression coefficients of the second-order polynomial regression equation. The significant coefficients are written in bold ($p<0.05$).

	PC	FL	BX	ABTS	DPPH	FRAP	TY
β_0	**21.2**	**1.3**	**0.2**	**9.9**	**10.8**	**40.9**	**42.8**
β_1	4.0×10^{-3}	5.7×10^{-5}	1.1×10^{-6}	-5.0×10^{-4}	$\mathbf{9.6\times10^{-3}}$	$\mathbf{3.7\times10^{-2}}$	-9.1×10^{-4}
β_1^2	-2.2×10^{-6}	-3.9×10^{-7}	$\mathbf{-1.1\times10^{-7}}$	$\mathbf{5.8\times10^{-6}}$	-5.2×10^{-6}	$\mathbf{-4.1\times10^{-5}}$	$\mathbf{-2.2\times10^{-5}}$
β_2	1.5×10^{-1}	-1.9×10^{-2}	-7.3×10^{-4}	6.5×10^{-3}	5.8×10^{-2}	$\mathbf{-5.5\times10^{-1}}$	-8.4×10^{-2}
β_2^2	-3.4×10^{-3}	3.7×10^{-4}	1.3×10^{-5}	1.8×10^{-3}	1.1×10^{-3}	$\mathbf{2.1\times10^{-2}}$	1.9×10^{-3}
β_3	$\mathbf{1.1\times10^{-1}}$	$\mathbf{2.5\times10^{-2}}$	$\mathbf{2.3\times10^{-3}}$	$\mathbf{1.4\times10^{-1}}$	$\mathbf{1.3\times10^{-1}}$	$\mathbf{3.6\times10^{-1}}$	$\mathbf{2.1\times10^{-1}}$
β_3^2	$\mathbf{-3.0\times10^{-3}}$	$\mathbf{-2.4\times10^{-4}}$	$\mathbf{-5.0\times10^{-5}}$	$\mathbf{-2.3\times10^{-3}}$	$\mathbf{-2.4\times10^{-3}}$	$\mathbf{-6.3\times10^{-3}}$	$\mathbf{-5.2\times10^{-3}}$
β_{12}	-1.0×10^{-4}	1.9×10^{-6}	4.0×10^{-7}	$\mathbf{-8.9\times10^{-5}}$	$\mathbf{-2.2\times10^{-4}}$	$\mathbf{-4.3\times10^{-4}}$	1.7×10^{-4}
β_{13}	$\mathbf{6.9\times10^{-5}}$	5.3×10^{-6}	$\mathbf{1.3\times10^{-6}}$	1.2×10^{-5}	$\mathbf{-6.7\times10^{-5}}$	2.2×10^{-5}	$\mathbf{1.9\times10^{-4}}$
β_{23}	6.4×10^{-4}	6.1×10^{-5}	-3.4×10^{-6}	-1.3×10^{-4}	2.1×10^{-4}	5.5×10^{-4}	-7.8×10^{-4}

In regression coefficients, 0 means constant, 1 pressure, 2 extraction time and 3 ethanol concentration. TY: extraction yields; ABTS, DPPH and FRAP: antioxidant activity by the ABTS, DPPH and FRAP methods; PC: total phenolic compounds; FL: Flavonoids; BX: betaxanthins.

The coefficient values obtained were generally low due to the large number of experiences performed. The model intercept coefficient (β_0) and ethanol coefficients (β_3 and β_3^2) was statistically significant for all cases ($p<0.05$). Pressure coefficients were significate for all models except for phenolic compounds and flavonoids. The regression coefficients that were statistically significant match to those reported in Table 7, Table 8, Table 9 and Table 10.

3.7. Optimum extraction conditions and validation of models

By means of partial derivatives of each model equation as a function of each of the independent variables studied (pressure, extraction time and ethanol concentration), the optimum extraction conditions and optimal extraction values for each model were determined (Bezerra et al., 2008). In order to validate the models, experimental extractions under optimum conditions were also performed and results are in Table 14.

Table 14. Optimum extraction conditions, predicted optimum values (POV), experimental optimum values (EOV) and average variation between experimental and predicted results.

	PC	FL	BX	ABTS	DPPH	FRAP	TY
P (MPa)	600	496	174	600	118	303	94
t (min)	16	5	5	30	30	30	5
E (%)	26	60	25	31	26	30	22
POV (mg St. Eq./g DW) [1]	26	1.97	0.238	14	16	53	45
EOV (mg St. Eq./g DW) [1]	27±1	1.81±0.02	0.236±0.002	15±0	17±1	52±1	44±1
Average variation of predicted values (%)	5	9	1	7	8	3	2

1: mg standard equivalent/g DW except for total yields, which are presented in %; TY: extraction yields; PC means total phenolic content; FL means Flavonoids; BX means betaxanthins.

The optimum conditions are strongly dependent of the parameter to be analyzed. Extraction time was the variable with lower impact in the responses and sometimes this impact was not significate. Thus, the optimum extraction time changed between 5 min to flavonoids, betaxanthins and total extraction yields and 30 min obtained for antioxidant activity. Optimum ethanol concentration was usually very low changing between 22 and 31%, excepted for total flavonoids that were better extracted with 60% of ethanol. The optimum pressure was the variable that more changed between all parameters. The optimum predicted extraction values for each parameter were very close to the experimental results obtained experimentally under optimum conditions defined by each model. Results differ ones from each other's less than 9%. Moreover, for betaxanthins, total extraction yields and antioxidant activity (FRAP) models, results only differ 1%, 2% and 3%, respectively.

3.8. High pressure extraction *versus* Soxhlet extraction

To compare the experimental optimum values with a traditional extraction method, a Soxhlet extraction was performed according to section 2.4. and results are summarized in Table 15.

Table 15. Comparison of the experimental optimum values by HPE (EOV) and Soxhlet extraction values (SE). Different letters indicate significant differences ($p<0.05$) between conditions (a and b).

	PC	FL	BX	ABTS	DPPH	FRAP	TY
SE (mg St. Eq./g DW)[1]	23 ± 1^{a}	2.33 ± 0.04^{b}	0.109 ± 0.002^{a}	10 ± 0^{a}	14 ± 1^{a}	51 ± 1^{a}	45 ± 0^{a}
EOV (mg St. Eq./g DW)[1]	27 ± 1^{b}	1.81 ± 0.02^{a}	0.236 ± 0.002^{b}	15 ± 0^{b}	17 ± 1^{a}	52 ± 1^{a}	44 ± 1^{a}

1: mg standard equivalent/g DW except for total yields, which are presented in %; TY: extraction yields; PC means total phenolic content; FL means Flavonoids; BX means betaxanthins.

High pressure extraction allowed a significant higher extraction of total phenolic compounds, betaxanthins and antioxidant activity evaluated by ABST when compared with Soxhlet extraction. Moreover, extraction time was also significantly lower. Betacyanins, anthocyanins and tannins were also not detected in extracts performed using Soxhlet and CV values of quantified compounds were lower than 10%.

3.9. Antibacterial activity

The microorganisms selected are widely found in food sources. *Escherichia coli* is a Gram-negative bacterium considered a model-organism, and the *Listeria innocua* is a Gram-positive non-pathogenic bacterium that is ubiquitous of pathogenic *Listeria monocytogenes*. To study the antimicrobial activity against these bacteria were selected the extracts obtained under the optimum extraction conditions and results are summarized in (Table 16) (see photos in Appendix C).

Table 16. Inhibition halos (mm) of extracts obtained under optimum extraction conditions. Different letters indicate significant differences ($p<0.05$) between different extraction conditions (lower cases) and between different microorganisms (upper cases).

Nomenclature	Extract		Ampicillin		Water		TY
	E. coli	*L. innocua*	*E. coli*	*L. innocua*	*E. coli*	*L. innocua*	(%)
P600/t16/E26	n.d.	17 ± 1^{a}	20 ± 1^{aA}	34 ± 1^{abB}	n.d.	n.d.	47.3±3.6
P496/t5/E60	10 ± 0^{bA}	21 ± 1^{bB}	20 ± 1^{aA}	33 ± 1^{aB}	n.d.	n.d.	36.2±4.2
P174/t5/E25	8 ± 1^{aA}	18 ± 1^{aB}	20 ± 2^{aA}	34 ± 1^{abB}	n.d.	n.d.	47.3±3.9
P600/t30/E31	8 ± 1^{aA}	18 ± 1^{aB}	20 ± 1^{aA}	35 ± 1^{bB}	n.d.	n.d.	54.1±0.9

n.d.: not detected inhibition halos; TY: extraction yields.

Both bacteria were inhibited by the prickly pear peels extracts. Inhibition halos obtained for *Listeria innocua* vary between 17 and 21 mm, while for *Escherichia coli* changed between 8 and 10 mm. However, in both cases it was for the extract P496/t5/E60 that was obtained the highest inhibition halo being statistically different from the obtained with the other extracts. Only the extracted performed at 600 MPa, during 16 min using 26% of ethanol did not present a visible inhibition halo against *Escherichia coli*. Similar results were obtained by Casquete et al. (2015) when studied the effect of high pressure on antimicrobial activity of citrus peels. In terms of total extraction yields, it was for P496/t5/E60 extract that was obtained the lowest extraction yield, but also the highest inhibition halos. Maybe in such extraction conditions, some individual compound with a relevant impact in bacterial inactivation was extracted in higher amount in relation to the obtained with different conditions. However, to prove this individual compounds should be analyzed by HPLC-MS.

4. Conclusions and Future Work

The three variables studied (ethanol concentration, pressure level and extraction time) significantly influenced the extractions of total compounds, independently and interactively. Ethanol concentration was the variable that showed the highest effect on extraction yields, followed by high pressure effects and then extraction time had the lowest impact. In general, high pressure extraction increased extraction yields between 6 and 17% and the high correlation of mathematical models indicated that the quadratic polynomial models could be employed to optimize extraction conditions. The fitness and adequacy of models were high since the R^2 obtained were higher than 0.83 for all models, except for total flavonoids (0.76). Moreover, the predictive values were very close to the experimental results indicating a good adequacy of models. The optimum extraction conditions were established and predict and experimental results differed less than 10%. The selected extracts showed antibacterial activity against *Escherichia coli* and *Listeria innocua*. High pressure extraction provided higher extraction yields or at least similar than Soxhlet extraction with the main advantage of extraction time reduction.

Agricultural processing inevitably goes along with the production of large amounts of agro-residues, which may represent a major residues disposal problem. The prickly pear peels are industrial residues that are rich in bioactive compounds and the optimizations obtained in this work make the high pressure technology applied to extraction process a promising process for scale up. However, pilot plant tests at higher scale will be necessary to ponder the economic viability of the process.

For further studies as future work, it would be interesting to:

1. Confirm if fermentation of the prickly peels degrades tannins, because this fruit is very rich in these compounds.
2. Characterize individually all phenolic compounds, flavonoids and betaxanthins present in the extracts under optimum conditions by HPLC and comparing to 0.1 MPa to have a more detailed analyses.
3. Test the antimicrobial activity of the selected extracts under optimum conditions against human pathogenic bacterium relevant in food industry and from hospital environment such as *Bacillus* spp, *Salmonella* spp, *Streptococcus* spp, *Staphylococcus* spp and *Methicillin-resistant Staphylococcus aureus,* among others.

4. Perform studies concerning the extraction of betalains by high pressure due to the lack of literature.
5. Study other interesting biological activities, such as anti-inflammatory and anti-cancer activities, among others.
6. Study the extraction of bioactive compounds and essential oils from prickly pear seeds, because they are also a good source of very interesting compounds and constitute a food residue from prickly pear fruit transformation by the food industry.

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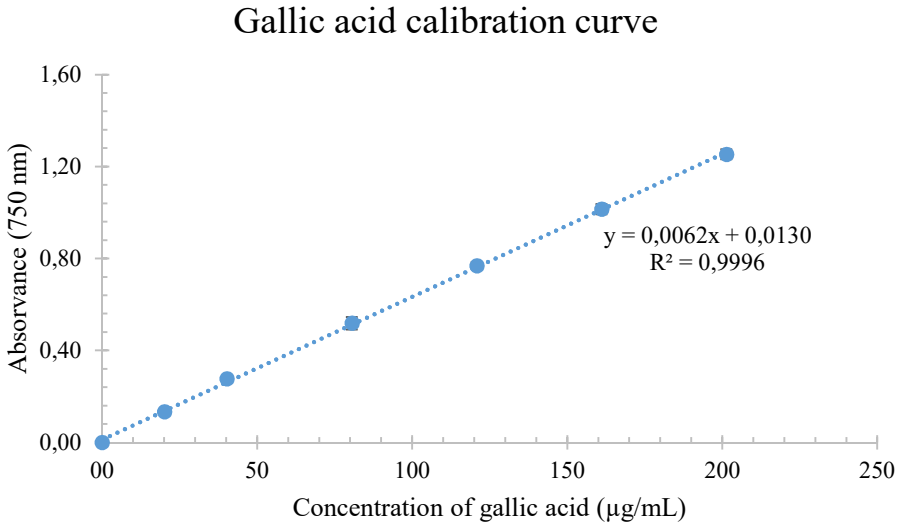
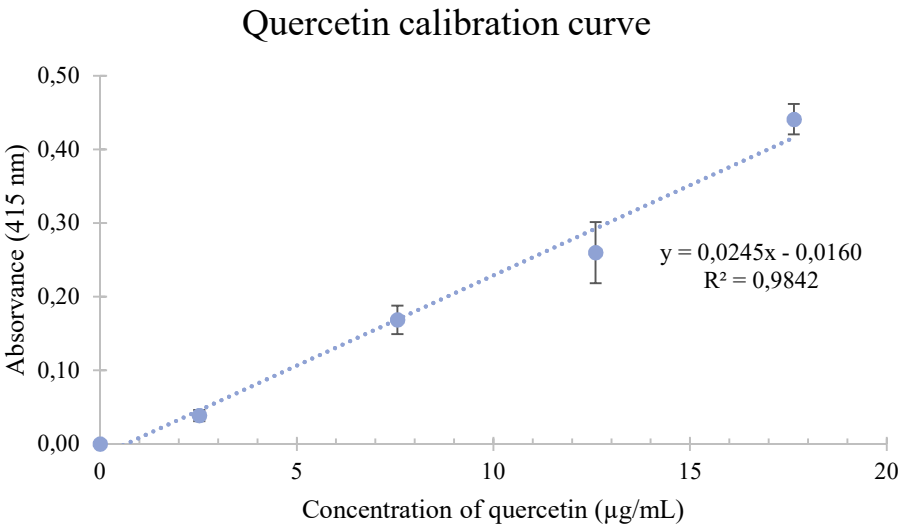
6. Appendices

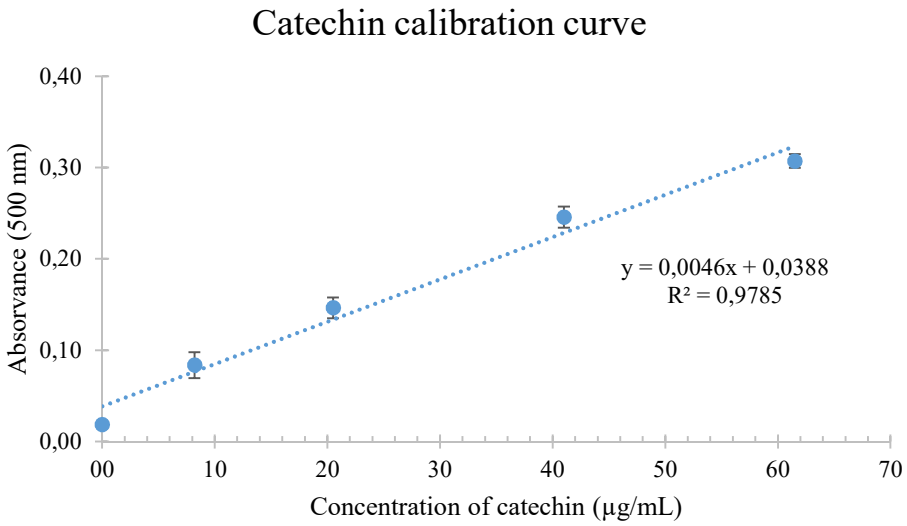
Appendix A. External calibration curves for the extracts characterization



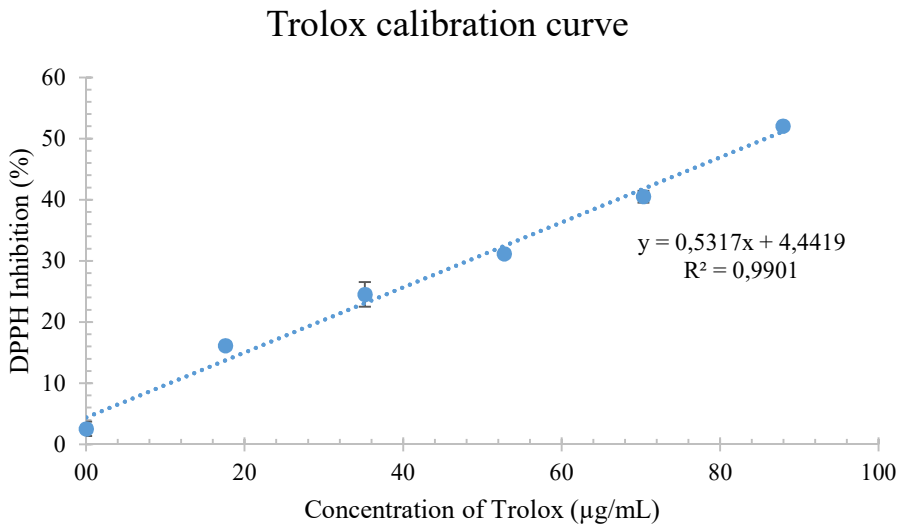
A1. Gallic acid external calibration curve for determination of total phenolic compounds.



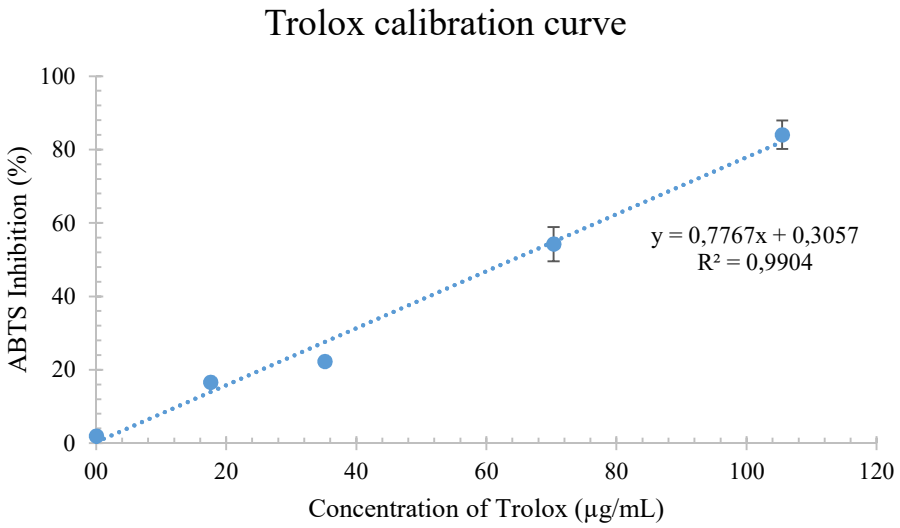
A2. Quercetin external calibration curve for determination of total flavonoids.



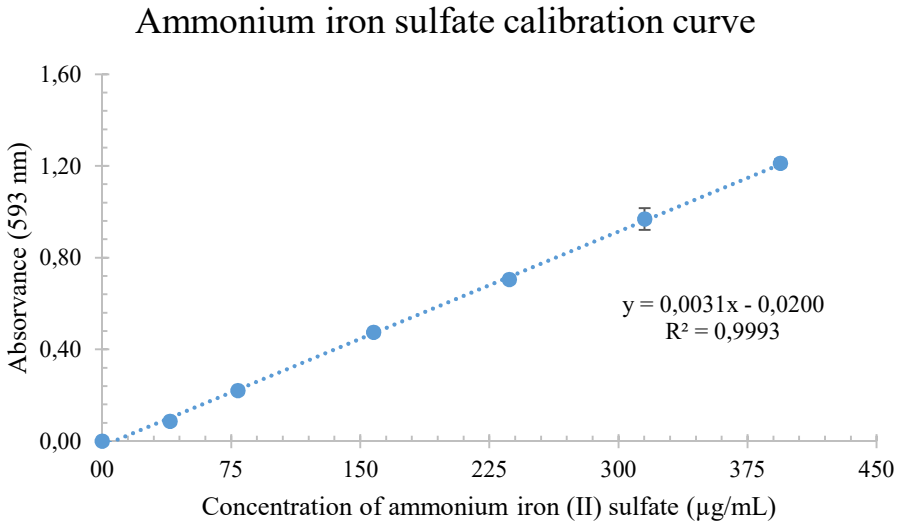
A3. Catechin external calibration curve for determination of total condensed tannins.



A4. Trolox external calibration curve for determination of total antioxidant activity by DPPH assay.



A5. Trolox external calibration curve for determination of total antioxidant activity by ABTS assay.



A6. Ammonium iron sulfate external calibration curve for determination of total antioxidant activity by FRAP assay.

Appendix B. Experimental (Exp) and predicted (Pre) values for bioactive compounds, antioxidant activity assays and total yields.

Extraction conditions	**PC** (mg/g DW)[1]		**FL** (mg/g DW)[1]		**BX** (mg/g DW)[1]		**ABTS** (mg/g DW)[1]		**DPPH** (mg/g DW)[1]		**FRAP** (mg/g DW)[1]		**TY** (%)	
	Exp	**Pre**	**Exp**	**Pre**	**Exp**	**Pre**	**Exp**	**Pre**	**Exp**	**Pre**	**Exp**	**Pre**	**Exp**	**Pre**
P0.1/t5/E0	23.19±0.48	21.84	1.10±0.06	1.18	0.21±0.00	0.21	10.61±0.39	10.00	11.58±1.40	11.16	38.67±0.01	38.70	41.34±0.56	42.46
P0.1/t5/E40	22.23±0.65	21.44	1.89±0.05	1.84	0.23±0.00	0.22	12.46±0.58	11.92	11.38±0.91	12.45	42.22±0.73	42.90	42.94±0.98	42.60
P0.1/t5/E80	10.79±0.54	11.51	1.46±0.03	1.73	0.07±0.00	0.08	6.46±0.34	6.47	7.11±0.29	5.99	23.65±0.36	26.80	26.60±0.76	26.22
P0.1/t17.5/E0	23.04±0.26	22.83	1.13±0.05	1.05	0.20±0.00	0.21	11.19±0.07	10.58	11.29±0.63	12.18	43.08±0.12	37.92	39.98±0.45	41.92
P0.1/t17.5/E40	21.19±1.63	22.74	1.82±0.02	1.73	0.21±0.01	0.22	11.45±0.80	12.44	13.59±0.62	13.57	41.08±0.20	42.39	39.78±0.45	41.68
P0.1/t17.5/E80	11.82±0.86	13.13	1.70±0.03	1.66	0.07±0.00	0.07	7.24±0.48	6.92	7.08±0.70	7.22	27.80±0.84	26.57	25.92±0.70	24.90
P0.1/t30/E0	22.26±0.21	22.76	1.12±0.06	1.03	0.22±0.00	0.20	11.74±0.34	11.72	14.33±0.90	13.53	43.91±0.50	43.84	44.70±3.78	41.97
P0.1/t30/E40	22.81±0.84	23.00	1.62±0.06	1.74	0.22±0.00	0.21	12.67±0.88	13.51	14.88±0.39	15.03	46.34±1.18	48.59	42.44±0.50	41.33
P0.1/t30/E80	15.62±0.59	13.70	1.83±0.06	1.70	0.06±0.00	0.06	7.64±0.41	7.92	8.68±0.12	8.78	34.01±0.47	33.05	23.54±3.34	24.16
P300/t5/E0	21.84±0.12	22.69	1.04±0.05	1.17	0.19±0.00	0.20	8.77±0.64	10.23	13.30±0.28	13.24	42.95±1.31	45.55	41.01±0.48	40.50
P300/t5/E40	23.52±0.77	23.12	2.14±0.11	1.88	0.25±0.01	0.23	11.41±0.04	12.30	12.38±0.50	13.72	50.97±2.39	50.01	48.11±2.53	42.87
P300/t5/E80	15.12±0.08	14.01	2.07±0.08	1.85	0.10±0.00	0.10	7.61±0.71	6.99	6.30±0.11	6.46	36.98±0.61	34.18	27.21±0.85	28.72
P300/t17.5/E0	22.64±0.29	23.30	1.09±0.09	1.04	0.21±0.00	0.19	10.03±0.19	10.48	10.26±0.63	13.44	38.90±0.71	43.14	42.83±0.28	40.61
P300/t17.5/E40	24.20±1.45	24.04	1.71±0.08	1.79	0.22±0.00	0.22	12.22±0.62	12.48	16.12±0.74	14.02	47.86±1.94	47.88	39.00±1.71	42.59
P300/t17.5/E80	13.10±0.88	15.25	1.89±0.04	1.78	0.11±0.00	0.09	6.04±0.39	7.11	5.28±0.30	6.86	34.86±2.07	32.32	29.70±0.96	28.04
P300/t30/E0	21.75±1.64	22.86	1.18±0.02	1.03	0.18±0.00	0.19	11.12±0.23	11.28	15.34±0.64	13.96	45.24±0.56	47.44	39.71±1.01	41.30
P300/t30/E40	25.28±1.37	23.92	1.14±0.02	1.80	0.26±0.00	0.22	15.34±0.45	13.22	12.82±0.17	14.65	52.58±0.54	52.46	42.71±1.50	42.89
P300/t30/E80	16.20±1.96	15.44	1.94±0.02	1.83	0.09±0.00	0.09	8.60±0.20	7.78	6.44±0.48	7.59	35.10±1.04	37.18	26.70±0.35	27.94
P600/t5/E0	21.49±1.53	23.14	1.06±0.09	1.08	0.17±0.00	0.17	11.35±0.21	11.50	15.72±0.23	14.38	47.33±1.16	44.95	34.10±2.64	34.67
P600/t5/E40	23.35±0.94	24.39	1.85±0.09	1.86	0.21±0.00	0.22	13.17±0.56	13.71	14.20±0.99	14.06	47.86±0.55	49.67	36.93±0.65	39.27
P600/t5/E80	16.71±2.40	16.10	1.83±0.04	1.89	0.10±0.00	0.10	8.40±0.19	8.54	5.46±0.14	5.98	36.24±0.48	34.11	26.40±0.61	27.34
P600/t17.5/E0	26.30±1.06	23.37	0.92±0.09	0.96	0.17±0.01	0.17	13.21±0.66	11.41	13.43±0.26	13.75	41.38±0.27	40.92	37.68±0.30	35.42
P600/t17.5/E40	24.70±0.43	24.93	1.94±0.04	1.77	0.22±0.00	0.21	13.08±0.13	13.56	11.78±0.30	13.53	43.83±0.69	45.92	39.77±0.41	39.62
P600/t17.5/E80	18.59±1.99	16.96	1.50±0.03	1.83	0.08±0.00	0.09	8.12±0.04	8.33	5.61±0.37	5.56	24.19±0.79	30.63	28.93±0.53	27.30
P600/t30/E0	22.83±0.33	22.55	0.87±0.05	0.96	0.17±0.01	0.17	11.05±0.19	11.88	13.84±0.36	13.45	44.59±0.22	43.60	34.24±1.72	36.75
P600/t30/E40	23.75±1.75	24.43	2.19±0.04	1.80	0.22±0.00	0.21	14.57±0.85	13.96	11.54±0.36	13.33	51.25±0.86	48.88	43.24±2.07	40.56
P600/t30/E80	14.94±1.97	16.78	1.93±0.04	1.88	0.10±0.00	0.09	8.62±0.36	8.67	7.92±0.06	5.47	35.87±0.52	33.87	27.46±0.83	27.85
P300/t17.5/E40	24.48±2.00	24.04	1.71±0.05	1.79	0.23±0.00	0.23	12.07±0.59	12.48	15.74±1.31	14.02	48.34±2.31	47.88	43.85±2.20	42.59
P300/t17.5/E40	24.78±0.63	24.04	1.82±0.09	1.79	0.23±0.00	0.23	13.49±1.03	12.48	15.40±1.39	14.02	49.40±0.92	47.88	42.58±0.51	42.59
P300/t17.5/E40	23.83±1.04	24.04	1.75±0.08	1.79	0.21±0.00	0.23	12.60±0.29	12.48	16.62±0.65	14.02	50.60±2.58	47.88	39.84±0.45	42.59
Variation (%)[2]	**5**		**9**		**5**		**6**		**10**		**5**		**4**	

1: Results are expressed as mg of standard equivalents/g DW; 2: Avarege varaiation from the predicted values; P: Pressure (MPa); t: time (min); E: Ethanol percentage (%); Total yields (TY); ABTS, DPPH and FRAP: antioxidant activity by the ABTS, DPPH and FRAP methods; total phenolic compounds (PC); flavonoids (FL) and betaxanthins (BX).

Appendix C. Response surfaces for total phenolic compounds.

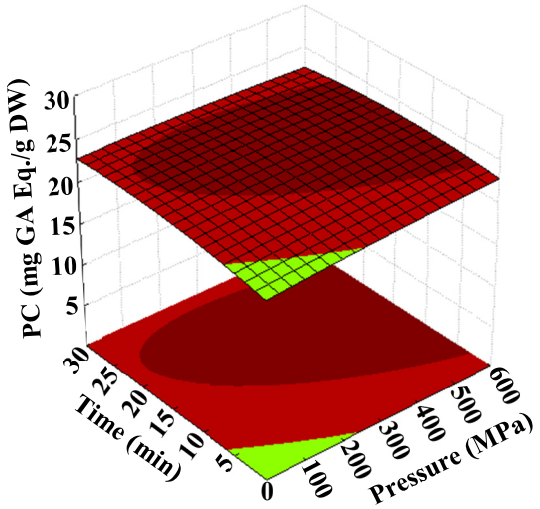


Figure C1. Response surface of phenolic compounds extraction for ethanol 0%.

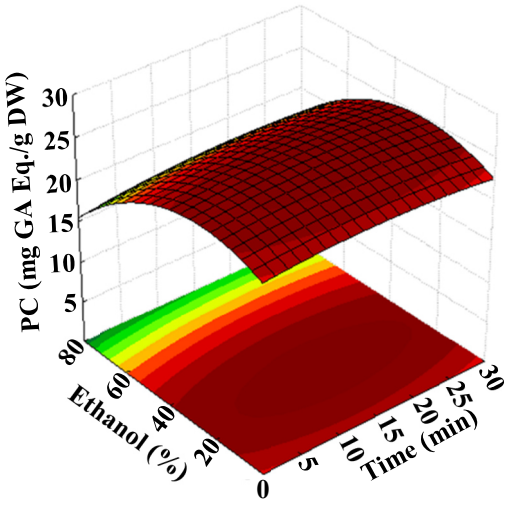


Figure C2. Response surface of phenolic compounds extraction for 600 MPa.

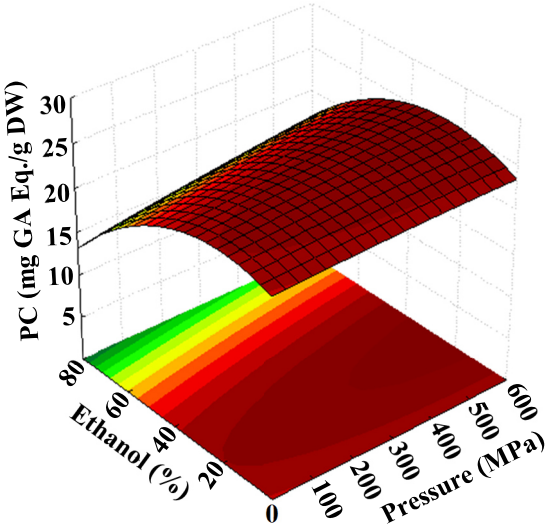


Figure C3. Response surface of phenolic compounds extraction for 17.5 min.

Appendix D. Response surfaces for total flavonoids.

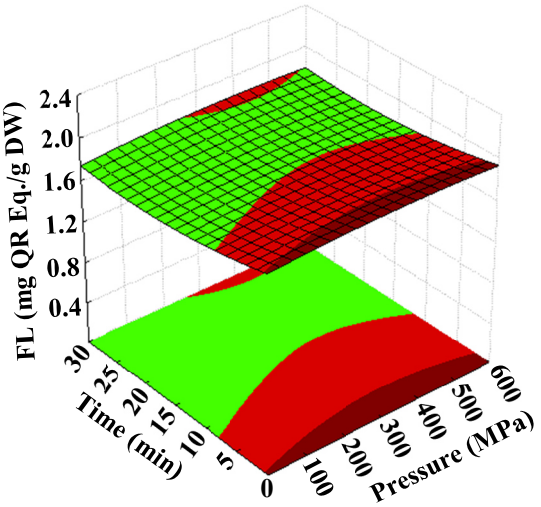


Figure D1. Response surface of flavonoids extraction for ethanol 40%.

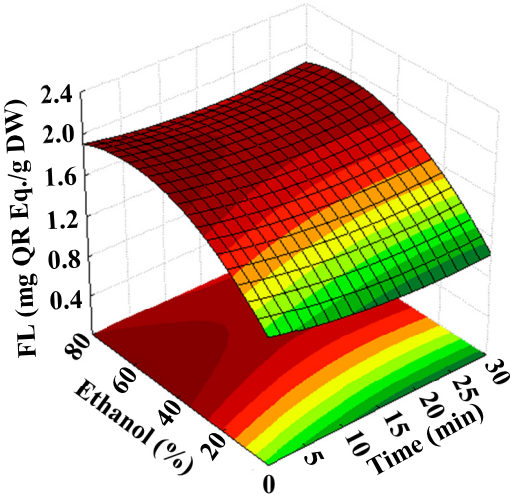


Figure D2. Response surface of flavonoids extraction for 300 MPa.

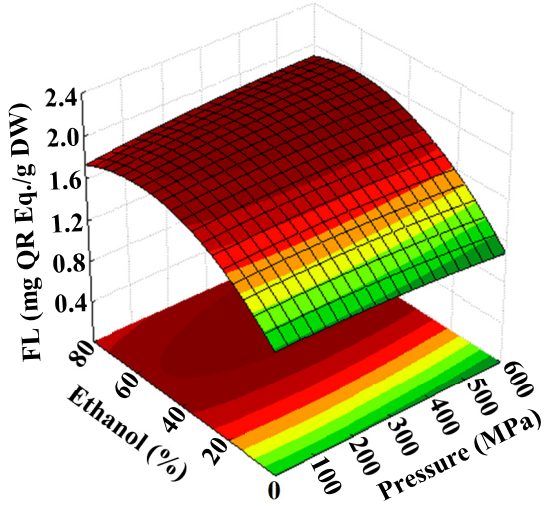


Figure D3. Response surface of flavonoids extraction for 5 min.

Appendix E. Response surfaces for total betaxanthins.

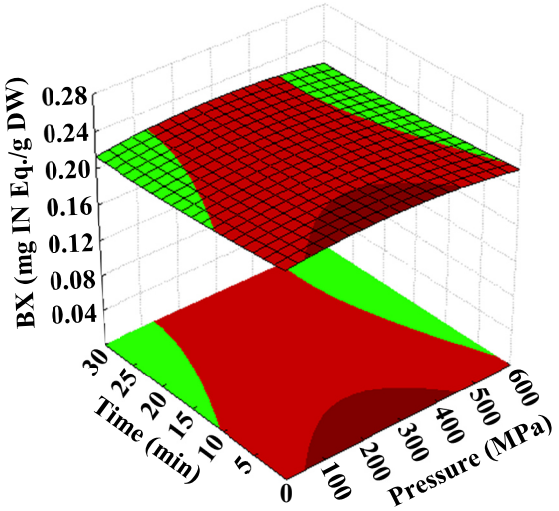


Figure E1. Response surface of betaxanthins extraction for ethanol 40%.

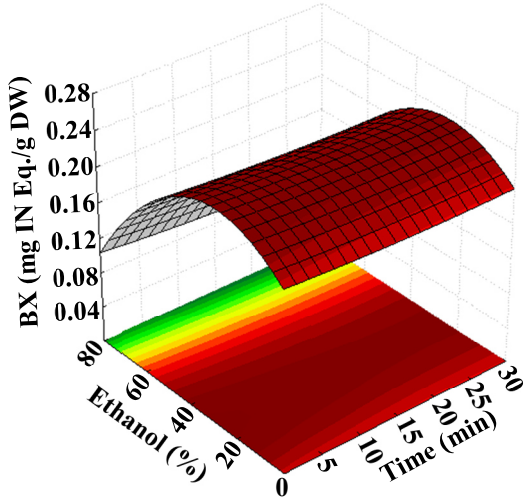


Figure E2. Response surface of betaxanthins extraction for 300 MPa.

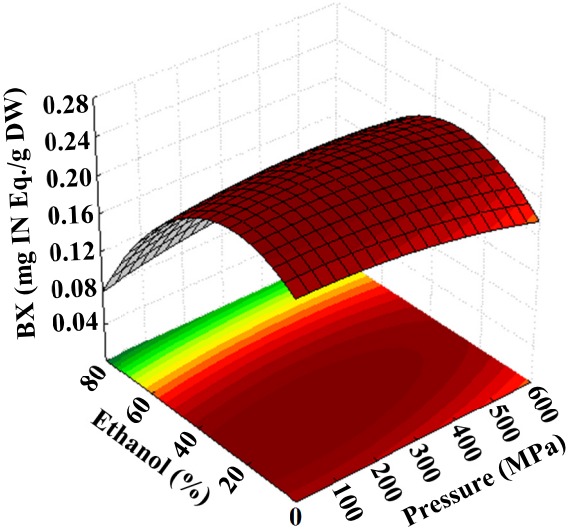


Figure E3. Response surface of betaxanthins extraction for 5 min.

Appendix F. Response surfaces for total ABTS antioxidant activity.

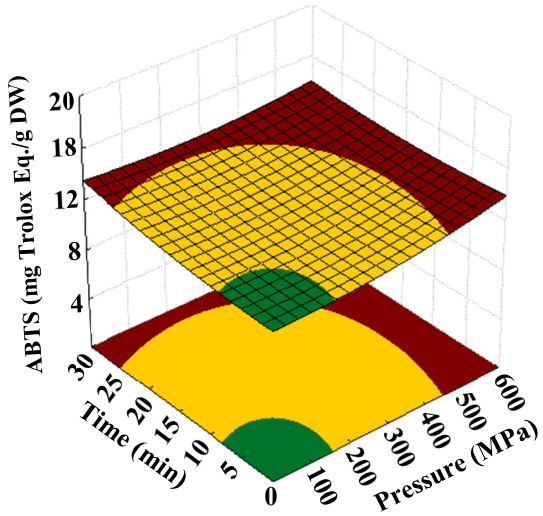


Figure F1. Response surface of ABTS antioxidant activity for ethanol 40%.

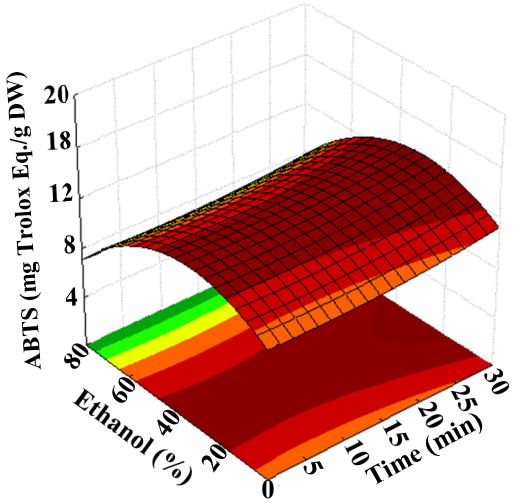


Figure F2. Response surfacc of ABTS antioxidant activity for 300 MPa.

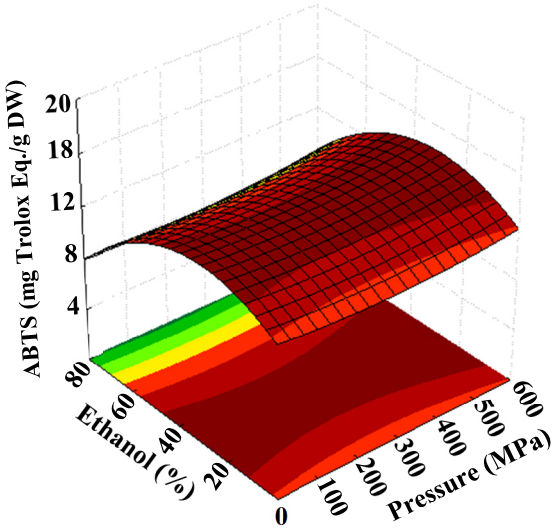


Figure F3. Response surfacc of ABTS antioxidant activity for 30 min.

Appendix G. Response surfaces for total DPPH antioxidant activity.

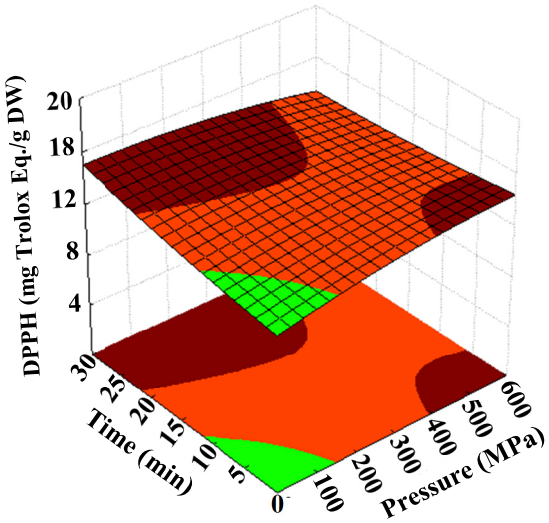


Figure G1. Response surface of DPPH antioxidant activity for ethanol 40%.

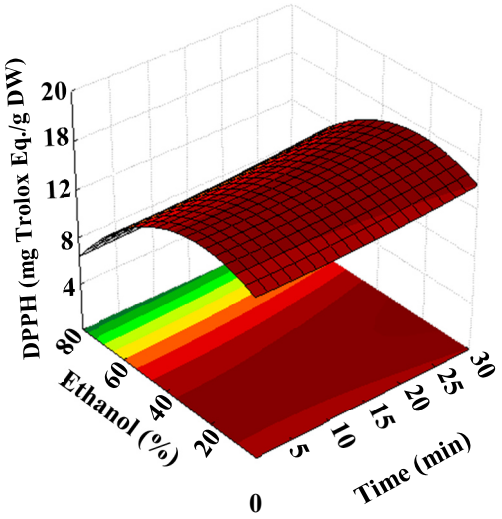


Figure G2. Response surface of DPPH antioxidant activity for 300 MPa.

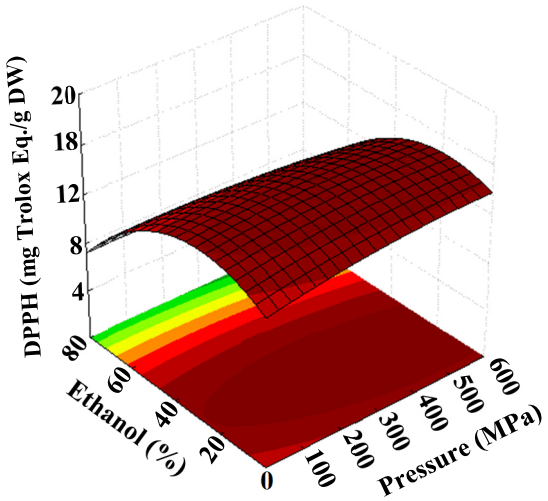


Figure G3. Response surface of DPPH antioxidant activity for 17.5 min.

Appendix H. Response surfaces for total FRAP antioxidant activity.

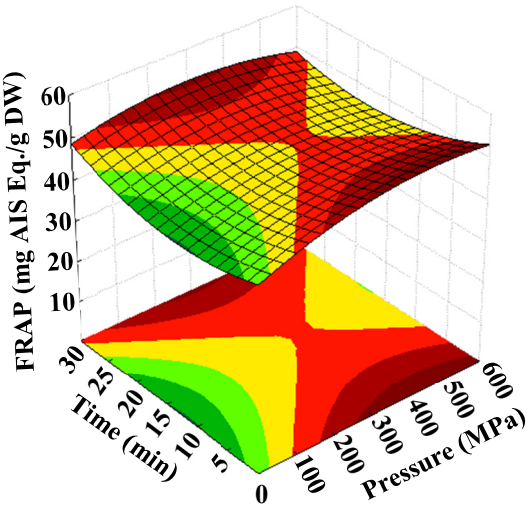


Figure H1. Response surface of FRAP antioxidant activity for ethanol 40%.

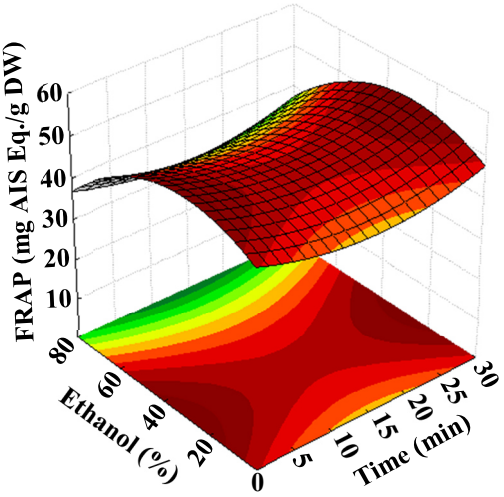


Figure H2. Response surface of FRAP antioxidant activity for 300 MPa.

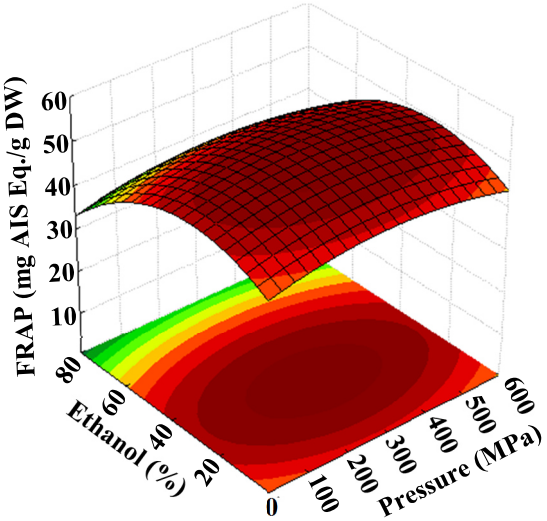


Figure H3. Response surface of FRAP antioxidant activity for 30 min.

Appendix I. Response surfaces for total extraction yields.

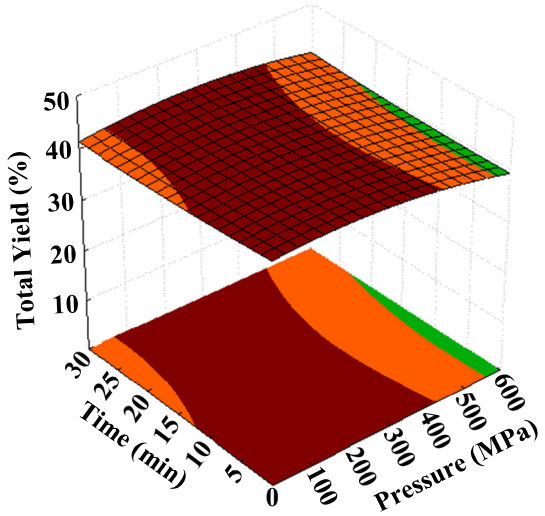


Figure I1. Response surface of extraction yields for ethanol 40%.

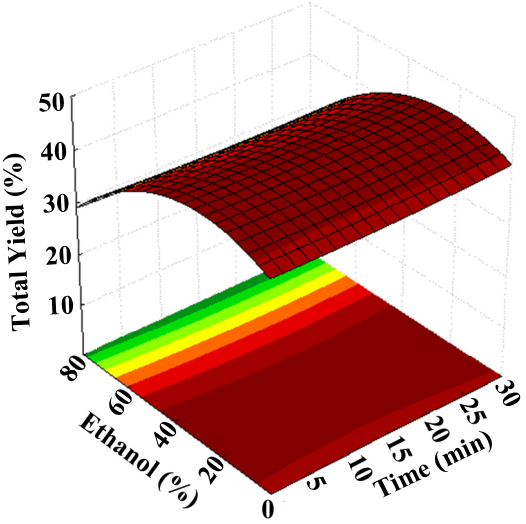


Figure I2. Response surfacc of cxtraction yields for 300 MPa.

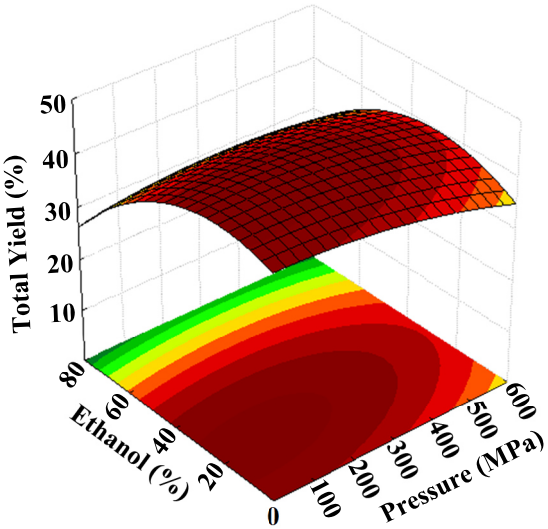


Figure 13. Response surface of extraction yields for 5 min.

Appendix J. Inhibitions zones for a) total phenolic compounds (P600/t16/E26), b) flavonoids (P496/t5/E60), c) betaxanthins (P174/t5/E25) and d) ABTS antioxidant activity (P600/t30/E31) extracts under optimum conditions against *Escherichia coli* (left column) and *Listeria innocua* (right column).