Effects of high-intensity pulsed electric fields on the bioactive compounds stability and enzymes of broccoli juice

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Doctoral Thesis

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Dedico esta tesis a

Mi esposa e hijos,

A mis padres y hermanos

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CONTENT

ABSTRACT

Epidemiological studies have suggested that a high consumption of broccoli may protect humans against chronic diseases. Broccoli is an important source of bioactive compounds, including vitamin C, phenolic compounds, glucosinolates, carotenoids, and chlorophylls. New broccoli-based products, such as blends of broccoli and apple juices have been suggested to further increase its consumption. Overall, vegetable juices represent an interesting alternative for incorporating bioactive compounds in the diet and a special attention has been focused on broccoli juice due to its elevated content of bioactive compounds that provide important health benefits.

Traditionally, juices have been processed by heat to avoid the detrimental effects of enzymes and microorganisms. However, thermal processing causes losses of nutritional and sensorial characteristics of foods. High-intensity pulsed electric field (HIPEF) is a nonthermal preservation technology for liquid foods that has been developed as alternative to thermal processing. HIPEF technology has shown promising results for microbial reduction and enzymatic inactivation. Besides, some studies have demonstrated that HIPEF processing is effective for maintaining bioactive compounds of liquid foods. Through a better understanding of the stability of bioactive compounds it is possible obtaining broccoli juice processed by HIPEF with elevated nutritional value.

For this reason, this doctoral thesis was focused on evaluating the influence of HIPEF-processing parameters, such as electric field strength (15 – 35 kV/cm), treatment time (500 – 2000 μs) and polarity (monopolar or bipolar mode) on bioactive compounds (chlorophylls, carotenoids, vitamin C and total phenolic compounds), minerals, amino acids and enzymes (myrosinase, polyphenol oxidase and lipoxygenase) were evaluated. Additionally, results obtained from HIPEF-processed broccoli juice were compared with those of thermally treated (90 °C/60 s) and untreated juices.

The HIPEF parameters electric field strength, treatment time and polarity significantly influenced the relative content (RC) of lutein, β-carotene, total phenolics (TP), vitamin C, minerals and amino acids, relative antioxidant capacity (RAC), as well as the RC of chlorophylls degradation compounds. Chl *a* and Chl *b* were only influenced by electric field strength and treatment time. However, polarity did not exert influence neither on Chl *a* nor on Chl *b*. In the case of chlorophylls and carotenoids, as electric field strength and treatment time augmented, the RC of the pigments increased. Maximum RC of Chl *a* (116.0%), Chl *b* (120.7%), lutein (121.2%), β-carotene (130.5%), TP (96.1%), vitamin C (90.1%) and RAC (95.9%) was reached between 25 and 35 kV/cm and from 2000 μs to 500 μs. Also, the RC of bioactive compounds and the RAC were higher in HIPEF treatments applied in bipolar mode with respect to monopolar mode, except for vitamin C. At the strongest HIPEF conditions (35 kV/cm for 2000 μs) in bipolar mode, the lowest RC of chlorophylls degradation compounds, as well as an increment or not significant changes in the mineral content in comparison to fresh broccoli juice was observed. Changes in the content of free amino acids depended of HIPEF treatment conditions applied and the amino acid evaluated.

Regarding enzymes, chlorophyllase (Chlase), polyphenol oxidase (PPO) and lipoxygenase (LOX) were affected by electric field strength, treatment time and polarity.

The residual activity (RA) of all enzymes decreased as electric field strength and treatment time increased. HIPEF treatments applied in bipolar mode led to the highest enzyme inactivation. Thus, the lowest RA for Chlase (26.3%), LOX (68.71%) and PPO (36.1%) was reached at 35 kV/cm for 2000 μs in bipolar mode. Within the range of assayed conditions, broccoli juice LOX was more resistant to HIPEF treatment, followed by PPO and Chlase. Respect to color difference (*ΔE*), it was significantly influenced by the electric field strength and treatment time. In contrast, polarity did not influence on the broccoli juice color. The maximum *ΔE* (2.03) *was achieved at* the strongest HIPEF conditions (35 kV/cm for 2000 μs in bipolar mode).

Overall, HIPEF-treated broccoli juice exhibited superior content of chlorophylls, carotenoids, TP, vitamin C, minerals, amino acids, and antioxidant capacity than those thermally treated or untreated juice. Also, the highest Δ*E* (7.89) was observed in the broccoli juice processed by heat.

These outcomes demonstrated that HIPEF processing could be a suitable technology for maintaining the bioactive compounds, preserving the antioxidant quality, the color, and inactivating degradative enzymes in broccoli juice.

RESUMEN

Estudios epidemiológicos han sugerido que un alto consumo de brócoli puede proteger a los humanos contra enfermedades crónicas. El brócoli es una fuente importante de compuestos bioactivos, incluyendo vitamina C, compuestos fenólicos, glucosinolatos, carotenoides y clorofilas. Nuevos productos a base de brócoli, tales como mezclas de zumos de brócoli y manzana se han sugerido para aumentar aún más su consumo. En general, los zumos de vegetales representan una alternativa interesante para la incorporación de compuestos bioactivos en la dieta y una especial atención se ha centrado en el zumo de brócoli, debido a su elevado contenido de compuestos bioactivos que proporcionan importantes beneficios a la salud.

Tradicionalmente, los zumos han sido procesados por calor para evitar los efectos perjudiciales de enzimas y microorganismos. Sin embargo, el tratamiento térmico provoca pérdidas de las características nutricionales y sensoriales de los alimentos. Los pulsos eléctricos de alta intensidad de campo (PEAIC) son una tecnología de conservación no térmica para los alimentos líquidos, que se ha desarrollado como alternativa al procesamiento térmico. Los PEAIC han mostrado resultados prometedores en la reducción microbiana y la inactivación enzimática. Además, algunos estudios han demostrado que el procesamiento con PEAIC es eficaz para preservar los compuestos bioactivos en alimentos líquidos. A través de una mejor comprensión de la estabilidad de los compuestos bioactivos es posible la obtención de zumo de brócoli procesado con PEAIC de un valor nutritivo elevado.

Por esta razón, esta tesis doctoral se centró en la evaluación de la influencia de los parámetros de procesamiento con PEAIC, tales como intensidad de campo eléctrico (15 - 35 kV / cm), tiempo de tratamiento (500 - 2000 μs) y polaridad (modo monopolar o bipolar) sobre compuestos bioactivos (clorofilas, carotenoides, vitamina C y compuestos fenólicos totales), minerales, aminoácidos y enzimas (mirosinasa, polifenol oxidasa y la lipoxigenasa). Además, los resultados obtenidos del procesamiento de zumo de brócoli con PEAIC, se compararon con los resultados del zumo tratado térmicamente (90 °C/60 s) y los zumos no tratados.

Los parámetros de procesamiento PEAIC intensidad de campo eléctrico, tiempo de tratamiento y polaridad influyeron significativamente sobre el contenido relativo (RC) de luteína, β-caroteno, fenoles totales (TP), vitamina C, minerales y aminoácidos capacidad antioxidante relativa (RAC), así como el RC de compuestos de degradación de las clorofilas. Chl *a* y Chl *b* sólo se vieron influidos por la intensidad de campo eléctrico y el tiempo de tratamiento. Sin embargo, la polaridad no ejerció influencia ni en Chl *a* ni en Chl *b*. En el caso de las clorofilas y carotenoides, como la intensidad de campo eléctrico y tiempo de tratamiento aumentaron, el RC de los estos pigmentos aumentó. El RC máximo de Chl *a* (116,0%), Chl *b* (120,7%), luteína (121,2%), β-caroteno (130,5%), TP (96,1%), vitamina C (90,1%) y la RAC (95,9%) fue alcanzado entre 25 y 35 kV/cm y de 2000 μs a 500 μs. Además, el RC de compuestos bioactivos y la RAC fueron mayores en los tratamientos de PEAIC aplicados en modo bipolar con respecto al modo monopolar, con excepción de la vitamina C. En las condiciones más fuertes de PEAIC (35 kV/cm durante 2000 μs) en el modo bipolar, el RC mas bajo de compuestos de degradación de las clorofilas, además de

que el contenido de minerales presentaron incrementos y algunos permanecieron sin cambios significativos en comparación con el zumo de brócoli fresco. Los cambios en el contenido de aminoácidos libres dependieron de las condiciones de PEAIC aplicadas y el ácido amino evaluado.

En cuanto a las enzimas, clorofilasa (Chlase), polifenol oxidasa (PPO) y lipoxigenasa (LOX) se vieron afectadas por la intensidad de campo eléctrico, el tiempo de tratamiento y la polaridad. La actividad residual (RA) de todas las enzimas disminuyó a medida que la intensidad de campo eléctrico y tiempo de tratamiento aumentaron. Los tratamientos con PEAIC aplicados en el modo bipolar condujeron a la más alta inactivación de las enzimas. Por lo tanto, la RA más baja para Chlase (26,3%), LOX (68,71%) y PPO (36,1%) se alcanzó a 35 kV / cm durante 2000 μs en modo bipolar. Dentro de la gama de las condiciones ensayadas, LOX de zumo de brócoli fue la más resistente al tratamiento con PEAIC, seguida de PPO y Chlase. Con respecto a la diferencia de color (ΔE), fue significativamente influenciado por la intensidad de campo eléctrico y tiempo de tratamiento. En contraste, la polaridad no influyó sobre el color del zumo de brócoli. La máxima ΔE (2.03) se logró a las condiciones más fuertes de PEAIC (35 kV/cm durante 2000 μs en modo bipolar).

En general, el zumo de brócoli tratado con PEAIC exhibió un contenido superior de clorofilas, carotenoides, TP, vitamina C, minerales, aminoácidos, y capacidad antioxidante que los zumos tratados térmicamente o sin procesar. Además, la mayor ΔE (7,89) se observó en el zumo de brócoli procesado por calor.

Estos resultados demostraron que el procesamiento de PEAIC podría ser una tecnología adecuada para la conservación de los compuestos bioactivos, preservando la calidad antioxidante, el color e inactivando las enzimas degradantes en el zumo de brócoli.

RESUM

Estudis epidemiològics han suggerit que un alt consum de bròquil pot protegir als humans contra malalties cròniques. El bròquil és una font important de compostos bioactius, incloent vitamina C, compostos fenòlics, glucosinolats, carotenoides i clorofil·les. Nous productes a força de bròquil, com ara barreges de sucs de bròquil i poma s'han suggerit per augmentar encara més el seu consum. En general, els sucs de vegetals representen una alternativa interessant per a la incorporació de compostos bioactius en la dieta i una especial atenció s'ha centrat en el suc de bròquil, degut al seu elevat contingut de compostos bioactius que proporcionen importants beneficis a la salut.

Tradicionalment, els sucs han estat processats per calor per evitar els efectes perjudicials d'enzims i microorganismes. No obstant això, el tractament tèrmic provoca pèrdues de les característiques nutricionals i sensorials dels aliments. Els polsos elèctrics d'alta intensitat de camp (PEAIC) són una tecnologia de conservació no tèrmica per als aliments líquids, que s'ha desenvolupat com a alternativa al processament tèrmic. Els PEAIC han mostrat resultats prometedors en la reducció microbiana i la inactivació enzimàtica. A més, alguns estudis han demostrat que el processament amb PEAIC és eficaç per preservar els compostos bioactius en aliments líquids. A través d'una millor comprensió de l'estabilitat dels compostos bioactius és possible l'obtenció de suc de bròquil processat amb PEAIC d'un valor nutritiu elevat.

Per aquesta raó, aquesta tesi doctoral es va centrar en l'avaluació de la influència dels paràmetres de processament amb PEAIC, com ara intensitat de camp elèctric (15-35 kV/cm), temps de tractament (500-2000 μs) i polaritat (modo monopolar o bipolar) sobre compostos bioactius (clorofil·les, carotenoides, vitamina C i compostos fenòlics totals), minerals, aminoàcids i enzims (mirosinasa, polifenol oxidasa i la lipoxigenasa). A més, els resultats obtinguts del processament de suc de bròquil amb PEAIC, es van comparar amb els resultats del suc tractat tèrmicament (90 °C/60 s) i els sucs no tractats.

Els paràmetres de processament PEAIC intensitat de camp elèctric, temps de tractament i polaritat van influir significativament sobre el contingut relatiu (RC) de luteïna, β-carotè, fenols totals (TP), vitamina C, minerals i aminoàcids capacitat antioxidant relativa (RAC), així com el RC de compostos de degradació de les clorofil·les. Chl *a* i Chl *b* només es van veure influïts per la intensitat de camp elèctric i el temps de tractament. No obstant això, la polaritat no va exercir influència ni en Chl *a* ni a Chl *b*. En el cas de les clorofil·les i carotenoides, com la intensitat de camp elèctric i temps de tractament van augmentar, el RC dels aquests pigments va augmentar. El RC màxim de Chl *a* (116,0%), Chl b (120,7%), luteïna (121,2%), β-carotè (130,5%), TP (96,1%), vitamina C (90,1%) i la RAC (95,9%) va ser aconseguit entre 25 i 35 kV/cm i de 2000 μs a 500 μs. A més, el RC de compostos bioactius i la RAC van ser majors en els tractaments de PEAIC aplicats en mode bipolar fa a la manera monopolar, amb excepció de la vitamina C. En les condicions més forts de PEAIC (35 kV/cm durant 2000 μs) en la manera bipolar, el RC mes baix de compostos de degradació de les clorofil·les, a més que el contingut de minerals van presentar increments i alguns van romandre sense canvis significatius en comparació amb el suc de bròquil fresc. Els canvis en el contingut d'aminoàcids lliures van dependre de les condicions de PEAIC aplicades i l'àcid amino avaluat.

Quant als enzims, clorofilasa (Chlase), polifenol oxidasa (PPO) i lipoxigenasa (LOX) es van veure afectades per la intensitat de camp elèctric, el temps de tractament i la polaritat. L'activitat residual (RA) de tots els enzims disminuir a mesura que la intensitat de camp elèctric i temps de tractament van augmentar. Els tractaments amb PEAIC aplicats en la manera bipolar van conduir a la més alta inactivació dels enzims. Per tant, la RA més baixa per Chlase (26,3%), LOX (68,71%) i PPO (36,1%) es va aconseguir a 35 kV / cm durant 2000 μs en mode bipolar. Dins de la gamma de les condicions assajades, LOX de suc de bròquil va ser la més resistent al tractament amb PEAIC, seguida de PPO i Chlase. Pel que fa a la diferència de color (ΔE), va ser significativament influenciat per la intensitat de camp elèctric i temps de tractament. En contrast, la polaritat no va influir sobre el color del suc de bròquil. La màxima ΔE (2.03) es va aconseguir a les condicions més forts de PEAIC (35 kV/cm durant 2000 μs en mode bipolar).

En general, el suc de bròquil tractat amb PEAIC va exhibir un contingut superior de clorofil·les, carotenoides, TP, vitamina C, minerals, aminoàcids, i capacitat antioxidant que els sucs tractats tèrmicament o sense processar. A més, la major ΔE (7,89) es va observar en el suc de bròquil processat per calor.

Aquests resultats van demostrar que el processament de PEAIC podria ser una tecnologia adequada per a la conservació dels compostos bioactius, preservant la qualitat antioxidant, el color i inactivant els enzims degradants en el suc de bròquil.

I INTRODUCTION

1. Broccoli

Broccoli is an italian word from the latin "*brachium"*, meaning an arm or branch (Gray 1982). Broccoli is a compact, fast-growing floral vegetable with a head of fleshy tight flower heads (curds) or buds, usually green in color arranged in a tree-like fashion on branches sprouting from an edible stalk (Bhattacharjee & Singal, 2011).

1.1. World production of broccoli

The broccoli (*Brassica oleracea* L. var *italica*) is a vegetable that belongs to the *Brassicaceae* family, which also includes cauliflower, Brussel's sprouts, cabbage, among others (Campas-Baypoli et al. 2009).

Table 1. Top production of broccoli and cauliflower of the principal producer countries

Rank	Country	Production (Tonnes)	Area harvested (Ha)	Yield (Hg/Ha)
1	China	9 030 990	446524	202250
2	India	6745000	369000	182 791
3	Spain	527500	31169	169238
4	Mexico	427884	29010	147495
5	Italy	420989	17637	238696
6	France	334170	19700	169629
7	USA	325180	15150	214640
8	Poland	297649	14948	199122
9	Pakistan	227591	13103	173693
10	Egypt	201201	7234	278132

[From FAOSTAT, (2011)]

Currently, China, India, Spain, Mexico, and Italy are the five worldwide major producers of broccoli (FAOSTAT, 2011) (Table 1). The broccoli was introduced in Spain in the 70's (Baixauli-Soria & Romeu-Iborra 2007) and actually is cultivated in an area of about 31000 hectares, with a production of around 500 000 tonnes (FAOSTAT, 2011). Indeed, Spain is the most important broccoli producer in Europe.

As a result of various studies linking consumption of broccoli with health benefits, such as the reduction of the risk of cancer, broccoli has emerged as one of the top consumed vegetables (Moreno et al. 2006, Talalay & Fahey 2001). The per capita

utilization of fresh and frozen broccoli in the United States in 2008 was about 2.8 Kg and 1.2 Kg, respectively (Bhattacharjee & Singal, 2011).

1.2. General composition of broccoli

The consumption of vegetables is indispensable for human health. Numerous epidemiological studies have demonstrated that the intake of vegetables reduced the risk of chronic diseases, heart attack and certain types of cancer (Kushi et al., 2012). As well, vegetables act as a low-calorie, low-sodium, and satiating food (Eilat-Adar et al., 2013). Particularly, broccoli is a vegetable with an elevated content of essential nutrients (vitamins, minerals) and bioactive compounds (carotenoids, chlorophylls, phenolics, and glucosinolates) (Moreno et al., 2006). For instance, in comparison with other vegetables of *Brassicaceae* family, broccoli has the highest vitamin C, vitamin E, folate, chlorophylls, carotenoids and glucoraphanin content (Table 2). Furthermore, broccoli has a greater content of vitamins (vitamin C, K and E), minerals (Ca, Fe), and phenolics, respect to other common vegetables, like green pepper and tomato (Table 2).

Recommended Dietary Allowances (RDA), created by the USDA (2006), suggested the quantity of essential nutrients to get an optimum health state (Table 3). Comparing the values of RDA (Table 3) with those of the broccoli composition (Table 2), it can be observed that the consumption of 100 g per day of this vegetable insurance the nutritional requirements for all population as follow: 46% of vitamin A, 33% of vitamin E and 18% of folate, and exceeds 46% of vitamin C.

Table 2. Comparative composition of common vegetables (mg/100 g edible portion)

Compound*	Broccoli	Cauliflower	White cabbage	Brussels sprouts	Iceberg Lettuce	Green Pepper	Tomato
Vitamins							
Vitamin E	0.46-4.93	0.21-1.28	0.06-0.27	0.51-1.25	0.33	0.08	0.68
Vitamin C	57.35-131.35	13.8-78	5.66-47	14.6-109	15	128	20-25
Vitamin K	76.6-205	5-300	60-149	177-570	15.6-36.2	6.8-7.8	2.2-3.1
Folate[a]	111-114	82-125	30-31	94-182	51-75	55-60	11-50
Pigments							
Chl *a*	5.77-71.6		1.13	2.4-4.61	3.32-35.65		-
Chl *b*	1.49-29.9		0.40	1.14			-
Lutein	0.41-8.4	0.004-0.15	0.02-0.26	0.18-0.9	0.11-0.64	0.34	0.04-0.34
β-carotene	0.48-5.0	0.01-0.1	0.01-0.41	0.07-1.7	0.048	0.21	0.52
Minerals							
K	289	221	266	425	170-360	124-178	249-319
Mg	18.1	14.5	14	20.7	8.3-21	11	10.8-22.4
Ca	27.2	17.5	44	35.6	20-56	10	10.8-20.1
Fe	0.87	0.50	0.14	0.76	300-670	340-1700	0.49-3.51
Se[a]	0.7	0.9	0.6-2.5	0.6	160-2000	1.1-19	1
Amino acids							
Arginine	190	92-120	100-110	240-300	56-69	23	18
Histidine	63	43-54	21-28	80-140	20-23	14	13
Isoleucine	130	100-110	36-43	170-270	48-83	45	23
Leucine	160	130-180	51-61	160-340	75-79	45	30
Valine	170	130-180	34-54	220-320	60-68	32	23

Compound	Broccoli	Cauliflower	White cabbage	Brussels sprouts	Iceberg Lettuce	Green Pepper	Tomato
Tryptophan	37	22-44	-	40-60	9-13	9	6
Threonine	120	70-130	36-41	130-190	51-59	49	23
Methionine	50	13-69	7.0-18	30-50	4.0-22	16	7
Phenylalanine	120	42-91	22-44	110-170	46-64	54	24
Lys	150	130-150	45-96	240-390	48-81	50	29
Glucosinolates							
Glucoraphanin	28.9-190.2	0-190.1	0.2-4.0	0.4-22.6			
Glucobrassicine	22.8-101	13.6-162.3	9.3-200	45.3-469.4			
Neo-glucobrassicin	2.4-26.2	1.1-32	400-900	1.9-34.3			
Glucoiberin	0-327.2	0-327.2	5.0-279.8	0-154.2			
4-HGlucobrassicin	5.07	4.95	1.0-2.0	7.46			
Total GLS	102-448.6	30.2-520.4	17.7-602.6	138-600			
AC[b]	23-208	62-152	23-146		56	54-300	33-112
TPP	34.5-337	27.8-274	3.8-15.3	35-90.7	5.1-22	4.4-47.6	3.5-36.8

[a] Are expressed as µg/100g

[b] AC = Actioxidant capacity. TE = Trolox equivalent obtained by the ORAC method expressed as µmol TE/100g

*Values are expressed as mg/100g

Table 3. Dietary reference intakes: Estimated average requirements

Nutrient	Children 4-8 years	Males			Females			Pregnancy
		9–18 years	19-50 years	51-70 years	9–18 years	19-50 years	51-70 years	6-9 months
Vitamin A[a]	400	600-900	900	9000	600-700	700	700	770
Vitamin C[b]	25	45-75	90	90	45-65	75	75	85
Vitamin E[b]	7	11-15	15	15	11-15	15	15	15
Folate[c]	200	300-400	400	400	300-400	400	400	600
Potassium[d]	3.8	4.7	4.7	4.7	4.7	4.7	4.7	4.7
Magnesium[b]	130	240-410	420	420	240-360	320	320	360
Selenium[e]	30	40-55	55	55	40-55	55	55	60

[a]Values expressed as retinol activity equivalents (RAEs). 1 RAE = 1 µg retinol, 12 µg β-carotene, 24 µg α-carotene, or 24 µg β cryptoxanthin. [b] Values expressed as mg/day. [c] As dietary folate equivalents (DEF). 1 DEF = 1 µg food folate = 0.6 µg of folic acid from fortified food.[d] Values expressed as g/day. [e] Values expressed as µg/day.

1.2.1. Bioactive compounds contained in broccoli

The beneficial effects on human health of broccoli have been associated with the elevated content of bioactive compounds (Mehta et al., 2010). Some of that are described below.

1.2.2. *Chlorophylls*

Chlorophylls (Chls) are one of the most abundant and widely distributed natural pigments. Chls are defined as the green tetrapyrrole pigments derivate of dihydroporphirin chelated with a centrally located magnesium ion (Fig. 1) with photosynthetic functions (Schwartz & Lorenzo 1990, Simpson 1985, Wilska-Jeszka 2007).

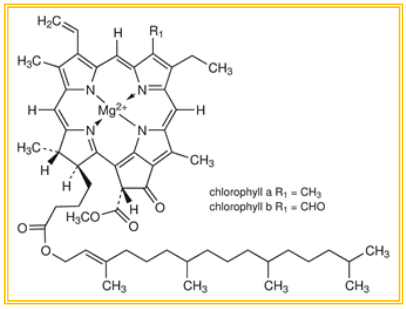

Figure 1. Chlorophyll structure

Table 4. Nomenclature of chlorophyll derivatives

Compound	Characteristics
Chlorophyll *a*	It is a magnesium-chelated tretrapyrrole structure with methyl substitutions at the 1, 3, 5 and 8 positions, vinyl at the 2, ethyl at the 4, propionate esterified with phytyl alcohol at the 7, keto at the 9, and carbomethoxy at the 10 position.
Chlorophyll *b*	Chlorophyll *b* has the same configuration as chlorophyll *a* except that in the 3 position there is a formyl group rather than a methyl group.
Phytol	A 20-carbon alcohol with an isoprenoid structure.
Chlorophyllide	The acid derivative resulting from enzymatic or chemical hydrolysis of the C_7 propionate ester.
Pheophytin	The magnesium-free derivatives of the chlorophylls.
Pheophorbide	The products containing a C_7 propionic acid resulting from removal of magnesium and hydrolysis of the phytyl ester.
Chlorophyllase	The enzyme present in leaves which catalyzes hydrolysis of the C_7 propionate ester.

(Simpson 1985, Von Elbe & Schwartz 2000)

In higher plants and algae (except the blue-green algae), Chls are found in the thylakoid membrane of chloroplasts, where they are associated with polypeptides, phospholipids, and tocopherols (Davídek et al. 1990a, Heaton & Marangoni 1996).

There are two Chls: Chl *a* (blue-green) and Chl *b* (yellow-green), occurring in plants in a ratio about 3:1, which is in function of growth conditions and environmental factors, particularly high-light and sun exposure (Belitz et al. 2009a, Schwartz & Lorenzo 1990, Wilska-Jeszka 2007). Differences between Chl *a* and Chl *b* are mentioned in Table 4.

1.2.2.1. *Biological activity of chlorophylls*

Chls and their commercial-grade derivatives since many years have been used for therapeutic purposes, such as for treating anemia and hypertension, as a healing agent and in oral hygiene (Kephart 1955). Also, Chls has been investigated for their antioxidant and anti-inflammatory properties (Endo et al. 1985, Larato & Pfau 1970), wound healing (Edwards 1954), but above all for its possible influence on cancer prevention (Ferruzzi & Blakeslee, 2007). In addition, Chls and their derivatives have demonstrated *in vitro* antimutagenic and anticarcinogenic effects against some substances known or suspected to cause cancer, such as polycyclic aromatic hydrocarbons found in smoke, heterocyclic amines cooked meats, as well as aflatoxin B1. It is speculated that one possible mechanism is the formation of complexes between the mutagen or carcinogen and the Chl or chlorophyllin through strong interaction between their planar unsaturated cyclic rings (Lai et al. 1980, Wilska-Jeszka 2007).

Endo et al. (1985) proposed that Chl derivatives may be acting as electron donors by their capability to reduce 1,1,dipheniyl-2-picrylhydrazyl (DPPH). Both the porphyrin structure as well as the presence and nature of the central metal are considered important to antioxidant activity of chlorophyll derivatives. A clear structural relationship within porphyrins for inhibition of lipid hydroperoxide formation was reported (Ferruzzi & Blakeslee 2007).

Furthermore, Chls are able to sequestering dietary mutagens (Hartman & Shankel 1990), modulate endogenous xenobiotic detoxification enzymes (Singh et al. 1996) and induces apoptosis in cancerous cells (Chiu et al. 2005).

1.2.3. *Carotenoids*

Carotenoids are one of the most widespread pigments in the nature. Their basic structure is a symmetrical tetraterpene skeleton, formed by head-to-tail condensation of two C_{20} units, which is modified by cyclization, addition, elimination, rearrangement, and substitution, as well as oxidation. Carotenoids contain a system of conjugated double bonds which influences their physical, biochemical and chemical properties (Van Den Berg et al. 2000, Wilska-Jeszka 2007). In green chloroplast, carotenoids are associated with chlorophyll in the photosynthetic pigment protein complexes (Van Den Berg et al. 2000).

Based on their composition, carotenoids are divided into two classes:

- *Carotenes*. Those contain in their structure only carbon and hydrogen atoms: lycopene and bicyclic β-, α- and λ-carotene (Fig. 2).
- *Xantophylls or oxocarotenoids*. Contain at least one oxygen in the form of hydroxyl (lutein), epoxy (violaxanthin), and oxo (canthaxanthin) groups (Fig. 2).

Carotenoids are membranal pigments: apolar carotenes are immersed in membranes and show limited mobility, whereas xanthophylls have a variable position and mobility in membranes. Carotenes have high antioxidant activity against radicals generated inside the membranes. The structure of carotenoids, in particular the length of the polyene chain, significantly influences its antioxidant properties.

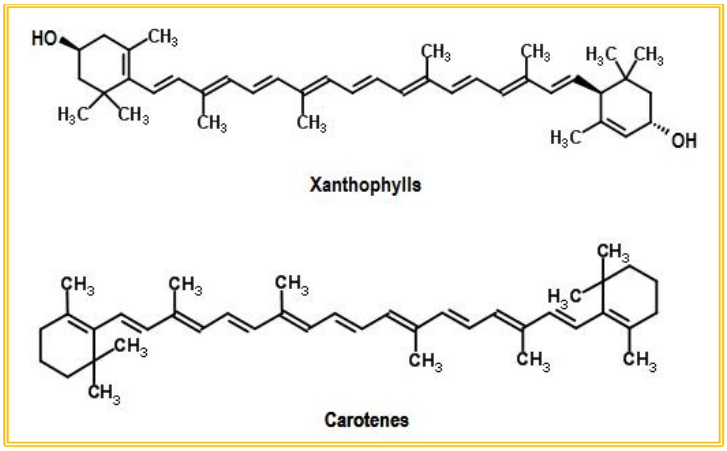

Figure 2. Xanthophyll and carotene structures.

With their large number of conjugated double bonds, the carotenoids contain a reactive electron-rich system, which is susceptible to reaction with electrophilic compounds. This structure is responsible for the high sensitivity of carotenoids to oxygen and light. Food processing and storage can cause isomerization of carotenoids and affect the color because increasing the number of *cis* bonds results in gradual lightening of the color (Wilska-Jeszka 2007).

1.2.3.1. *Biological activity of carotenoids*

In all photosynthetic organisms, carotenoids have two major functions: as accessory pigments for light harvesting (they absorb photons and transfer the energy to Chl) and in the prevention of phooxidative damage (that are caused by the excited triplet state of Chl). In human body, the best documented and established function of some of the carotenoids is their provitamin A activity (Parker 1996).

Carotenoids have diverse biological functions and actions (Berg et al., 2000):

Provitamin A activity	β-carotene, α-carotene, β-cryptoxanthin
Antioxidant	All carotenoids
Cell communication	β-carotene, canthaxanthin, crytoxanthin
Immune function enhancers	β-carotene
UV skin protection	β-carotene, lycopene
Macula protection	Lutein, zeaxanthin

Some carotenoids are precursors of a separate class of bioactive compounds, the retinoids, which can regulate cell growth and differentiation in various cell types. Pathological processes, including cancer and cardiovascular disease, are commonly associated with an oxidative stress condition. Carotenoids have a considerably high antioxidant activity, which has most often been the focus of its role in preventing disease initiation and propagation (Wilska-Jeszka 2007).

Carotenoids molecules with nine or more conjugated carbon-carbon double bonds, can absorb triplet-state energy from Chl, and thus prevent the formation of harmful singlet oxygen (Wilska-Jeszka 2007). Epidemiological studies have also shown an inverse association between carotenoid intake and the risk of cataract and age-related macular degeneration. Lutein and zeaxanthin are the principal components of macular pigments (Wilska-Jeszka 2007).

1.2.4. Vitamin C

Vitamin C is one of the most important water soluble antioxidants from food. It is found in two L-isomeric forms: ascorbic acid (Vitamin C) in the reduced state and dehydroascorbic acid in the oxidized state. The term vitamin C is used as the generic descriptor for all compounds exhibiting the biological activity of ascorbic acid (L-ascorbic acid and dehydroascorbic acid) in fruits and vegetables (Ball 2006, Johnson 2003).

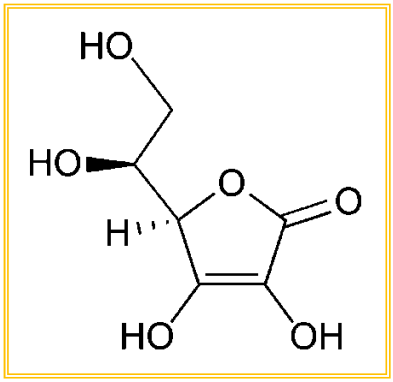

Figure 3. L-Ascorbic acid structure

L-ascorbic acid (L-AA) (Fig. 3) is used extensively in food technology as a stabilizer for the processing of beverages, wines, and meat products (Combs 2008). In the present document, both terms will be used interchangeably. The content of vitamin C in foods is in function of genetic variation, maturity, climate, sunlight, method of harvesting, and storage, among others (Ball 2006, Johnson 2003). L-AA is very susceptible to chemical and enzymatic oxidation during processing, storage, and cooking of foods (Ball 2006, Johnson 2003). In the presence of trace amounts of cupper, iron and alkali it is easily oxidized, but stable when dry (Ball 2006, Davey et al. 2000).

1.2.4.1. Biological activity of vitamin C

Vitamin C is an essential nutrient with uncountable functions for human health. The most important biological functions of vitamin C are listed in the Table 5. Although some of these functions have become widely accepted, the evidence remains incomplete for many. The only wholly unquestionable function of vitamin C is the prevention of scurvy (Johnson 2003).

Vitamin C can act as an enzyme cofactor, a radical scavenger, and as a donor/acceptor in electron transport at the plasma membrane (Davey et al. 2000). The body requires it to form and maintain bones, blood vessels, and skin, thus, it must be replenished daily. Moreover, vitamin C is a potent water-soluble antioxidant in biological fluids. L-AA losses electrons easily, and because of its reversible monovalent oxidation to the ascorbyl radical, it can be serve as a biochemical redox system. Furthermore, there is a growing evidence that vitamin C provide protective effects against cancer (Johnson 2003). Therefore, adequate intake of vitamin C from foods is vital for normal functioning of the human body.

Table 5. Metabolic functions of vitamin C

Metabolic function	Specific function	
Electron transport	Ascorbic acid losses electrons easily, and, because of its reversible monovalent oxidation to the ascorbyl radical, it can serve as a biochemical redox system.	
Antioxidant functions	L-AA can act as an antioxidant owing to its ability to react with free radicals. Yield a relatively poor reactive intermediate (ascorbyl radical); reducing the toxic, reactive oxygen species superoxide anion ($O_2^{\bullet -}$) and hydroxyl radical ($OH^{\bullet}$), as well as organic ($RO_2^{\bullet}$) and nitrogen ($NO_2^{\bullet}$) oxy radicals. Is able to reduce the semistable chromanoxyl radical, thus regenerating the metabolically active form of the lipid antioxidant vitamin E.	
Enzyme cofactor	Collagen synthesis Catecholamine synthesis Peptide hormone biosynthesis Carnitine synthesis Drug and steroid metabolism Tyrosine metabolism	Functions as a cofactor for at least eight enzymes that are either monooxygenases or dioxygenases.
Nonenzymatic functions	Cellular antioxidation	Prevention of lipid peroxidation, protein, nitric oxide and DNA oxidation.
	Metal ion metabolism	Increases the bioavailability of iron in foods. Reduces the toxicities of elements whose reduced forms are poorly absorbed or more rapidly excreted (Se, Ni, Pb, V, and Cd). Stimulate mechanisms to preserve tissue copper stores, which are required for several enzymes.
Health effects	Antihistamine reactions	Is involved in histamine metabolism, acting with Cu^{2+} to inhibit its release and enhance its degradation. Circulating histamine concentration is known to be reduced by high doses of vitamin C.
	Immune function	Stimulate the production of interferons, the protein that protect cell against viral attack. Stimulate the positive chemotactic and proliferative responses of neutrophils. Protect against free-radical mediated protein inactivation associated with the oxidative burst of neutrophils. Stimulate the synthesis of humoral thymus factor and antibodies of the IgG and IgM classes. Has shown protective effects against common cold, *Helicobacter pylori*, and herpes.

	Cardiovascular disease	Has been associated to an anti-atherogenic function in reducing the oxidation of low-density lipoproteins (LDLs).
	Exercise tolerance	Antioxidant supplementation can alleviate muscle damage and protein oxidation induced by exercise; due to the protection of NO, a mediator of endothelium-dependent vasodilatation.
	Diabetes	The vitamin C supplementation reduce the glycosylation of plasma proteins; suggesting that it may have a role in preventing diabetic complications. Prevents arterial hemodynamic changes induced by hyperglucemia.
	Neurologic function	The brain and spinal cord are the richest tissues in ascorbic acid contents. Has been associated with memory performance in patients with dementia.
	Skin health	Is essential in collagen synthesis. It is well documented that vitamin C-deficient animals show prolonged wound-healing times.
	Cancer	Reduce the binding of polycyclic aromatic carcinogens to DNA Delay the tumor formation in several animal models. Inhibits the nitrosamine-induced carcinogenesis, functioning as a nitrite scavenger. Protects against cancers of the esophagus, larynx, oral cavity, pancreas, stomach, colon-rectum, and breast.

(Combs 2008, Davey et al. 2000, Thurnham 1994)

1.2.5. Phenolics

Phenolics are chemical compounds widely distributed at the plant kingdom with more than 8000 phenolic structures currently known and defined as secondary metabolites of plants that appear as response to pathogens and ultraviolet radiation (Manach et al. 2004, Vermerris & Nicholson 2006). Natural polyphenols can range from simple molecules (phenolic acids), to highly polymerized compounds (tannins). Generally, the compounds that present more than one phenolic group are named as phenolics. Their classification is complicated due to diversity of structures (Manach et al. 2004), but the most accepted classification is that proposed by Harborne (1989), based on the number of carbon atoms presents in the phenolic compounds (Table 6).

Table 6. Classification of phenolics

Number of carbon atoms	Structure	Classification
6	C_6	Simple phenolics
7	$C_6 - C_1$	Phenolic acids and related compounds
8	$C_6 - C_2$	Acetophenones and phenylacetic acids
9	$C_6 - C_3$	Cinnamic acids, coumarins, isocoumarins, and chromones
10	$C_6 - C_4$	Chalcones, aurones, dihydrochalcones
13	$C_6 - C_1 - C_6$	Xanthones
14	$C_6 - C_2 - C_6$	Stilbenes
15	$C_6 - C_3 - C_6$	Flavonoids
18	$(C_6 - C_3)_2$	Lignans, neolignans
30	$(C_6 - C_3 - C_6)_2$	Biflavonoids
n	$(C_6)_n$	Catecolamines
	$(C_6 - C_3)_n$	Lignin
	$(C_6 - C_3 - C_6)_n$	Condensed tannins

1.2.5.1. Biological activity of phenolics

Phenolics are indispensable compounds for food quality and human health. Indeed, they are associated to the color, flavour, astringency, and bitterness of foods and beverages (Haard & Chism 2000).

Polyphenols have been recognized as one of the most important nutrients due to them exhibits many biologically significant functions, such as protection against oxidative stress and degenerative diseases (Hollman, 2001). Many of these functions and the ways followed by the human body against oxidative stress are listed in Fig. 4.

As previously discussed, polyphenols perform numerous biological functions in the body; however, the health effects of polyphenols are in function of the amount consumed and on their bioavailability (Manach et al. 2004).

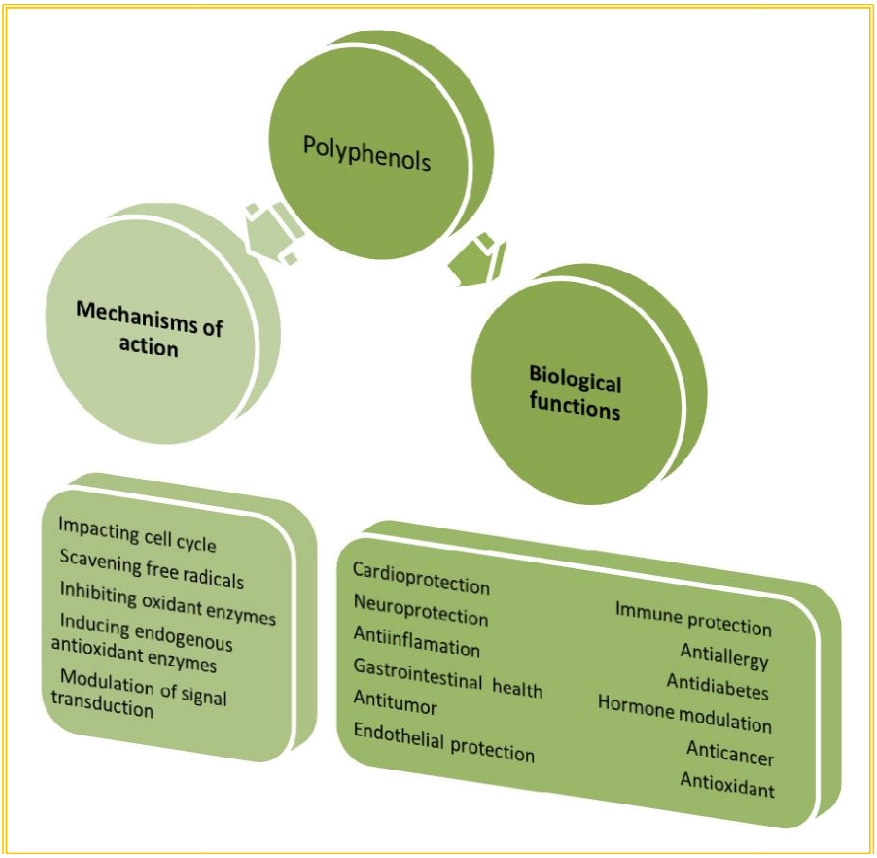

Figure 4. Biological activities of dietary polyphenols [Adapted from Han et al. (2007)]

1.2.6. Glucosinolates and their hydrolysis products

There are 16 families of glucosinolates (GLS) containing angiosperms. The *Brassicaceae* family, which include the cruciferous vegetables, are an important source of GLS (Fahey et al. 2001). Over 120 naturally occurring GLS have been identified and quantities of these compounds in broccoli range from 10 to 21.4 µmol/g dry weight; whereas McNaughton and Marks (2003) developed a database for the GLS content of cruciferous vegetables, ranging from 19.3 to 127.5 mg/100g for raw broccoli. Variation in the GLS content is in function of upon cultivar, agricultural practices, climate, among other factors (Charron et al. 2005, Fenwick et al. 1983, Kushad et al. 1999, Vallejo et al. 2002b). In Table 7 an overview is given of common GLS presents in broccoli.

GLS are very stable, water-soluble compounds stored within cell vacuoles of all cruciferous vegetables. GLS share a common basic structure containing a β-D-thioglucoside group, a sulphonate doxime moiety and a side chain derived from

methionine, phenylalanine, tryptophane or branched-chain amino acids (Figure 5) (Fahey et al. 2001, Hertog et al. 1997, Rafter 2002, Śmiechowska et al. 2010). In function on the amino acid precursors, GLS are grouped in three chemical classes: aliphatic, aromatic, or indol GLS, according to whether their amino acid precursor is methionine, phenylalanine, or tryptophan, respectively (Bheemreddy & Jeffery 2006).

GLS are not bioactive compounds until they have been fragmented to various bioactive products (isothiocyanates) by the endogenous plant enzyme myrosinase (Keck & Finley 2004). Myrosinase (β-thioglucoside glucohydrolase, E.C. 3.2.3.1) is present in so-called myrosin cells (idioblasts) separately from GLS (Śmiechowska et al. 2010). When vegetables are damaged, GLS are released and rapidly hydrolyzed by myrosinase to glucose, biologically active isothiocyanates (ITC), thiocyanates, nitriles and cyanoepithioalkanes, among others (Bones & Rossiter 2006, Fahey et al. 2001, Moreno et al. 2006). Most of these compounds are volatiles provoking a strong meal and flavour, associated with herbivore and microbial defence (Keck & Finley 2004). When myrosinase is heat inactivated during food processing, humans can efficiently convert GLS to ITC through the action of the microflora of the gastrointestinal tract (Shapiro et al. 2001).

Table 7. Glucosinolates contained in broccoli.

Trivial name	Chemical names of R-groups	Clasification	Range of amount (µmol/g dw)
Glucoraphanin	4-(Methylsulfinyl) butyl	Aliphatic	0.3 – 38.4
Glucoiberin	3-(Methylsulfinyl) propyl	Aliphatic	0 – 7.8
Glucoalyssin	5-(Methylsulphinyl) pentyl	Aliphatic	0 – 5.9
Progoitrin	2(R)-2-Hydroxy-3-butenyl	Aliphatic	0.1 – 16.1
Gluconapin	3-Butenyl	Aliphatic	0 – 1.0
Glucobrassicanapin	4-Pentenyl	Aliphatic	0 – 0.6
Glucobrassicin	Indol-3-ylmethyl	Indole	1.1 – 33.4
Neoglucobrassicin	1-Methoxyindol-3-ylmethyl	Indole	0.2 – 19.9
4-Hydroxyglucobrassicin	4-Hydroxy-3-indolylmethyl	Indole	0 – 0.6
4-Methoxyglucobrassicin	4-Methoxyindol-3-ylmethyl	Indole	0.2 – 2.0

(Fahey et al. 2001, Latté et al. 2011)

The formation and relative amount of ITC strongly depend of many factors, like plant species and cultivar, the site of hydrolysis (inside the plant or in the gut), the tissue, the kind of side chain of the glucosinolate, the presence of cofactors (vitamin C, ferrous ions, epithiospecifier protein) and the environmental conditions (pH, temperature, moisture) (Keck & Finley 2004; Cieslik et al., 2007).

Under physiological pH, the major hydrolysis products generally are stable ITC (Cartea & Velasco 2008). Whereas nitriles are formed at acid conditions, which might be

promoted by the millimolar concentrations of iron or the presence of epithiospecifier protein (ESP) (Bheemreddy & Jeffery 2006). ESP present in broccoli, is a co-factor of myrosinase and recent studies have shown that ESP is able to shift the hydrolysis of glucoraphanin in favour of sulforaphane (SFN) (Fenwick & Heaney 1983).

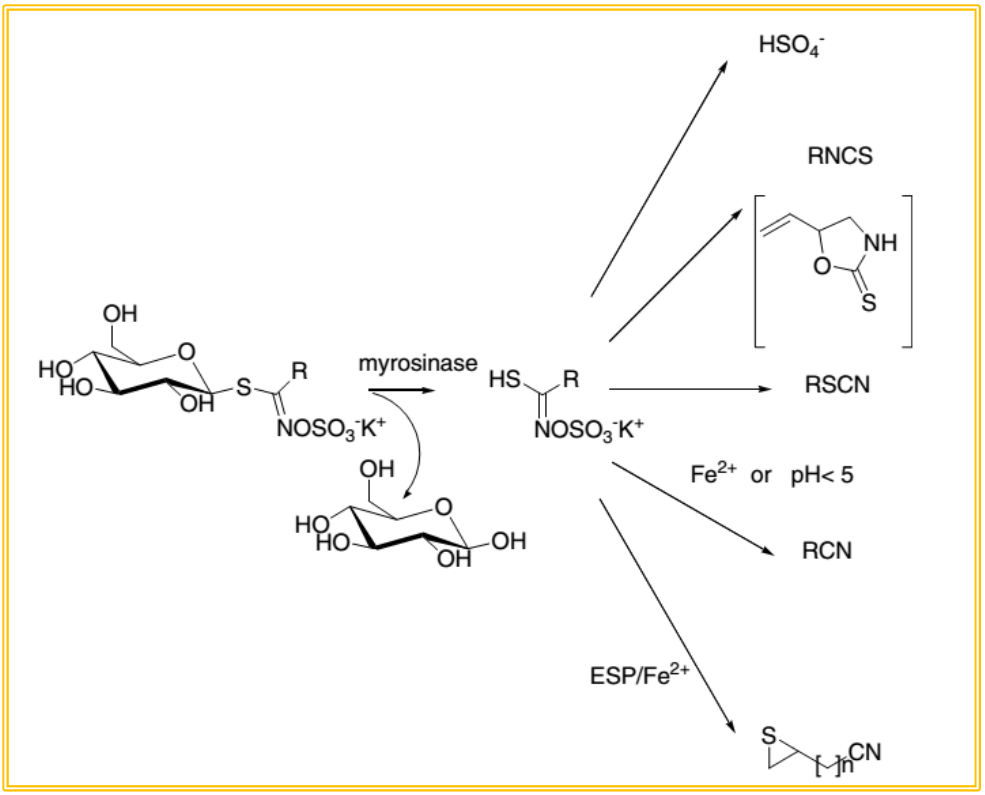

Figure 5. General hydrolysis of glucosinolates (Bones & Rossiter 2006).

1.2.6.1. Biological activity of glucosinolates and their hydrolysis products

A growing number of epidemiological studies have shown that a high dietary intake of *Brassica* vegetables is associated with the protection against a wide range of different types of cancers (London et al. 2000, Talalay & Fahey 2001, Verhoeven et al. 1996). This means that many cancers and deteriorative diseases may be prevented modifying dietary habits. In the same way, diverse studies have exposed that the consumption of three or more half-cup servings of broccoli or other *Brassica* genus vegetables, per week significantly reduced the risk for prostate cancer by 40% compared to ingestion of one or fewer servings per week (Cohen et al. 2000). Verhoeven et al. (1996) demonstrated an inverse relation between ingestion of total *Brassica* vegetables and cancer risk. The anticarcinogenic properties of broccoli have been attributed to their relatively high content of glucosinolates (GLS) (Verhoeven et al. 1997).

One of these bioactive compounds, resulting from the hydrolysis of glucoraphanin (4-methyl-sulfinyl-butylglucosinolate), is sulforaphane, a potent inducer of phase II enzymes, which representthe most important group of detoxication enzymes of the human organism, protecting the cells against carcinogenesis and mutagenesis (Galgano et al., 2007).

1.2.7. Minerals

Minerals represent from 0.2% to 0.3% of the total intake of all nutrients in the diet and they are classified, depending on the amounts needed for human beings, into macro (Na, K, Ca, Mg, Cl, P) and microminerals (Fe, I, F, Zn, Se, Cu, Mn, Cr, Mo, Co, Ni) (Belitz et al. 2009b, Nabrzyski 2007).

1.2.7.1. Biological activity of minerals

Minerals participate in a wide number of biochemical and physical processes important for human health (Biziuk & Kuczyńska 2007). Specifically, minerals play essential roles in cell membrane transport, stabilize plasma membranes and nucleic acids, regulate the osmotic pressure, and are cofactors of enzymes and constituents of vitamins, hormones and hemoglobin, among other functions as are shown in Table 8.

Special interest has been focused on selenium (Se) due to its possible anticarcinogenic properties (Navarro-Alarcon & Cabrera-Vique 2008, Silvera & Rohan 2007). Furthermore, Se status is able to modify mental functions. That is, a low selenium status leads to depressed mood, while high dietary or supplementary Se improves the mood. Low Se status is related with an increased incidence of depression, anxiety, confusion, and hostility, and with senility and cognitive decline in elderly people. Subjects with head or neck cancers, urinary tract cancers, or with rheumatoid arthritis also have a depleted concentration of Se in the blood. The brain Se level in Alzheimer patients is only 60% of that in control groups. An accumulated toxic level of selenium evokes severe selenosis, with such symptoms as malodorous breath, dermatitis, loss of hair, and neurological abnormalities (Borawska 2007).

Brassica vegetables contain low amounts of Se (0.1-0.3 μg Se/g DW). Nonetheless, when broccoli grows on Se enriched soils, have the unique ability to accumulate concentrations of Se many orders of magnitude above normal (Keck & Finley 2004).

Table 8. The biological functions of some minerals

Element	Biological function
Iron (Fe)	Constituent of haemoglobin, myoglobin and many enzymes.
Potassium (K)	Osmotic pressure, water balance, acid-base balance, nerve stimulation, muscle contraction, synthesis of protein, glycogen formation.
Magnesium (Mg)	Component of bones and teeth, activation of many enzymes, nerve stimulation, muscle contraction.
Calcium (Ca)	Bone and tooth formation, blood clotting, cell permeability, nerve stimulation, muscle contraction, enzyme activation.
Copper (Cu)	Necessary for iron utilization and haemoglobin formation, constituent of cytochrome oxidase, and involved in bone and elastic tissue development, present in important proteins and enzymes.
Manganese (Mn)	In the aging process has a role as an antioxidant (Mn-superoxide dismutase), cofactor of a large number of enzymes, important for normal brain function, for reproduction, and for bone structure.
Sodium (Na)	Osmotic pressure, water balance, acid-base balance, nerve stimulation, muscle contraction, cell permeability.
Phosphorus (P)	Bone and tooth formation, energy metabolism, component of ATP and ADP, protein synthesis, component of DNA and RNA, fat transport, acid-base balance, glycogen formation.
Zinc (Zn)	Constituent of many enzyme systems, carbon dioxide transport, vitamin A utilization.
Selenium (Se)	Protects against a number of cancers, associated to proteins (selenoproteins) defending against oxidative stress, regulating thyroid hormone metabolism and the redox status of vitamin C.

From Nabrzyski (2007); Goldhaber (2003)

1.2.8. *Amino acids*

Of the over 300 amino acids described in the nature, only ten are considered essentials for human being (Massey et al. 1998). Amino acids are considered *essentials* since human metabolic pathways are insufficient to synthesize them at adequate rates from other precursors and they must be supplied in the diet (Table 9).

Table 9. Essential and non-essential amino acids in humans.

Essential amino acids	Non-essential amino acids
Methionine	Alanine
Threonine	Asparagine
Valine	Aspartic acid
Isoleucine	Cysteine
Phenylalanine	Glutamic acid
Tryptophan	Glutamine
Leucine	Glycine
Lysine	Histidine
Histidine	Proline
Arginine	Serine
	Tyrosine

However, arginine (Arg) and histidine (His) are essential only during periods of high anabolic activity, such as tissue growth during childhood (Massey et al. 1998). Broccoli is a rich source of AA and the concentration of that depends of the soil composition, cultivars, and growing seasons among others (Gomes & Rosa 2001).

1.2.8.1. Biological activity of amino acids

AA are associated with many biological functions which can be grouped in categories as is shown in Table 10. AA participate in gene expression, collagen structure and function, activation of enzymes, neurotransmitter, inhibites the production of inflammatory cytokines and superoxide, regulate the function of nervous system, and act as efficient antioxidants and detoxification agents, among other functions (Martínez-Tomé et al. 2001, Wu 2009). Moreover, many low-molecular-weight hormones are synthesized from specific AA. For instance, Tyr (or Phe) is the precursor for the synthesis of epinephrine, norepinephrine, dopamine, and thyroid hormones. L-arginine stimulate the secretion insuline, growth hormone, prolactine, glucagon, and progesterone (Wu 2009).

Table 10. Biological functions of amino acids

Biological function	Description	Amino acid associated
Synthesis and secretion of hormones	Precursors for the synthesis of epinephrine, norepinephrine, dopamine, and thyroid hormones.	Tyr, Phe, Arg, Leu
Nutrient metabolism and oxidative defense	Participate in cell signaling, cell-specific metabolism of nutrients, and efficiency of utilization of dietary protein.	Arg, Glu, Asp, Ala, Phe
Intracellular protein turnover	Balance between the synthesis and degradation of proteins in tissues	Leu, Arg
Immune function	Synthesis of cytotoxic products to pathogenic microorganisms and viruses.	Arg, Met, Cys, Trp, Pro
Reproduction	Important role in spermatogenesis.	Arg
Pathologies	Reduction of obesity, diabetes, and the metabolic syndrome.	Arg

From Wu (2009)

1.3. New broccoli-based products

In general, broccoli is consumed as whole processed vegetable; however, novel broccoli-based products have been proposed. For instance, byproducts of broccoli (leaves and stalks), a rich source of isothiocyanates, phenols, minerals and carotenoids, have been used as ingredients in novel functional foods. Dominguez-Perles et al. (2011) designed a novel beverage combining the health-promoting properties of organic green tea with broccoli byproducts to provide functional ingredients, thus adding value to food products and reducing agricultural wastes. Later, it was observed that broccoli added to green tea resulted in a beverage with potential antitumoral activity (Domínguez-Perles et al. 2012). Campas-Baypoli et al. (2009) obtained flours made with broccoli crop remains to be used as a source of nutrients, and as a dietary supplement. A blend of apple and broccoli juice was also proposed to increase the broccoli consumption (Houška et al. 2006). Novembrino et al. (2011) evaluated the influence of the encapsulated fruit and vegetable juice powder concentrates (containing broccoli juice) on oxidative status in heavy smokers, resulting in positive metabolic modifications.

1.3.1. Vegetable juices

Fruit and vegetable juices have been defined as those beverages that are composed exclusively of an aqueous liquid or liquids extracted from one or more fruits or vegetables with no added caloric sweeteners (Popkin et al. 2006).

Based on the pH value, vegetable juices can be classified in four categories:

1. Juices prepared from normally acidic produces (tomato, rhubarb).
2. Acidified vegetable juices. The major raw material is a low-acid or slightly acidic vegetable. The acidifying agent can be an organic acid (citric acid), or a fruit juice or another vegetable juice with higher acidity (citrus, pineapple, tomato juices).
3. Acidic juices obtained from fermented vegetables. Lactic bacteria are commonly involved in the fermentation process that reduces the pH.
4. Non-acidified low-acid vegetable juices, which must be processed at a relatively high temperature to kill bacterial spores (carrot, asparagus, broccoli juices) (Ahmed & Alam, 2011).

Although the fruit juices are the most consumed at worldwide, the number of commercially available vegetable juices are rising (Wootton-Beard et al. 2011). Indeed, vegetable juice is among the major processed vegetable products (Wu & Shen, 2011). Actually, many of these vegetable based beverages are available to the consumers in supermarkets, given the emergence of brands such as V8 (Campbell Foods, Belgium). Moreover, diverse studies have been carried out having vegetables as raw material. Ruxton et al. (2006) reviewed a wide number of studies associated to effect of nutritional characteristics of fruits and vegetables and their juices, concluding that the juices, in spite of being deficient in fiber, they also contribute to the prevention of the cancer and coronary heart disease prevention (Ruxton et al. 2006). In a study conducted by Shenoy et al. (2009), it was evaluated the suitability of vegetable juice to improve the vegetable intake andreduce the cardiovascular risk factors in adults, concluding that just 1-2 cups of vegetable juice/day could significantly reduce the blood pressure in hypertensive subjects. Furthermore, it has been observed that supplementation with mixed fruit and vegetable juice concentrates effectively increased plasma levels of important antioxidant nutrients and folate (Kiefer et al. 2004). Juices are an excellent source of nutrients and are considered a natural, healthy and suitable way to consume fruits and vegetables (Caswell 2009).

1.3.2. Broccoli juice

The commonly existing vegetable juices include tomato, *aloe vera,* carrot, beetroot, mixed vegetables and a great variety of fruit and vegetable juices; nonetheless, the interest in new and nutritious vegetables is increasing. For instance, broccoli juice is attracting the attention of both food manufacturers and scientist due to its nutritional

characteristics. In Thailand, a pasteurized broccoli juice is currently marketed (Tipco Foods Public Company Limited) and some studies has evaluated the health promoting effects of broccoli juice (Sun et al. 2007). Additionally, new broccoli juice-based products have been developed to increase its consumption. Houška et al. (2006) proposed a blend of broccoli and apple juices in different proportions to improve the acceptability by consumers and at the same time, preserve the content of bioactive compounds (sulforaphane) after processing.

1.4. Relevant enzymes in broccoli juice processing

Broccoli is characterized by its elevated content of vitamins, minerals, and amino acids content. Also, it is a good source of health-promoting compounds, such as polyphenols, chlorophylls, carotenoids and glucosinolates (Moreno et al., 2006). Many of these compounds are susceptible to the activity of deteriorative enzymes, such as polyphenol oxidase, lipoxygenase, among others, which may impact adversely on the antioxidant and sensorial status of broccoli (Table 11). Therefore, is important apply a technological processing to along the shelf-life of foods through inactivating enzymes.

Enzymes, such as polyphenol oxidase (PPO) or also known as tyrosinase, phenolase, catecholase, *o*-diphenol oxidase, monophenol oxidase, act on two general types of substrates: a) monohydroxyphenols (EC 1.14.18.1), and b) *o*-dihydroxyphenols (EC 1.10.3.1) (Ramírez et al., 2003). PPO is of great importance in determining the quality attributes of some fruits and vegetables its detrimental aspects caused by the browning of bruised and broken plants tissues are well known.

Other enzyme with relevant importance in food technology is the lipoxygenase (LOX). In the presence of molecular oxygen, LOX catalyzes the conversion of linoleic and linolenic acids into acids with a *cis,trans*-1,3-butadiene hydroperoxide system. Chls appears to be bleached by co-oxidation during this chain reaction. Studying the yellowing of vegetables, it was concluded that first the chloroplast lipids are hydrolyzed by the lipolytic acyl hydrolase liberating unsaturated fatty acids; then the LOX catalyzes the formation of fatty acids hydroperoxides, which cause the degradation of chlorophylls (Gross 1991).

Chlorophyllase (Chlase) (EC 3.1.1.14) is a thylakoid membrane glycoprotein that catalyzes hydrolysis of the phytol side chain of Chl (no change in color), although it appears that hydrolysis is followed by oxidative degradation of the tetrapyrrole involving a peroxidase (chlorophyll:H_2O_2oxidoreductase) (Sikorski & Haard 2007). Chlase catalyzes the hydrolysis of the ester linkage between the 7-propionic acid residue at ring D of the macrocyclic ring system and phytol, in chlorophylls and pheophytins (Gross 1991).

Table 11. Detrimental effects of enzymes in broccoli juice

Enzyme	Substrate	Adverse effects
Polyphenol oxidase	Phenols	Browning
Lipoxygenase	Fatty acids	Rancidity
Chlorophyllase	Chlorophylls	Colorless

1.5. Food preservation

The main purpose of food preservation is to maintain foods with the desired properties or nature for as long as possible (Shafiur-Rahman 1999). Among the techniques used for food preservation, heat treatment is still the most used process. Nowadays, the pasteurization is the most comun process for food preservation, which is based on the use of heat (From 60 to 140 ^{o}C) to extend the shelf-life through the inactivation of enzymes and elimination of microorganisms that might cause deterioration or endanger the consumer's health. The severity of the health treatment and the resulting extension of shelf-life are determined mostly by the pH of the food. In low-acid foods (pH >4.5) the main objective is the elimination of pathogenic bacteria; whereas below pH 4.5, destruction of spoilage microorganisms or enzymes inactivation is usually more important (Ahmed & Alam, 2011; Ho & Mittal 2000).

During the preservation of low-acid vegetable juice for long-term storage, high-temperature sterilization is often applied. Thermal processing commonly causes serious quality deteriorations such as loss of chlorophyll in green vegetable juices, off-flavor, off-taste, coagulation, floculation and precipitation.

Acidification may convert low-acid juice to an acidic juice and allow the use of milder sterilization conditions, and in many cases improve the quality of the product. Unfortunately, the reduction of pH may cause some detrimental slide effects, like the accelerated destruction of chlorophylls in green vegetable juices (Ahmed & Alam, 2011).

During juice elaboration the fruit and vegetable, the tissues have been destroyed; therefore, nutrients and deteriorative enzymes had enter into contact, being necessary to apply an effective preservation method in order to inactivate this enzymes.

1.5.1. Food preservation by high-intensity pulsed electric fields

1.5.1.1. Background

The principle of applying electric fields for food preservation is not new. At the end of the 19th appeared the first method using electricity to pasteurize milk, Electro-pure; however, it was based on the use of heat by ohmic resistance (220 – 4200 V) (Anderson & Finkelstein 1919, Toepfl et al. 2007).

Around 1949, Flaumenbaum reported the use of electric fields to increase the permeability of plant tissues to facilitate the subsequent extraction of juice, which is actually an important application of electric pulses (Toepfl et al. 2007). Gilliland and Speck (1967) introduced an electrohydrolytic method to eliminate microorganisms suspended in liquid systems, and during the same period, Sale and Hamilton (1967) and Sale and Hamilton (1968) evaluated the bactericidal effects of electric fields on microorganisms.

In the 1980s, several research groups developed studies on the use of electric pulses as a novel food preservation technology (Grahl & Märkl 1996, Mizuno & Hori 1988, Palaniappan et al. 1990).

1.5.1.2. HIPEF technology

The objective of HIPEF treatment is to ensure the food safety and extend storage life, to maintain "fresh-like" sensorial and nutritional quality operating at moderate temperature. HIPEF technology comprises the application of high-voltage energy (10-80 kV/cm) in form of short pulses (<10 μs) to a food placed between two electrodes (Barsotti et al. 1999, Olajide et al. 2006).

An electrical pulse consists of a burst of electrical energy. The great electric field intensities are achieved through storing a large amount of energy in a capacitor from a direct current (DC) power supply, which is then discharged in the form of high-voltage pulses.

1.5.1.3. HIPEF components

The major components of the HIPEF system include:

A). High voltage power supply

It supplies electrical energy at the selected voltage. It can either be of direct current (DC) or alternating current (AC) (Barbosa-Cánovas & Altunakar 2006, Mittal & Griffiths 2005).

B). Energy storage capacitor

Power supply is temporarily stored in the capacitor and is discharged through the food material to generate the necessary electrical fields in the food. Energy stored can be discharged almost instantaneously (in a millionth of second) at very high levels of power. The energy stored in the capacitor is given by

$$Q = 0.5\, C_0 V^2 \qquad \text{Eq. 2}$$

where V is the charging voltage and C_0 is defined as capacitance of the energy store capacitor, which is defined as

$$C_0 = \frac{t}{R} = t\sigma\frac{A}{d} \quad \text{Eq. 3}$$

where *t* is the pulse duration, *R* is the resistance, σ is the conductivity of the food, *d* is the gap between the electrodes, and *A* the area of electrode surface (Rastogi 2003).

C). Discharge switch

The discharge switch delivers the energy to the electrodes and to the sample. The switch must resist the maximum voltage across the capacitors (Barsotti et al. 1999, Mittal & Griffiths 2005).

D). Treatment chamber

Treatment chambers can be either batch-type or continuous. Consist of two parallel electrodes made generally of stainless steel encased in an insulating material. The basic function of the treatment chamber is to contain the food while the electric fields are applied (Barsotti et al. 1999, Mittal & Griffiths 2005, Zhang et al. 1995).

1.5.2. Critical process factors during HIPEF processing

1.5.2.1. HIPEF system variables

A). Electric field intensity

Liquid foods are considered electrical conductors because they contain large concentrations of ions as electrical charge carriers. To generate a high-intensity pulse electric field within a food, a large flux of current must flow in a very short period of time, because the time between pulses is much longer than the pulse width, the generation of pulses involves slow charging and fats discharging of the capacitor (Barbosa-Cánovas et al. 1999a). In addition, to achieve a maximum electric field strength, for a given energy input and treatment time, the energy requirement is inversely proportional to the electrical conductivity of the liquid food (Góngora-Nieto et al. 2002, Zhang et al. 1995).

When an electric field is generated between two parallel-plate electrodes, the electric field strength, is given by

$$E = \frac{V}{d} \quad \text{Eq. 4}$$

where V is the voltage (kV) and d (m) the gap between the electrodes.

B). Pulse width

Commonly, pulse width (τ) is fixed and its value is in function of both the system circuitry and the resistivity of the material being treated. For longer treatment times, the number of pulses (*n*) must increase (Mittal & Griffiths 2005).

C). Treatment time

The treatment time is the length of time the liquid food is exposed to HIPEF, which is defined by:

$$t = n \cdot \tau \qquad \text{Eq. 5}$$

where *n* is the number of pulses and τ is the pulse width (Mittal & Griffiths 2005).

D). Pulse wave shape

Electrochemical reactions and the formation of deposits at the electrodes occur during HIPEF processing. It is reduced through the use of alternating polarity and very short duration pulses (Mittal & Griffiths 2005, Morren et al. 2003). Bipolar pulses have simultaneous positive and negative amplitude per pulse, which were reported to reduce the amount of deposits on the electrode surfaces (Evrendilek et al. 1999).

In electric pulses with the same energy, the exponential waves would have a higher peak and greater width, whereas square waves the electric field strength is maintained over the pulse width to effect cell destruction (Mittal & Griffiths 2005).

The lethal effects of polarity are associated to the increased pulse period (Mittal & Griffiths 2005).

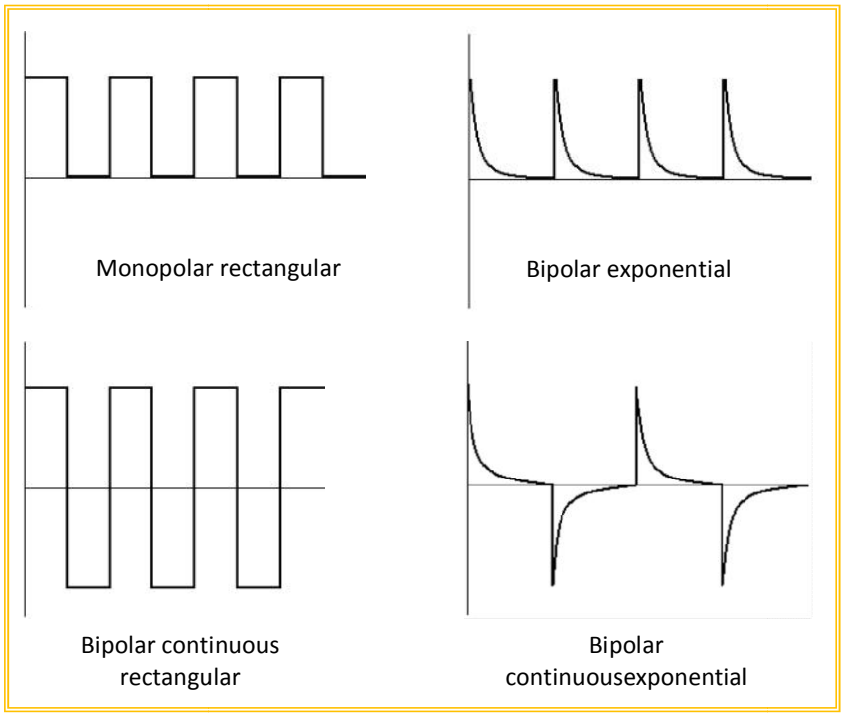

Figure 7. The commonly used wave forms for HIPEF

Electric field pulses may be applied in the form of exponentially decaying, square-wave, oscillatory, bipolar, or instant-reverse charges (Fig. 7). Oscillatory pulses are the least efficient for microbial inactivation, and square-wave pulses are more energy and lethally efficient than exponentially decaying pulses (Barbosa-Cánovas et al., 1999). In

terms of pulse polarity, these pulses can be applied in bipolar or monopolar mode (Alvarez et al., 2006).

E). Treatment temperature

Increments in temperature could occur either as consequence of the heat generated during HIPEF processing or in combination with HIPEF. Generally, the combination of HIPEF and temperature is designated as the influence of the inlet temperature of the treatment product on the HIPEF lethality (Mittal & Griffiths 2005). A significant synergic effect on microorganisms (Bazhal et al. 2006, Pothakamury et al. 1996) and enzymes (Min et al. 2003, Yang et al. 2004) was observed. The benefits of this synergistic lethal effect is the chance of reduce the intensity of HIPEF treatment and save energy (Heinz et al. 2003). However, the combined effect of HIPEF-heat could result in very high final temperature, due to ohmic heating, which may modify the nutritional and/or sensorial food characteristics.

1.5.3. Medium Characteristics

A). Conductivity

The conductivity (σ) is defined as the ability of a food to conduct the electric current. Conductivity is inverse of resistivity (Barbosa-Cánovas et al. 1999b, Keener 2007, Rastogi 2003) and it is expressed in siemens per meter (S/m), according to the International System of Units (SI). The conductivity of certains foods are shown in Table 12.

Conductivity is a function of medium temperature. In fact, it increases with a corresponding rise of temperature (Keener 2007, Quitão-Teixeira et al. 2008).

Liquid food materials are usually considered electrical conductors because they contain large concentration of ions as electrical charge carriers. The ionic strength depends of the amount of positive and negative ions presents in the liquid food, which is related to high conductivity or low resistivity. The presence of ions enhance the strength of HIPEF treatments; however foods with large concentrations of ions may hinder the process due they generate peak electric fields across the treatment chamber (Barbosa-Cánovas et al. 1999b, Mittal & Griffiths 2005).

The relation between inactivation rate and medium conductivity is not clear. Medium conductivity did not affect the polyphenol oxidase and peroxidase activity (Van Loey et al. 2001). In contrast, Giner et al. (2001) observed that the higher medium conductivity led to lower polyphenol oxidase inactivation.

Table 12. Electrical conductivity of various liquids

Sample	Conductivity (S/m)	Testing temperature (°C)
Drinking water[i]	0.005 – 0.05	-
Sea water[i]	5	-
Orange juice[b,h]	0.333 – 0.369	22 °C
Apple juice[b,h]	0.219 – 0.259	22 °C
Grape juice[f]	0.21	20 °C
Pear juice[h]	0.485	22 °C
Carrot juice[j]	0.972	25 °C
Melon juice[g]	0.523	-
Strawberry juice[c,h]	0.326 - 0.53	22 °C
Watermelon juice[d]	0.336	-
Broccoli juice	0.9	22 °C
Tomato juice[c,h]	0.430 - 0.65	-
Milk (raw)[b]	0.385 - 0.455	15 - 25 °C
Whole milk[e]	0.55	25 °C
1.5% fat milk[e]	0.585	25 °C
Skim milk[b,e]	0.603	25 °C
Liquid egg[i]	0.59	21°C

[From [a]Keener (2007); [b]Barbosa-Cánovas et al. (1999b); [c,d]Aguiló-Aguayo et al. (2009), (2010); [e]Sobrino-López et al. (2006); [f]Marsellés-Fontanet et al. (2013); [g,h]Mosqueda-Melgar et al. (2007), (2012); [i]Olajide et al. (2006), [j]Quitão-Teixeira et al. (2008)]

B). Uniformity of electric field

Air bubbles can be released due to temperature increase or electrochemical reactions (Toepfl et al. 2005). The presence of particles or air bubbles during HIPEF processing represent the nonuniform distribution of the applied electric field, as well as the dielectric breakdown, arcing or sparks (Barbosa-Cánovas et al. 1999b, Barsotti et al. 1999, Góngora-Nieto et al. 2003).

The electric field inside an air bubble can be more than 5 times that of the applied electric field. If the applied electric field surpasses the dielectric strength of the air bubbles, discharges inside the bubbles take place, volatizing the liquid and producing more vapour so that the bubbles grown even larger. When elongated under the applied electric field, the bubbles become big enough to bridge the gap between the two electrodes and a spark is produced (Barbosa-Cánovas et al. 1999a).

The electric breakdown is characterized by

- A large electric current in a narrow channel
- A bright luminous spark
- Evolution of bubbles
- Formation of pits on the electrodes
- Impulsive pressure through the liquid with an accompanying explosive sound

Dielectric breakdown and the presence of air bubbles can be minimized by vacuum degassing,

1.6. Effects of thermal and non-thermal food preservation on bioactive compounds in broccoli juice

1.6.1. *Effects on chlorophylls*

Fruit and vegetable quality and freshness are strongly associated with color. Color is an important quality attribute of vegetables, therefore this parameters will influence on their acceptance or reject of consumers. Chl pigments are unstable and may be used as indicator of health and ripeness of different plant material as well as of processing condition.

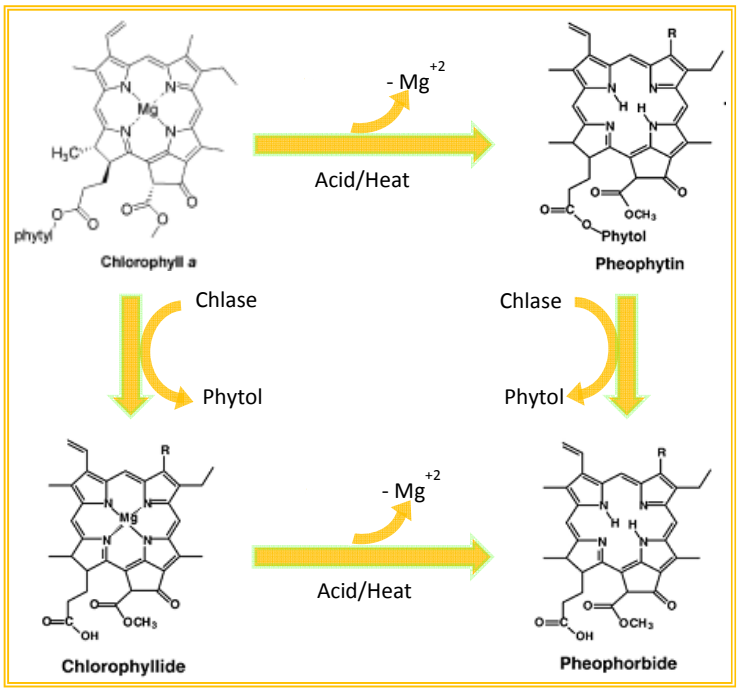

Figure 8. Mechanisms of chlorophyll degradation

Thermal treatment, the most common method for food processing, storage, among other factors, induces the Chl degradation. The participation of different oxidative enzymes, such as lipoxygenase and its isoenzymes has also implicated in Chl degradation (Sikorski & Haard 2007). Figure 8 describes the possible mechanisms for Chl decomposition.

The degradation pathway involves the chlorophyllase (EC 3.1.1.14) action on Chls causing the green chlorophyllide (Chlide) formation. Chlide is transformed in olive-brown pheophorbide (Phb) by the high temperature and acidic removal of Mg. Replacing Mg by Fe or Sn ions yields grayish-brown compounds, while Cu or Zn ions retain the green color. On the other hand, Chls are transformed to olive-brown pheophytin (Phe) by the elimination of Mg under heat or acidic conditions. The removal of Mg^{+2} may also be acomplished by the enzyme magnesium dechelatase (Heaton & Marangoni 1996). Afterwards, the enzymatic hydrolysis of Phe leads to Phb creation (Fig. 4). Table 3 describes the characteristics of Chls and their derivative compounds.

Chls and Phe are lipophilic due to the presence of the phytol group. While, the phytol chain elimination causes an increment in the polarity of the Chl molecule. This would result in a disruption of protein-lipid interactions, and possibly a release of Chl-protein complexes (Belitz et al. 2009a, Heaton & Marangoni 1996, Schwartz & Lorenzo 1990, Wilska-Jeszka 2007).

Murcia et al. (2001) evaluated the influence of different blanching times on broccoli Chl and its derivatives, comparing it with frozen and canned broccoli. They observed that reductions in the Chl content depended on thermal processing applied. Higher losses of Chl were observed when stems or florets were canned or frozen. Thus higher concentration of Phe was noted in canned broccoli. Similarly, Turkmen et al. (2005) reported that the retention of Chls depended on the vegetable type and cooking method (boiling, steaming or microwaving) and Phe increased in all vegetables after cooking. Moreover, Chl *a* was found more heat resistant compared with Chl *b* of squash, green beans, leak, spinach and broccoli.

Information related to the influence of non-thermal technologies on Chls is scarce. Nowadays, only the high hydrostatic pressure has been tested. In fact, in the literature the information available on this topic is referred to high hydrostatic pressure, but the effect of HIPEF processing on Chls have not been studied at this time. Stability of broccoli juice Chls under different pressure-temperature combinations were evaluated by Van Loey et al. (1998), reporting significant reductions in Chl content when pressure was combined with temperatures up to 50 °C. The degradation rates increased with temperature increments; whereas high pressure treatments at room temperature not caused significant changes neither Chl *a* nor Chl *b*. Chl *a* was more sensible to high pressure-temperature treatments than Chl *b*.

1.6.2. *Effects on carotenoids*

The stability of carotenoids is different for each food, even when the same processing conditions are applied. Indeed, carotenoids have different susceptibilities to

degradation, and optimum conditions during processing differ from one food to another (Rodriguez-Amaya 1997).

Thermal processing is reported to increase carotenoids concentration, perhaps owing to greater extractability, enzymatic degradation and unaccounted losses of moisture and soluble solids that concentrate the sample per unit weight (Rodriguez-Amaya 1997). The magnitude of these changes is in function of vegetable, temperature and time conditions of thermal treatment. The carotenoids degrade with longer time in processing and higher temperatures (Chandler & Schwartz 1988, Rodriguez-Amaya 1997).

Among the thermal processing methods assayed, microwave is less destructive than steaming and boiling (Rodríguez-Amaya 1997). In contrast, non-thermal technologies, such as high pressure and HIPEF have shown effective preservation of carotenoids. Plaza et al. (2011) reported a significant increment on total carotenoid in high pressure treated orange juice (49.19%), with respect to unprocessed juice; whereas no significant changes for juices processed by HIPEF and low pasteurization treatment (70 ºC for 30 s). In the same context, thermal and HIPEF processing enhanced some individual carotenoids and total carotenoids of tomato juice (Odriozola-Serrano et al. 2009). Furthermore, Odriozola-Serrano et al. (2007) reported increments in the content of lycopene between 1 and 46% in tomato processed by HIPEF at 35 kV/cm for 1000 µs, varying the frequency (50 – 250 Hz), pulse width (1 – 7 µs)and polarity (mono- or bipolar).

1.6.3. *Effects on vitamin C*

Vitamin C is used as an index of the nutritional value of foods, since, compared to other beneficial compounds, it is more sensitive to degradation by processing and storage. Vitamin C is thermosensitive and highly susceptible to oxidation in the presence of oxygen and metal ions (Mesías-García et al. 2010). The first change in vitamin C concentration take place prior to processing. That is, significant losses occur during any post-harvest storage period, particularly in over-ripe and damaged fruits and vegetables where enzyme Systems induce important oxidative changes (Davídek et al. 1990b, Gregory III 2000).

Ascorbate oxidase (EC 1.10.3.3) is the enzyme that catalyzes the oxidation of ascorbic acid to dehydroascorbic acid (Ball 2006). When the tissues are disrupted, the enzymes enter into contact with the vitamin C and begin to destroy it. This enzyme exhibits maximum activity at 40 ^{o}C and is almost completely inactivated at 65 ^{o}C; whereas maintaining cool conditions during transport and storage can markedly reduce vitamin C loss in some vegetables (Favell 1998).

The most relevant food preservation techniques are canning, freezing and dehydration for their availability for elimination of unwanted microorganisms and enzymes. However, these heat treatments also have consequences for labile nutrients such as vitamin C (Gregory III 2000).In contrast, non-thermal technologies such as HIPEF have shown a good retention of vitamin C in liquid foods. HIPEF-processed foods always have shown higher vitamin C retention than those treated by heat. Hence, during HIPEF processing, very low or negligible reductions in the vitamin C content of orange (Hodgins et al. 2002) and grape (Wu et al. 2005) juices were described. In the same way, Elez-

Martínez and Martín-Belloso (2007) reported that HIPEF-treated orange juice and "gazpacho", a cold vegetable soup, retained higher vitamin C (until 98.2 and 97.1%, respectively) than those treated by heat (82.4 and 79.2%). Similarly, tomato juice processed by HIPEF showed higher concentration of vitamin C than the thermally processed juice(Odriozola-Serrano et al. 2008). Odriozola-Serrano et al. (2007) reported that the vitamin C retention of HIPEF-treated tomato juice ranged between 58.2% and 99.0%; which depended on the treatment conditions.

1.6.4. Effects on polyphenols

Polyphenols are susceptible to undergo numerous desirable and undesirable enzymatic and chemical reactions during postharvest food storage and processing. Factors such as temperature, light, and humidity could cause some harmful reactions on the content of polyphenols (Gebczyński & Lisiewska 2006, Sikora et al. 2008). The influence of food processing on the content of polyphenols is in function of their concentration, chemical structure, oxidation state, localization in the cell and type of thermal treatment applied (van Boekel et al. 2010).

Some studies have reported reductions, increments or minor changes in the polyphenols content. The application of three different cooking method (boiling, steaming and frying) for carrots, zucchinis and broccoli led to significant losses of polyphenol compounds (Miglio et al. 2008). Significant losses of broccoli polyphenols after boiling and microwave in comparison to fresh broccoli was observed (Zhang & Hamauzu 2004). Similar results were reported by Ismail et al. (2004) for blanched and boiled vegetables.

On the contrary, Roy et al. (2009) reported an increment (6.39 and 18.19%) in the content of total polyphenols in steam-treated broccoli during 5 and 10 min. Increments of phenolic compounds between 66 and 94%, depending on the cooking method applied, was observed in artichoke heated by boiling, steaming, and frying. The increment in the content of phenolic compounds was justified by the isomerization and hydrolysis reactions that take place during heating process; leading to a re-distribution of phenolic acid concentrations (Ferracane et al. 2008).

The influence of non-thermal technologies, such as high-intensity pulsed electric fields has been tested on the content of polyphenols that currently occurs in fruit and vegetable juices.

Odriozola-Serrano et al. (2008) did not find significant differences in total phenolic content amongst fresh and HIPEF-processed tomato juices (35 kV/cm for 1500 μs in bipolar mode). Furthermore, HIPEF-treated tomato juice showed higher total phenolic compounds throughout the storage period than that treated at 90 ^{o}C for 60 s. Likewise, no significant differences in total phenolic compounds content between fresh and HIPEF treated (35 kV/cm for 1500 μs in bipolar mode) carrot juices were observed (Quitão-Teixeira et al. 2009).

1.6.5. *Effects on glucosinolates*

The nutritional quality of broccoli and their derivatives, such as broccoli juice depends not only on the nutrient content when the vegetable is harvested but also on the changes that take place during postharvest handling, storage conditions, processing and preparation (Howard et al. 1999).

Chewing, chopping, blending, and juicing are common steps in food preparation prior to food processing (industrial or domestic), which leads to cellular disruption and subsequent hydrolysis of glucosinolates (GLS) to form isothiocyanates (ITC) by the action of myrosinase enzyme (MYR). These processes affect the content of GLS and the flavour and aroma of the final product (Verkerk et al. 2009). Song and Thornalley (2007) reported that fine shredded *Brassica* vegetables reduced the content of GLS after 6 h of storage at ambient temperature. Losses up to 75% were observed in broccoli and cauliflower.

Thermal treatments have shown substantial losses in the content of GLS. Reductions between 50 and 74%of total GLS were observed in blanched in two cultivars of *Brassica* vegetables (Wennberg et al. 2006). Similarly, boiling of broccoli, cauliflower, Brussels sprouts and green cabbage for up to for 30 min led to reduction of GLS content between 58 and 77% (Song & Thornalley 2007). GLS and some of their hydrolysis compounds are water-soluble; therefore, the reductions in their content are due to leaching. In the same context, the amount of decreases is in function of the nature of GLS, the type of vegetable, and cooking conditions (Ciska & Kozłowska 2001, Oerlemans et al. 2006, Vallejo et al. 2002a).

Non-thermal technologies, such as high-hydrostatic pressure have been applied to Brassica vegetables, in order to evaluate the influence of this technology on GLS. Vallejo et al. (2002a) observed a reduction of 52% in GLS content when broccoli florets where high-pressure processed. Posteriorly, the pressure/temperature performance of broccoli juice ITC was tested; observing that during traditional thermal treatments ITC, such as sulforaphane was easily degraded. In contrast, a high-pressure treatment in combination with mild temperature was able to maintain the sulforaphane content in broccoli juice (Van Eylen et al. 2007).

1.6.6. Effects on minerals

The effect of food processing on minerals depends of the kind of food cooked, the time of thermal treatment, the stability of the element analyzed, the addition or not of water (López-Berenguer et al. 2007, Nabrzyski 2007).

Severi et al. (1998) tested the influence of cooking (100 ^{o}C during 10 min) on the retention of minerals in cauliflower. These authors observed losses that ranged between 8 and 37% in the minerals analyzed. Studies applying microwave treatments (700 W) revealed that different cooking conditions did not significantly affect the mineral content (Ca, Mg, Na, Mn) in broccoli inflorescences (López-Berenguer et al. 2007). Non-thermal technologies, such as HIPEF have been useful in the retention of nutrients of fruit juices. Akin and Evrendilek (2009) processed a carrot juice-based beverage by HIPEF, observing

that the HIPEF parameters electric field strength (from 13 to 27 kV/cm) and treatment time (from 82 to 262 μs) did not affected the mineral content. In contrast, there was an increment in the content of Cr, Zn, Fe and Mn when the samples of beer treated by HIPEF, which was associated with the metal ion migration from electrodes (Evrendilek et al. 2004).

1.6.7. Effects on amino acids

The most common food processing applied to vegetables is the blanching, which is carried out in water close to boiling point or in steam at 100 °C with the objective to inactivate enzymes, eliminate intercellular gases and reduce the number of bacteria before to dehydration or frozen (Murcia et al. 1999). The same group of authors evaluated the influence of food processing, including freezing (with a previous blanching at 94 °C from 60 to 150 s) and bottling treatment (121 °C for 30 min) on broccoli. These authors observed that the losses of total amino acids in broccoli florets decreased as the blanching time increased. Whereas, the blotted broccoli showed higher retention of amino acids than those frozen after being exposed to the longest blanching times (Murcia et al. 2001). In the same line, Garde-Cerdán et al. (2007) assessed the effect of thermal (90 °C during 1 min) and HIPEF processing (35 kV/cm for 1 ms, 4 μs and 1000 Hz) on grape juice; observing that both treatments did not influenced on the total concentration of amino acids. On the contrary, a significant increment in the total free amino acids was obtained when green tea infusion was HIPEF-processed with increasing electric field strength from 20to 40 kV/cm (Zhao et al. 2009).

1.7. **Effects of thermal and non-thermal food preservation on enzymes of relevant importance in broccoli juice**

By the protein nature of enzymes, they are highly sensitive to high temperatures. Indeed, it has been reported a total enzyme inactivation when heat has been applied. Marszałek et al., (2015) reported that polyphenol oxidase and peroxidase of strawberry puree were inactivated 98 and 100%, respectively, when strawberry puree was processed by conventional thermal processing (90 °C for 15 min).

The influence of nonthermal technologies, such as HIPEF on enzymes has been also tested. Several studies exist on the effects of HIPEF on enzymes suspended in aqueous solutions and in real foods like fruit juices and milk, which are products of great importance to the food industry. The studied enzymes have been pectin methyl esterase (PME), polygalacturonase (PG), polyphenol oxidase (PPO), peroxidase (POD), lipoxygenase (LOX), among others. Depending on the particular enzyme, the medium where it is suspended and the HIPEF treatment conditions, most enzymes are almost completely inactivated, while others show resistance to HIPEF processing. Electric field strength, treatment time, number of pulses, pulse width, field polarity, frequency and treatment temperature are HIPEF factors that have significant effects on enzyme inactivation.

The enzyme inactivation by HIPEF is higher when electric field strength and treatment time are increased (Elez-Martínez et al., 2006; Giner et al., 2000, 2002; Aguiló-Aguayo et al., 2010). The pulse polarity is another factor that has been demonstrated affect the enzyme inactivation. Different results have been reported depending on the enzyme. HIPEF treatments applied in bipolar mode led to greater polyphenol oxidase (PPO) and pectin methyl esterase (PME) inactivation than those applied in monopolar mode (Giner et al., 2002; Elez-Martínez et al., 2007), while monopolar pulses were more effective to inactivate peroxidase (POD) (Elez-Martínez et al., 2006a). On the other hand, Giner et al. (2000) did not observe any effect of pulse polarity on PME inactivation. Temperature during HIPEF processing is an important aspect to be considered in enzyme inactivation (Martín-Belloso and Elez-Martínez, 2005). The majority of the studies concluded that enzyme activity was diminished by increasing the temperature during PEF processing (Vega-Mercado et al., 1997; Van Loey et al., 2002; Yeom et al., 2002; Min et al., 2003a; Rodrigo et al., 2003; Yang et al., 2004a and b). Nevertheless, Castro et al. (2001) did not observe any effect of temperature on the inactivation of alkaline phosphatase. The type of enzyme, enzyme source and enzyme concentration have a significant effect on the level of enzyme inactivation by HIPEF processing. The sensitivity of enzymes to HIPEF treatment varies from enzyme to enzyme (Grahl and Märkl, 1996; Ho et al., 1997; Yang et al., 2004a). Giner et al. (2001) observed that PPO from pear was more resistant to PEF treatments than PPO from apple.

Variable enzyme inactivation has been reported, which was ranged from total inactivation to enzyme activation (Van Loey et al., 2002; Ho et al., 1997). The precise mechanism of enzyme inactivation by HIPEF is still unclear. Nonetheless, an explanation of effects of HIPEF on enzymes has been based on enzyme structure. In fact, Yeom, Zhang, and Dunne (1999) found the a-helix of secondary structure of HIPEF-treated papain was decomposed. Zhong, Hu, Zhao, Chen, and Liao (2005) and Zhong et al. (2007) reported the inactivation of HIPEF-treated horseradish peroxidase, peroxidase and polyphenol oxidase was related to the loss of a-helix of secondary structure. Yang et al. (2004a) reported the inactivation of pepsin by HIPEF was correlated with the alteration of the secondary structure (b-sheet dominant structure) of pepsin.

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II OBJECTIVES

GENERAL OBJECTIVE

Evaluate the influence of high-intensity pulsed electric field parameters on bioactive compounds stability and enzymes of broccoli juice

SPECIFIC OBJECTIVES

- Study the influence of high-intensity pulsed electric field parameters (electric field strength, treatment time and polarity) on antioxidant compounds
- Analyze the effect of high-intensity pulsed electric field parameters on degradative enzymes of broccoli juice
- Evaluate the effect of HIPEF or heat processing on color of broccoli juice
- Compare the influence of processing by HIPEF or heat on bioactive compounds, degradative enzymes and color of broccoli juice

III MATERIALS & METHODS

3.1. Juice extraction

Broccoli (*Brassica oleraceae* var. italica) was purchased at commercial maturity in a local grocery (Lleida, Spain). The broccoli florets were separated from the main stem and cut into pieces. The juice was obtained with a juice extractor. The resulting juice was filtered through cheesecloth and vacuum-degassed during 10 min. to avoid the presence of bubbles, after being filtrated with cheesecloth. Afterwards, broccoli juice was divided in three batches; one for HIPEF processing, the second for thermal treatment and the last was maintained unprocessed (Fig. III.1).

Figure III.1. Extraction and processing of broccoli juice

3.2. Broccoli juice treatment

3.2.1. HIPEF processing

HIPEF treatments were carried out using a continuous-flow bench-scale system (OSU-4F, Ohio State University, Columbus, OH) that held monopolar or bipolar square wave pulses. The treatment system consisted of eight co-field flow chambers in series, each one containing two stainless steel electrodes separated by a gap of 2.92 mm. Temperatures of inlet and outlet of each pair of chambers were monitored during HIPEF treatment and never exceeded 35 °C. The temperature was maintained by using a cooling coil connected between each pair of chambers and submerged in an ice-water shaking

bath. The treatment flow was controlled by a variable-speed pump (model 75210-25, Cole Parmer Instruments Company, Vernon Hills, IL, USA).

3.2.2. Thermal processing

Broccoli juice was thermally treated through a continuous system using a tubular heat exchange coil immersed in a hot water bath (University of Lleida, Spain) at 90 °C for 60 s. The juice was immediately cooled in a heat exchange coil immersed in an ice water-bath.

3.3. Analytical determinations

3.3.1. Reagents

Magnesium carbonate hydroxide hydrate, metaphosphoric acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), phenylmethylsulfonyl fluoride (PMSF), cysteine, linoleic acid, chlorogenic acid, Tween 20, ethyl acetate, Triton X-100, hydrochloric acid, acetic acid, and stock solutions of lutein, β-carotene, chlorophyll *a* and *b*, norvaline, and sarcosine were purchased from Aldrich Chemical Co. (St. Louis, MO, USA); sodium sulfate anhydrous, Na_2HPO_4, $NaHPO_4$, ascorbic acid, Folin-Ciocalteu reagent, gallic acid and diethyl ether were supplied by Scharlau Chemie S.A. (Barcelona, Spain). Methanol, propanone and sodium hydroxide were from Teknokroma (Barcelona, Spain), and polyvinylpolypyrrolidone (PVPP), was obtained from Acros Organics (Fair Lawn, NJ, USA).

3.3.2. Characterization of broccoli juice

The fresh squeezed broccoli juice was characterized. The physicochemical properties such as color (Minolta CR-400 colorimeter, Konica Minolta Sensing, Inc., Osaka, Japan), pH (Crison 2001 pH-meter; Crison Instruments SA, Alella, Barcelona, Spain), soluble solids (Atago RX-1000 refractometer; Atago Co. Ltd., Japan) and electrical conductivity (Testo GmBh & Co., Lenzkirch, Germany) were determined.

3.3.3. Chlorophylls and carotenoids analysis

Extraction

The extraction procedure was based on the method described by Cano (1991) with some modifications. A portion of 20 mL of broccoli juice was added to 20 mL of chilled propanone and then magnesium carbonate (1 g) and sodium sulphate (10 g) were poured. This mixture was vigorously homogenized and filtered. The residues were washed with

chilled propanone until colorless. The filtrate was reduced until 5 mL by rotoevaporation, and then it was transferred to a separatory funnel where diethyl ether (30 mL) and saturated sodium solution (20 mL) were added. This operation was repeated three times, and then the mixture was vigorously shaken. The organic phase was dehydrated with analytical grade sodium sulphate anhydride and the diethyl ether was eliminated by rotoevaporation. A portion of 4 mL of propanone was added to reconstitute the sample containing the Chls and carotenoids (Chls-carotenoids extract), for its subsequent chromatographic analysis.

An aliquot of 25 µL of the carotenoids extract was injected into the HPLC equipment using a reverse-phase C18 Spherisorb® ODS2 (5 µm) stainless steel column (4.6 x 250 mm). A gradient elution was composed of methanol/water (75:25), as eluent A, methanol (100%), as eluent B (cleaning solution), and ethyl acetate, eluent C (Table 1). The flow rate was fixed at 1.0 mL/min and the column temperature was maintained at 30 °C. The 2996 Waters Photodiode Array Detector, (Milford, MA) was adjusted at 440 nm. Carotenoids and Chls were quantified by comparison with external standards and expressed as mg of Chl *a*, Chl *b*, lutein or β-carotene per 100 mL of broccoli juice. Pheophytin (Phe) was preparated by acidification with hydrochloric acid (1 N) of propanone solution of the respective standard solution. Chlorophyllide (Chlide) and pheophorbide (Phb) were identified according to their chromatographic characteristics and absorption spectra.

Table 1. Mobil phase gradient for determination of carotenoids by HPLC

Time (min)	Flow (mL/min)	A (%)	B (%)	C (%)
0	1	80	0	20
5	1	77.5	0	22.5
22.5	1	50	0	50
27.5	1	50	0	50
30	1	20	0	80
35	1	20	0	80
40	1	0	0	100
45	1	0	0	100
50	1	0	100	0
70	1	0	100	0
71	1	80	0	20
80	1	80	0	20

A: Methanol/Water (75:25). B: Methanol (100%). C: Ethyl acetate (100%).

3.3.4. Vitamin C

Vitamin C content of broccoli juice was analyzed by HPLC. The extraction procedure was based on a method validated by Odriozola-Serrano et al. (2007). Briefly, broccoli juice was mixed with 4.5% metaphosphoric solution in a proportion 1:1. The mixture was homogenized and centrifuged at 4000 rpm for 10 min at 4 °C (Centrifuge AVANTI^{TM} J-25, Beckman Instruments Inc., Fullerton, CA, USA). Then, the vacuum-filtered sample was passed through a Millipore 0.45 µm membrane to be injected in the HPLC system.

An aliquot of 20 µL of extract was injected into the HPLC system using a reverse-phase C18 Spherisorb ODS2 (5 µm) stainless-steel column (4.6 mm x 250 cm). The mobile phase was a solution of acidified water adjusted to pH = 2.6 with sulphuric acid (1 mL/L). The flow rate was 1 mL/min at room temperature. Detection was performed with a 486 Absorbance Detector (Waters, Milford, MA) set at 245 nm. Identification of the ascorbic acid was carried out comparing the retention time and UV–visible absorption spectrum of the juice samples with those of the standards (ascorbic acid). The results were expressed as mg of ascorbic acid per 100 mL of juice.

3.3.5. Total polyphenols

Total polyphenols (TP) were determined by the colorimetric method described by Singleton et al. (1998) using the Folin-Ciocalteu reagent. A portion of 0.5 mL of broccoli juice was mixed with 0.5 mL of Folin-Ciocalteu reagent and 10 mL of saturated Na_2CO_3 solution. Samples were kept at room temperature in darkness for 1 h. Afterwards, the absorbance was measured at 725 nm using a CECIL 2021 spectrophotometer (Cecil Instruments Ltd., Cambridge, UK). Concentrations were determined by comparing the absorbance of the samples with a calibration curve built with galic acid. Results were expressed as mg of galic acid per 100 mL of juice.

3.3.6. Antioxidant capacity

Antioxidant capacity (AC) was determined through the evaluation of free radical scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH) following the method described by Odriozola-Serrano et al. (2007). Broccoli juice (0.01 mL) was mixed with 3.9 mL of methanolic DPPH (0.025 g/L) and 0.090 mL of distilled water. The homogenate was shaken vigorously and kept in darkness for 30 min. Absorption of the samples was measured using a CECIL 2021 spectrophotometer (Cecil Instruments Ltd., Cambridge, UK) at 515 nm against a blank of methanol without DPPH. Antioxidant capacity was calculated from the DPPH inhibition values of broccoli juice related to the initial absorbance of the methanolic DPPH solution.

3.3.7. Minerals

Mineral profile in broccoli juice was measured following the procedure proposed by Alwakeel and Al-Humaidi (2008) with some modifications. Broccoli juice (20 mL) was mixed with 10 mL of HCl in a volumetric flask and made up to 100 mL with water. The mixture was vigorously shaken, transferred to centrifuge tubes, centrifuged to remove solid particles and then analyzed by atomic absorption using ICP-OES Optical Emission Spectrometer, Horiba JobinYvon, Activa (USA). Ca, Cu, Fe, K, Mg, Mn, P, S, Zn, Na and Se were determined at wavelengths of 210.3, 324.8, 259.9, 766.5, 333.2, 257.6, 213.6, 180.7, 213.9, 589.6 and 196.0 nm, respectively. Quantification of the different elements was done using the calibration curves of the respective standard solution for each mineral.

3.3.8. Amino acids

Analysis of amino acids was performed by the method described by López et al. (2012) with some modifications. Free amino acids were analysed by reverse phase HPLC using a Hewlett Packard Series 1100 liquid chromatograph equipped with an ALS automatic liquid sampler (Hewlett Packard 1100 Series), an Agilent 1100 fluorometric detector (FLD) and a Hewlett Packard UV-DAD 1100 Series detector (DAD). 5 mL of sample (previously centrifuged, 4000 rpm, 10 min) was mixed with 100 µL of norvaline (internal standard to quantify all amino acids except proline) and 100 µL of sarcosine (internal standard to quantify proline). The mixture was filtered through a 0.45 µm OlimPeak pore filter (Teknokroma, Barcelona, Spain) and submitted to an automatic precolumn derivatization with *o*-phthaldialdehyde (OPA Reagent, Agilent Technologies, Palo Alto, CA) for primary amino acids and with 9-fluorenylmethylchloroformate (FMOC Reagent, Agilent Technologies) for secondary amino acids. The injected amount from the derivated sample was 10 µL and a constant temperature of 40 °C was maintained. All separations were performed on a Hypersil ODS (250 x 4.0 mm, I.D. 5 µm) column (Agilent Technologies). Solvents and gradient conditions for amino acids analysis are described below.

Two eluents were used as mobile phases: eluent A: 75 mM sodium acetate, 0.018% triethylamine (pH 6.9) + 0.3% tetrahydrofuran; eluent B: water, methanol, and acetonitrile (10:45:45, v/v/v). All reagents were first filtered with Millipore filters (0.45µm). The gradient profile was: 0−15 min, 0%-47.5% B, 1.630 mL/min; 15−15.01 min, 47.5% B, 0.800 mL/min; 15.01−25 min, 47.5%-60% B, 0.800 mL/min; 25-25.01 min, 60% B, 1.630 mL/min; 25.01-26.01 min, 60%-100% B, 1.630 mL/min; 26.01-26.51 min, 100% B, 2.500 mL/min; 26.51-34.01 min, 100% B, 1.630 mL/min; 34.01-36.01 min, 100%-0% B, 1.630 mL/min. Detection was performed by fluorescence FLD detector (λ excitation = 340 nm, λ emission = 450 nm for primary amino acids, and λ excitation = 266 nm, λ emission = 305 nm for secondary amino acids) and DAD detector (λ= 338 nm for primary amino acids and λ= 262 nm for secondary amino acids). Identification of compounds was carried out by comparison of their retention times with those of pure reference standards. The pure reference compounds and internal standards were from Sigma (St. Louis, MO, USA).

Water was obtained from a Milli-Q purification System (Millipore, USA). Quantification of amino acids was performed with an internal standard method.

3.3.9. Expression of results

All the results of bioactive compounds analysed were expressed as relative content (RC) or relative antioxidant capacity (RAC) values with respect to untreated juice (Equation 3).

$$RC\ or\ RAC(\%) = \frac{C_t}{C_0} x100 \qquad \text{Eq. 3}$$

where C_t and C_0 were the concentration of the bioactive compounds, the DPPH inhibition for AC or the areas of Chlide, Phe and Phb of the treated and untreated broccoli juice, respectively.

3.4. Enzyme activity determination

3.4.1. Chlorophyllase (Chlase)

Chlase activity in broccoli juice was measured using the method described by Costa et al. (2006) with some modifications.

Enzyme extraction

The enzymatic extract for the determination of Chlase activity was obtained by homogenization of 10 mL of broccoli juice with 20 mL of extraction buffer, which contained 0.1M Na_2HPO_4, 0.1M NaH_2PO_4, 2mL/L Triton X-100, 30 g/L polyvinylpolypyrrolidone (PVPP), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mM cysteine, pH 6.0. The mixture was stirred for 1 hour at 4 °C in darkness and centrifuged at 9000*xg* for 15 min at 4 °C (Centrifuge AVANTI™ J-25, Beckman Instruments Inc., Fullerton, CA, USA). The resulting supernatant constituted the enzymatic extract.

Enzyme activity determination

The assay is based on the formation of chlorophyllide from chlorophyll degradation. Chlase activity was assayed mixing 2 mL of enzyme extract, 0.1M of sodium phosphate buffer (pH 7.0), 0.15% Triton X-100, 10 µM chlorophyll *a*, 16% (v/v) of propanone in a total volume of 13 mL. The mixture was incubated for 1 hour at 40 °C and 2 mL were taken every 15 min from 0 up to 60 min and poured into tubes with 5 mL of pre-cooled hexane-propanone (7:3 v/v) solution. Then it was vigorously shaken until the formation of an emulsion. Afterwards, the emulsion was centrifuged at 6000*xg* for 5 min at 4 °C. The supernatant was utilized to measure the Chlase activity. The progress of the Chlase activity was followed by measuring the absorbance at 663 nm using a spectrophotometer

(CECIL CE2021 Instruments Ltd, Cambridge, UK). One unit of Chlase activity was defined as a change in absorbance at 663 nm/min per mL of enzymatic extract.

3.4.2. Lipoxygenase (LOX)

LOX activity in broccoli juice was determined using the method described by Anese and Sovrano (2006) with some modifications by continuously monitoring the formation of conjugated dienes from linoleic acid. LOX was extracted by mixing 5 mL of the juice with 2 mL of sodium phosphate buffer (70.95 g/L, pH 6.5) and 0.5% Triton X-100 in a centrifuge tube. The homogenate was centrifuged for 15 min at 10000*g* at 4 °C (Centrifuge AVANTI™ J-25, Beckman Instruments Inc., Fullerton, CA, USA) and the pellet was discarded. The substrate consisted of 10 µL of linoleic acid, 4 mL of distilled water, 1 mL of 0.05 mol/L NaOH and 5 µL of Tween 20. The mixture was shaken and diluted to 25 mL with distilled water. Activity measurements were carried out at 25 °C. Each quartz cuvette contained 2.7 mL of 0.2 mol/L phosphate buffer (pH 6.5) and 40 µL of substrate. The reaction started by adding 100 µL of enzyme extract and the increase in absorbance was followed spectrophotometrically (Cecil Instruments Ltd, Cambridge, UK) at 234 nm for 3 min at 22 °C. LOX activity was calculated from the slope of the linear portion of the curve obtained. One unit of LOX activity was defined as a change in absorbance at 234 nm/min per mL of enzymatic extract.

3.4.3. Polyphenoloxidase (PPO)

PPO activity was assayed according to the method described by Sorensen et al., 1999. Enzyme extracts were obtained by homogenization of 5 mL broccoli juice with 50 mL 0.2 mol/L acetic acid. Then, the homogenate was centrifuged at 10000 rpm, 10 min (4 °C) (Centrifuge AVANTI™ J-25, Beckman Instruments Inc., Fullerton, CA, USA). The supernatant was filtered through a Whatman No 1 paper and the collected liquid was the enzyme extract. PPO activity was determined spectrophotometrically by placing 1 mL of 0.1 mM chlorogenic acid dissolved in 20 mM phosphate buffer (pH 7.0), 1.45 mL of 0.2 mM phosphate buffer (pH 7.0) and 200 µL of the enzymatic extract in a 1 cm path cuvette. Absorbance was read at 470 nm using a CECIL CE 2021 spectrophotometer (Cecil Instruments Ltd, Cambridge, UK). PPO activity was determined by measuring the initial rate of reaction, which was computed from the linear portion of the plotted curve. One unit of PPO activity was defined as a change in absorbance at 470 nm/min per mL of enzymatic extract.

3.4.4. Myrosinase (MYR)

The procedure to myrosinase extraction from broccoli juice was based on the method described by Van Eylen et al. (2007). Whereas the determination of broccoli juice

myrosinase activity was assayed according with the method proposed by Ludikhuyze, Ooms, Weemaes, and Hendrickx (1999) with some modifications.

Enzyme extraction

The juice was centrifuged at 17000g and 4 °C for 5 min (Centrifuge AVANTI™ J-25, Beckman Instruments Inc., Fullerton, CA, USA) to reduce the original glucose concentration present in the broccoli juice, which was too high to use this coupled enzymatic procedure directly. The resultant pellet was washed with 1 mL of phosphate buffer (pH 7.5, 50 mM), and the centrifugation procedure was applied a second time under the same conditions. The pellet was dissolved in 250 µL of phosphate buffer (pH 7.5, 50 mM).

Enzyme activity determination

This method is based on the NADPH formation from $NADP^+$ using as substrate the glucose formed during the myrosinase catalyzed hydrolysis of sinigrin. The amount of NADPH formed is stoichiometric to the amount of glucose.

A Boehringer Mannheim test kit for glucose determination was used. The reaction mixture consisted of 0.9 ml of a water solution containing 0.05 g/L $MgCl_2$ and 1 g/L ascorbic acid, 0.5 mL ATP/$NADP^+$ solution (test kit solution 1), 10 µl hexokinase/glucose-6-P-dehydrogenase (test kit solution 2) and 50 µL of sample containing myrosinase solution. After homogenization, 50 µL substrate (sinigrin, 0.3 g/mL, Sigma, St. Louis, MO) was added and the formation of NADPH was spectrophotometrically followed at 340 nm and 23 °C for 10 min. The enzyme activity was determined based on the slope of the initial linear part of the curve of absorbance versus reaction time. One unit of myrosinase was defined as the amount of enzyme that forms 1 µmol glucose per min at 23 °C and pH 7.6 when sinigrin is used as a substrate.

3.4.5. Expression of results

The relative residual activity of the enzymes, RA (%), was defined by Equation 4:

$$RA = \frac{A_t}{A_0} x100 \qquad \text{Eq. 4}$$

where, A_t and A_0 were the enzyme activity in treated and untreated broccoli juice, respectively.

3.5. Color measurement

The color of broccoli juice was measured using a Colorimeter (Minolta CR-400, Konica Minolta Sensing, Inc., Osaka, Japan) at room temperature. CIE *L**, a* and b* coordinates were determined. These values were then used to calculate the total color differences (*ΔE*) indicating the color variations in processed broccoli juice respect to the untreated juice through Equation. 1:

$$\Delta E = \sqrt{(L - L_0)^2 + (a - a_0)^2 + (b - b_0)^2} \quad \text{Eq. 1}$$

where a_0, b_0 and $L_0$are the CIE-Lab values of the untreated broccoli juice

3.6.1. Experimental design

A response surface methodology was used to evaluate the effect of the different HIPEF treatment variables on bioactive compounds (Chls, carotenoids, vitamin C, polyphenols), Chls degradation compounds (Chlide, Phe, and Phb content) and enzyme activities (Chlase, PPO, LOX and MYR). A central composite design with three faced centred factors was proposed. Numerical variables were: treatment time (500, 1250 and 2000 μs), and electric field strength (15, 25 and 35 kV/cm), while a categorical variable was pulse polarity (monopolar or bipolar), keeping the pulse width (4 μs) and frequency (100 Hz) constant.

The experiment design was conducted in duplicate and every analytical analysis was carried out in triplicate. The order of assays was randomized. Experimental data were fitted to a polynomial response surface. The second order response function was predicted by Equation 2:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X^2{}_{i_i} + \sum_{i=1}^{2} \sum_{i=i+1}^{3} \beta_{ij} X_i X_j \quad \text{Eq. 2}$$

were Y is the response, β_0, β_i, β_{ii}, and β_{ij}, are the constant, linear, quadratic and interaction regression coefficients, respectively. X_i represented the independent variables. Response surface methodology was employed for experimental design, data analysis, model building and plot generation using Design Expert 6.01 software (Stat Ease Inc., Minneapolis, Minn., USA).

7.2. Statistical analysis for minerals and amino acids

Each processing condition was assayed by duplicate and two replicate analyses were carried out in order to obtain the mean value for minerals and amino acids determination. Analysis of variance (ANOVA) and the least significance difference test (LSD) at the 5% significance level was performed for the determination of significance differences among HIPEF-processed, thermally-treated and fresh broccoli juice, using Statgraphics Plus v5.1 Windows package (Statistical Graphics Co., Rockville, MD, USA).

3.6.2. Validation and optimization of the second order models

A set of 52 experiments was carried out to validate the models. The correlation coefficients between the predicted and the experimental data were taken as indicator of the prediction accuracy.

An optimization in the range of the studied parameters was carried out according to the method described by Derringer and Suich (1980). The highest desirability represented the most adequate condition to reach the highest levels of bioactive compounds and the lowest enzyme residual activity. Heat-processed, HIPEF-treated under optimized conditions and untreated broccoli juices were compared in order to determine differences amongst treatments, using an one-way ANOVA followed by least significant difference (LSD)-test.

3.6.3. Correlation between bioactive compounds

Correlations between bioactive compounds, enzymes and color were evaluated with Pearson's test. r value indicated the relatively strong relationship between the response variables. These analyses were carried out using the Statgraphics Plus v5.1 Windows package (Statistical Graphics Co., Rockville, MD, USA).

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IV PUBLICATIONS

1 EFFECTS OF HIGH-INTENSITY PULSED ELECTRIC FIELDS PROCESSING PARAMETERS ON THE CHLOROPHYLL CONTENT AND ITS DEGRADATION COMPOUNDS IN BROCCOLI JUICE

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ABSTRACT

The influence of high-intensity pulsed electric fields (HIPEF) parameters including electric field strength (15 – 35 kV/cm), treatment time (500–2000 μs), and polarity (monopolar or bipolar mode) on the content of chlorophylls (Chls), pheophytin (Phe), chlorophyllide (Chlide), and pheophorbide (Phb), and chlorophyllase activity (Chlase) in broccoli juice were assessed. A significant effect of HIPEF parameters on Chlase, Chls and Chls degradation compounds was observed through a response surface methodology design. However, polarity did not exert influence neither on Chl *a* nor on Chl *b*. The optimum HIPEF treatment was found to be 35 kV/cm for 1980 μs in bipolar mode, where the highest content of Chls was kept, the lowest Chlase residual activity was reached, and the minimal quantities of Chls degradation compounds content were formed. Additionally, at these HIPEF conditions, broccoli juice exhibited greater content of Chls than thermally treated or untreated juice. These outcomes demonstrated that HIPEF processing could be a suitable technology to maintain the Chls content in broccoli juice.

Keywords: High-intensity pulsed electric fields; Broccoli juice; Chlorophyll; Chlorophyllase; Chlorophyll degradation compounds.

INTRODUCTION

Epidemiological studies have suggested that a high consumption of vegetables from the *Brassicaceae* family, such as broccoli, may protect human organism against chronic diseases (Jeffery & Araya 2009) because it is an important source of bioactive compounds, including vitamin C, phenolic compounds, glucosinolates, carotenoids and chlorophylls (Moreno et al. 2006).

Chlorophylls (Chls) are among the most abundant pigments in the nature and they are located in the thylakoid membrane of chloroplast (Mínguez-Mosquera et al. 2006). Chls (Chl *a* and Chl *b*) play an important role in the appearance and acceptability of green vegetables. However, factors such as acidic medium, light, free radicals, food processing, as well as chlorophyllase activity (Chlase) accelerate Chls destruction (Davídek et al. 1990).

The degradation of Chls involves the action of Chlase (EC 3.1.1.14) on Chls, causing the formation of chlorophyllide (Chlide). Subsequently, Chlide is transformed to pheophorbide (Phb) at high-temperature and acidic conditions. On the other hand, Chls are converted to pheophytin (Phe) by the magnesium ion removal of Chls under heat or acidic conditions. Afterwards, Phe is transformed to Phb by the action of Chlase (Drazkiewicz 1994, Heaton & Marangoni 1996, Schwartz & Lorenzo 1990).

Nowadays, broccoli juice is attracting the attention of both food manufacturers and scientist due to its nutritional characteristics. In fact, a pasteurized broccoli juice is now marketed in Thailand (Tipco Foods Public Company Limited). New broccoli juice-based products have been also developed to increase its consumption. For instance, Houška et al. (2006) proposed a blend of broccoli and apple juices in different proportions.

Additionally, scientific research concerning the sensorial characteristics of broccoli juice has been reported by Weemaes et al. (1999a), who observed no significant changes in color of broccoli juice treated by high pressure (800 MPa and 30-40 ºC), whereas notable color changes were observed with increasing the temperature (up to 70-80 °C). Loss of green color in broccoli juice due to the influence of heat has been also reported by Weemaes et al. (1999b).

Traditionally, fruit and vegetable juices have been thermally processed to ensure their microbiological and enzymatic stability. However, heat treatment has drawbacks, such as loss of flavor, pigments and nutrients, resulting in a reduction of food quality (Fennema 1985). Besides, it has been reported that prolonged heat treatments degrade Chls to Chlide, Phe and Phb (Canjura & Schwartz 1991, Schwartz & Lorenzo 1990). In the past few years, nonthermal preservation methods have been investigated as alternative to conventional processing. In this context, high-intensity pulsed electric fields (HIPEF) have been reported to maintain the nutritional and sensorial qualities as well as the microbial safety of liquid foods (Elez-Martínez et al. 2005, Mosqueda-Melgar et al. 2008, Ortega-Rivas 2007). Furthermore, HIPEF processing inactivates degradative enzymes (Aguiló-Aguayo et al. 2008b, Giner et al. 2001). In comparison to the wide range of research carried out on enzyme and microorganism inactivation by HIPEF, there are few studies related to the effect of HIPEF treatment on pigments and bioactive compounds of juices. In the same way, Odriozola-Serrano et al. (2007)reported an increment of 46% in the content of lycopene in tomato juice after applying HIPEF treatment. Similarly, a rise in

carotenoids concentration was noted in HIPEF-treated orange-carrot juice (Torregrosa et al. 2005). A raise in anthocyanin content (2.1%) was observed when strawberry juice was processed by HIPEF at 35 kV/cm (Odriozola-Serrano et al. 2009). Other nonthermal technologies, such as high pressure have been used for the processing of broccoli juice, with the objective of evaluating whether this technology improves the retention of bioactive compounds (Mandelová & Totusek 2007, Mandelová & Totušek 2006, Trska et al. 2007, Van Eylen et al. 2007, Van Loey et al. 1998) and sensorial characteristics (Weemaes et al. 1999a). However, to the best of our knowledge, there are no studies about the effect of HIPEF on Chls and Chlase activity in fruit and vegetable juices. Therefore, the aim of this research was to evaluate the influence of HIPEF parameters, such as electric field strength, treatment time, and polarity pulse on the relative content (RC) of Chls, Chlide, Phe, Phb and the residual activity (RA) of Chlase in broccoli juice. Moreover, the effects of HIPEF treatment were compared to those of traditional thermal processing.

MATERIALS AND METHODS

Reagents

Magnesium carbonate hydroxide hydrate, phenylmethylsulfonyl fluoride (PMSF), cysteine, ethyl acetate, Triton X-100, and stock solutions of chlorophyll *a* and *b* were purchased from Aldrich Chemical Co. (St. Louis, MO, USA); sodium sulfate anhydrous, Na_2HPO_4, $NaHPO_4$, and diethyl ether were supplied by Scharlau Chemie S.A. (Barcelona, Spain). Methanol was from Teknokroma (Barcelona, Spain), and polyvinylpolypyrrolidone (PVPP) was obtained from Acros Organics (Fair Lawn, NJ, USA).

Broccoli juice

Broccoli (*Brassica oleracea* var. *italica*) was purchased at commercial maturity in a grocery store at Lleida (Spain). Subsequently, broccoli was chopped and crushed. The resulting juice was filtered through cheesecloth and vacuum degassed for 10 min.

The physical characteristics of fresh squeezed broccoli juice were as follows: pH 6.49 ±0.11 (Crison 2001 pH-meter; Crison Instruments SA, Alella, Barcelona, Spain), soluble solids = 8.62 ±0.14 °Brix (Atago RX-1000 refractometer; Atago Co. Ltd., Japan) and color: L^*= 33.81 ±0.27, a^*= -8.61 ±0.20, of b^*= 9.84 ±0.32 (Minolta CR-400 colorimeter, Konica Minolta Sensing, Inc., Osaka, Japan).

HIPEF equipment

HIPEF treatments were carried out using a continuous-flow bench-scale system (OSU-4F, Ohio State University, Columbus, OH, USA) that held monopolar or bipolar square wave pulses. The treatment system consisted of eight co-field flow chambers in series, each one containing two stainless steel electrodes separated by a gap of 2.92 mm. The inlet and outlet temperatures of each pair of chambers were monitored during HIPEF treatment. The temperature was kept below 35 °C by using a cooling coil connected between each pair of chambers and submerged in an ice-water shaking bath. The treatment flow was controlled by a variable-speed pump (model 75210-25, Cole Parmer Instruments Company, Vernon Hills, IL, USA).

Thermal treatments

Broccoli juice was heat treated in a tubular stainless steel heat exchange coil immersed in a hot water shaking bath using a gear pump (Universitat de Lleida, Lleida, Spain) at 90 °C for 60 s. After thermal processing, the juice was immediately cooled in a heat exchange coil immersed in an ice water-bath. Thermal treatment equipment consists of a continuous system. It was designed to get comparable results with respect to the HIPEF continuous system. It was assumed that the temperature did not depend on treatment time, once the steady state was reached. Broccoli juice was passed through a tubular heat exchange coil (2mm internal diameter and 9.5 m of length) immersed in a hot water bath. Taking into account that the pipe wall area was very large in comparison with its thickness, it could be assumed that the temperature distribution will remain unchanged during the time of heat processing.

Chlorophyllase activity determination

Chlase activity in broccoli juice was measured using the method described by Costa et al. (2006), with some modifications.

Enzyme extraction

The enzymatic extract for the determination of Chlase activity was obtained by homogenization of 10 mL of broccoli juice with 20 mL of extraction buffer, which contained 0.1 M Na_2HPO_4, 0.1 M NaH_2PO_4, 2 mL/L Triton X-100, 30 g/L PVPP, 1 mM PMSF and 5 mM cysteine, pH 6.0. The mixture was stirred for 1 h at 4 °C in darkness and centrifuged at 9000*xg* for 15 min at 4 °C (Centrifuge AVANTI^{TM} J-25, Beckman Instruments Inc., Fullerton, CA, USA). The resulting supernatant constituted the enzymatic extract.

Chlorophyllase activity measurement

The assay is based on the formation of chlorophyllide from chlorophyll degradation. Chlase activity was assayed by mixing 2 mL of enzyme extract, 0.1 M of sodium phosphate buffer (pH 7.0), 0.15 % Triton X-100, 10 µM chlorophyll *a*, 16 % (*v*/*v*) of propanone in a total volume of 13 mL. The mixture was incubated for 1 h at 40 °C and 2 mL was taken every 15 min from 0 up to 60 min and poured into tubes with 5 mL of precooled hexane-propanone (7:3 *v*/*v*) solution. Then, it was vigorously shaken at 60 rpm for 1 h (Mini orbital shaker OVAN®, USA) until the formation of an emulsion. Afterwards, the emulsion was centrifuged at 6000*xg* for 5 min at 4 °C. The supernatant was utilized to measure the Chlase activity. The progress of the Chlase activity was followed by measuring the absorbance at 663 nm using a spectrophotometer (CECIL CE2021 Instruments Ltd., Cambridge, UK). One unit of Chlase activity was defined as a change in absorbance at 663 nm/min/mL enzymatic extract. The influence of stirring and shaking on Chlase activity could be considered negligible, taking into consideration the findings reported by Ganesh et al. (2000).

The RA of Chlase activity (%) was defined by Eq. 1:

$$RA = \frac{A_t}{A_0}\, x100 \qquad (1)$$

where A_t and A_0 were the Chlase activity in treated and untreated broccoli juice, respectively.

Determination of Chlorophylls

Chlorophylls Extraction

The extraction procedure was based on the method described by Cano (1991), with some modifications. A portion of 20 mL of broccoli juice was added to 20 mL of chilled propanone, and then magnesium carbonate and sodium sulphate were poured. This mixture was vigorously homogenized and filtered. The residues were washed with chilled propanone until colorless. The filtrate was reduced until 5 mL by rotoevaporation (pressure, < 70 mbar; temperature, 35 °C), and then it was transferred to a separatory funnel where diethyl ether and saturated sodium solution were added. This operation was repeated three times, and then the mixture was vigorously shaken at 1200 rpm for 1 min (Mini shaker IKA® Works Inc., Wilmington, NC, USA). The organic phase was dehydrated with analytical grade sodium sulphate anhydride, and diethyl ether was eliminated by rotoevaporation. A portion of 4 mL of propanone was added to reconstitute the sample containing Chls (Chls extract), for its subsequent chromatographic analysis.

Chromatographic procedure

An aliquot of 25 µL of the Chls extract was injected into the high-performance liquid chromatography (HPLC) equipment using a reverse-phase C18 Spherisorb® ODS2 (5 µm) stainless steel column (4.6x250 mm). A gradient elution was composed of methanol/water (75:25), as eluent A; methanol (100%) as cleaning solution, eluent B and ethyl acetate, as eluent C (Table 1). The flow rate was fixed at 1.0 mL/min and the column temperature was maintained at 30 °C. The 2996 Waters Photodiode Array Detector (Milford, MA, USA) was adjusted at 440 nm.

Table 1. Mobile phase gradient for the determination of chlorophylls and Chl degradation compounds by HPLC.

Time (min)	Flow (mL/min)	A (%)	B (%)	C (%)
0	1	80	0	20
5	1	77.5	0	22.5
22.5	1	50	0	50
27.5	1	50	0	50
30	1	20	0	80
35	1	20	0	80
40	1	0	0	100
45	1	0	0	100
50	1	0	100	0
70	1	0	100	0
71	1	80	0	20
80	1	80	0	20

A Methanol/Water (75:25), *B* methanol (100%), *C* ethyl acetate (100%).

Chl *a* and Chl *b* were quantified by comparison with external standards. Pheophytin (Phe) was prepared by acidification with hydrochloric acid (1 N) of propanone solution of the respective standard solution. Chlorophyllide (Chlide) and pheophorbide (Phb) were identified according to their chromatographic characteristics and absorption spectra. Results of Chls concentration and Chlide, Phe, and Phb chromatographic areas were expressed as RC values with respect to the untreated juice (Eq. 2):

$$RC\ (\%) = \frac{C_t}{C_0}\ x100 \qquad (2)$$

where C_t and C_0 were the concentration of Chl *a* and *b* or the areas of Chlide, Phe, and Phb of the treated and untreated broccoli juice, respectively.

Experimental design

A response surface methodology (RSM) was used to evaluate the effect of the different HIPEF treatment variables on Chls, Chlide, Phe, and Phb content and Chlase activity. A central composite design with three faced centred factors was proposed. Numerical variables were treatment time (500, 1250 and 2000 µs), and electric field strength (15, 25 and 35 kV/cm), while a categorical variable was pulse polarity (monopolar or bipolar), keeping the pulse width (4 µs) and frequency (100 Hz) constant. RSM was chosen to analyze the experimental results because it offers a large amount of information with less number of experiments, with respect to other traditional experimental designs, and it allows observing the interaction effect of the independent parameters on the response (Baş & Boyaci 2007).

The experiment design was conducted in duplicate and every analytical analysis was carried out in triplicate. The order of assays was randomized. Experimental data were fitted to a polynomial response surface. The second order response function was predicted by Eq. 3:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X^2{}_{i_i} + \sum_{i=1}^{2} \sum_{i=i+1}^{3} \beta_{ij} X_i X_j \qquad (3)$$

were Y is the response, β_0, β_i, β_{ii}, and β_{ij}, are the constant, linear, quadratic and interaction regression coefficients, respectively, and X_i represents the independent variables. RSM was employed for the experimental design, data analysis, model building and plot generation using Design Expert 6.01 software (Stat Ease Inc., Minneapolis, MN, USA).

Validation and optimization of the predictive models

A set of 52 experiments were carried out to validate the developed predictive models. The correlation coefficients between the predicted and the experimental data were taken as indicator of prediction accuracy.

An optimization in the range of the studied parameters was carried out according to the method described by Derringer and Suich (1980). The highest desirability represented the most adequate condition to reach the highest levels of Chls and the lowest Chlase RA. Heat-processed, HIPEF-treated broccoli juice under optimized conditions, and untreated broccoli juices were compared in order to determine differences amongst treatments, using an one-way analysis of variance (ANOVA) followed by least significant difference test.

Correlations between Chlase, Chls and Chl degradation compounds were evaluated with Pearson's test. The *r* value indicated the relatively strong relationship between the

response variables. These analyses were carried out using the Statgraphics Plus v5.1 Windows package (Statistical Graphics Co., Rockville, MD, USA).

RESULTS AND DISCUSSION

Effects of HIPEF parameters on chlorophyllase activity

Table 2. Central composite response surface methodology design for Chls, their degradation compounds, and Chlase activity on HIPEF-treated broccoli juice.

Variables						
Assay no[a]	Electric field strength (kV/cm)	Treatment time (µs)	Polarity	Chlase RA (%)[b]	Chl a	Chl b
					RC (%)[b]	
1	15	500	Monopolar	64.8 ±1.4	89.10 ± 2.9	74.67 ±1.6
2	35	500	Monopolar	54.5 ±2.5	105.54 ± 3.1	103.90 ±1.2
3	15	2000	Monopolar	49.3 ±1.7	89.55 ± 2.4	87.91 ±0.9
4	35	2000	Monopolar	40.4 ±3.3	110.29 ± 4.3	114.61 ±2.3
5	15	1250	Monopolar	57.1 ±2.6	95.15 ± 2.1	85.35 ±1.1
6	35	1250	Monopolar	33.5 ±1.8	109.20 ± 4.5	112.03 ±0.8
7	25	500	Monopolar	51.8 ±2.9	92.67 ± 3.7	95.72 ±2.1
8	25	2000	Monopolar	33.8 ±3.2	102.16 ± 5.4	108.00 ±1.7
9	25	1250	Monopolar	37.2 ±3.8[c]	100.39± 3.6[c]	103.72 ±2.4[c]
10	15	500	Bipolar	52.8 ±2.4	81.84 ± 2.8	79.29 ±2.1
11	35	500	Bipolar	43.5 ±2.9	110.58 ± 3.3	109.20 ±1.4
12	15	2000	Bipolar	62.6 ±1.9	83.31 ± 3.7	86.27 ±1.7
13	35	2000	Bipolar	26.3 ±2.1	116.01 ± 4.5	120.71 ±2.6
14	15	1250	Bipolar	51.0 ±3.2	86.66 ± 4.8	82.00 ±2.9
15	35	1250	Bipolar	30.9 ±2.8	112.10 ± 3.2	111.06 ±1.4
16	25	500	Bipolar	41.5 ±1.9	95.16 ± 2.9	98.15 ±2.3
17	25	2000	Bipolar	34.5 ±2.0	107.14 ± 4.2	106.17 ±2.1
18	25	1250	Bipolar	35.7 ±2.4[c]	105.01 ±2.8[c]	103.09 ±1.9[c]

Chlase exhibited an initial activity of 0.035 ΔAbs 663 nm/min/mL in broccoli juice. Chlase activity was in the range of that reported by Costa et al. (2006b). RA results of Chlase (%) in HIPEF-treated broccoli juice are shown in Table 2.

Table 2. Continued

Variables						
Assay no[a]	Electric field strength (kV/cm)	Treatment time (µs)	Polarity	Chlide	Phe	Phb
				RC (%)[b]		
1	15	500	Monopolar	138.2 ±4.2	126.0 ±6.0	132.0 ±5.1
2	35	500	Monopolar	135.4 ±5.0	119.5 ±5.3	128.3 ±5.5
3	15	2000	Monopolar	125.3 ±3.9	102.9 ±5.1	107.9 ±6.2
4	35	2000	Monopolar	122.7 ±4.7	95.7 ±4.8	98.8 ±6.1
5	15	1250	Monopolar	134.9 ±5.2	122.0 ±6.2	124.4 ±5.3
6	35	1250	Monopolar	130.1 ±3.6	111.9 ±5.6	121.9 ±4.8
7	25	500	Monopolar	137.0 ±4.8	122.1 ±4.3	129.3 ±6.7
8	25	2000	Monopolar	123.9 ±3.9	98.3 ±4.1	104.3 ±5.8
9	25	1250	Monopolar	132.3 ±4.1[c]	118.7 ±5.8[c]	122.5 ±6.6[c]
10	15	500	Bipolar	135.1 ±5.9	120.5 ±4.2	116.0 ±4.3
11	35	500	Bipolar	130.0 ±4.5	111.4 ±6.0	115.6 ±3.9
12	15	2000	Bipolar	118.1 ±3.7	98.2 ±5.5	100.7 ±4.2
13	35	2000	Bipolar	116.9 ±4.1	91.2 ±6.3	95.2 ±5.9
14	15	1250	Bipolar	127.8 ±5.9	110.3 ±5.7	113.0 ±4.7
15	35	1250	Bipolar	124.7 ±3.6	105.7 ±4.9	109.5 ±6.3
16	25	500	Bipolar	133.6 ±4.2	118.7 ±5.4	116.9 ±6.1
17	25	2000	Bipolar	117.8 ±5.1	96.3 ±6.1	98.4 ±6.8
18	25	1250	Bipolar	126.1 ±3.6[c]	108.0 ±4.8[c]	111.0 ±5.8[c]

RA = Residual activity (%). RC = Relative content (%). Chl a = Chlorophyll a; Chl b = Chlorophyll b; Chlase = Chlorophyllase; Chlide = Chlorophyllide; Phe = Pheophytin; Phb = Pheophorbide.
[a] Order of the assays was randomized.
[b] Values are expressed as mean ± SD of two treatment repetitions; each assay was performed by triplicate.
[c] Data shown are mean of the central points with five repetitions

When broccoli juice was processed by HIPEF, Chlase activity decreased within the range of electric field strength and treatment time assayed in monopolar or bipolar mode treatments. Minimal RA (26.3%) was observed when applying pulses at 35 kV/cm for 2000 µs in bipolar mode.

A second-order response surface function fitted properly the experimental data (p<0.0001) with a determination coefficient (R^2) of 0.90 and a nonsignificant lack of fit (Table 3). Owing to polarity, a categorical variable which significantly influenced the RA of Chlase (p< 0.05), the model that describes the RA of the enzyme exposed to HIPEF was expressed in two different polynomial equations for monopolar (Eq. 4) and bipolar (Eq. 5) modes:

$$RA_B = 118.18 - 4.05 * E - 0.02 * t - 4.3x10^{-4} * E * t + 0.08 * E^2 + 8.9x10^{-6} * t^2 \quad (4)$$

$$RA_M = 114.68 - 4.44 * E - 0.01 * t - 4.3x10^{-4} * E * t + 0.08 * E^2 + 8.9x10^{-6} * t^2 \quad (5)$$

where RA is the residual activity of Chlase enzyme in monopolar (M) or bipolar (B) mode, *E* is the electric field strength (kV/cm), and *t* the treatment time (μs).

Table 3. Analysis of variance of the second-order polynomial models for Chlase, Chl and degradation compounds.

	F value					
Source[a]	Chlase	Chl a	Chl b	Chlide	Phe	Phb
Model	18.58[d]	21.91[d]	102.14[d]	236.00[d]	55.76[d]	204.84[d]
E	61.86[d]	145.81[d]	669.84[d]	69.29[d]	32.88[d]	33.41[d]
t	20.18[c]	8.61[b]	85.10[d]	1307.58[d]	307.32[d]	968.10[d]
P	5.97[b]	1.74	0.56	466.71[d]	76.20[d]	490.38[d]
E^2	20.93[c]	1.11	41.53[b]	0.090	0.65	$1.2x10^{-3}$
t^2	8.80[b]	4.49[b]	1.38	24.18[d]	21.24[c]	89.13[d]
E x t	5.13[b]	0.78	0.13	4.89[b]	0.049	8.85[b]
E x P	2.77	9.72[b]	2.53	0.11	0.18	1.98
t x P	5.84[b]	0.13	2.04	9.73[b]	0.58	32.02[d]
Lack of fit	2.88	1.43	1.49	2.54	3.23	1.06
Desv. Std	3.98	3.30	1.96	0.67	2.23	1.23
Mean	42.01	100.52	100.35	128.60	110.91	114.60
C.V.	9.48	3.28	1.96	0.52	2.01	1.07
R^2	0.90	0.91	0.98	0.99	0.96	0.99
Adjusted R^2	0.85	0.87	0.97	0.98	0.95	0.98

[a] *E* = Field strength, *t* = Treatment time; *P* = Polarity. Chlide = Chlorophyllide; Phe = Pheophytin; Phb = Pheophorbide.
[b] Significant at $p< 0.05$
[c] Significant at $p< 0.001$
[d] Significant at $p< 0.0001$

HIPEF treatments applied in bipolar mode to broccoli juice led to lower Chlase residual activity than those operated in monopolar mode. In agreement with these results, Quitão-Teixeira et al. (2008) observed that the bipolar mode induced more carrot juice peroxidase inactivation than the monopolar mode after applying HIPEF treatments at 35 kV/cm for 1000 μs. Moreover, Giner et al. (2002) and Aguiló-Aguayo et al. (2010a)reported that pulses applied in bipolar mode caused a great inactivation of polyphenol oxidase enzyme from peach and strawberry juice, respectively. In the same way, the application of HIPEF in bipolar mode improved the orange juice pectin methyl esterase inactivation (Elez-Martínez et al. 2007b).

According to Eq. 4 and 5, treatment time is a determining factor in the broccoli Chlase inactivation. Bipolar pulses have simultaneous positive and negative amplitudes per pulse, which could cause changes in the direction of charged molecules (Barbosa-

Cánovas et al. 1999, Barsotti & Cheftel 1999). When the duration of the electric pulses is long enough, the influence of HIPEF could lead to conformational changes of the enzymes, resulting in its inactivation (Elez-Martínez et al. 2007a, Zhao et al. 2007, Zhong et al. 2005). Therefore, pulses of identical voltage for a given treatment time in bipolar mode inactivate Chlase more efficiently than those in monopolar mode. Also, the linear coefficient in both electric field strength and treatment time was negative, meaning that the higher electric field strength and treatment time, the lower the Chlase RA.

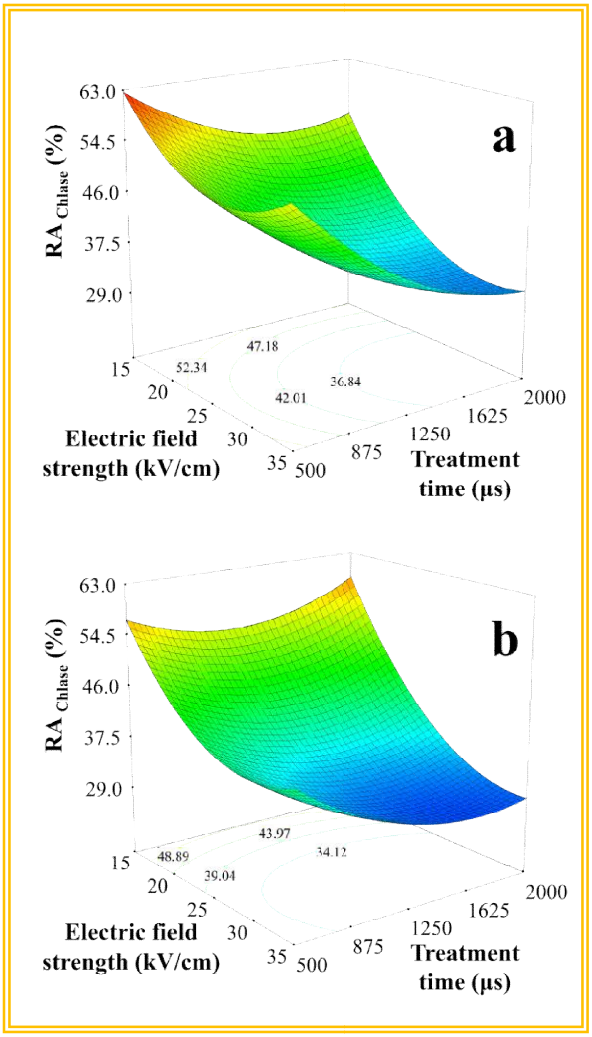

Figure 1. Effect of electric field strength and treatment time on chlorophyllase activity in monopolar (**a**) and bipolar (**b**) modes in broccoli juice

The difference in effectiveness attributed to polarity could be explained by the fact that a separation of particles with electric charge takes place when food is exposed to an electric field. These particles may cover the electrodes and lead to the formation of a shielding layer in their surface, which modifies the electric field and, therefore, reduces the efficiency of HIPEF treatment. Bipolar pulses minimize the deposit of charged

molecules, avoiding the creation of a shielding layer (Barbosa-Cánovas et al. 1998, Min et al. 2007).

Chlase was also affected by the electric field strength and treatment time. In fact, increasing both variables, the RA was reduced. Thus, the lowest RA (26.3%) was observed when HIPEF processing was performed at the highest treatment time (2000 μs) and electric field strength (35 kV/cm) in bipolar mode. Nonetheless, the positive quadratic terms of *t* and *E* indicated that a minimum Chlase activity value was reached when pulses from 30 to 35 kV/cm, for treatment times between 1620 μs and 2000 μs in bipolar mode were applied (Fig. 1).

These results are in accordance with other studies, where the effectiveness of HIPEF treatments on orange juice peroxidase and pectin methyl esterase, and horseradish peroxidase inactivation increased as the electric field strength and treatment time augmented (Elez-Martínez et al. 2006, Elez-Martínez et al. 2007b, Zhong et al. 2005). Similarly, Min et al. (2003) reported a major inactivation of tomato juice lipoxygenase as electric field strength (from 15 to 35 kV/cm) and treatment time (from 20 to 70 μs) increased.

Diverse theories have been postulated to explain enzyme behaviour when food is subjected to an electric field. Some authors have suggested that HIPEF treatment causes conformational changes in enzymes, such as the loss of its α-helix (Zhong et al. 2005). Barsotti and Cheftel (1999) proposed that HIPEF treatment destabilizes the covalent or non-covalent interactions, protein unfolding, and denaturation. Therefore, these findings might explain the Chlase inactivation observed in this research.

But, the incomplete reduction of Chlase activity by HIPEF in broccoli juice could be associated to the fact that thylakoid, chloroplast and cell membranes protected Chlase against HIPEF. This hypothesis is supported by Hornero-Méndez and Mínguez-Mosquera (2001) and Matile et al. (1997) who demonstrated that Chlase is an enzyme bounded to the chloroplast membrane.

Effects of HIPEF parameters on Chl a and Chl b

The initial concentrations of Chl *a* and Chl *b* in untreated broccoli juice were 8.33 and 3.25 mg/100 mL, respectively. To the best of our knowledge, there are no works that report about the concentration of Chl in broccoli juice. However, Van Loey et al. (1998) observed that the proportion of Chl *a* and Chl *b* in broccoli juice was 3:1. Similarly, in the present study, it was demonstrated that the ratio is kept when broccoli juice is obtained.

The influence of HIPEF parameters on Chl *a* and Chl *b* contents in broccoli juice is shown in Table 2. The RC of Chl *a* and Chl *b* in HIPEF-processed broccoli juice ranged between 81.8-116.0 and 74.7-120.7%, respectively. An increment in the content of Chl *a* and Chl *b* occurred when broccoli juice was treated in the electric field strength ranging from 25 to 35 kV/cm and 1250 μs in both monopolar and bipolar modes. However, the RC of Chl did not increase at 15 kV/cm and at any treatment time.

At the lowest electric field strength applied to broccoli juice, incomplete enzyme inactivation occurred, resulting in Chl losses, as shown in Table 2. Results of Pearson test

(Table 4) showed that there was a significant negative correlation between Chlase and Chls (Chl *a* and Chl *b*), meaning that, when Chlase activity increased, the RC of both Chl *a* and Chl *b* decreased. In this sense, Yin et al. (2007) associated the microorganism and enzyme elimination by HIPEF to the maintenance of spinach puree Chls and its color.

The ANOVA indicates that a second-order model described with accuracy the Chl *a* and Chl *b* RC of HIPEF-treated broccoli juice ($p < 0.0001$) (Table 3). The determination coefficient (R^2) was 0.91 and 0.98 for Chl *a* and Chl *b*, respectively, and a nonsignificant lack of fit was observed.

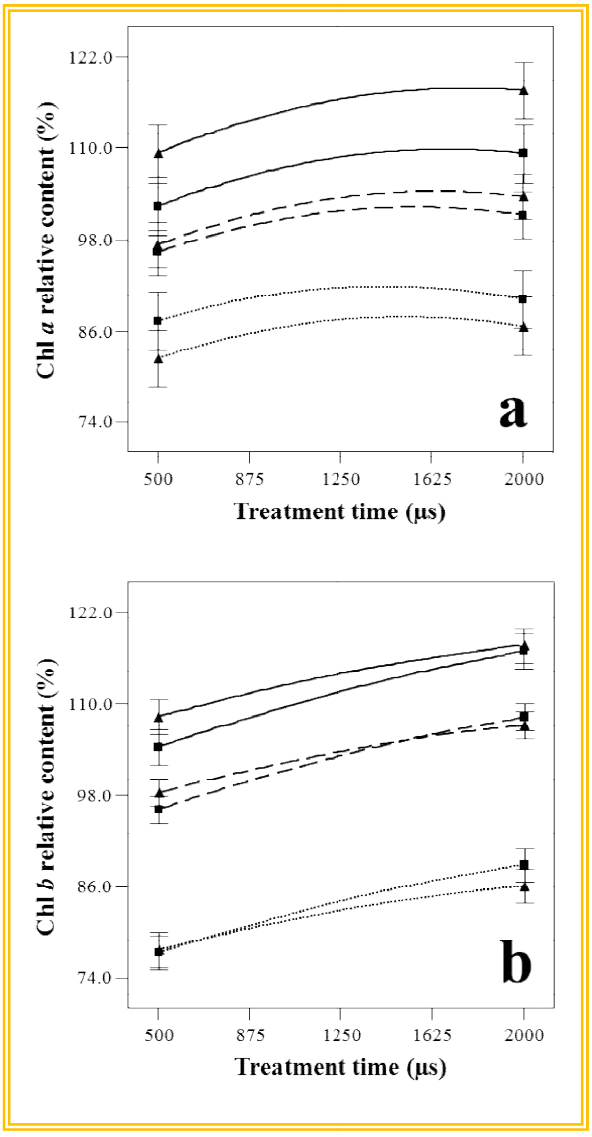

Figure 2. Effect of treatment time (µs) on chlorophyll *a* (**a**) and chlorophyll *b* (**b**) RC at 35 (—), 25 (- -) and 15 kV/cm (···) in monopolar (filled squares) or bipolar mode (filled triangles).

Notwithstanding polarity did not significantly affect the RC of Chl *a* and Chl *b*, the interaction electric field strength-polarity showed a significant influence on Chl *a* (Table 3). Thus, the model was reduced to a function that can be used for either monopolar or bipolar mode pulses (Eqs. 6 and 7).

$$Chl\ a = 102.57 + 11.51 * E + 2.80 * t - 2.98 * t^2 \quad (6)$$

$$Chl\ b = 103.29 + 14.67 * E + 5.23 * t - 5.38 * E^2 \quad (7)$$

where Chl *a* and Chl *b* were chlorophyll *a* and chlorophyll *b* RC, respectively, *E* was the electric field strength (kV/cm) and *t* the treatment time (μs).

Electric field strength and treatment time had significant effects on both Chl *a* and Chl *b* RC. Figure 2 shows that, when treatment time and electric field strength (from 25 to 35 kV/cm) rose, the RC of Chl *a* and Chl *b* augmented. The increment in the content of Chls achieved at electric field strength from 25 to 35 kV/cm could be explained by the fact that an activation of specific enzymes related to the Chl biosynthesis pathway could take place due to the effect of HIPEF processing. For instance, the Mg-chelatase is an enzyme that catalyzes the insertion of Mg^{+2} into protoporphyrin IX to yield Mg-protoporphyrin IX, which is then further metabolized into chlorophyll (Mochizuki et al. 2010, Reinbothe et al. 2010). Several studies have also shown enzyme activation when exposed to HIPEF. In this context, watermelon, tomato and strawberry juice lipoxygenase (Aguiló-Aguayo et al. 2008a, Aguiló-Aguayo et al. 2009, 2010b), tomato juice hidroperoxide lyase (Aguiló-Aguayo et al. 2009) and strawberry juice β-glucosidase (Aguiló-Aguayo et al. 2008a) activation by HIPEF were reported.

On the other hand, broccoli is a vegetable with an elevated content of magnesium (18.1-27.3 mg/100g) (Kmiecik et al. 2007, López-Berenguer et al. 2007) and it has been demonstrated that the synthesis of Chls is favored at elevated Mg^{+2} concentration (Nakayama et al. 1998). In line with the results reported in this research, Torregrosa et al. (2005) observed that the concentration of carotenoids in orange-carrot juice increased after applying pulses at 25-30 kV/cm for 60-340 μs, respect to untreated juice. Odriozola-Serrano et al. (2008) observed a lycopene increment in tomato juice as the treatment time (from 100 to 2000 μs) and electric field strength (from 20 to 35 kV/cm) increased, compared to the fresh tomato juice.

Effects of HIPEF parameters on Chls degradation compounds

Table 2 shows the RC of Chlide, Phe and Phb of HIPEF-treated broccoli juice. The RC of Chlide, Phe and Phb in broccoli juice processed by HIPEF ranged between 116.9-138.2%, 91.2-126.0% and 95.2-132.0% for Chlide, Phe and Phb, respectively.

The experimental data of RC of Chlide, Phe and Phb were properly modeled by a second–order response surface function ($p < 0.0001$) with a nonsignificant lack of fit (Table 3). Pulse polarity was considered as a categorical factor. Hence, the RC of the

products of Chls degradation in broccoli juice can be fitted through Eqs. 8, 10 and 12 for monopolar and 9, 11 and 13 for bipolar mode pulses:

$$Chlide_M = 144.83 - 0.21 * E - 4.06x10^{-3} * t - 2.5x10^{-6} * t^2 + 7.0x10^{-5} * E * t \quad (8)$$

$$Chlide_B = 140.81 - 0.20 * E - 5.68x10^{-3} * t - 2.5x10^{-6} * t^2 + 7.0x10^{-5} * E * t \quad (9)$$

$$Phe_M = 130.47 - 4.43x10^{-2} * E + 3.16x10^{-3} * t - 7.8x10^{-6} * t^2 \quad (10)$$

$$Phe_B = 119.84 - 9.84x10^{-3} * E + 4.46x10^{-3} * t - 7.8x10^{-6} * t^2 \quad (11)$$

$$Phb_M = 131.38 - 0.049 * E + 8.87x10^{-3} * t - 8.79x10^{-6} * t^2 - 1.7x10^{-4} * E * t \quad (12)$$

$$Phb_B = 111.47 - 0.051 * E + 1.42x10^{-2} * t - 8.79x10^{-6} * t^2 - 1.7x10^{-4} * E * t \quad (13)$$

where *Chlide*, *Phe* and *Phb* were chlorophyllide, pheophytin and pheophorbide RC, respectively in monopolar (M) or bipolar (B) mode, *E* was the electric field strength (kV/cm) and *t* the treatment time (µs).

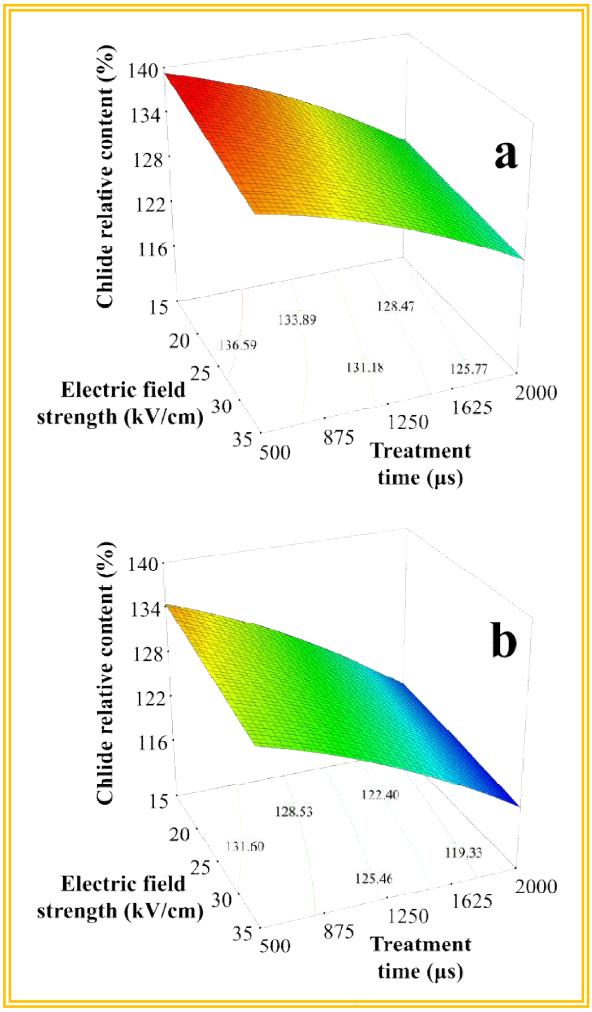

Fig 3. Effect of electric field strength and treatment time on chlorophyllide RC in monopolar (**a**) and bipolar (**b**) modes in broccoli juice.

Polarity significantly influenced (p< 0.0001) the content of Chls degradation products (Table 3). Monopolar treatments led to higher Chlide, Phe and Phb content than those applied in bipolar mode in HIPEF-treated broccoli juice.

On the other hand, electric field strength (E) and treatment time (t) significantly influenced on RC of Chlide, Phe and Phb (Table 3). That is, the minimum RC of Chlide, Phe and Phb were observed when the broccoli juice was treated with the strongest electric field strength (35 kV/cm)and treatment time (2000 µs). In contrast, at the lowest HIPEF conditions (15 kV/cm for 500 µs) the greatest RC of Chls degradation compounds were observed (Fig. 3-5).

These findings could be associated to incomplete inactivation of degradative enzymes of Chls, such as Chlase, among others. Correlation of Pearson values (Table 4) displayed that there was a significant negative correlation between Chls and their degradation products (Chlide, Phe and Phb), indicating that when Chls degradation rose, the RC of Chlide, Phe and Phb augmented. Furthermore, the lowest Chlase RA achieved during HIPEF processing of broccoli juice was well correlated to the minimum Chlide and Phb content.

Both residual Chlase activity and HIPEF processing could have a significant influence on the Chl degradation. Moreover, during HIPEF treatment the electrodes are in direct contact with the food product. Electrochemical reactions can occur which lead to metal release and oxidation reactions could take place when electric pulses are applied (Roodenburg et al. 2005).

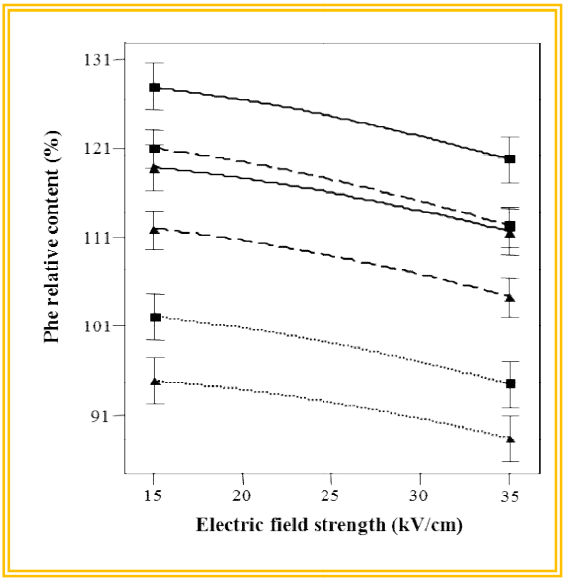

Figure 4. Effect of the electric field strength on pheophytin relative content at (—) 500, (- -), 1250 and (···) 2000 µs of treatment time in monopolar (filled squares) or bipolar mode (filled triangles).

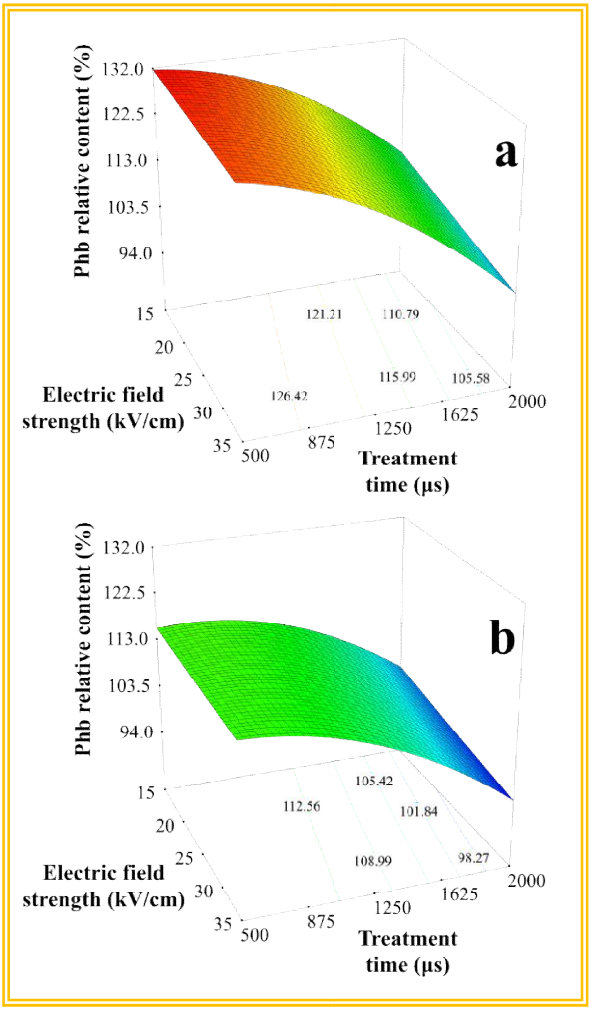

Fig 5. Effect of electric field strength and treatment time on pheophorbide RC in monopolar (**a**) and bipolar (**b**) modes in broccoli juice.

To the best our knowledge, there is not available information related to HIPEF treatment and the formation of Chls degradation compounds. However, studies applying other non-thermal technologies, such as high hydrostatic pressure (150 MPa) in combination to heat (75-80 °C), reported a slight increment in the Chls degradation products (Weemaes et al. 1999a). The same authors observed that as the pressure level rose, the Chls loss augmented. In contrast to high-hydrostatic pressure treatment, when the HIPEF parameters augmented, an increment in Chls content as well as reduction of Chlide, Phe and Phb content were observed.

Validation and optimization of HIPEF-processing conditions

Table 4. Correlation coefficients between HIPEF Chlorophyllase, Chlorophyll *a* and *b,* Chlorophyllide, Pheophytin and Pheophorbide of broccoli juice.

	Chlase	Chl a	Chl b	Chlide	Phe	Phb
Chlase	-	-0,8389[a]	-0,8766[a]	0,8925[a]	0,3510	0,6351[a]
Chl a	-0,8389[a]	-	0,2449	-0,8733[a]	-0,8979[a]	-0,8367[a]
Chl b	-0,8766[a]	0,2449	-	-0,8649[a]	-0,8645[a]	-0,9631[a]
Chlide	0,8925[a]	-0,8733[a]	-0,8649[a]	-	0,1967	0,3910
Phe	0,3510	-0,8979[a]	-0,8645[a]	0,1967	-	0,5811
Phb	0,6351[a]	-0,8367[a]	-0,9631[a]	0,3910	0,5811	-

[a] Significant differences at p< 0.05 using Pearson's correlation coefficients.

The correlation coefficients between the observed and predicted Chlase residual activity, RC of Chl a, Chl b, Chlide, Phe, and Phb data were 0.93, 0.85, 0.95, 0.98, 0.89 and 0.98, respectively, meaning that 2nd-order expressions obtained for each assay were adequate to fit the experimental results.

In the range of the HIPEF parameters studied, the treatment condition that exhibited the maximum Chl *a* and Chl *b* content, as well as, the minimum Chlide, Phe, Phb content and Chlase activity, was achieved at 35 kV/cm for 1980 µs in bipolar mode (Table 5). The desirability of HIPEF treatment was 0.97, which was taken as an indicator of accuracy between the polynomial model predictions and the experimental data.

Comparison between HIPEF and thermal treatment

Table 5 describes the effects of thermal treatment (90 °C/60 s) and the optimal HIPEF condition (35 kV/cm for 1980 µs in bipolar mode) on Chlase, Chls and Chls degradation compounds of broccoli juice, regarding the untreated juice.

It is important to highlight that Chls content after applying HIPEF processing was higher than in that treated by heat. When the broccoli juice was processed by heat, the RC of Chl*a* andChl*b* decreased until 18.28 and 24.23%, respectively (Table 5); whereas the RC of Chlide, Phe and Phb increased. On the contrary, the HIPEF-processed broccoli juice displayed increments in both Chl*a* andChl*b* content. Thus, lower RC of Phe (90.52%) and Phb (94.97%) than heat treatment (186.31 and 142.98%, respectively) was reached. In contrast, no significant differences in the RC of Chlide among treatments were observed (Table 5). This fact could be attributed to the formation of Chlide by the Chlase enzyme action (Schwartz & Lorenzo 1990), but also by high temperature treatment effect(Canjura & Schwartz 1991). Traditional pasteurization of broccoli juice inactivated Chlase but Chlide formation was observed due to the high temperature applied (90 °C). HIPEF treatment

was performed at temperature below to 35 °C; therefore, Chlide apparition is explained by the action of residual activity of Chlase.

Table 5.Effect of HIPEF and thermal treatment on residual activity of Chlase, relative content of Chl and Chl degradation compounds of broccoli juice.

Parameters[a]	HIPEF	TT
Chlase	30.39±2,8[b]	0,5 ±3,4[c]
Chl a	117.66±3, 6[b]	24.23±4,9[c]
Chl b	117.41±2,1[b]	18.28±3,5[c]
Chlide	116,39±4,3[b]	113.79 ±5,0[b]
Phe	90,52±5,7[b]	186.31 ±6,9[c]
Phb	94,97±6,3[b]	142.98 ±5,4[c]

[a]Values are expressed as mean ±SD of two repetitions; each assay was performed by triplicate. *b* and *c* letters indicate significant differences ($p< 0.05$). HIPEF = High intensity pulsed electric fields 35 kV/cm, 1980µs in bipolar mode; TT = Thermal treatment (90ºC for 60 s).

The most harmful reaction during Chls degradation is the formation of Phe, which reduces the visual quality of food due to the apparition of olive-brown color(Coultate 2009, Simpson 1985). Additionally, it has been suggested that the loss of green color in vegetables during heat treatment is due to the increase in the permeability of hydrogen ions across cell membrane, replacing Mg^{+2} by two protons and producing Phe and Phb formation (Von Elbe & Schwartz 2000). In contrast, HIPEF treatments at optimum conditions increased the RC of Chl*a* and Chl*b* and decreased the formation of Phe and Phb. Chlase inactivation and low temperatures observed during HIPEF processing improves the protection of Chls in broccoli juice.

CONCLUSION

The RC of Chls increased and residual activity of Chlase diminished when broccoli juice was HIPEF-processed within the studied range. In general, results demonstrated that when electric field strength and treatment time increased, the content of Chl*a* andChl*b* augmented; while Chlase activity, Chlide, Phe and Phb content diminished. Furthermore the application of bipolar pulses raised the RC of Chls and reduced the residual activity of Chlase, as well as the RC of Chlide, Phe and Phb more than those applied in monopolar mode. The RC of broccoli juice Chl*a* andChl*b* rose when HIPEF processing was applied from 25 kV/cm.

At the assayed conditions, the optimum HIPEF combination to reduce the Chlase residual activity, RC of Chlide, Phe and Phb, as well as to achieve the highest Chl*a* and Chl*b*

was 35 kV/cm in bipolar mode for 1980 µs. Moreover, HIPEF processing led to higher Chls content than thermal processing.

Therefore, these results demonstrated that HIPEF technology could be an alternative to thermal treatment for obtaining broccoli juice with high nutritional and visual quality.

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2 INFLUENCE OF HIGH-INTENSITY PULSED ELECTRIC FIELD PROCESSING PARAMETERS ON ANTIOXIDANT COMPOUNDS OF BROCCOLI JUICE

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ABSTRACT

The influence of high-intensity pulsed electric field (HIPEF) processing parameters (electric field strength, treatment time, and polarity) on broccoli juice carotenoids, vitamin C, and total phenolic (TP) content, as well as antioxidant capacity (AC) was evaluated. Results obtained from HIPEF-processed broccoli juice were compared with those of thermally treated (90 °C/60 s) and untreated juices. HIPEF processing parameters influenced the relative content (RC) of bioactive compounds, and the relative AC (RAC). Maximum RC of lutein (121.2%), β-carotene (130.5%), TP (96.1%), vitamin C (90.1%) and RAC (5.9%) was reached between 25 and 35 kV/cm and from 2000 μs to 500 μs. The highest RAC and RC of bioactive compounds were observed in HIPEF treatments applied in bipolar mode, except for vitamin C. HIPEF-treated broccoli juice exhibited greater RC of bioactive compounds and RAC than juice treated by heat. HIPEF technology could be considered a promising option for preserving the antioxidant quality of broccoli juice.

Keywords: High-intensity pulsed electric fields; broccoli juice; carotenoids; vitamin C; total phenolic compounds; antioxidant capacity.

INTRODUCTION

Broccoli (*Brassica oleraceae* var. *italica*) is a green vegetable with a high content of chlorophylls, glucosinolates and other bioactive compounds, such as carotenoids (Singh, Upadhyay, Prasad, Bahadur, & Rai, 2007), vitamin C (Combs, 1998; Heinonen & Meyer, 2002), and phenolic compounds (Faller & Fialho, 2010; Mattila & Hellström, 2007). Epidemiological studies have demonstrated that these bioactive compounds stimulate the immune system (Combs, 1998; Harrison & May, 2009), prevent cardiovascular diseases (Vermerris & Nicholson, 2006), and exert chemoprotection against different types of cancer (Huang, Ho, Wang, Ferraro, Finnegan-Olive, Lou, et al., 1992; Jeffery & Araya, 2009).

Recently, the consumption of broccoli has increased worldwide (Latté, Appel, & Lampen, 2011). As a result, new broccoli-based products, such as blends of broccoli and apple juices have been suggested to further increase its consumption (Houška, Strohalm, Kocurová, Totušek, Lefnerová, Tříska, et al., 2006). Overall, vegetable juices represent an interesting alternative for incorporating bioactive compounds in the diet and a special attention has been focused on broccoli juice due to its elevated content of bioactive compounds that provide important health benefits (Lee, Kim, Son, Lee, Park, Kim, et al., 2013; Mandelová & Totusek, 2007).

Traditionally, juices have been processed by heat to avoid the harmful influence of enzymes and microorganisms. Nevertheless, thermal processing causes losses of nutritional and sensorial characteristics of foods. As alternatives to thermal processing, nonthermal technologies have been developed. High-intensity pulsed electric fields (HIPEF) is a nonthermal preservation technology for liquid foods that has shown promising results for microbial reduction (Mosqueda-Melgar, Elez-Martínez, Raybaudi-Massilia, & Martín-Belloso, 2008) and enzymatic inactivation (Odriozola-Serrano, Aguiló-Aguayo, Soliva-Fortuny, & Martín-Belloso, 2013). Besides, some studies have demonstrated that HIPEF processing is effective for maintaining bioactive compounds of liquid foods. In the same way, Odriozola-Serrano, Aguiló-Aguayo, Soliva-Fortuny, Gimeno-Añó, and Martín-Belloso (2007) reported that bioactive compounds such as, lycopene and vitamin C concentration, as well as increased antioxidant levels in HIPEF-treated tomato juice.

The effects of HIPEF processing on bioactive compounds in fruit juices have been widely reviewed (Odriozola-Serrano, Aguiló-Aguayo, Soliva-Fortuny, & Martín-Belloso, 2013; Soliva-Fortuny, Balasa, Knorr, & Martín-Belloso, 2009). Odriozola-Serrano, Aguiló-Aguayo, Soliva-Fortuny, and Martín-Belloso (2013) observed as general tendency that bioactive compounds, such as vitamin C, carotenoids and phenolics were better retained in HIPEF-processed juices than in those thermally-treated. However, literature related to the effect of HIPEF on bioactive compounds present in green vegetable juices is still scarce (Sánchez-Vega, Elez-Martínez, & Martín-Belloso, 2014). Therefore, the purpose of this study was to evaluate the effect of HIPEF processing parameters (electric field strength, treatment time, and polarity) on the content of carotenoids (lutein and β-carotene), vitamin C, total phenolic compounds (TP) and antioxidant capacity (AC) of broccoli juice. Furthermore, the effects of HIPEF and traditional thermal pasteurization on relative

content (RC) of bioactive compounds and relative antioxidant capacity (RAC) of broccoli juice were also compared.

MATERIALS AND METHODS

Reagents.

Magnesium carbonate, ethyl acetate, metaphosphoric acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), stock solutions of lutein and β-carotene were purchased from Aldrich Chemical Co (St Louis MO, USA); sodium sulphate anhydrous, ascorbic acid, Folin-Ciocalteu reagent, gallic acid and diethyl ether were supplied by Scharlau Chemie, SA (Barcelona, Spain). Methanol and propanone were obtained in Teknokroma (Barcelona, Spain).

Sample preparation

Broccoli (*Brassica oleraceae* var. italica) was purchased at commercial maturity in a local supermarket (Lleida, Spain). Broccoli was cut (discarding the leaves and stalk) and crushed. The resulting juice was filtered through cheesecloth and vacuum degassed for 10 min. The electrical conductivity of broccoli juice was analyzed (Testo 240 conductivimeter; Testo GmBh & Co, Lenzkirch, Germany), resulting in 0.9 S/m. Afterwards, broccoli juice was divided in three batches; one for HIPEF processing, the second for thermal treatment and the third was maintained unprocessed.

HIPEF equipment

HIPEF treatments were carried out using a continuous-flow bench-scale system (OSU-4F, Ohio State University, Columbus, OH) that held monopolar or bipolar square wave pulses. The treatment system consisted of eight collinear chambers serially connected. Each chamber consisted of two stainless steel electrodes separated by a gap of 2.92 mm, whose treatment volume was 0.012 cm^3. The treatment flow was controlled by a variable-speed pump (model 75210-25, Cole Parmer Instruments Company, Vernon Hills, IL, USA). The maximum temperature registered in the chamber outlet during HIPEF processing of broccoli juice never exceeded 35 ^oC. This temperature was maintained through a cooling coil connected between each pair of chambers and submerged in an ice-water shaking bath.

The input of electrical energy density (Q, J/m^3) supplied to the samples was calculated through Eq. 1 (Martin, Zhang, Castro, Barbosa-Cánovas & Swanson, 1994):

$$Q = \frac{V_0 * I * t}{v} \qquad \text{Ec. 1}$$

where V_0 is the peak voltage (V), I the intensity of the current (A), t the treatment time (s), and v the volume of all treatment chambers (m^3).

Thermal treatments.

Broccoli juice was heat-treated (90 ºC for 60 s) in a tubular stainless steel heat exchange coil immersed in a hot water shaking bath using a gear pump (University of Lleida, Spain). Afterwards, the juice was immediately cooled in an ice water-bath through immersion of the heat exchange coil.

Bioactive compounds

Carotenoids

Table 1. Mobil phase gradient for determination of carotenoids by HPLC.

Time (min)	Flow (mL/min)	A (%)	B (%)	C (%)
0	1	80	0	20
5	1	77.5	0	22.5
22.5	1	50	0	50
27.5	1	50	0	50
30	1	20	0	80
35	1	20	0	80
40	1	0	0	100
45	1	0	0	100
50	1	0	100	0
70	1	0	100	0
71	1	80	0	20
80	1	80	0	20

A: Methanol/Water (75:25). B: Methanol (100%). C: Ethyl acetate (100%).

The extraction of carotenoids was based on the method described by Cano (1991) with some modifications. A portion of 20 mL of broccoli juice was mixed with 20 mL of chilled propanone and then magnesium carbonate (1 g) and sodium sulphate (10 g) were added. This mixture was vigorously homogenized for 1 minute and filtered. The residues

were washed with chilled propanone until colorless. The filtrate volume was reduced to 5 mL by rotoevaporation and transferred to a separatory funnel where diethyl ether (30 mL) and saturated sodium chloride solution (20 mL) were added. The washing procedure was repeated 3-5 times and then it was vigorously shaken. The organic phase was dehydrated with analytical grade sodium sulphate anhydride and the diethyl ether was eliminated by rotoevaporation. An aliquot of 4 mL of propanone was added to reconstitute the sample containing the carotenoids. Then this solution was filtered (0.45 µm, Millipore Iberica S.A., Spain) and analyzed by HPLC.

A portion of 25 µL of the carotenoids extract was injected into the HPLC equipment using a reverse-phase C18 Spherisorb® ODS2 (5 µm) stainless steel column (4.6 x 250 mm). A gradient elution was composed of methanol/water (75:25), as eluent A, methanol (100%), as eluent B (cleaning solution), and ethyl acetate, eluent C (Table 1). The flow rate was fixed at 1.0 mL/min and the column temperature was maintained at 30 °C. The 2996 Waters Photodiode Array Detector, (Milford, MA) was adjusted at 440 nm. Carotenoids were quantified by comparison with external standards and expressed as mg of lutein or β-carotene per 100 mL of broccoli juice.

Vitamin C

Vitamin C content of broccoli juice was determined by HPLC. The extraction procedure was based on a method validated by Odriozola-Serrano, Aguiló-Aguayo, Soliva-Fortuny, Gimeno-Añó, and Martín-Belloso (2007). Briefly, broccoli juice was mixed with 4.5% metaphosphoric solution in a proportion 1:1. The mixture was homogenized and centrifuged at 2401*g* for 10 min at 4 °C (Centrifuge AVANTI™ J-25, Beckman Instruments Inc., Fullerton, CA, USA). Then, the vacuum-filtered sample was passed through a Millipore 0.45-µm membrane to be injected in the HPLC system.

An aliquot of 20 µL of extract was injected into the HPLC system using a reverse-phase C18 Spherisorb ODS2 (5 µm) stainless-steel column (4.6 mm x 250 cm). The mobile phase was a 0.01% sulphuric acid solution adjusted to pH = 2.6. The flow rate was 1 mL/min at room temperature. Detection was performed with a 486 Absorbance Detector (Waters, Milford, MA) set at 245 nm. Identification of the ascorbic acid was carried out comparing the retention time and UV-visible absorption spectrum of the juice samples with those of the standards (ascorbic acid). The results were expressed as mg of ascorbic acid per 100 mL of juice.

Total phenolics

Total phenolics (TP) were determined by the colorimetric method described by Singleton, Orthofer, and Lamuela-Raventós (1998) using the Folin-Ciocalteu reagent. A portion of 0.5 mL of broccoli juice was mixed with 0.5 mL of Folin-Ciocalteu reagent and 10 mL of saturated Na_2CO_3 solution. Samples were kept at room temperature in darkness for 1 h. Afterwards, the absorbance was measured at 725 nm using a CECIL 2021

spectrophotometer (Cecil Instruments Ltd., Cambridge, UK). Concentrations were determined by comparing the absorbance of the samples with a calibration curve built with gallic acid. The results were expressed as mg of gallic acid per 100 mL of juice.

Antioxidant capacity

Antioxidant capacity (AC) was determined through the evaluation of the free radical scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH) following the method described by Odriozola-Serrano, Aguiló-Aguayo, Soliva-Fortuny, Gimeno-Añó, and Martín-Belloso (2007). Broccoli juice (0.01 mL) was mixed with 3.9 mL of methanolic DPPH (0.025 g/L) and 0.090 mL of distilled water. The homogenate was shaken vigorously and kept in darkness for 30 min. Absorption of the samples was measured using a CECIL 2021 spectrophotometer (Cecil Instruments Ltd., Cambridge, UK) at 515 nm against a blank of methanol. Antioxidant capacity was calculated from the DPPH-inhibition values of broccoli juice related to the initial absorbance of the methanolic DPPH solution.

Expression of results

The results of carotenoids, vitamin C, and TP concentration were expressed as relative content (RC) (Eq. 2).

$$RC(\%) = \frac{C_t}{C_o} x100 \qquad \text{Eq. 2}$$

where C_t and C_0 are the concentration of bioactive compounds of the treated and untreated broccoli juice, respectively. Whereas, AC was expressed as relative AC (RAC) with respect to untreated broccoli juice (Eq. 3).

$$RAC(\%) = \frac{AC_t}{AC_o} x100 \qquad \text{Eq. 3}$$

where AC_t and AC_0 are DPPH inhibition for AC of the treated and untreated broccoli juice, respectively.

Experimental design

A response surface methodology was used to evaluate the effect of the different HIPEF treatment variables on the relative content of carotenoids, vitamin C, total phenolic compounds and antioxidant capacity. A central composite design with three faced centred factors was proposed. Numerical variables were treatment time (500-2000 μs), and

electric field strength (15-35 kV/cm), while polarity (mono- or bipolar) was a categorical variable, keeping the pulse width (4 μs) and frequency (100 Hz) constant. The intensity of the current corresponded to 9135, 6525 and 3915 Amperes (A) for 35, 25 and 15 kV/cm, respectively. The input of electrical energy density supplied to the samples ranged from 88.7 to 1931.7 MJ/m3 (Table 2).

The experimental design along with each experimental condition is shown in Table 2. All HIPEF treatments were conducted in duplicate and each analysis was carried out by triplicate (n=6). Experimental data were fitted to a polynomial model equation. The second order response function was predicted by Eq. 4:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X^2{}_{i_i} + \sum_{i=1}^{2} \sum_{i=i+1}^{3} \beta_{ij} X_i X_j \qquad \text{Eq. 4}$$

were Y is the response, β_0, β_i, β_{ii}, and β_{ij}, are the constant, linear, quadratic and interaction regression coefficients, respectively and X_i represent the encoded values of the variables. Response surface methodology was employed for experimental design, data analysis, model building and plot generation using Design Expert 6.01 software (Stat Ease Inc., Minneapolis, MN, USA).

RESULTS AND DISCUSSION

The concentration of lutein, β-carotene, vitamin C, and total phenolic compounds (TP) in untreated broccoli juice was 4.9, 2.8, 118.9 and 77.1 mg/100 mL, respectively. Also, fresh broccoli juice exhibited an initial DPPH inhibition of 37.8%. As far as we know, there are no studies regarding the content of carotenoids, vitamin C and TP in broccoli juice. Nonetheless, diverse studies have reported similar ranges for carotenoids (Ibrahim & Juvik, 2009), vitamin C (Vallejo, Tomás-Barberán, & Garcia-Viguera, 2002), TP (Koh, Wimalasiri, Chassy, & Mitchell, 2009) and AC (Turkmen, Sari, & Velioglu, 2005) in whole broccoli.

The highest relative content (RC) of both lutein (121%) and β-carotene (130%) were reached at 35 kV/cm and 2000 μs in bipolar mode (Fig. 1). In agreement with the present research, Torregrosa, Cortés, Esteve, and Frígola (2005) observed a RC up to 187% of lutein and 117% of β-carotene in orange-carrot juice treated by HIPEF at 35 kV/cm for 200 μs.

On the other hand, HIPEF-treated broccoli juice displayed losses in the vitamin C content within the assayed conditions (Fig. 2). As can be seen in Table 2, RC of vitamin C ranged from 67.0 to 90.1%. The maximum RC of vitamin C was kept at 35 kV/cm and 500 μs in monopolar mode. Consistently, Torregrosa, Esteve, Frígola, and Cortés (2006) observed that the RC of vitamin C ranged between 83 and 97% in HIPEF-treated orange juice, varying the electric field strength (from 25 to 40 kV/cm) and treatment time (from 30 to 340 μs).

Regarding TP, the highest RC of TP (96.1%) was reached at 25 kV/cm for 1250 μs in bipolar mode. On the contrary, the lowest RC of TP (79.6%) was obtained at 35 kV/cm for

2000 μs in monopolar mode (Fig. 3). Odriozola-Serrano, Soliva-Fortuny, and Martín-Belloso (2008b) also reported reductions in TP content of strawberry juice, after applying HIPEF treatments at similar electric field strength (35 kV/cm) and treatment time (1700 μs) than those used in the present research.

The lowest reduction of broccoli juice AC (RAC of 95.9%) was obtained at 35 kV/cm for 1250 μs in bipolar mode. In contrast, broccoli juice processed at 15 kV/cm for 500 μs in monopolar mode exhibited the highest AC diminution (RAC of 71.9%) (Fig. 4). Similarly, Odriozola-Serrano, Aguiló-Aguayo, Soliva-Fortuny, Gimeno-Añó, and Martín-Belloso (2007) reported a reduction in the RAC of HIPEF-processed tomato juice. The authors observed that the highest RAC (92.3%) of tomato juice was kept after applying HIPEF treatments at 35 kV/cm for 1000 μs with pulses of 4 μs at 150 Hz.

The analysis of variance indicated that a second order polynomial model described with accuracy (p<0.0001) the changes in bioactive compounds content and AC of broccoli juice treated by HIPEF. The determination coefficients of models (R^2) were 0.98, 0.97, 0.83, 0.91 and 0.84 for lutein, β-carotene, vitamin C, TP and AC, respectively. Moreover, the lack of fit of each model was not significant, meaning that the models were adequate for predicting the response within the range of the assayed conditions (Table 3).

Table 2. Central composite response surface design for relative content of lutein, β-carotene, vitamin C, total phenolics and relative antioxidant capacity on HIPEF-treated broccoli juice.

Parameters					Variables[a]				
Assay No[b]	Point type	*E* (kV/cm)	*t* (µs)	Polarity	Lutein (%)	β-carotene (%)	Vitamin C (%)	TP (%)	AC (%)
1	Factorial	15	500	Monopolar	65.6 ±2.3	50.7 ±3.5	71.8 ±3.5	85.4 ±2.3	71.9 ±2.2
2	Factorial	35	500	Monopolar	93.9 ±3.5	103.5 ±2.9	90.1 ±3.6	86.1 ±1.9	87.7 ±2.5
3	Factorial	15	2000	Monopolar	78.6 ±2.5	64.4 ±4.2	67.2 ±4.7	84.3 ±1.7	85.5 ±1.2
4	Factorial	35	2000	Monopolar	104.6 ±3.1	104.7 ±3.7	74.6 ±4.5	79.6 ±1.4	82.8 ±1.7
5	Axial	15	1250	Monopolar	75.5 ±1.8	60.4 ±4.8	68.9 ±3.6	85.5 ±2.5	72.0 ±3.3
6	Axial	35	1250	Monopolar	103.2 ±4.2	109.5 ±3.2	82.2 ±4.0	80.4 ±2.1	78.8 ±2.9
7	Axial	25	500	Monopolar	85.5 ±3.4	74.6 ±3.7	75.6 ±3.1	92.3 ±2.4	79.9 ±2.5
8	Axial	25	2000	Monopolar	99.9 ±2.4	96.8 ±2.3	72.9 ±3.3	90.8 ±1.2	87.5 ±2.4
9	Central[c]	25	1250	Monopolar	95.7 ±2.1	88.9 ±2.2	73.3 ±3.2	90.8 ±1.7	88.3 ±2.6
10	Factorial	15	500	Bipolar	71.3 ±3.6	58.4 ±1.5	71.8 ±2.2	93.5 ±3.7	79.3 ±3.4
11	Factorial	35	500	Bipolar	111.9 ±2.8	112.3 ±2.2	73.4 ±3.4	85.7 ±3.1	89.8 ±2.3
12	Factorial	15	2000	Bipolar	85.6 ±1.9	75.8 ±1.1	78.1 ±4.2	91.0 ±4.3	92.7 ±1.3
13	Factorial	35	2000	Bipolar	121.2 ±4.1	130.5 ±2.1	69.2 ±2.7	82.4 ±3.7	90.1 ±1.8
14	Axial	15	1250	Bipolar	81.1 ±3.5	69.7 ±3.8	74.2 ±3.1	90.0 ±2.5	81.2 ±1.4
15	Axial	35	1250	Bipolar	112.9 ±3.8	117.2 ±2.1	72.6 ±3.7	86.3 ±4.4	95.9 ±2.7
16	Axial	25	500	Bipolar	91.3 ±2.3	81.1 ±2.3	70.8 ±2.6	95.7 ±2.5	85.3 ±2.3
17	Axial	25	2000	Bipolar	109.2 ±2.8	107.8 ±2.9	67.1 ±4.3	93.2 ±3.0	89.9 ±1.6
18	Central[c]	25	1250	Bipolar	105.6 ±2.4	99.1 ±1.96	67.0 ±2.8	96.1 ±2.8	95.1 ±1.9
Thermal treatment (90 ºC/60 s)					51.2 ±2.9	30.6 ±2.6	68.0 ±3.4	71.8 ±3.2	72.4 ±2.7

[a]Values are expressed as relative content (%) ± standard deviation of two repetitions; each assay was performed by triplicate. *E*= Field strength, *t*= Treatment time; *TP*= Total phenols. *AC*= Antioxidant capacity. [b]Order of the assays was randomized. [c] Data shown are mean of the central points with five repetitions.

Effect of electric field strength and treatment time

Electric field strength and treatment time significantly influenced the RC of lutein, β-carotene, vitamin C and TP, as well as the RAC in broccoli juice (Table 3). The RC of lutein and β-carotene, they increased as the electric field strength and treatment time augmented. The increments in the carotenoids content reported in the present work agreed with previous studies in different food products. For instance, Odriozola-Serrano, Soliva-Fortuny, Hernández-Jover, and Martín-Belloso (2009) reported a raise in total carotenoids when tomato juice was processed by HIPEF at 35 kV/cm for 1700 μs. Similarly, the RC of lutein and β-carotene corresponded to 187% and 117%, respectively, when orange-carrot juice was treated by HIPEF at 35 kV/cm during 200 μs (Torregrosa, Cortés, Esteve, & Frígola, 2005). Taking into consideration the results obtained in this research and those of literature, it could be started that the retention of carotenoids depends of HIPEF treatment conditions, as well as food matrix. Thus, juices treated at the same condition of electric field strength (35 kV/cm) showed different values of carotenoids RC (between 121.2 and 130.5% for broccoli juice; 104.26% for tomato juice; and between 117 and 187% for orange-carrot juices).

Odriozola-Serrano, Aguiló-Aguayo, Soliva-Fortuny, Gimeno-Añó, and Martín-Belloso (2007) proposed that HIPEF processing could trigger the biosynthesis of carotenoids, resulting in an increment of some of them. In addition, HIPEF treatment is able to inactivate oxidative enzymes (Aguiló-Aguayo, Odriozola-Serrano, Quitão-Teixeira, & Martín-Belloso, 2008; Elez-Martínez, Aguiló-Aguayo, & Martín-Belloso, 2006), avoiding the reduction in the content of carotenoids in broccoli juice.

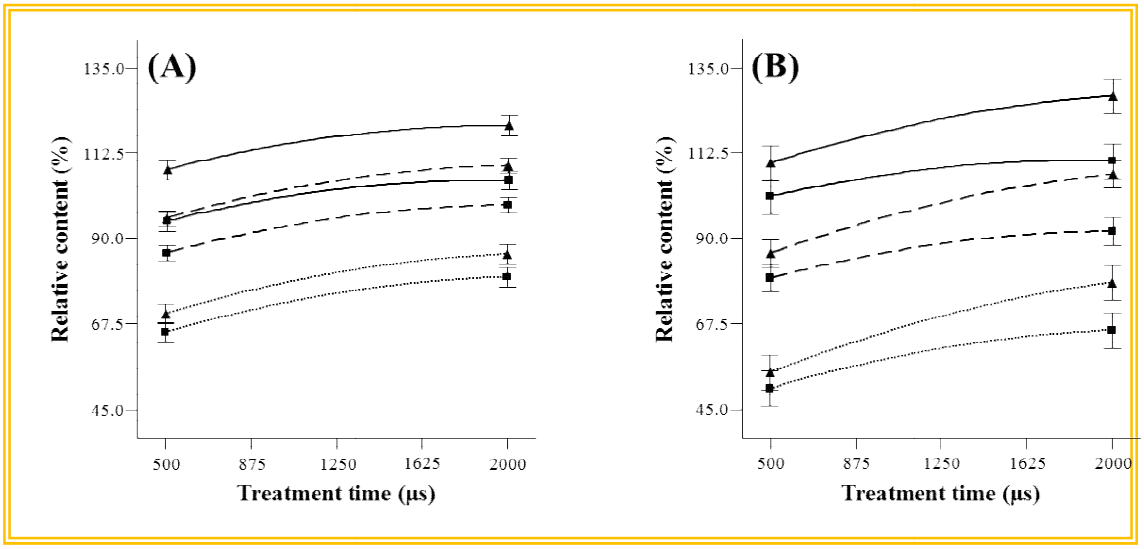

Figure 1. Effect of treatment time (μs) on lutein (A) and β-carotene (B) relative content of broccoli juice at 15 (···), 25 (- -) and 35 kV/cm (—) in monopolar (■) or bipolar (▲) mode pulses.

On the other hand, a significant reduction in the content of carotenoids (RC of 65.5% for lutein and RC of 50.7% for β-carotene) from broccoli juice was observed at the lowest HIPEF treatment conditions (15 kV/cm for 500 μs) applied in the present work. These results suggest that the elimination of microorganisms and oxidative enzymes are not completely achieved at 15 kV/cm for 500 μs. As a result, bioactive compounds such as carotenoids are reduced by the action of remaining microorganisms and enzymes. This hypothesis is supported by the literature where it has been demonstrated that the lower the electric field strength and the treatment time, the lower the microbial and enzyme inhibition by HIPEF processing (Barsotti & Cheftel, 1999; Pedro Elez-Martínez, Martín-Belloso, Rodrigo, & Sampedro, 2007; Espachs-Barroso, Barbosa-Cánovas, & Martín-Belloso, 2003; Martín-Belloso & Elez-Martínez, 2005; Min, Evrendilek, & Zhang, 2007).

A reduction of vitamin C RC was observed when HIPEF treatments at 35 kV/cm from 500 μs to 2000 μs were applied (Fig. 2). This trend was not followed after applying bipolar mode pulses at 15 kV/cm, where vitamin C RC diminished as treatment time decreased. Different combinations of electric field strength and treatment time allow obtaining the same RC of vitamin C, either in monopolar or bipolar mode. For example, 70% of vitamin C RC can be reached by processing broccoli juice at 18 kV/cm for 556 μs or at 34 kV/cm for 1270 μs in bipolar mode, as well as at 25 kV/cm and 1800 μs in monopolar mode (Fig. 2). The degradation of vitamin C observed in this study could be justified by the electrochemical reactions due to the electric current flowing between electrodes during HIPEF processing (Morren, Roodenburg, & de Haan, 2003). In this sense, Ottaway, Ottaway, and Associates Ltd (2010) reported that the contact of food with traces of heavy metal ions such as Fe and Zn causes the degradation of vitamin C. Additionally, an incomplete inactivation of oxidative enzymes could catalyze the vitamin C losses. Although HIPEF is able to reduce the enzymatic activity in diverse fruit and vegetable juices (Aguiló-Aguayo, Soliva-Fortuny, & Martín-Belloso, 2009; Quitão-Teixeira, Aguiló-Aguayo, Ramos, & Martín-Belloso, 2008) there are HIPEF resistant enzymes, as has been demonstrated in other studies (Aguiló-Aguayo, Oms-Oliu, Soliva-Fortuny, & Martín-Belloso, 2009; Van Loey, Verachtert, & Hendrickx, 2001).

HIPEF treatments carried out at 35 kV/cm and any treatment time in monopolar mode, led to the lowest reduction of vitamin C content in broccoli juice (Fig. 2). On the contrary, P. Elez-Martínez and Martín-Belloso (2007), Odriozola-Serrano, Aguiló-Aguayo, Soliva-Fortuny, Gimeno-Añó, and Martín-Belloso (2007), Odriozola-Serrano, Soliva-Fortuny, and Martín-Belloso (2009) and Oms-Oliu, Odriozola-Serrano, Soliva-Fortuny, and Martín-Belloso (2009) observed an inverse trend in comparison with that observed in the present study. These authors reported that the loss of vitamin C content in “gazpacho”, tomato, orange, strawberry and watermelon juices augmented as electric field strength and treatment time increased. However, it is important to take into consideration that each food contains specific quality-related enzymes, which could be more or less resistant to HIPEF treatment, and bioactive compounds, that could exert synergistic/antagonistic interactions among themselves or with other food constituents.

The RC of TP in broccoli juice depended on the HIPEF treatment conditions applied. As shown in Figure 3, broccoli juice processed by HIPEF at 25 kV/cm for 1250 μs retained

the highest content of TP (96.1%). In agreement with the present study, Odriozola-Serrano, Soliva-Fortuny, and Martín-Belloso (2008b) and Aguilar-Rosas, Ballinas-Casarrubias, Nevarez-Moorillon, Martín-Belloso, and Ortega-Rivas (2007) reported reductions in TP content after HIPEF processing of strawberry and apple juices, respectively. An incomplete inactivation of polyphenol oxidase, among other enzymes, can catalyse the polyphenol degradation (Giner, Ortega, Mesegué, Gimeno, Barbosa-Cánovas, & Martín, 2002). Hence, residual polyphenol oxidase activity might reduce the content of phenolic compounds in HIPEF-processed broccoli juice.

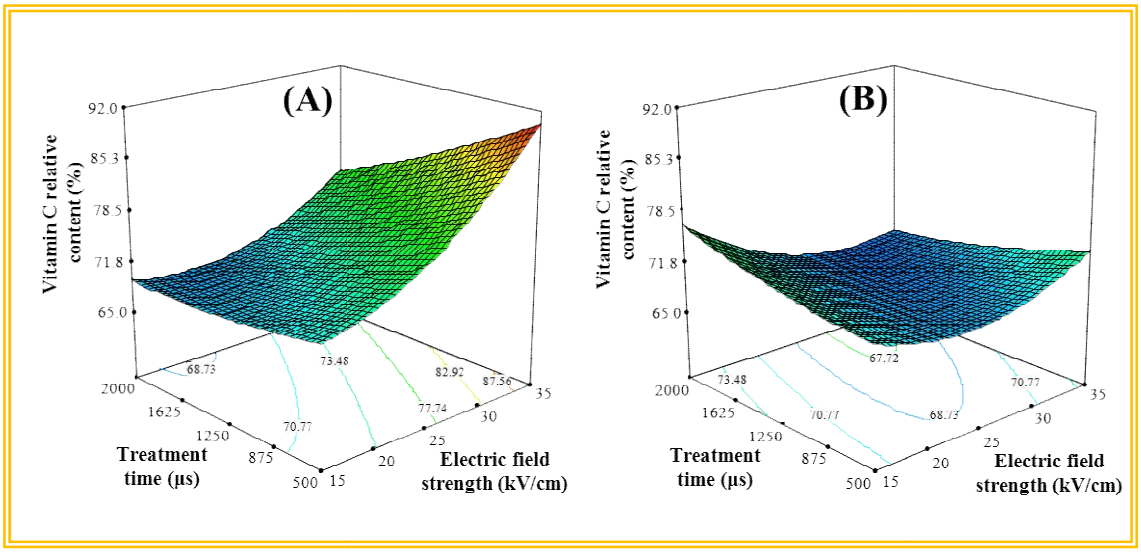

Figure 2. Effect of electric field strength and treatment time on vitamin C relative content of broccoli juice in monopolar (A) or bipolar (B) mode pulses.

Regarding AC, it was observed a reduction in the RAC of broccoli juice, irrespectively of the HIPEF treatment applied. Maximum RAC (95.9%) was achieved at 35 kV/cm for 1250 µs, although no significant differences were observed when broccoli juice was treated by HIPEF at 35 or 25 kV/cm for 1250 µs (Table 2). In this sense, Odriozola-Serrano, Soliva-Fortuny, Gimeno-Añó, and Martín-Belloso (2008) reported reductions in the AC of HIPEF-treated tomato juice. The authors showed that the highest AC was reached at 20 kV/cm in tomato juice, followed by that when processing at 35 kV/cm. The pattern observed in the present research could be associated to losses of some antioxidant compounds during HIPEF processing. Broccoli is a vegetable with a high AC (Kurilich, Jeffery, Juvik, Wallig, & Klein, 2002), and it has been suggested that the AC in broccoli and other vegetables depends on the scavenger substances found at high levels in foods (Sies & Stahl, 1995). In this context, among the bioactive compounds determined in the present work, vitamin C was the most abundant, followed by the phenolic compounds. However, it has been reported that the contribution of vitamin C to the total AC of broccoli was only 12% (Chu, Sun, Wu, & Liu, 2002). Other authors observed that the AC of broccoli juice was mainly ascribed to the phenolic compounds (Sun, Powers, & Tang, 2007). Likewise, the

HIPEF conditions (25 kV/cm for 1250 µs in bipolar mode) where the lowest RC of vitamin C (67%) was observed coincided with a high RAC (95.1%). In the same way, the greatest RC of TP (96.1%) corresponded to the most elevated RAC values (95.1%). Therefore, the reductions of AC in HIPEF-treated broccoli juice could be mainly attributed to the extent to which this treatment may modify the retention or losses of TP.

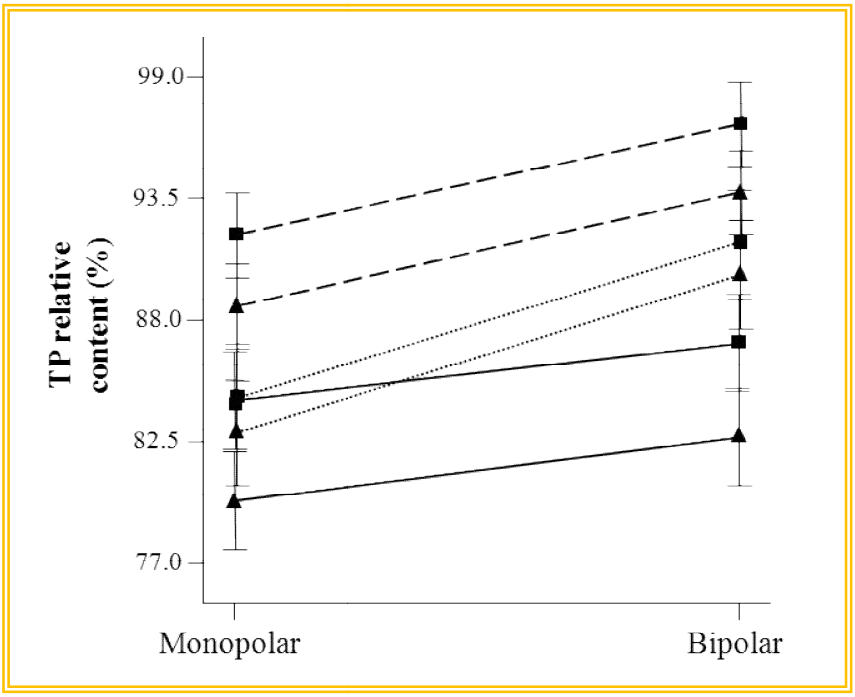

Figure 3. Effect of polarity on total phenolics relative content of broccoli juice at 15 (⋯), 25 (- -) and 35 kV/cm (–) for (■) 500 and (▲) 2000 µs.

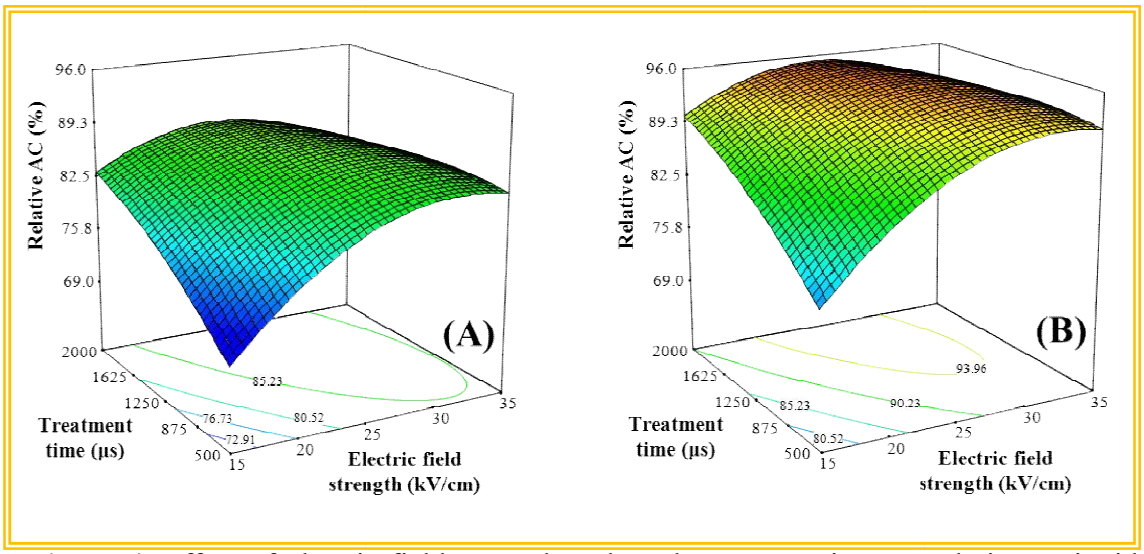

Figure 4. Effect of electric field strength and total treatment time on relative antioxidant capacity of broccoli juice in monopolar (A) or bipolar (B) mode pulses.

Effect of pulse polarity

As can be seen in Table 3, RC of lutein, β-carotene, vitamin C and TP, as well as the RAC, were significantly influenced by pulse polarity. An increment in the content of lutein (RC of 111.9%) and β-carotene (RC of 112.3%) was observed when broccoli juice was treated by HIPEF in bipolar mode; while applying pulses in monopolar mode, the RC of lutein diminished up to 93.9% and β-carotene augmented up to 103.5%. In agreement with the results reported in this research, HIPEF treatments in bipolar mode led to higher carotenoid content in HIPEF-treated tomato juice than that of monopolar mode (Odriozola-Serrano, Aguiló-Aguayo, Soliva-Fortuny, Gimeno-Añó, & Martín-Belloso, 2007).

Table 3. Analysis of variance of the second-order polynomial models for lutein, β-carotene, vitamin C, total phenols and antioxidant capacity of HIPEF-treated broccoli juice.

Source[a]	F value				
	Lutein	β-carotene	Vitamin C	TP	AC
Model	116.84[d]	72.78[d]	10.32[d]	20.75[d]	6.12[d]
E	609.74[d]	465.77[d]	9.85[b]	19.29[c]	7.75[b]
t	106.06[d]	51.75[d]	6.45[b]	6.79[b]	5.17[b]
P	125.92[d]	47.08[d]	16.26[c]	37.44[d]	16.84[c]
E^2	43.20[d]	4.86[b]	9.18[b]	84.08[d]	8.13[b]
t^2	9.64[b]	ns	ns	ns	ns
E x t	ns	ns	7.66[b]	ns	6.47[b]
E x P	11.41[b]	ns	24.31[c]	ns	ns
t x P	ns	ns	4.98[b]	ns	ns
Lack of fit	ns	ns	ns	ns	ns
Desv. Std	2.22	3.99	2.85	1.91	4.40
Mean	96.06	90.69	32.43	89.86	87.19
C.V.	2.32	4.4	8.79	2.13	5.05
R^2	0.98	0.97	0.83	0.91	0.84
Adjusted R^2	0.97	0.96	0.75	0.86	0.76

[a]*E*= Electric field strength, *t*= Treatment time; *P*= Polarity. TP = Total phenols; AC = Antioxidant capacity. [b]Significant at $p< 0.05$. [c]Significant at $p< 0.001$. [d]Significant at $p< 0.0001$. ns = Non-significant.

Fig. 3 shows the influence of polarity on RC of TP. Broccoli juice treated with pulses in bipolar mode led to greater TP content than those processed using monopolar mode pulses at any electric field strength and treatment time condition. For example, when HIPEF treatment in bipolar mode (at 25 kV/cm for 1250 μs) was applied to broccoli juice, the RC of TP was 96.1±2.8%, whereas the juice treated in monopolar mode exhibited a RC of 90.8±1.7%. Similar results were observed by Odriozola-Serrano, Soliva-Fortuny, and

Martín-Belloso (2009), who reported higher anthocyanin (a group of plant phenolic compounds) retention in strawberry juice after HIPEF treatments applied at 35 kV/cm for 1000 μs, in bipolar mode in comparison with monopolar mode.

In the same way, the RAC was higher in broccoli juice treated by HIPEF in bipolar mode than that of monopolar mode. For instance, broccoli juice exhibited a RAC of 81% when HIPEF treatments were applied at 15 kV/cm for 1250 μs with pulses in bipolar mode; however, at the same conditions of electric field strength and treatment time, the RAC was 72% in monopolar mode. The highest RAC observed in broccoli juice processed by HIPEF in bipolar mode coincided with the greatest RC of TP and carotenoids. Therefore, the RAC is a function of retention or reduction of the bioactive compounds in HIPEF-processed broccoli juice. These results agreed with the retention of TP and carotenoids observed after applying HIPEF treatments at the same polarity (bipolar mode) in watermelon juice (Oms-Oliu, Odriozola-Serrano, Soliva-Fortuny, & Martín-Belloso, 2009), tomato juice (Odriozola-Serrano, Aguiló-Aguayo, Soliva-Fortuny, Gimeno-Añó, & Martín-Belloso, 2007) and strawberry juice (Odriozola-Serrano, Soliva-Fortuny, Hernández-Jover, & Martín-Belloso, 2009).

It has been reported that oxidative reactions could take place during HIPEF processing. HIPEF treatments applied in monopolar mode may favor the occurrence of this phenomenon. In contrast, the application of electric pulses in bipolar mode reduces the occurrence of oxidative reactions (Morren, Roodenburg, & de Haan, 2003). This fact could explain why the highest retention of carotenoids and TP in broccoli treated by HIPEF in bipolar mode was reached.

On the contrary, HIPEF treatments in monopolar mode were found to be more effective in keeping the broccoli juice vitamin C than those applied in bipolar mode, except for treatments carried out at 15 kV/cm, where bipolar mode led to higher or similar RC of vitamin C than monopolar mode pulses (Fig. 2). For instance, at 35 kV/cm for 1250 μs vitamin C RC of broccoli juice was almost 10% higher in monopolar mode treatments than that observed in bipolar mode.

According to these results, it could be hypothesized that the enzymes associated to vitamin C degradation, such as ascorbate oxidase (Davey, Van Montagu, Inzé, Sanmartin, Kanellis, Smirnoff, et al., 2000), could be greatly inactivated after HIPEF treatments in monopolar mode than those applied in bipolar mode. In this context, other oxidative enzymes such as orange juice peroxidase (P. Elez-Martínez, Aguiló-Aguayo, & Martín-Belloso, 2006) and strawberry juice lipoxygenase (Aguiló-Aguayo, Sobrino-López, Soliva-Fortuny, & Martín-Belloso, 2008) were more efficiently inactivated when HIPEF treatments were applied in monopolar mode, as compared with that applied in bipolar mode.

The results obtained in this study were consistent with those reported by Odriozola-Serrano, Aguiló-Aguayo, Soliva-Fortuny, Gimeno-Añó, and Martín-Belloso (2007) and Oms-Oliu, Odriozola-Serrano, Soliva-Fortuny, and Martín-Belloso (2009) in HIPEF-treated tomato juice (at 35 kV/cm for 1000 μs) and watermelon juice (varying the treatment time, 50-2050 μs; and electric field strength, 25-35 kV/cm) , respectively. These authors (Odriozola-Serrano, Aguiló-Aguayo, Soliva-Fortuny, Gimeno-Añó, & Martín-Belloso, 2007; Oms-Oliu, Odriozola-Serrano, Soliva-Fortuny, & Martín-Belloso, 2009) concluded that RC

of vitamin C after applying bipolar pulses was lower in comparison to that of monopolar mode.

Comparison between thermal and HIPEF processing effects

The RC of antioxidant compounds and AC of HIPEF-processed broccoli juice was compared with that of thermally treated juice (90 ºC/60 s). In general, reduction in the lutein, β-carotene, vitamin C, and TP content, as well as the diminution of AC was greater in thermally treated broccoli juice than that processed by HIPEF (Table 2).

In agreement with the results reported in this study, Odriozola-Serrano, Soliva-Fortuny, Hernández-Jover, and Martín-Belloso (2009) observed a better preservation of tomato juice carotenoids after HIPEF processing than after thermal treatment. Min, Jin, and Zhang (2003), Sánchez-Moreno, Plaza, Elez-Martínez, De Ancos, Martín-Belloso, and Cano (2005), Zulueta, Esteve, and Frígola (2010) and P. Elez-Martínez and Martín-Belloso (2007) reported higher vitamin C losses in thermally processed tomato and orange juice, orange juice-milk beverage, and "gazpacho" respectively, with regard to the products treated by HIPEF.

On the other hand, Odriozola-Serrano, Soliva-Fortuny, Hernández-Jover, and Martín-Belloso (2009) and Quitão-Teixeira, Odriozola-Serrano, Soliva-Fortuny, Mota-Ramos, and Martín-Belloso (2009) did not find significant differences in the content of TP between thermally and HIPEF-processed tomato and carrot juice, respectively. Other studies carried out in apple and tomato juices, demonstrated that TP content was lower after heat processing than HIPEF treatments (Aguilar-Rosas, Ballinas-Casarrubias, Nevarez-Moorillon, Martín-Belloso, & Ortega-Rivas, 2007; Odriozola-Serrano, Soliva-Fortuny, & Martín-Belloso, 2008a).

Cortés, Barba, Esteve, González, and Frígola (2008) compared the AC between orange juices treated at 30 kV/cm for 100 μs and heat processed, showing that the highest AC occurred in HIPEF-treated orange juice. In contrast, Odriozola-Serrano, Soliva-Fortuny, and Martín-Belloso (2008a) reported that heat treatment had a similar AC respect to HIPEF-treated tomato juice. This fact could be related to the formation of Maillard reaction products during heat treatment, which could have antioxidant properties (Odriozola-Serrano, Soliva-Fortuny, & Martín-Belloso, 2008a).

In general, the application of heat to broccoli juice resulted in reduced content of bioactive compounds and thus diminution of AC, in comparison to HIPEF-treated and untreated broccoli juice. The AC depletion can be directly related to the losses of heat-sensitive nutrients such as vitamin C, phenolics, and carotenoids during juice processing (Odriozola-Serrano, Soliva-Fortuny, & Martín-Belloso, 2008b; Plaza, Sánchez-Moreno, Elez-Martínez, De Ancos, Martín-Belloso, & Cano, 2006). In addition, it has been demonstrated that foods processed by heat have lower nutritional value than their respective fresh foods, due to the loss of antioxidant compounds (Klopotek, Otto, & Böhm, 2005; Nicoli, Anese, & Parpinel, 1999; Richardson & Finley, 1985).

Differences between treatments (heat and HIPEF) could be also attributed to the oxidative reactions that take place during thermal processing and to the susceptibility of

most of the bioactive compounds contained in broccoli juice (vitamin C, TP, carotenoids, among others) to heat (Shoji, 2007). Additionally, it is important to highlight that throughout HIPEF treatments, the temperature of broccoli juice, at the inlet and outlet of each HIPEF chamber, never exceeded 35 °C. Therefore, bioactive compounds were better preserved by HIPEF than by heat.

Conclusion

The relative contents (RC) of lutein, β-carotene, vitamin C, total phenolic compounds and relative antioxidant capacity (RAC) of broccoli juice treated by HIPEF were significantly influenced by electric field strength, treatment time and polarity mode. HIPEF treatments applied in bipolar mode led to higher relative content of lutein, β-carotene, total phenolic and RAC than those applied in monopolar mode. In contrast, RC of vitamin C was greater when broccoli juice was treated by HIPEF in monopolar mode in comparison with bipolar mode. The RC of carotenoids (lutein and β-carotene) augmented when the broccoli juice was HIPEF-processed at the strongest conditions (35 kV/cm for 2000 μs in bipolar mode). The results demonstrated that the bioactive compounds of broccoli juice were better retained by HIPEF processing as compared with that treated by heat. Therefore, HIPEF technology could be considered as a good alternative to preserve the antioxidant characteristics of broccoli juice. Further research focused on the mechanistic insight of the changes in bioactive compounds during HIPEF processing of foods is required.

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3 HIGH-INTENSITY PULSED ELECTRIC FIELDS AND THERMAL TREATMENT OF BROCCOLI JUICE: THE EFFECTS OF PROCESSING ON MINERALS AND FREE AMINO ACIDS

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ABSTRACT

This research was focused on the influence of high-intensity pulsed electric fields (HIPEF) parameters, electric field strength (from 15 to 35 kV/cm), treatment time (from 500 to 2000 µs) and polarity (mono- or bipolar mode) on minerals and free amino acids content of broccoli juice. Additionally, HIPEF-processed broccoli juice was compared with thermally-treated (90 °C/60 s) and untreated juices. In general, HIPEF parameters significantly influenced the content of minerals and free amino acids, except Cu, S, P, Trp, Ala, His, and Tyr. At the strongest HIPEF conditions (35 kV/cm for 2000 µs) in bipolar mode, an increment or not significant changes in the mineral content in comparison to fresh broccoli juice was observed. Changes in the content of free amino acids depended of HIPEF treatment conditions applied and the amino acid evaluated. Significant losses of minerals and free amino acids were reached after thermal processing of broccoli juice. Overall, HIPEF-treated broccoli juice had superior mineral and free amino acids content than those thermally treated.

Keywords: High-intensity pulsed electric fields; thermal treatment; broccoli juice; minerals; free amino acids.

INTRODUCTION

Broccoli (*Brassica oleracea*) is a vegetable that belongs to *Brassicaceae* family and whose consumption has increased due to its multiple health benefits (Moreno, Carvajal, López-Berenguer, & García-Viguera, 2006). Broccoli is an excellent source of antioxidant compounds such as vitamin C, glucosinolates, polyphenols, as well as minerals and amino acids (Hounsome, Hounsome, Tomos, & Edwards-Jones, 2008; Moreno, Carvajal, López-Berenguer, & García-Viguera, 2006). In fact, broccoli has one of the highest concentration of some minerals (K, Mg, Ca) (Ekholm, Reinivuo, Mattila, Pakkala, Koponen, Happonen, et al., 2007) and amino acids (Arg, Leu, and Met) (Hounsome, Hounsome, Tomos, & Edwards-Jones, 2008), among diverse common vegetables consumed in the daily diet. Macro- (Ca, K, Na, Mg) and microminerals (Fe, Cu, Zn) are considered indispensable to maintain the physiological functions (Biziuk & Kuczyńska, 2007). Specifically, minerals participate in cell membrane transport, stabilize plasma membranes and nucleic acids, regulate the osmotic pressure, are cofactors of enzymes and constituents of vitamins, hormones and haemoglobin (Biziuk & Kuczyńska, 2007). Particularly selenium acts as an efficient antioxidant and may have cancer preventive effects (Navarro-Alarcon & Cabrera-Vique, 2008; Silvera & Rohan, 2007). On the other hand, the essential amino acids play important roles on human health. The principal function of amino acids are linked to peptide formation (Massey, Blakeslee, & Pitkow, 1998), are precursor of glucosinolates (Fahey, Zalcmann, & Talalay, 2001), serotonin and histamine (Wu, 2009). Moreover, amino acids have immune functions, controls blood coagulation, reduces the infarct size, exhibits oxygen free radical scavenging properties and some amino acids have been used in the treatment of osteoporosis, phenylketonuria, herpetic lesions, migraine headaches and respiratory infections, among others (Wu, 2009).

The major method commonly used to commercially preserve liquids foods is high temperature process. Application of heat is able to eliminate microorganisms and inactivate enzymes, which are responsible of foods deterioration. Nonetheless, the use of thermal treatments presents disadvantages, such as depletion of nutrients, and degradation of compounds responsible of color and flavour presents in foods (Fennema, 1985). Furthermore, consumers are increasingly claiming for safety, healthy and high quality foods (Linnemann, Benner, Verkerk, & Van Boekel, 2006). Hence, food preservation technologies, which allow obtaining foodstuff with high nutrient quality and natural freshness, have been developed. In fact, the treatment of liquid foods by high intensity pulsed electric fields (HIPEF) had shown beneficial effects on nutritional compounds as well as preserving their sensorial characteristics (Elez-Martínez, Escolá - Hernández, Soliva-Fortuny, & Martín-Belloso, 2005; Ortega-Rivas, 2007). Thus, HIPEF technology is able to provide products with adequate safety to consumers (Martín-Belloso & Elez-Martínez, 2005), a long shelf life (Elez-Martínez, Soliva-Fortuny, & Martín-Belloso, 2006; Qiu, Sharma, Tuhela, Jia, & Zhang, 1998), with minimal losses of nutrients (Odriozola-Serrano, Soliva-Fortuny, & Martín-Belloso, 2009). To the best our knowledge, there are scarce studies about how minerals and amino acids are affected by HIPEF. Pifarré, Martín, De Portela, Langini, Weisstaub, Greco, et al. (2006) reported no significant

influence on minerals (sodium, potassium, calcium, magnesium, and zinc) in a beverage based on orange juice and whey powder processed by HIPEF processing at 29 kV/cm. In this sense, Morales-de la Peña, Salvia-Trujillo, Rojas-Graü, and Martín-Belloso (2011) demonstrated that minerals as calcium and sodium did not suffer significant changes after HIPEF processing, compared to fresh and thermally treated juices, whereas other minerals increased or diminished during non-thermal treatments.

There was a significant increment in the total amino acid concentration of HIPEF treated-green tea infusions increasing electric field strength from 20 to 40 kV/cm (Zhao, Yang, Wang, & Lu, 2009). In contrast, Garde-Cerdán, Arias-Gil, Marsellés-Fontanet, Ancín-Azpilicueta, and Martín-Belloso (2007) did not find difference in total concentration of amino acid between thermal and HIPEF processing of grape juice. Therefore, this research attempts to evaluate the influence of high-intensity pulsed electric fields on broccoli juice minerals and amino acids concentration and compare them with those untreated and thermally-processed.

MATERIALS AND METHODS

Sample preparation.

Broccoli (*Brassica oleraceae* var. Italica) was purchased from a local grocery (Lleida, Spain). The vegetal was cut and the juice was obtained with a juice extractor. The juice was vacuum-degassed during 10 min, after being filtrated with cheesecloth. Broccoli juice was immediately treated by HIPEF or heat.

HIPEF equipment.

HIPEF treatments were carried out using a continuous-flow bench-scale system (OSU-4F, Ohio State University, Columbus, OH) that held monopolar or bipolar square wave pulses. The treatment system consisted of eight co-field flow chambers in series, each one containing two stainless steel electrodes. Temperatures of inlet and outlet of each pair of chambers were monitored during HIPEF treatment and never exceeded 35 °C. The temperature was maintained by using a cooling coil connected between each pair of chambers and submerged in an ice-water shaking bath. The treatment flow was controlled by a variable-speed pump (model 75210-25, Cole Parmer Instruments Company, Vernon Hills, IL, USA). Numerical variables were treatment time (500-2000 μs), and electric field strength (15-35 kV/cm), while a categorical variable was the polarity (mono- or bipolar), keeping the pulse width (4 μs) and frequency (100 Hz) constant.

Thermal treatments

Broccoli juice was thermally treated through a continuous system using a tubular heat exchange coil immersed in a hot water bath (University of Lleida, Spain) at 90 ºC for 60 s. The juice was immediately cooled in a heat exchange coil immersed in an ice water-bath.

Analysis of minerals

Mineral profile in broccoli juice was measured following the procedure proposed by Alwakeel and Al-Humaidi (2008) with some modifications. Broccoli juice (20 mL) was mixed with 10 mL of HCl in a volumetric flask and made up to 100 mL with water. The mixture was vigorously shaken, and centrifuged (10000*g* for 30 min at 4 ºC) and then analyzed by atomic absorption using ICP-OES Optical Emission Spectrometer, Horiba Jobin Yvon, Activa (USA). Ca, Cu, Fe, K, Mg, Mn, P, S, Zn, Na and Se were determined at wavelengths of 210.3, 324.8, 259.9, 766.5, 333.2, 257.6, 213.6, 180.7, 213.9, 589.6 and 196.0 nm, respectively. Quantification of the different elements was done using the calibration curves of the respective standard solution for each mineral.

Analysis of free amino acids

Analysis of amino acids was performed by the method described by López, Tenorio, Gutiérrez, Garde-Cerdán, Garijo, González-Arenzana, et al. (2012) with some modifications. Free amino acids were analysed by reverse phase HPLC using a Hewlett Packard Series 1100 liquid chromatograph equipped with an ALS automatic liquid sampler (Hewlett Packard 1100 Series), an Agilent 1100 fluorometric detector (FLD) and a Hewlett Packard UV-DAD 1100 Series detector (DAD). 5 mL of sample (previously centrifuged, 4000 rpm, 10 min) was mixed with 100 µL of norvaline (internal standard to quantify all amino acids except proline) and 100 µL of sarcosine (internal standard to quantify proline). The mixture was filtered through a 0.45 µm OlimPeak pore filter (Teknokroma, Barcelona, Spain) and submitted to an automatic precolumn derivatization with *o*-phthaldialdehyde (OPA Reagent, Agilent Technologies, Palo Alto, CA) for primary amino acids and with 9-fluorenylmethylchloroformate (FMOC Reagent, Agilent Technologies) for secondary amino acids. The injected amount from the derivated sample was 10 µL and a constant temperature of 40 ºC was maintained. All separations were performed on a Hypersil ODS (250 x 4.0 mm, I.D. 5 µm) column (Agilent Technologies). Solvents and gradient conditions for amino acids analysis are described below.

Two eluents were used as mobile phases: eluent A: 75 mM sodium acetate, 0.018% triethylamine (pH 6.9) + 0.3% tetrahydrofuran; eluent B: water, methanol, and acetonitrile (10:45:45, v/v/v). All reagents were first filtered with Millipore filters (0.45 µm). The gradient profile was: 0−15 min, 0%-47.5% B, 1.630 mL/min; 15−15.01 min, 47.5% B, 0.800 mL/min; 15.01−25 min, 47.5%-60% B, 0.800 mL/min; 25-25.01 min, 60% B, 1.630 mL/min;

25.01-26.01 min, 60%-100% B, 1.630 mL/min; 26.01-26.51 min, 100% B, 2.500 mL/min; 26.51-34.01 min, 100% B, 1.630 mL/min; 34.01-36.01 min, 100%-0% B, 1.630 mL/min. Detection was performed by fluorescence FLD detector (λ excitation = 340 nm, λ emission = 450 nm for primary amino acids, and λ excitation = 266 nm, λ emission = 305 nm for secondary amino acids) and DAD detector (λ= 338 nm for primary amino acids and λ= 262 nm for secondary amino acids). Identification of compounds was carried out by comparison of their retention times with those of pure reference standards. The pure reference compounds and internal standards were from Sigma (St. Louis, MO, USA). Water was obtained from a Milli-Q purification System (Millipore, USA). Quantification of amino acids was performed with an internal standard method.

Minerals and amino acids were expressed as relative content (RC) values respect to untreated juice (Equation 1).

$$RC(\%) = \frac{C_t}{C_0} x\ 100 \qquad \text{Eq. 1}$$

where C_t and C_0 are the content of the treated and untreated broccoli juice, respectively.

Statistical analysis

Each processing condition was assayed by duplicate and two replicate analyses were carried out in order to obtain the mean value for minerals and amino acids determination. Analysis of variance (ANOVA) and the least significance difference test (LSD) at the 5% significance level was performed for the determination of significance differences among HIPEF-processed, thermally-treated and fresh broccoli juice, using Statgraphics Plus v5.1 Windows package (Statistical Graphics Co., Rockville, MD, USA).

RESULTS AND DISCUSSION

Effects of HIPEF and thermal treatments on minerals of broccoli juice

The concentration for each mineral analyzed in untreated broccoli juice is displayed in Table 1. In descending order K, S, P, Na, Ca and Mg were the most abundant elements, among the minerals analysed. Values reported in the present research were in the range of those published in the literature for whole broccoli (Ekholm, et al., 2007; Kmiecik, Lisiewska, & Korus, 2007; López-Berenguer, Carvajal, Moreno, & García-Viguera, 2007; Rosa, Haneklaus, & Schnug, 2002; Sezgin, Esringu, Turan, Yildiz, & Ercisli, 2010).

The influence of HIPEF parameters (electric field strength, treatment time and polarity) on the relative content (RC) of minerals in HIPEF-processed broccoli juice is showed in Table 2. The content of all minerals increased or was maintained unchanged under some HIPEF conditions. Indeed, Fe, Mn and Zn exhibited the highest raises.

Table 1. Initial mineral and free amino acids concentration in untreated broccoli juice

Minerals (mg/100 mL)		Amino acids (mg/100 mL)			
Iron (Fe)	0.47 ±0.09	Isoleucine (Ile)	7.57±0.74	Alanine (Ala)	26.89 ±0.76
Potassium (K)	490.97 ±4.52	Leucine (Leu)	10.97 ±1.61	Asparagine (Asn)	18.47 ±2.36
Magnesium (Mg)	24.94 ±0.87	Lysine (Lys)	1.91 ±0.46	Aspartic acid (Asp)	15.81 ±2.04
Calcium (Ca)	36.19 ±1.14	Methionine (Met)	2.50 ±0.32	Glutamic acid (Glu)	20.43 ±2.47
Copper (Cu)	0.11 ±0.02	Phenylalanine (Phe)	5.81 ±0.95	Glycine (Gly)	3.80 ±0.73
Manganese (Mn)	0.16 ±0.01	Threonine (Thr)	10.91 ±1.46	Proline (Pro)	20.61 ±2.86
Sodium (Na)	45.31 ±1.79	Tryptophan (Trp)	1.31 ±0.26	Serine (Ser)	15.31 ±1.83
Phosphorus (P)	61.72 ±1.01	Valine (Val)	16.96 ±1.20	Tyrosine (Tyr)	3.90 ±0.64
Sulfur (S)	96.80 ±0.68	Arginine (Arg)	53.67 ±4.90		
Zinc (Zn)	0.63 ±0.1	Histidine (His)	19.84 ±3.43	Total amino acids	256.66 ±13.28
Selenium (Se)	0.0022 ±0.00				

[a] Data are means ± the standard deviation.

Polarity markedly influenced on RC of minerals, except for Cu. In general, monopolar pulses increased the content of Fe, Mn and Zn. For instance, after HIPEF treatments in monopolar mode at 35 kV/cm for 2000 µs the RC of Fe and Mn was 214.2 and 114.3%. Whereas, applying pulses in bipolar mode at the same conditions, the RC of those minerals was 203.3 and 103.6%, respectively. During HIPEF processing corrosion of stainless steel electrodes could occur, liberating Fe, Mn and Zn. Similarly, Evrendilek, Li, Dantzer, and Zhang (2004) observed a significant augment in the concentration of Fe, Cr, Zn and Mn in HIPEF-treated beer at 41 kV/cm for 175 µs. In this context, Roodenburg, Morren, Berg, and de Haan (2005) and Morren, Roodenburg, and de Haan (2003) reported that the release of minerals was a consequence of electrochemical reactions that happen on electrodes, depending on current magnitude, fluid composition, as well as pulse shape and duration (Roodenburg, Morren, Berg, & de Haan, 2005). Furthermore, monopolar pulses may cause higher corrosion of electrodes, thus the increment in the content of Fe, Mn and Zn could be explained. In the same way, Morren, Roodenburg, and de Haan (2003) reported that corrosion of electrodes can be limited by applying bipolar pulses.

On the other hand, electric field strength and treatment time had significant influence on RC of minerals with the exception of Cu, P and S. While S was neither influenced by electric field strength nor treatment time.

Se is one of the most important minerals contained in broccoli juice due to its antioxidant and anticarcinogenic properties. Se content increased 7% when electric field strength changed from 15 to 25 kV/cm (for 1250 µs in bipolar mode) and 9% when treatment time augmented from 500 to 2000 µs (at 15 kV/cm in bipolar mode). These results demonstrated that these HIPEF conditions afforded and/or improved the preservation of Se in broccoli juice. In the same line, Morales-de la Peña, Salvia-Trujillo, Rojas-Graü, and Martín-Belloso (2011) observed that the treatment time conditions tested (800 and 1400 µs) significantly affected the content of Cu, Mn, Zn, Ca and Mg of a fruit juice-soymilk beverage. In contrast, Akin and Evrendilek (2009) and Altuntas, Evrendilek,

Sangun, and Zhang (2010) reported that electric field strength and treatment time did not change significantly the mineral content of a formulated carrot juice-based beverage and sour cherry juice.

Regarding thermal treatment, the content of minerals was influenced by the effect of heat, except for K and P, which did not exhibit significant changes. Likewise, Pifarré, et al. (2006) did not found differences in the content of Na, K, Ca, Mg and Zn among HIPEF-processed (59 kV/cm for 59 μs), heat (75 ºC for 15 min) and untreated beverage based on orange juice and whey powder. Overall, there was a decrease of Fe, Ca, Mn, Na, Zn, Se content in heated broccoli juice, compared to some HIPEF conditions and just prepared juice. The trend observed in these elements suggests changes in their solubility due to thermal treatment as reported by Koplík, Mestek, Komínková, Borková, and Suchánek (2004), whom observed a diminution in the mineral content of peas after been cooked. These authors also proposed that thermal treatment could lead to denaturation of metallobiomolecules or liberation of the metal ions and formation of insoluble inorganic compounds. However, the extent of change in the content of minerals depends on the mineral stability. In contrast, Mg, Cu and S content incremented in thermally processed broccoli juice (Table 2), being Cu the mineral with the highest increment. The noticeable rise in Cu observed in this study could be associated to the release of this mineral from the tubular heat exchanger (made from copper) towards broccoli juice during this treatment. Additionally, the high temperature of this process (90 ºC) increasing the content of some minerals, such as Mg and S. In line with these results, Gutzeit, Winterhalter, and Jerz (2008) observed losses and increments in mineral content in heat-processed sea buckthorn juice, suggesting as causes of this tendencies to the processing steps. Morales-de la Peña, Salvia-Trujillo, Rojas-Graü, and Martín-Belloso (2011) observed significant changes in the Mn and Fe content in a fruit juice-soymilk beverage processed by heat. However, these authors did not find differences in the content of Cu, Ca and Mg between heat treatment, HIPEF (800 μs) and unprocessed beverage.

Effects of HIPEF and thermal treatments on amino acids

Table 1 display the free amino acid profile of untreated broccoli juice. The total free amino acid concentration was 256.66 mg/100 mL, of which Arg represented 21%.

Following Arg, other minerals such as Ala, Pro, Glu, His, Asn, Val, Asp, Ser, Leu, and Thr were the most abundant amino acids; while Ile, Phe, Tyr, Gly, Met, Lys and Trp were in lower concentrations. Similar values of individual amino acids in whole fresh broccoli were reported by Murcia, López-Ayerra, Martínez-Tomé, and García-Carmona (2001). In contrast, other studies have shown higher amino acid content than that reported in the present research (Kmiecik, Słupski, & Lisiewska, 2010). These differences could be associated to climatic conditions, growing seasons, and cultivars (Gomes & Rosa, 2001). Farming practices are an important factor in the amino acid content in broccoli. Indeed, soil fertilized with selenium showed noticeable increments in the amino acids content of broccoli florets (Lee, Finley, & Harnly, 2005).

The impact of HIPEF parameters (electric field strength, treatment time and polarity) and thermal treatment on the RC of free amino acids in broccoli juice is displayed in Table 3.

His and Lys were the amino acids with the highest RC (159.3 and 157.8%) when broccoli juice was HIPEF-treated at 25 kV/cm for 500 μs in bipolar mode and 15 kV/cm for 2000 μs in monopolar mode, respectively. Whereas the free amino acids content remained unchanged or even increased, respect to the fresh juice after treatments at 25 kV/cm in bipolar mode. On the contrary, Lys, Ser and Asn reached the lowest RC (17.8, 20.4 and 23.5%, respectively) when HIPEF treatments at 35 kV/cm for 2000 μs in monopolar mode were applied. In contrast, Garde-Cerdán, Arias-Gil, Marsellés-Fontanet, Ancín-Azpilicueta, and Martín-Belloso (2007) evaluated the effect of HIPEF processing on (35 kV/cm for 1 ms) on the grape juice amino acids, observing non-significant changes in the total concentration of amino acids after HIPEF treatment, respect to untreated juice.

Among HIPEF parameters evaluated, polarity influenced the RC of free amino acids. The highest RC for all amino acids was observed in monopolar mode at 15 kV/cm for 2000 μs (except for His). Conversely, the lowest RC was observed at the same polarity (excluding Pro) at the highest electric field strength and treatment time (35 kV/cm and 2000 μs). At this treatment condition a noticeable influence of HIPEF parameters interaction took place. Treatments applied in bipolar mode both at 25 and 35 kV/cm led to higher RC of amino acids than those in monopolar mode. An example, treatments at 35 kV/cm for 2000 μs in monopolar mode the content of Arg diminished a 66%, but at the same treatment conditions in bipolar mode, Arg content increased a 5.6%.

During HIPEF processing, electrochemical reactions could occur. In addition, treatments performed in monopolar mode might rise the reactions of oxidation (Barbosa-Cánovas, Pothakamury, Palou, & Swanson, 1998). This fact could explain the findings observed in the present research, where treatments in monopolar mode and at the strongest conditions (35 kV/cm for 2000 μs) led to the highest reductions in the amino acids content. Furthermore, oxidation reactions could result in the formation of free radicals, which react with amino acids reducing their content.

Electric field strength had significant influence on the RC of all free amino acids evaluated in broccoli juice. However, the magnitude of changes in the free amino acids content was a function of electric field intensity and the amino acid evaluated. For instance, when electric field strength augmented from 15 to 25 kV/cm (for 1250 μs in bipolar mode), the RC of Leu increased from 85.2 to 104.4%. Nonetheless, when electric field varied from 25 to 35 kV/cm, the content of Leu diminished from 104.4 to 74.6% (at the same treatment time and polarity). At the same HIPEF conditions, different tendencies were observed for Met. Met content diminished 6.2% as electric field strength changed from 15 to 25 kV/cm and 13.3% from 25 to 35 kV/cm (for 1250 μs in bipolar mode). In the same way, Zhao, Yang, Wang, and Lu (2009) assayed the effect of three different electric field strengths (20, 30 and 40 kV/cm) on green tea infusions. They reported that there was a significant increment in the total free amino acids concentration as electric field strength increased from 20 to 40 kV/cm. However, amino acids as Asp, Glu, Ser, Gly, Thr, among others, showed reductions in their concentration at 30 and/or 40 kV/cm.

Treatment time significantly influenced the RC of amino acids, except Trp and Ala. The content of almost all amino acids decreased as treatment time rose from 500 to 1250 μs at 15 kV/cm in monopolar mode; nevertheless, from 1250 to 2000 μs a noticeable increment in their content occurred (Table 3). Morales-de la Peña, Salvia-Trujillo, Garde-Cerdán, Rojas-Graü, and Martín-Belloso (2012) reported that immediately after HIPEF treatments at 35 kV/cm with 4 μs bipolar pulses at 200 Hz for 800 μs, the free amino acid content of a fruit juice-soymilk beverage remained with no significant changes, respect to fresh beverage. Nonetheless, when treatment time increased until 1400 μs, the concentration of amino acids decreased. Reductions in the amino acids content of HIPEF processed broccoli juice could be due to reactions of oxidation that inevitably occur on the electrode. In fact, the highest losses in the amino acid content at the strongest HIPEF conditions in monopolar mode were observed. At this polarity the electrochemical reactions and generation of free radicals are more common.

Table 2. Relative content (RC) of mineral in untreated, HIPEF and thermally processed broccoli juice.

Parameters[a]			RC (%)[b]										
Polarity mode	E (kV/cm)	t (µs)	Fe	K	Mg	Ca	Cu	Mn	Na	P	S	Zn	Se
Monopolar	15	500	187.1±3.0*cde*	97.2±2.9*a*	98.0±2.2*a*	98.5±1.7*defg*	98.0±2.7*a*	115.0±3.3*hij*	96.1±3.4*a*	98.7±1.9*abcde*	100.4±2.5*bcde*	118.2±4.0*de*	100.4±3.2*c*
		1250	197.7±1.0*f*	98.2±1.9*abc*	100.0±2.7*abcde*	96.5±3.6*cde*	99.5±3.1*abc*	116.3±4.4*ij*	96.3±2.8*a*	100.9±2.7*bcdef*	105.8±3.8*i*	119.2±3.0*de*	103.0±2.0*def*
		2000	204.6±3.2*i*	99.1±3.9*abcd*	100.2±2.3*abcde*	100.6±1.6*fgh*	100.2±2.1*abc*	117.7±1.5*j*	98.8±1.3*abc*	105.2±5.1*gh*	101.1±1.4*bcdefg*	130.9±3.3*h*	106.0±4.3*g*
	25	500	187.7±2.1*cde*	97.8±2.0*a*	98.5±1.5*ab*	94.3±2.2*bc*	98.8±3.8*abc*	109.5±2.2*de*	97.7±1.5*ab*	98.9±1.2*abcde*	101.1±1.4*bcdefg*	125.4±1.7*fg*	96.7±1.9*b*
		1250	198.9±4.3*fg*	100.3±2.5*abcde*	101.8±1.6*bcdef*	93.5±1.6*bc*	99.0±1.6*abc*	111.5±1.0*efg*	98.7±1.2*abc*	101.6±2.1*def*	104.5±1.9*hi*	128.5±1.9*gh*	101.7±0.8*cde*
		2000	213.5±3.6*j*	100.5±3.6*abcde*	100.3±3.1*abcde*	96.6±4.7*cde*	100.0±2.1*abc*	112.8±1.2*fgh*	103.8±1.6*ef*	105.8±3.2*h*	104.2±2.2*ghi*	137.2±1.8*i*	104.4±1.0*efg*
	35	500	189.0±3.2*de*	97.9±3.9*ab*	99.1±1.5*abc*	88.1±2.3*a*	99.0±1.1*abc*	111.1±2.0*ef*	100.1±1.4*bcd*	98.5±1.6*abcd*	103.8±1.3*fghi*	130.0±2.6*h*	106.9±1.9*ghi*
		1250	199.6±1.5*fgh*	100.6±1.9*abcde*	101.6±2.4*bcdef*	92.3±3.4*b*	99.9±3.5*abc*	112.9±0.5*fgh*	99.5±0.7*bcd*	102.0±1.3*efg*	103.9±0.7*fghi*	135.1±5.5*i*	105.4±0.9*fg*
		2000	214.2±3.0*j*	101.0±2.4*abcde*	102.1±2.2*cdef*	94.9±2.1*bcd*	101.9±1.1*c*	114.3±1.5*ghi*	105.6±2.5*f*	103.1±2.4*fgh*	104.2±1.9*ghi*	148.4±1.4*k*	109.1±1.1*hij*
Bipolar	15	500	184.1±2.9*c*	98.1±2.9*abc*	100.9±3.6*abcde*	100.9±1.3*fgh*	98.9±1.2*abc*	108.7±1.5*de*	99.5±2.4*bcd*	97.1±2.5*a*	99.4±3.0*abc*	114.2±1.1*c*	100.5±1.0*cd*
		1250	187.5±1.8*cde*	98.3±1.3*abc*	100.6±2.2*abcde*	100.5±2.8*fgh*	99.1±0.9*abc*	109.8±2.1*de*	103.7±1.5*ef*	97.7±2.5*ab*	103.4±0.9*efghi*	115.7±1.6*cd*	101.7±1.1*cde*
		2000	197.2±3.7*f*	101.7±2.0*bcde*	103.4±1.6*efg*	102.3±2.4*h*	100.2±3.3*abc*	110.2±1.3*ef*	103.7±1.5*ef*	99.3±2.3*abcde*	100.9±1.10*bcdef*	121.8±3.8*ef*	109.5±1.9*ij*
	25	500	184.4±3.5*c*	98.8±2.3*abcd*	101.9±2.9*cdef*	96.8±1.3*cde*	98.3±2.1*ab*	106.9±1.2*d*	99.7±3.0*bcd*	97.8±1.5*ab*	101.7±1.6*cdefgh*	116.0±2.4*cd*	105.9±1.4*g*
		1250	188.6±1.8*de*	101.9±3.5*cde*	104.9±2.4*fgh*	100.5±2.7*fgh*	99.1±1.7*abc*	110.0±2.7*ef*	101.7±2.2*de*	98.4±1.7*abcd*	97.1±1.9*a*	121.2±2.9*e*	106.4±2.1*gh*
		2000	202.7±1.7*ghi*	102.5±2.5*de*	106.0±1.9*gh*	101.7±2.3*gh*	100.4±1.2*abc*	110.1±2.4*ef*	100.8±1.3*cd*	99.9±2.5*abcdef*	99.7±2.6*abc*	127.8±1.0*gh*	109.4±1.2*ij*
	35	500	185.1±2.9*cd*	99.0±1.3*abcd*	103.2±1.1*defg*	95.1±3.22*bcd*	99.4±3.6*abc*	100.8±2.7*bc*	106.0±2.7*fg*	97.2±2.6*a*	100.7±1.5*bcdef*	144.1±1.1*j*	109.7±1.7*j*
		1250	190.1±3.2*e*	102.3±1.4*de*	105.9±2.1*gh*	94.9±1.7*bcd*	100.3±3.0*abc*	103.2±0.9*c*	108.5±2.1*gh*	98.1±2.8*abc*	100.1±4.4*abcd*	144.7±1.6*j*	109.0±1.0*hij*
		2000	203.3±1.8*hi*	104.0±3.3*e*	107.4±1.0*h*	98.0±2.9*def*	101.6±1.2*bc*	103.6±2.9*c*	109.1±1.5*h*	99.9±1.6*abcdef*	98.3±3.0*ab*	145.1±1.5*jk*	106.3±1.9*gh*
Thermal processing (90°C/60s)			82.5±4.4*a*	99.2±4.7*abcd*	105.6±5.1*gh*	86.7±3.1*a*	123.7±3.6*d*	84.5±2.4*a*	96.5±2.2*a*	101.3±2.6*cdef*	103.2±3.8*defghi*	90.8±2.7*a*	84.0±3.2*a*
Untreated broccoli juice			100.0*b*	100.0*abcd*	100.0*abcd*	100.0*efgh*	100.0*abc*	100.0*b*	100.0*bcd*	100.0*abcdef*	100.0*abcd*	100.0*b*	100.0*c*

Data are means ± the standard deviation. Different letters in the same column indicate significant difference among treatments ($p < 0.05$; LSD test).

[a] E=Electric field strength; t=Treatment time. [b]Fe = Iron, K = Potassium, Mg = Magnesium, Ca = Calcium, Cu = Copper, Mn = Manganese, Na = Sodium, P = Phosphorus, S = Sulfur, Zn = Zinc, Se = Selenium.

Table 3. Relative content (RC) of free amino acids in untreated, HIPEF and thermally processed broccoli juice.

Parameters			RC (%)									
Polarity mode[a]	E (kV/cm)	t (μs)	Ile	Leu	Lys	Met	Phe	Thr	Trp	Val	Arg	His
Monopolar	15	500	119.2±2.2*j*	121.4±7.4*l*	125.2±1.7*gh*	104.3±5.6*ghi*	117.6±3.4*i*	122.4±2.6*j*	122.1±3.1*ij*	111.2±0.4*jk*	117.7±1.2*i*	154.5±1.1*g*
		1250	92.0±1.9*cde*	91.2±0.2*fgh*	82.3±6.1*de*	89.0±10.2*def*	89.7±2.1*de*	103.1±3.7*fgh*	119.3±5.9*ij*	93.5±2.5*def*	97.7±0.1*cde*	109.5±7.0*bcdef*
		2000	130.5±1.8*k*	135.0±3.1*m*	157.8±1.9*j*	120.5±11.6*j*	137.0±1.7*j*	129.0±1.0*j*	131.7±14.3j	125.0±1.6*l*	139.6±5.8*j*	145.3±0.9*g*
	25	500	101.5±0.4*gh*	88.1±3.0*fg*	89.7±6.4*ef*	102.4±8.5*j*	91.2±1.8*def*	99.6±0.2*fg*	94.0±5.3*cdefg*	106.4±1.4*hij*	117.9±6.3*i*	123.4±6.8*ef*
		1250	86.7±2.5*bcd*	87.0±3.4*f*	86.0±0.6*e*	111.0±10.6*hij*	90.0±1.6*de*	90.4±0.1*de*	85.6±1.7*cde*	107.1±1.7*hijk*	108.9±1.0*gh*	111.9±4.2*cdef*
		2000	83.7±10.5*b*	78.6±3.6*e*	70.6±3.3*cd*	110.2±4.4*hij*	83.0±2.0*c*	85.1±3.3*cd*	79.3±0.8*c*	86.2±9.7*bcd*	100.0±3.7*def*	107.7±19.0*bcde*
	35	500	96.8±3.3*efg*	78.6±4.0*e*	60.4±9.6*c*	76.7±8.8*cd*	87.4±0.1*cd*	84.3±2.5*cd*	78.2±3.1*c*	88.7±2.6*cde*	88.9±1.4*b*	98.4±4.3*bc*
		1250	85.5±3.2*bc*	58.3±1.4*c*	59.3±9.4*c*	62.0±10.3*bc*	61.0±1.1*b*	85.9±0.9*d*	60.2±5.2*b*	84.3±3.9*bc*	94.2±2.5*bcd*	96.8±2.9*bc*
		2000	43.8±4.0*a*	35.1±0.2*a*	17.8±1.5*a*	48.8±4.2*ab*	38.8±0.5*a*	36.8±5.1*a*	33.2±4.6*a*	47.0±2.9*a*	34.0±1.8*a*	40.7±2.3*a*
Bipolar	15	500	92.4±0.5*cdef*	94.6±1.4*hi*	116.8±19.4*g*	94.8±1.3*efg*	95.1±0.6*efg*	103.1±3.8*fgh*	93.2±0.9*cdef*	97.9±2.0*fg*	104.3±5.9*efg*	106.5±1.3*bcd*
		1250	92.7±2.2*cdef*	85.2±3.3*f*	94.3±7.2*ef*	104.9±3.8*ghi*	96.0±4.1*efgh*	95.9±5.5*ef*	109.4±1.0*ghi*	80.5±8.7*b*	99.3±1.6*def*	107.7±10.3*bcde*
		2000	101.7±1.7*gh*	94.1±0.3*ghi*	88.5±2.7*ef*	116.6±3.6*ij*	101.6±3.1*h*	109.3±10.8*hi*	112.1±1.8*hi*	102.4±1.3*ghi*	108.4±5.6*gh*	110.8±9.0*cdef*
	25	500	120.7±4.3*j*	103.1±4.0*k*	139.8±0.6*i*	118.4±6.1*ij*	116.5±3.8*i*	112.0±3.1*i*	97.5±2.5*defgh*	114.6±5.1*k*	120.2±0.8*i*	159.3±18.4*g*
		1250	113.7±1.9*ij*	104.4±0.1*k*	133.6±0.2*hi*	98.7±1.2*efgh*	115.8±1.0*i*	110.2±0.2*hi*	96.9±4.0*defgh*	112.6±1.7*jk*	114.7±2.6*hi*	100.1±6.0*bc*
		2000	108.4±4.9*hi*	101.7±3.8*jk*	116.3±4.7*g*	93.9±3.8*efg*	118.2±6.8*i*	105.8±1.2*ghi*	111.3±10.7*hi*	109.1±3.3*ijk*	109.4±0.1*gh*	95.9±2.8*bc*
	35	500	102.1±3.0*gh*	96.6±0.6*hij*	59.7±7.1*c*	94.9±14.3*efg*	97.0±4.2*fgh*	96.4±0.1*ef*	101.8±10.0*fgh*	105.8±3.7*hij*	106.4±0.6*fg*	120.9±7.4*def*
		1250	90.0±3.6*bcde*	74.6±0.6*de*	33.8±4.6*b*	85.4±3.6*def*	81.3±5.2*c*	85.8±3.7*d*	83.3±0.2*cd*	95.7±0.7*efg*	105.6±5.4*fg*	124.3±4.7*f*
		2000	93.5±5.5*def*	69.0±1.5*d*	30.1±6.9*ab*	84.9±5.9*de*	82.0±4.5*c*	75.2±0.4*b*	121.6±7.5*ij*	102.4±1.6*ghi*	105.6±0.6*fg*	111.4±2.4*cdef*
Thermal processing (90 °C/60 s)			89.6±1.9*bcde*	46.4±4.6*b*	37.5±3.4*b*	42.5±1.6*a*	58.9±1.3*b*	77.9±1.6*bc*	82.9±0.5*cd*	84.5±1.0*bc*	91.3±5.4*bc*	94.0±8.2*b*
Untreated broccoli juice			100.0*fg*	100.0*ijk*	100.0*f*	100.0*fgh*	100.0*gh*	100.0*fg*	100.0*efgh*	100.0*fgh*	100.0*def*	100.0*bc*

Table 3. Continued

Parameters[a]			RC (%)[b]								
Polarity mode	E (kV/cm)	t (µs)	Ala	Asn	Asp	Glu	Gly	Pro	Ser	Tyr	Total
Monopolar	15	500	99.4±4.1*ef*	120.3±0.1*ij*	101.5±2.0*cd*	134.1±2.2*j*	125.1±0.4*i*	112.4±2.4*i*	126.0±1.5*j*	128.2±4.3*i*	120.6±0.6*lm*
		1250	86.9±1.7*c*	94.5±2.1*c*	104.2±4.9*cde*	110.4±0.4*fg*	98.2±0.4*ef*	100.9±0.7*gh*	99.8±3.5*defg*	102.2±1.4*fgh*	98.1±1.5*efg*
		2000	130.3±1.8*i*	139.2±3.8*k*	140.2±6.7*j*	137.4±1.2*j*	132.8±1.7*j*	148.3±1.4*j*	144.2±0.6*k*	144.4±6.1*j*	140.1±5.8*n*
	25	500	105.6±1.8*fgh*	112.8±1.5*hi*	133.3±2.4*ij*	120.7±0.5*hi*	97.2±1.5*ef*	99.1±3.3*efgh*	104.8±4.1*fg*	101.3±6.4*fgh*	110.1±0.2*jk*
		1250	102.8±7.1*efgh*	107.2±1.9*efgh*	115.4±1.7*efg*	113.5±0.3*fgh*	101.4±4.6*fg*	94.6±4.0*bcde*	102.3±5.1*efg*	94.7±1.6*efg*	105.0±1.0*hij*
		2000	102.3±3.5*efg*	96.8±1.3*cd*	86.5±10.4*b*	93.2±3.9*cd*	95.4±4.2*ef*	96.6±2.0*defg*	92.7±3.2*cde*	91.3±4.8*ef*	94.6±2.0*def*
	35	500	89.2±1.2*cd*	103.2±2.3*def*	93.7±5.5*bc*	88.3±3.9*bc*	95.3±0.7*ef*	104.3±2.7*h*	104.7±1.6*fg*	78.7±0.4*cd*	91.7±4.4*cd*
		1250	89.3±2.2*cd*	85.2±3.5*b*	99.3±3.2*cd*	80.5±2.9*b*	92.9±1.1*de*	91.8±3.4*bcd*	80.2±4.6*b*	64.0±6.1*ab*	85.6±1.3*bc*
		2000	49.8±1.9*a*	23.5±1.3*a*	50.8±0.1*a*	34.3±1.9*a*	31.9±4.6*a*	167.3±3.1*k*	20.4±1.4*a*	52.0±3.0*a*	44.0±1.3*a*
Bipolar	15	500	96.6±3.3*de*	103.5±3.2*defg*	101.2±3.0*cd*	107.3±2.6*ef*	100.4±5.1*f*	95.1±3.2*bcdef*	102.3±3.6*efg*	100±5.8*fgh*	101.9±2.2*ghi*
		1250	101.0±4.8*ef*	83.4±6.5*b*	93.4±2.1*bc*	97.2±4.7*d*	95.9±5.7*ef*	94.5±3.9*bcde*	80.1±9.1*b*	97.3±6.0*fgh*	93.4±1.3*de*
		2000	98.8±7.7*ef*	102.8±6.9*cdef*	106.4±6.9*def*	109.4±0.8*fg*	96.4±1.9*ef*	100.1±0.8*fgh*	98.1±4.7*def*	102.1±0.7*fgh*	107.5±5.0*ij*
	25	500	95.2±1.9*de*	121.5±0.4*j*	127.9±4.0*hi*	135.4±3.4*j*	107.3±4.4*gh*	104.3±3.9*h*	121.9±5.8*ij*	106.9±12.6*gh*	123.0±3.3*m*
		1250	110.2±2.0*h*	112.1±3.6*hi*	116.9±9.1*fgh*	125.0±0.6*i*	119.2±2.7*i*	99.9±2.8*fgh*	115.3±1.3*hi*	107.4±3.7*h*	114.8±6.2*kl*
		2000	109.6±1.5*gh*	111.8±4.6*gh*	97.9±5.1*cd*	116.7±5.4*gh*	108.8±1.4*h*	102.7±0.2*h*	109.0±5.7*gh*	101.5±0.3*fgh*	111.0±4.7*jk*
	35	500	85.0±1.4*c*	110.5±2.8*fgh*	123.0±2.6*ghi*	112.9±11.9*fgh*	93.0±2.3*de*	78.2±0.7*a*	108.3±6.5*gh*	93.0±12.7*ef*	100.6±2.1*fgh*
		1250	85.5±4.0*c*	105.1±1.7*defgh*	122.8±9.8*ghi*	109.5±0.5*fg*	81.8±5.3*bc*	90.4±0.4*b*	97.6±2.3*def*	83.1±3.9*de*	96.2±2.9*defg*
		2000	91.2±3.0*cd*	110.5±6.9*fgh*	128.1±3.4*hi*	108.5±5.1*f*	88.0±1.9*cd*	96.1±2.1*cdefg*	90.6±7.8*cd*	77.6±11.3*cd*	98.7±1.8*efgh*
Thermal processing (90 °C/60 s)			65.5±6.0*b*	83.5±9.7*b*	101.2±8.1*cd*	84.5±4.3*b*	80.2±3.5*b*	91.3±1.7*bc*	83.9±6.8*bc*	68.6±0.8*bc*	84.2±1.5*b*
Untreated broccoli juice			100.0*ef*	100.0*cde*	100.0*cd*	100.0*de*	100.0*f*	100.0*fgh*	100.0*defg*	100.0*fgh*	100.0*fgh*

Data are means ± the standard deviation. Different letters in the same column indicate significant difference among treatments ($p < 0.05$; LSD test).
[a]E = Electric field strength; t = Treatment time. [b]Ile = Isoleucine, Leu = Leucine, Lys = Lysine, Met = Methionine, Phe = Phenylalanine, Thr = Threonine, Trp = Tryptophan, Val = Valine, Arg = Arginine, His = Histidine, Ala = Alanine, Asn = Asparagine, Asp = Aspartic acid, Glu = Glutamic acid, Gly = Glycine, Pro = Proline, Ser = Serine, Tyr = Tyrosine.

Increments in the content of some free amino acids have been also reported by other authors. Garde-Cerdán, Arias-Gil, Marsellés-Fontanet, Ancín-Azpilicueta, and Martín-Belloso (2007) proposed that during HIPEF processing the formation of pores through the plasmatic membrane and organelle disruption took place. As a consequence, amino acid, as well as proteins and proteases were released. Thus these enter into contact leading to the protein degradation in smaller peptides and amino acids.

Furthermore, the characteristics of HIPEF equipment used, treatment conditions, as well as the complexity of the food matrix could be determinant factors in the differences between the results exposed in this study and the reported by other authors. Nevertheless, further research focused on the mechanisms of the increment and reduction in free amino acids in HIPEF-treated broccoli juice is needed.

On the other hand, in thermally treated broccoli juice significant reductions in the content of amino acids were observed, except for Asp. In agreement with the results showed in this research, Murcia, López-Ayerra, Martínez-Tomé, and García-Carmona (2001) reported that the amino acids concentration was reduced when the whole broccoli was treated by heat at different times.

When broccoli juice is heated a wide variety of chemical reactions on the amino acids can happen, such as carboxylation, dehydrogenation, isomerization, Maillard reaction. These reactions could lead to amino acid degradation by the effect of high temperature (Belitz, Grosch, & Schieberle, 2009; Feeney, Whitaker, Wong, Osuga, & Gershwin, 1985).

In contrast, Garde-Cerdán, Arias-Gil, Marsellés-Fontanet, Ancín-Azpilicueta, and Martín-Belloso (2007) did not found differences in the free amino acids concentration between thermally treated and untreated grape juice.

CONCLUSIONS

HIPEF parameters (electric field strength, treatment time and polarity) significantly influenced on the RC of minerals and amino acids of broccoli juice. Under certain HIPEF conditions there were increments of all minerals and amino acids. This pattern never happened when broccoli juice was processed by heat. In fact, thermal treatment caused significant losses of some minerals and free amino acids in broccoli juice. In general, the content of minerals was better retained when HIPEF processing was applied, in comparison to thermal treatment, having as reference the untreated broccoli juice. It has been demonstrated that HIPEF is a promising non-thermal technology, mainly in the preservation of minerals and amino acids of broccoli juice.

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4 BROCCOLI JUICE PROCESSED BY HIGH-INTENSITY PULSED ELECTRIC FIELDS: INFLUENCE ON OXIDATIVE ENZYMES AND COLOR

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ABSTRACT

The aim of this work was assess the influence of high-intensity pulsed electric field (HIPEF) processing parameters, [electric field strength (15-35 kV/cm), treatment time (500-2000 µs) and polarity (mono- or bipolar mode)] on color difference (Δ*E*), polyphenol oxidase (PPO) and lipoxygenase (LOX) activity in broccoli juice. Besides, the results of residual activities of PPO (RA_{PPO}) and LOX (RA_{LOX}), as well as the Δ*E* of broccoli juice processed by HIPEF were compared with those obtained from a traditional thermal treatment (90 °C/ 60 s). At the strongest HIPEF conditions (35 kV/cm for 2000 µs in bipolar mode), the maximum color difference (2.03) and the lowest RA_{LOX} (68.71%) and RA_{PPO} (36.11%) were reached. Broccoli juice processed by HIPEF in bipolar mode showed lower RA_{LOX} and RA_{PPO} than that treated in monopolar mode. In contrast, polarity did not influence on the broccoli juice color. Within the range of assayed conditions, broccoli juice LOX was more resistant to HIPEF treatment than PPO. The highest Δ*E* (7.89) was observed in the broccoli juice processed by heat, but also a complete LOX and PPO inactivation was reached. HIPEF processing was appropriate to preserve the color properties and reduce the enzyme activity of broccoli juice.

Keywords: High-intensity pulsed electric fields; broccoli juice; polyphenol oxidase; lipoxygenase, color.

INTRODUCTION

Broccoli is a good dietary source of vitamins, pigments and antioxidants, among others compounds (Moreno, Carvajal, López-Berenguer, & García-Viguera, 2006). This fact makes broccoli juice susceptible to oxidative enzymes action. Lipoxygenase (LOX, EC 1.13.11.12) and polyphenol oxidase (PPO, EC 1.14.18.1) are oxidoreductases (Ramírez et al., 2003) present in vegetables, such as broccoli. These enzymes are associated to off-color, off-flavours and nutrient losses in vegetables (Adams, 2010; Song, 2010). LOX is capable to catalyze the oxidation of polyunsaturated fatty acids producing hydroperoxides (O'Conor and O'Brien, 1999), the generation of free radicals (Robinson, Wu, Domoney, & Casey, 1995) and it has been also related to the degradation of chloroplast pigments (Gallardo-Guerrero, Jarén-Galân, Hornero-Méndez, & Mínguez-Mosquera, 2003), and ascorbic acid oxidation (Roy & Kulkarni, 1996). On the other hand, PPO participates in the phenolic oxidation forming brown pigments (Vámos-Vigyázó, 1981). Therefore, inactivation of LOX and PPO is desirable in order to maintain the quality and extent the shelf life of plant products.

Heat is the most common treatment applied to inactivate enzymes during juice processing; however, it leads to losses of chemical, physical and nutritional properties of foods (Odriozola-Serrano, Aguiló-Aguayo, Soliva-Fortuny, & Martín-Belloso, 2013). The increasing consumers demand for fresh appearance and nutritious products, as well as the mentioned drawbacks of thermal processing has contributed to the development of novel preservation technologies that are able to preserve the nutritional and sensory quality of liquid foods. High-intensity pulsed electric fields (HIPEF) is based on the application of short duration and high voltage pulses to liquid foods placed between two electrodes, where the heating produced in each pulse is minimal (Barbosa-Cánovas, Góngora-Nieto, Pothakamury, & Swanson, 1999). This technology is gaining the interest of food technologists and researchers because it has shown an efficient microbial inactivation in foods (Mosqueda-Melgar, Elez-Martínez, Raybaudi-Massilia, & Martín-Belloso, 2008; Yeom, Streaker, Zhang, & Min, 2000) without significant losses of their sensorial and nutritional characteristics (Ortega-Rivas, 2007).

Studies related to the effect of HIPEF on oxidative enzymes in fruit and vegetable juices have shown diverse grades of inactivation. Strawberry juice PPO was inactivated until 90% when it was exposed to HIPEF treatments (Aguiló-Aguayo, Soliva-Fortuny, & Martín-Belloso, 2010a). Giner, Gimeno, Barbosa-Cánovas, and Martín (2001) conducted experiments with apple and pear PPO extracts, where apple PPO extract exhibited an almost total inactivation (96.8%), whereas pear PPO extract showed 62% of activity reduction. In contrast, Van Loey, Verachtert, and Hendrickx (2001) described that HIPEF treatments resulted in less than 10% of inactivation of apple PPO and no inactivation of LOX from green pea juice. In tomato juice, maximum LOX inactivation (19.7%) was reached at 35 kV/cm for 1000 μs in monopolar mode (Aguiló-Aguayo, Soliva-Fortuny, & Martín-Belloso, 2009). A 80% of LOX inactivation was reported by Min, Min, and Zhang (2003) when tomato juice was HIPEF-treated at 35 kV/cm for 50 or 60 μs.

Among the physical characteristics of vegetables, the color stands out due to its importance in the rejection or acceptance of foods by consumers. Nonetheless, color

losses are linked to pigments degradation by enzyme action. Some authors have evaluated the influence of HIPEF technology on color properties of fruit and vegetable juices. For instance, Akin and Evrendilek (2009) did not find differences in color between fresh carrot juice and that processed by HIPEF. On the contrary, Cortés, Esteve, Rodrigo, Torregrosa, and Frígola (2006) observed high difference among HIPEF-treated orange juice and that unprocessed. Although there is available information about the HIPEF influence on enzymes and color in fruit juices (Aguiló-Aguayo et al., 2010; Aguiló-Aguayo et al., 2009; Aguiló-Aguayo et al., 2008; Quitão-Teixiera et al., 2008), the literature related to vegetable juices, mainly broccoli juice is scarce. Therefore, the objective of this research was to study the influence of HIPEF variables (electric field strength, treatment time and polarity) on LOX, and PPO activities, as well as color. Moreover, the outcomes of the HIPEF effect on LOX, PPO and color of broccoli juice were compared with that obtained from heat-treated juice, having as reference the unprocessed broccoli juice.

MATERIALS AND METHODS

Sample preparation

Broccoli (*Brassica oleraceae* var. italica) at commercial maturity was purchased in a local grocery (Lleida, Spain). The vegetable was cut and crushed. The resulting juice was filtered through cheesecloth and vacuum degassed during 10 min. Broccoli juice was immediately treated by HIPEF or heat.

HIPEF equipment

HIPEF processing was carried out using a continuous-flow laboratory-scale system (OSU-4F, The Ohio State University, Columbus) that provides square-wave pulses within eight co-field flow chambers in series, each one containing two stainless steel electrodes. The flow was controlled by a variable speed pump (model 75210-25, Cole Parmer, Vernon Hills, IL, USA). The inlet and outlet temperatures of each pair of chambers were monitored during HIPEF treatment. The temperature eas kept below 35 ^{o}C by using a cooling coil connected between each pair of chambers and submerged in an ice-water shaking bath.

Thermal treatments

Broccoli juice was processed at 90 ^{o}C for 60 s in a tubular heat exchanger coil immersed in a hot water shaking bath using a gear pump (Universitat de Lleida, Lleida, Spain). The juice was immediately cooled down to 7±1 °C, using a heat exchange coil immersed in an ice-water shaking bath.

Enzyme activity determination

Lipoxygenase (LOX)

LOX activity in broccoli juice was determined using the method described by Anese and Sovrano (2006) with some modifications by continuously monitoring the formation of conjugated dienes from linoleic acid. LOX was extracted by mixing 5 mL of the juice with 2 mL of sodium phosphate buffer (70.95 g/L, pH 6.5) and 0.5% Triton X-100 in a centrifuge tube. The homogenate was centrifuged for 15 min at 10000 *g* at 4 °C (Centrifuge AVANTI™ J-25, Beckman Instruments Inc., Fullerton, CA, USA) and the pellet was discarded. The substrate consisted of 10 µL of linoleic acid, 4 mL of distilled water, 1 mL of 0.05 mol L^{-1} NaOH and 5 µL of Tween 20. The mixture was shaken and diluted to 25 mL with distilled water. Activity measurements were carried out at 25 °C. Each cuvette contained 2.7 mL of 0.2 mol L^{-1} phosphate buffer (pH 6.5) and 40 µL of substrate. The reaction started by adding 100 µL of enzyme extract and the increase in absorbance was followed spectrophotometrically (Cecil Instruments Ltd, Cambridge, UK) at 234 nm for 3 min at 22 °C. LOX activity was calculated from the slope of the linear portion of the curve obtained. One unit of LOX activity was defined as a change in absorbance/min per mL of enzyme extract.

Polyphenoloxidase (PPO)

PPO activity was assayed according to the method described by Sorensen et al., (1999). Enzyme extracts were obtained by homogenization of 5 mL broccoli juice with 50 mL of 0.2 mol L^{-1} acetic acid. Then, the homogenate was centrifuged at 10000 rpm during 10 min (4 °C) (Centrifuge AVANTI™ J-25, Beckman Instruments Inc., Fullerton, CA, USA). The supernatant was filtered through a Whatman No 1 paper and the collected liquid was the enzyme extract. PPO activity was determined spectrophotometrically by placing 1 mL of 0.1 mM chlorogenic acid dissolved in 20 mM phosphate buffer (pH 7.0), 1.45 mL of 0.2 mM phosphate buffer (pH 7.0) and 200 µL of the enzymatic extract in a 1 cm path cuvette. Absorbance was read using a CECIL CE 2021 spectrophotometer (Cecil Instruments Ltd, Cambridge, UK). PPO activity was determined by measuring the initial rate of reaction, which was computed from the linear portion of the plotted curve. One unit of PPO activity was defined as a change in absorbance at 470 nm/min per mL of enzymatic extract.

The relative residual activity of LOX and PPO, RA (%), was defined by Equation 1:

$$RA\ (\%) = \frac{A_t}{A_0} x100 \qquad \text{Eq. 1}$$

where A_t and A_0 were the enzyme activities of treated and untreated samples, respectively.

Color measurements

The color of broccoli juice was measured using a Colorimeter (Minolta CR-400, Konica Minolta Sensing, Inc., Osaka, Japan) at room temperature. CIE *L**, a* and b* coordinates were determined. These values were then used to calculate the total color differences (*ΔE*), indicating the color variations in processed broccoli juice with respect to untreated juice (Eq. 2):

$$\Delta E = \sqrt{(L - L_0)^2 + (a - a_0)^2 + (b - b_0)^2} \qquad \text{Eq. 2}$$

where a_0, b_0 and L_0 are the CIE-Lab values of untreated broccoli juice. L, a and b correspond to the treated (by HIPEF or heat) broccoli juice.

Experimental design

A response surface methodology was used to evaluate the effect of the different HIPEF treatment variables on *ΔE*, PPO and LOX activity of broccoli juice. A central composite design with three faced centred factors was proposed. Numerical variables were treatment time (500-2000 µs), and electric field strength (15-35 kV/cm), while a categorical variable was the polarity (monopolar or bipolar), keeping the pulse width (4 µs) and frequency (100 Hz) constant. The experimental design along with each experimental condition is shown in Table 1.

The experiment design was performed twice and each analytical analysis was carried out in triplicate (n=6). Experimental data were fitted to a polynomial model equation. The second order response function was predicted by equation 3:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X^2{}_{i_i} + \sum_{i=1}^{2} \sum_{i=i+1}^{3} \beta_{ij} X_i X_j \qquad \text{Eq. 3}$$

were Y is the response, β_0, β_i, β_{ii}, and β_{ij}, are the constant, linear, quadratic and interaction regression coefficients, respectively and X_i represent the independent variables. Response surface methodology was employed for experimental design, data analysis, model building and plot generation using Design Expert 6.01 software (Stat Ease Inc., Minneapolis, Minn., USA). One-way ANOVA followed by LSD-test was used to determine differences amongst HIPEF and heated treated, as well as unprocessed broccoli juice. Statistical analyses were carried out using the Statgraphics Plus v5.1 Windows package (Statistical Graphics Co., Rockville, Md).

Validation and optimization of the predictive models

A set of 52 experiments was carried out to validate the developed predictive models. The correlation coefficient between the predicted and the experimental data was taken as an indicator of the prediction accuracy.

An optimization in the range of the HIPEF parameters studied was carried out according to the method described by Derringer and Suich (1980). The highest desirability represented the most adequate condition in order to select the lowest RA_{LOX}, RA_{PPO} and *ΔE*. The selected HIPEF condition was compared with those thermally treated and unprocessed juices.

RESULTS

Table 1. Central composite response surface design for polyphenol oxidase, lipoxygenase relative activity and color diference on HIPEF treated broccoli juice.

Assay No.[a]	Point type	*E* (kV/cm)	*t* (µs)	Polarity mode	Variables [b]		
					RA_{LOX} (%)	RA_{PPO} (%)	*ΔE*
1	Factorial	15	500	Monopolar	101.75 ±1.16	107.50 ±2.57	0.37 ±0.03
2	Factorial	35	500	Monopolar	96.18 ±1.35	67.54 ±1.21	0.76 ±0.04
3	Factorial	15	2000	Monopolar	93.13 ±2.61	60.41 ±1.74	0.75 ±0.02
4	Factorial	35	2000	Monopolar	82.13 ±1.18	50.68 ±2.05	2.11 ±0.07
5	Axial	15	1250	Monopolar	101.43 ±1.54	84.55 ±1.08	0.48 ±0.02
6	Axial	35	1250	Monopolar	76.48 ±2.02	57.01 ±0.91	1.13 ±0.04
7	Axial	25	500	Monopolar	92.13 ±2.45	71.2 ±1.14	0.48 ±0.02
8	Axial	25	2000	Monopolar	75.79 ±1.80	54.86 ±2.62	0.75 ±0.01
9	Central	25	1250	Monopolar	81.71 ±3.27[c]	61.92 ±3.03[c]	0.69 ±0.02[c]
10	Factorial	15	500	Bipolar	92.76 ±1.36	78.99 ±0.98	0.29 ±0.03
11	Factorial	35	500	Bipolar	85.28 ±2.06	50.78 ±1.43	0.89 ±0.01
12	Factorial	15	2000	Bipolar	102.52 ±1.24	57.51 ±0.87	0.64 ±0.02
13	Factorial	35	2000	Bipolar	68.71 ±1.66	36.11 ±0.94	2.03 ±0.03
14	Axial	15	1250	Bipolar	91.47 ±1.36	65.71 ±1.36	0.34 ±0.03
15	Axial	35	1250	Bipolar	73.09 ±2.75	48.35 ±1.44	1.61 ±0.02
16	Axial	25	500	Bipolar	83.56 ±3.05	67.69 ±1.16	0.37 ±0.01
17	Axial	25	2000	Bipolar	76.36 ±2.88	53.62 ±1.29	0.74 ±0.04
18	Central	25	1250	Bipolar	75.57 ±3.47[c]	46.42 ±1.05[c]	0.62 ±0.01[c]

[a] Order of the assays was randomized.
[b] Values are expressed as mean ± SD of two treatment repetitions; each assay was performed by triplicate.
[c] Data shown are mean of the central points with five repetitions.
E= Field strength, *t*= Treatment time; RA_{PPO}= Residual polyphenol oxidase activity; RA_{LOX}= Residual lipoxygenase activity.
ΔE= Color difference.

Table 1 summarizes the results of the effects of HIPEF treatment at different electric field strength (15-35 kV/cm), treatment time (500-2000 µs) and polarity (bipolar or monopolar) on the relative residual activity LOX (RA_{LOX}), PPO (RA_{PPO}) and color difference (ΔE) of broccoli juice.

Table 2. Analysis of variance of the second-order polynomial models for polyphenol oxidase, lipoxygenase and color difference of HIPEF-treated broccoli juice.

Source[a]	F value		
	LOX	PPO	ΔE
Model	18.59[d]	17.61[d]	21.38[d]
E	59.19[d]	48.93[d]	91.30[d]
t	16.25[c]	40.08[d]	42.12[d]
P	15.40[b]	32.30[d]	ns
E^2	22.35[c]	5.48[b]	17.10[c]
t^2	ns	ns	ns
E x t	8.75[b]	4.85[b]	11.70[b]
E x P	ns	ns	ns
t x P	ns	ns	ns
Lack of fit	ns	ns	ns
Desv. Std	3.80	5.95	0.17
Mean	83.82	79.78	0.78
R^2	0.89	0.89	0.91
Adjusted R^2	0.85	0.84	0.87

[a] *E* = Electric field strength, *t* = Treatment time; *P* = Polarity. LOX = Lipoxygenase; PPO = Polyphenoloxidase; ΔE = Color difference. [b] Significant at $p < 0.05$. [c] Significant at $p < 0.001$. [d] Significant at $p < 0.0001$. ns = Non-significant.

Lipoxygenase

The lowest RA_{LOX} (68.71%) in HIPEF-processed broccoli juice was reached at the strongest HIPEF conditions (35 kV/cm for 2000 µs in bipolar mode). In contrast, there was not LOX inactivation when broccoli juice was treated at 15 kV/cm for 500 and 1250 µs in monopolar mode, as well as at 15 kV/cm for 2000 µs in bipolar mode (Table 1).

The statistical analysis indicates that the second order model fitted properly the experimental data ($p < 0.0001$) (Table 2), where the determination coefficient (R^2) was 0.89 and the lack of fit was not significant, meaning that the model was adequate for predicting the response across the design space.

Polarity significantly influenced on the RA_{LOX} ($p < 0.05$). Hence, separate RA_{LOX} equations were obtained for mono- and bipolar mode (Eq. 4 and Eq. 5):

$$RA_{LOX_M} = 151.75 - 3.85 * E - 1.3x10^{-2} * t - 5.29x10^{-4} * E * t + 0.076 * E^2 \quad \text{Eq. 4}$$

$$RA_{LOX_B} = 146.53 - 4.15 * E - 7.57x10^{-3} * t - 5.29x10^{-4} * E * t - 0.076 * E^2 \quad \text{Eq. 5}$$

where RA_{LOX} is the residual activity of lipoxygenase, in monopolar (M) or bipolar (B) mode. *E* is the electric field strength (kV/cm) and *t* the treatment time (µs).

In general, HIPEF treatments in bipolar mode were more efficient for LOX inactivation than that applied in monopolar mode. For instance, the RA_{LOX} was 68.7% when broccoli juice was exposed to HIPEF processing in bipolar mode at 35 kV/cm for 2000 µs, while it was 82.1% applying the same treatment in monopolar mode.

Electric field strength and treatment time, as well as their interaction significantly influenced on the RA_{LOX}. RA_{LOX} diminished from 102.52 to 68.71% as electric field strength increased from 15 to 35 kV/cm for 2000 µs in bipolar mode. A similar tendency was observed at 35 kV/cm as treatment time increased. Besides, minimum RA_{LOX} values were observed varying the electric field strength for 500 µs and changing the treatment time at 15 kV/cm in both polarities (Fig. 1).

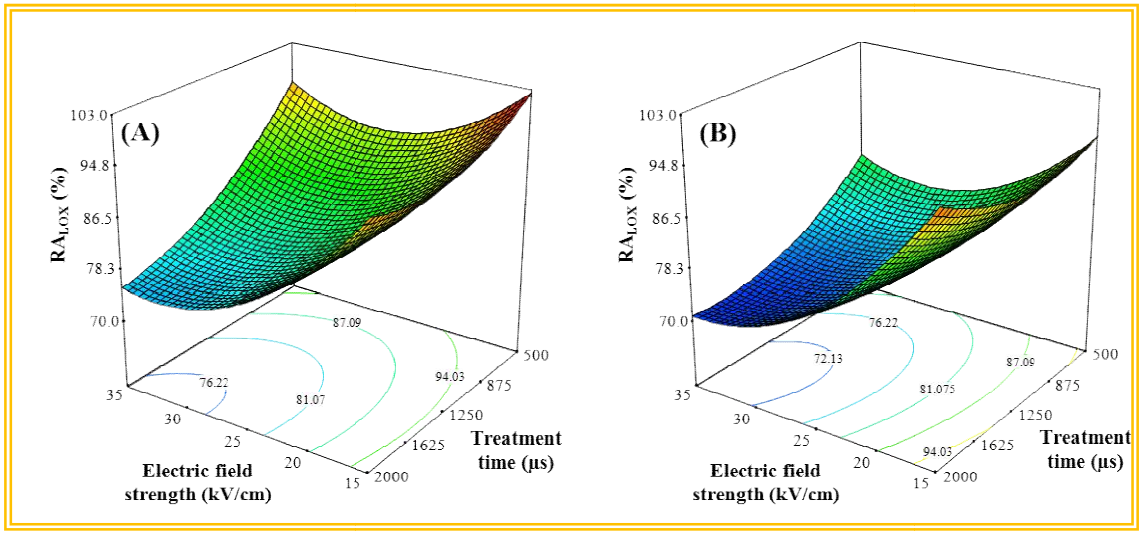

Figure 1. Influence of electric field strength and treatment time on residual lipoxygenase of HIPEF-treated broccoli juice in (A) monopolar or (B) bipolar mode.

Polyphenol oxidase

The statistical analysis indicated that the quadratic model was adequate for describing with accuracy (R^2=0.89) the RA_{PPO} of broccoli juice (Table 2). As polarity is a categorical variable and significant differences between mono- and bipolar mode were observed, the RA_{PPO} in HIPEF-treated broccoli juice was modelled through two different polynomial equations (Eqs. 6 and 7):

$$RA_{PPO\,M} = 183.45 - 5.02 * E - 5.05x10^{-2} * t + 6.18x10^{-4} * E * t + 0.059 * E^2 \quad \text{Eq. 6}$$

$$RA_{PPO\,B} = 157.56 - 4.85 * E + 4.38x10^{-2} * t + 6.18x10^{-4} * E * t + 0.059 * E^2 \quad \text{Eq. 7}$$

where RA_{PPO} is the residual activity of polyphenol oxidase, in monopolar (M) or bipolar (B) mode. *E* is the electric field strength (kV/cm), and *t* the treatment time.

HIPEF treatment applied in bipolar mode tended to cause lower RA_{PPO} than that of monopolar mode in broccoli juice.

The RA_{PPO} decreased as electric field strength and treatment time increased when HIPEF treatments in monopolar or bipolar mode were applied (Figure 2). Also, the interaction electric field strength – treatment time significantly influenced on the RA_{PPO}. Indeed, as the treatment time increased, the effect of the electric field becomes much more noticeable on RA_{PPO}. As the treatment time rose from 500 to 2000 µs at 15 kV/cm in bipolar mode, the RA_{PPO} decreased from 78.99 to 57.51%, but when electric pulses at 35 kV/cm were applied, the RA_{PPO} diminished from 50.78 to 36.11%, at the same interval of treatment time and polarity.

The minimal RA_{PPO} (36.11%) was reached at 35 kV/cm for 2000 µs in bipolar mode.

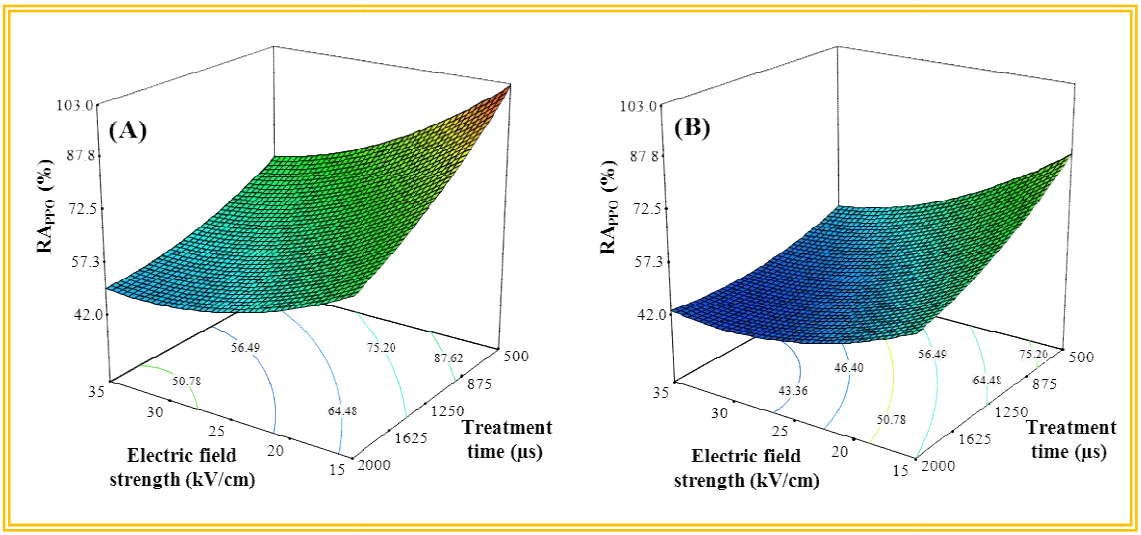

Figure 2. Influence of electric field strength and treatment time on residual polyphenol oxidase of HIPEF-treated broccoli juice in (A) monopolar or (B) bipolar mode.

Table 3. Effects of HIPEF and thermal treatment on oxidative enzymes and color parameters in broccoli juice

Parameters	Unprocessed	HIPEF	TT
L*	35.42 ± 0.37[a]	34.40 ± 0.61[b]	41.24 ± 1.26[c]
*a**	-9.89 ± 0.20[a]	-9.69 ± 0.75[b]	-13.81 ± 1.73[c]
*b**	11.76 ± 0.12[a]	11.58 ± 0.83[b]	15.37 ± 1.88[c]
ΔE	-	0.69 ± 0.04[b]	7.89 ± 0.71[c]
RA_{PPO} (%)	100[a]	47.37 ± 2.35[b]	0[c]
RA_{LOX} (%)	100[a]	74.39 ± 3.14[b]	0[c]

Values are expressed as mean ±SD of two repetitions; each assay was performed by triplicate. *a, b* and *c* letters indicate significant differences ($p < 0.05$) respect to untreated juice. (HIPEF = High intensity pulsed electric fields (26.35 kV/cm, 1235 µs in bipolar mode); TT = Thermal treatment (90 ºC for 60 s). RA_{LOX} (%) = Residual activity of lipoxygenase; RA_{PPO} (%) = Residual activity of polyphenol oxidase; L*, a* and b* are CIELab parameters. Δ*E* = Color difference.

Color

Color is considered one of the most important parameters of food quality, since it is the first attribute by which the consumer approves or rejects any food. In the present study color changes were expressed as total color difference (Δ*E*). Results of Δ*E* ranged from 0.29 to 2.11 units (Table 1). The maximum Δ*E* (2.11 units) was observed when broccoli juice was processed at 35 kV/cm for 2000 µs in monopolar mode.

Second order polynomial model described with accuracy the changes in Δ*E* of broccoli juice (R^2 = 0.91). The polarity did not have a significant influence on color difference. Therefore, the model was reduced to a unique expression that can be used for monopolar or bipolar mode (Eq. 8):

$$\Delta E = 0.63 + 0.47 * E + 0.32 * t + 0.22 * E * t + 0.31 * E^2 \quad \text{Eq. 8}$$

where *ΔE* is the color difference. *E* is the electric field strength (kV/cm), and *t* the treatment time.

Electric field strength and treatment time significantly influenced on the *ΔE* of HIPEF-treated broccoli juice. *ΔE* increased as the electric field strength (from 15 to 25 kV/cm) and treatment time rose (Fig 3). For example, when electric field strength was set at 35 kV/cm, from 500 to 2000 µs in bipolar mode, the broccoli juice *ΔE* augmented from 0.89 to 2.03 units. In addition, the interaction of both parameters had a remarkable effect on *ΔE*. Hence, the *ΔE* augmented from 0.29 to 0.64 as treatment time increased from 500 to 2000 µs at 15 kV/cm in bipolar mode, but when the highest electric field strength (35 kV/cm) was applied at the same interval of treatment time in bipolar mode, the *ΔE* rose from 0.89 to 2.03.

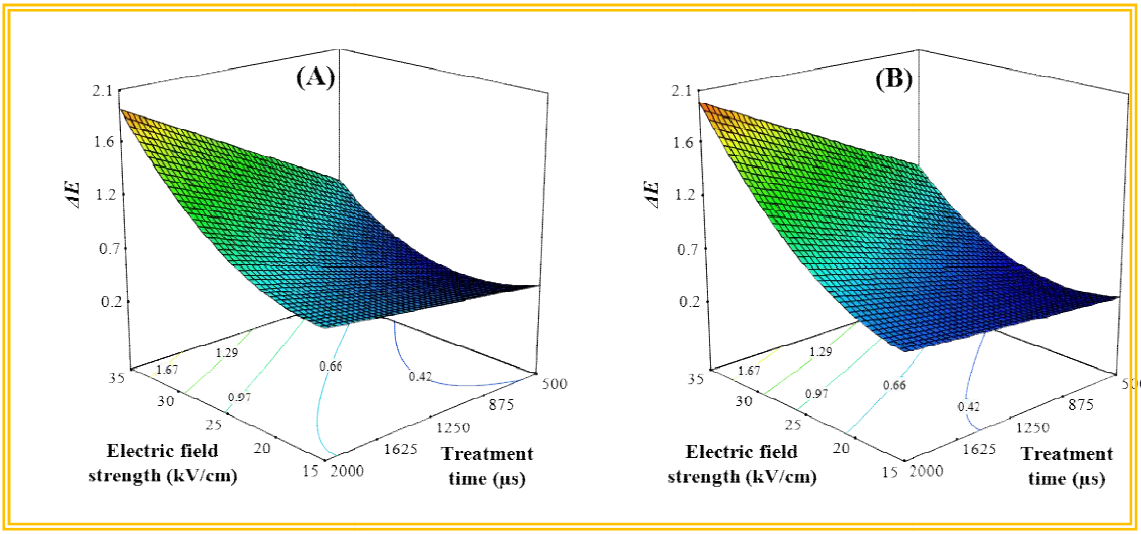

Figure 3. Influence of electric field strength and treatment time on color difference of HIPEF-treated broccoli juice in (A) monopolar or (B) bipolar mode.

Validation and optimization of HIPEF-processing conditions

In the range of the HIPEF parameters studied, an optimization was done in order to find the combination of HIPEF parameters that gives to the maximum LOX and PPO inactivation, as well as, the minimum color difference value. This condition was achieved at 26.35 kV/cm for 1235 µs in bipolar mode, where the lowest color difference was 0.69 and the highest LOX and PPO inactivation was 25.6 and 52.6%, respectively. The desirability of HIPEF treatment was 0.82, which was taken as a measure of accuracy between the polynomial model predictions and the experimental data (Fig. 4). The correlation coefficients between the observed and predicted data were 0.903, 0.802 and 0.920 for LOX, PPO and Δ*E* models, respectively. Therefore, 2nd-order expressions obtained for each assay were adequate to fit the experimental results.

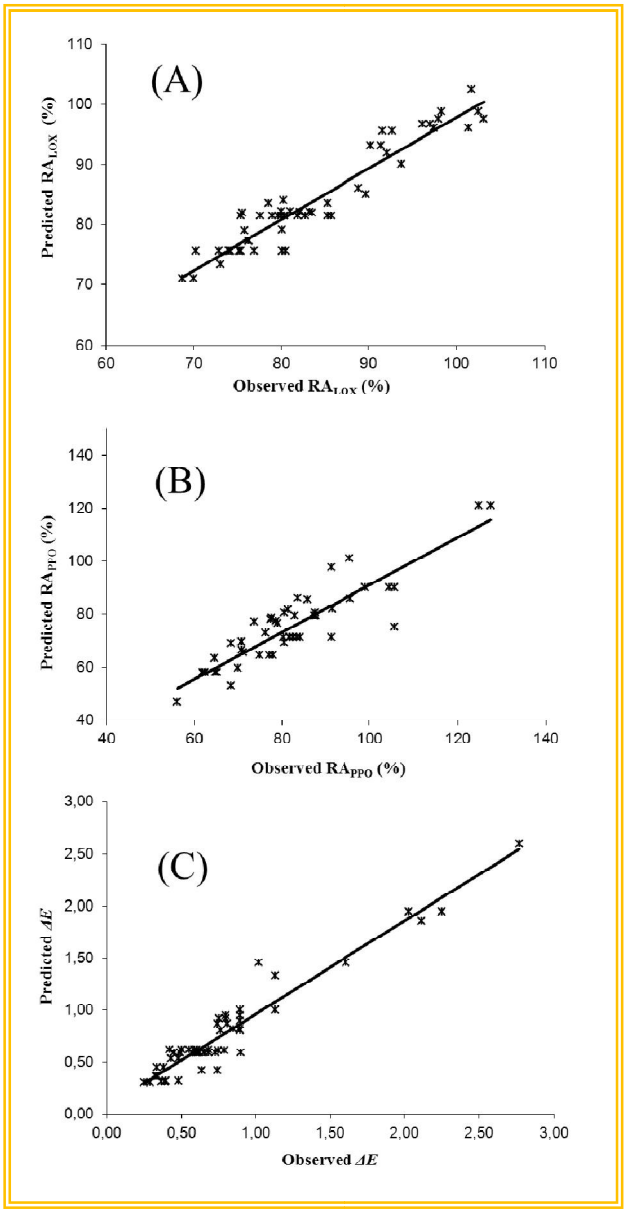

Figure 4. Scatter plots of the observed and predicted data of residual lipoxygenase (A), polyphenol oxidase (B) activities and color difference (C) of the validation trials.

DISCUSSION

According to the results obtained in this work, the HIPEF parameters electric field strength and treatment time influenced on the RA_{LOX}, RA_{PPO} and ΔE. Polarity had a significant effect on the RA of oxidative enzymes, but not on ΔE.

Pulse polarity is an important HIPEF variable which affected the enzyme activity in broccoli juice. A greater enzyme activity reduction of LOX and PPO was achieved after HIPEF treatments in bipolar mode in comparison with that of monopolar mode.

The mechanisms involved in enzyme inactivation caused by changes in the polarity during HIPEF treatment are not fully understood at this time. Nonetheless, it has been suggested that successive monopolar pulses separate particles with electric charge and the formation of shielding layer in the surface of electrodes take place, reducing the efficiency of the treatment. In contrast, bipolar pulses minimize polar deposit of charged molecules, avoiding the shielding layer development and thus, making the HIPEF treatment more uniform (Min, Evrendilek, & Zhang, 2007; Qin, Zhang, Barbosa-Canovas, Swanson, & Pedrow, 1994).

Studies about the effects of HIPEF on LOX activity in watermelon juice (Aguiló-Aguayo, Soliva-Fortuny, & Martín-Belloso, 2010b), PPO of both strawberry juice (Aguiló-Aguayo, Soliva-Fortuny, & Martín-Belloso, 2010a) and peach extracts (Giner, Ortega, Mesegué, Gimeno, Barbosa-Cánovas, & Martín, 2002) also demonstrated that treatments in bipolar mode induced higher enzyme inactivation than those applied in monopolar mode. Elez-Martínez, Suárez-Recio, and Martín-Belloso (2007) also observed the same trend in the orange juice pectin methyl esterase inactivation. In contrast, Aguiló-Aguayo, Soliva-Fortuny, and Martín-Belloso (2009) and Elez-Martínez, Aguiló-Aguayo, and Martín-Belloso (2006) reported higher rates of tomato juice LOX and orange juice POD inactivation in monopolar mode with respect to that of bipolar mode.

On the other hand, RA_{LOX} and RA_{PPO} were influenced by electric field strength and treatment time. The effect of HIPEF parameters was also showed by Aguiló-Aguayo, Sobrino-López, Soliva-Fortuny, and Martín-Belloso (2008), Elez-Martínez, Aguiló-Aguayo, and Martín-Belloso (2006) and Giner, Gimeno, Barbosa-Cánovas, and Martín (2001), who found that as HIPEF parameters increased, the enzyme activity decreased. In agreement with the findings obtained in the present study, other authors reported that the enzymatic inactivation by HIPEF was in function of the electric parameters and the type of enzyme (Aguiló-Aguayo, Soliva-Fortuny, & Martín-Belloso, 2009; Li, Chen, Liu, & Chen, 2008; Riener, Noci, Cronin, Morgan, & Lyng, 2008).

Different HIPEF susceptibility of LOX and PPO of broccoli juice was observed. For instance, a fixed HIPEF processing condition, 25 kV/cm for 1250 µs in bipolar mode, RA_{PPO} was 46.42%; whereas LOX only 75.57%. The differences in the ratio of inactivation between LOX and PPO of broccoli juice exposed to HIPEF processing, could be associated to the differences in their molecular weight. LOX is a monomer of 95-100 kDa (Williams & Harwood, 2008), while the PPO varies from 51 to 57 kDa (Gawlik-Dziki, Szymanowska, & Baraniak, 2007). Moreover, it has been proposed that enzyme inactivation depends on the type of enzyme, electric conductivity, and pH (Elez-Martínez, Martín-Belloso, Rodrigo, & Sampedro, 2007).

A notable resistance of broccoli juice LOX and PPO to HIPEF processing was observed. In fact, there was not PPO and LOX inactivation when broccoli juice was HIPEF-treated at 15 kV/cm and 500 µs in monopolar mode. The incomplete LOX and PPO inactivation by HIPEF in broccoli juice may be due to the presence of isoenzymes, which have different resistance to HIPEF treatment (Aguiló-Aguayo, Sobrino-López, Soliva-Fortuny, & Martín-Belloso, 2008; Min, Jin, & Zhang, 2003). Similarly, LOX of tomato juice showed low sensitivity (RA_{LOX}= 80.26%) to HIPEF when pulses were applied at 35 kV/cm, 150 Hz for 1000 µs and 1 µs of pulse width (Aguiló-Aguayo, Soliva-Fortuny, & Martín-

Belloso, 2009). Green pea LOX was not inactivated when pulses at 20 kV/cm for 400 µs were applied (Van Loey, Verachtert, & Hendrickx, 2001).

Nowadays, the precise mechanisms implicated in enzyme inactivation by HIPEF are not fully understood but it is believed that HIPEF causes changes in the conformational state of enzymes leading to their inactivation. Zhao and Yang (2008) observed a correlation between the loss of secondary and tertiary structures of lysozyme and their inactivation by HIPEF. In the same way, a loss of α-helix in secondary structure was observed when papain and lysozyme were subjected to HIPEF treatment (Yeom, Zhang, & Dunne, 1999; Zhao, Yang, Lu, Tang, & Zhang, 2007). Likely, HIPEF polarize the protein molecule and change its conformation, resulting in the protein inactivation (Perez & Pilosof, 2004), which could explain the LOX and PPO inactivation observed when broccoli juice was treated by HIPEF. However, further research is needed to elucidate the mechanisms of enzyme inactivation by HIPEF.

Both LOX and PPO were completely inactivated when broccoli juice was heated at 90 °C for 60 s (Table 3). It could be justified by the fact that enzymes are globular proteins sensitive to heat denaturation. In contrast, incomplete broccoli juice LOX and PPO inactivation was reached when HIPEF treatments were applied.

In the present work, the lowest Δ*E* value (0.29 units) was observed at 15 kV/cm for 500 µs in bipolar mode. Cortés, Esteve, Rodrigo, Torregrosa, and Frígola (2006) observed Δ*E* values of up to 7 units when orange juice was processed by HIPEF at 25 kV/cm for 340 µs. Yin, Han, and Liu (2007) described an increment in *a** parameter as electric field strength increased from 20 to 60 kV/cm, when spinach puree was HIPEF treated. On the contrary, Akin and Evrendilek (2009) reported that HIPEF treatments between 13-27 kV/cm from 82 to 262 µs did not display significant differences in *L**, *a** and *b** parameters of a formulated carrot juice, regarding to the unprocessed juice. Similarly, Quitão-Teixeira, Aguiló-Aguayo, Ramos, and Martín-Belloso (2008) reported that there were not differences in the color coordinates between HIPEF processed (shifting frequency from 50 to 250 Hz and pulse width from 1 to 7 µs, in bipolar or monopolar mode) and untreated carrot juice.

LaBorde & Von Elbe, (1994) observed the formation of metallocomplexes of chlorophyll derivatives during food processing of green vegetables. Also, electrode material may be transferred to liquid food, which could lead to electrode corrosion and the releasing of metals to the food (Roodenburg et al., 2005). Therefore, color changes in HIPEF-processed broccoli juice could be justified by the fact that metal ions released during HIPEF processing could replace the magnesium atom of chlorophyll or its derivative compounds, changing their optical properties and thereby generating a color change in broccoli juice.

From an industrial point of view, Δ*E* values between 1.1–2.8 and 2.8–5.6, correspond to rigorous and normal color tolerances, respectively. Values higher than 5.6 units are easily distinguished (Lozano, 1978). In this research *ΔE* of broccoli juice displayed less than 3 units; therefore, HIPEF processing did not have a significant influence on visual color appearance.

Within the range of assayed conditions, HIPEF-treated broccoli juice attained lower *ΔE* values (Table 1) than that of thermally treated, which could be associated to higher

retention of pigments, such as chlorophylls, phenols, and carotenoids during HIPEF processing, maintaining the color stability of broccoli juice after HIPEF treatment. The *ΔE* observed in HIPEF processed broccoli juice could be associated to the apparition of degradation compounds of chlorophylls, such as pheophytin and pheophorbide. Besides, residual activity of oxidative enzymes might catalyse the degradation of broccoli juice pigments. On the contrary, thermal processed broccoli juice exhibited higher brightness (*L**) than those treated by HIPEF. This pattern could be explained by the fact that broccoli juice was passed through a copper tube during thermal treatment. Therefore, regreening may happen when copper substitutes the magnesium atom of tetrapyrrole ring from chlorophyll (Simpson, 1985). This trend was not observed in juices that not contain chlorophylls. For instance, orange juice treated by HIPEF at 40 kV/cm for 97 μs, showed higher L and hue angles values than that thermally processed (Min, Min, & Zhang, 2003).

CONCLUSIONS

Broccoli juice LOX and PPO RA and Δ*E* were influenced by electric field strength and treatment time. Polarity had a significant effect on the PPO and LOX activities but not on Δ*E*. When electric field strength or treatment times augmented, the residual activity of oxidative enzymes and Δ*E* diminished. The strongest HIPEF conditions (35 kV/cm for 2000 μs in bipolar mode) increased the LOX and PPO inactivation of broccoli juice. Moreover, LOX was more resistant than PPO to HIPEF processing. The visual quality of broccoli juice was better retained by HIPEF processing (Δ*E* up to 2.11) in comparison to thermal treatment (Δ*E* up to 7.89). Therefore, HIPEF technology is able to reduce the enzymatic oxidation of broccoli juice, maintaining at the same time its visual quality.

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V GENERAL DISCUSSION

In the present research, the effects of HIPEF parameters (electric field strength, treatment time and polarity) on bioactive compounds and some relevant enzymes presents in broccoli juice were assessed. The effects of a conventional thermal treatment and that for HIPEF on bioactive compounds and enzymes presents in broccoli juice were compared.

The initial concentration of each bioactive compound analysed in broccoli juice was within the ranges reported in the literature. Some differences between values reported in the bibliography and that found in the present research could be associated with cultivars, climatic conditions, growing seasons, farming practices, extraction juice methods (Kaur et al. 2007), genotype of this vegetable, among others (Kurilich et al. 2002).

The literature related to the influence of some HIPEF parameters on bioactive compounds in vegetable juices is scarce. To the best of our knowledge there are no studies about the effect of HIPEF processing parameters on bioactive compounds in broccoli juice. However, plausible explanations of the findings in the present research were proposed.

A global analysis of correlation among the bioactive compounds, Chl degradation compounds, color difference and oxidative enzymes was developed through Pearson's test in order to analyze the overall interactions.

This study reports encouraging results about as bioactive compounds are better preserved by HIPEF processing in comparison to thermal treatment, demonstrating to be a good alternative to the liquids foods preservation.

Influence of HIPEF processing parameters on bioactive compounds of broccoli juice.

Maximum increments in the relative content (RC) of Chl *a* (RC of 116.0%), Chl *b* (RC of 120.7%), lutein (RC of 121.2%) and β-carotene (RC of 130.5%) were reached when broccoli juice was HIPEF processed at 35 kV/cm for 2000 μs in bipolar mode. The highest RC of vitamin C (90.1%) was reached at 35 kV/cm for 500 μs in monopolar mode. Whereas minimal reductions in the RC of TP content (RC of 96.1%) and relative antioxidant capacity (RAC) (RAC of 95.9%) were observed at 25 and 35 kV/cm for 1250 μs in bipolar mode, respectively. There were increments in the RC of some minerals and amino acids of broccoli juice at specific HIPEF conditions.

The results of the bioactive compounds evaluated in the present research (except for minerals and amino acids), were fit through a second order polynomial model. The determination coefficient (R^2) ranged between 0.83 and 0.99 and the lack of fit was not significant, meaning that the models were adequate for predicting the responses across the design space. Minerals and amino acids were analyzed using an ANOVA.

Through a response surface methodology (RSM), an optimization was developed to find the HIPEF conditions where higher RC of bioactive compounds, lower residual activity of deteriorative enzymes, as well as lower formation of Chls derivative compounds were

reached. Optimum HIPEF condition was 28 kV/cm for 2000 μs in bipolar mode with a desirability of 0.72.

Effect of polarity

Neither Chl *a* nor Chl *b* were influenced by the polarity. That is, similar increments or reductions in the Chl content were observed in both polarities; therefore the model was reduced to a unique function for mono and bipolar mode. In the same way, the cupper, histidine, alanine and tyrosine were not affected by polarity. On the contrary, polarity markedly influenced on the RC of Chl degradation compounds (Chlide, Phe and Phb), lutein, β-carotene, vitamin C, TP, minerals (except Cu), amino acids (excluding His, Ala and Tyr) and RAC.

Higher RC of lutein, β-carotene, TP, minerals (K, Mg, Ca, Na, Se), the amino acid His and the RAC was observed when electric pulses in bipolar mode were applied. For instance, an increment in the content of lutein (RC of 111.9%), β-carotene (RC of 112.3%), total amino acids (RC of 100.6%) and minerals such as Se (RC of 109.7%) was achieved when broccoli juice was treated by HIPEF in bipolar mode at 35 kV/cm for 500 μs; while applying monopolar mode pulses, the content of lutein and total amino acids diminished 6.1 and 8.3%, in that order. RC of β-carotene and Se augmented only 3.5% and 6.9%, respectively. In the same way, the RC of TP, and the RAC was 86.3% and 95.9% with bipolar mode pulses at 35 kV/cm for 1250 μs; however, in monopolar mode the RC and RAC was 80.4% and 78.8%. In agreement with the reported in the present research, greater lycopene and antioxidant capacity retention in tomato juice (Odriozola-Serrano et al. 2007) strawberry juice (Odriozola-Serrano et al. 2009b) and watermelon juice (Oms-Oliu et al. 2009) processed by HIPEF in bipolar mode than those in monopolar mode was reported.

On the contrary, monopolar pulses were found to be more effective in keeping the broccoli juice vitamin C, some minerals such as Fe, Mn, P, S, Zn and the amino acids than those applied in bipolar mode. The effect of polarity on vitamin C, minerals and amino acids depended on the conditions of electric field strength and treatment time applied. Indeed, HIPEF treatments set up at 15 kV/cm (at any treatment time) in bipolar mode pulses led to higher or similar RC of vitamin C than that in monopolar mode, while at 35 kV/cm for 1250 μs the vitamin C RC was 82.2% and 72.6% after applying bipolar and monopolar mode pulses, respectively. Likewise, monopolar pulses increased the content of Fe, Mn and Zn. For instance, after HIPEF treatments in monopolar mode at 35 kV/cm for 2000 μs the RC of Fe and Mn was 214.2 and 114.3%. Whereas, applying pulses in bipolar mode at the same conditions, the RC of those elements was 203.3 and 103.6%, respectively. In the case of amino acids, the highest increment in the content of amino acids was achieved at 15 kV/cm for 2000 μs in monopolar mode (except His). Nonetheless, increasing the electric field (35 kV/cm) at the same polarity, their RC diminished drastically (excluding Pro).

In general bipolar mode pulses retained more efficiently the bioactive compounds contained in broccoli. Bipolar pulses offer the advantages of reduce the undesirable food

electrolysis, minimize the deposition of solids on the electrode surface and limit the possibility of electrolytic reactions (Barbosa-Cánovas et al. 1999). Therefore, the oxidation of bioactive compounds is reduced. Moreover, it has been demonstrated that bipolar mode pulses are potentially more efficient compared to those in monopolar mode in reducing the oxidative enzyme activity (Aguiló-Aguayo et al. 2008a, Aguiló-Aguayo et al. 2010a, Giner et al. 2002).

In contrast, monopolar pulses separate charged particles in liquid foods. The separate charged particles may form a deposit on the electrode and their erosion (Loeffler 2006). Indeed, during HIPEF processing corrosion of stainless steel electrodes occur, liberating Fe, Mn and Zn, thus the increment in the content of Fe, Mn and Zn could be explained. In the same way, Morren et al. (2003) reported that corrosion of electrodes can be limited by applying bipolar pulses. Similarly, Evrendilek et al. (2004) observed a significant augment in the concentration of Fe, Cr, Zn and Mn in HIPEF-treated beer at 41 kV/cm for 175 μs. In this context, Roodenburg et al. (2005) and Morren et al. (2003) reported that the release of minerals was a consequence of electrochemical reactions that happen on electrodes, depending on current magnitude, fluid composition, as well as pulse shape and duration (Roodenburg et al. 2005).

Conversely, the content of vitamin C was maintained when pulses in monopolar mode were used. It could be explained by the fact that the enzymes associated to vitamin C degradation, such as ascorbate oxidase (Davey, Van Montagu, Inzé, Sanmartin, Kanellis, Smirnoff, et al., 2000), could be greatly inactivated after HIPEF treatments in monopolar mode than those applied in bipolar mode. In this context, other oxidative enzymes such as orange juice peroxidase (Elez-Martínez, Aguiló-Aguayo, & Martín-Belloso, 2006) and strawberry juice lipoxygenase (Aguiló-Aguayo, Sobrino-López, Soliva-Fortuny, & Martín-Belloso, 2008) were more efficiently inactivated when HIPEF treatments were applied in monopolar mode, as compared with that applied in bipolar mode. The results obtained in this study were consistent with those reported by Odriozola-Serrano, Aguiló-Aguayo, Soliva-Fortuny, Gimeno-Añó, and Martín-Belloso (2007) and Oms-Oliu, Odriozola-Serrano, Soliva-Fortuny, and Martín-Belloso (2009) in HIPEF-treated tomato juice (at 35 kV/cm for 1000 μs) and watermelon juice (varying the treatment time, 50-2050 μs; and electric field strength, 25-35 kV/cm) , respectively. These authors (Odriozola-Serrano, Aguiló-Aguayo, Soliva-Fortuny, Gimeno-Añó, & Martín-Belloso, 2007; Oms-Oliu, Odriozola-Serrano, Soliva-Fortuny, & Martín-Belloso, 2009) concluded that RC of vitamin C after applying bipolar pulses was lower in comparison to that of monopolar mode.

The losses of bioactive compounds both in bipolar and monopolar mode could be related to the fact that during HIPEF treatment the electrodes are in direct contact with the food product. Electrochemical reactions can occur which lead to metal release and oxidation reactions could take place when electric pulses are applied (Roodenburg et al. 2005).

Effect of electric field strength and treatment time

Electric field strength (*E*) and treatment time (*t*) significantly influenced on the RC of Chls, Chls degradations compounds, carotenoids, vitamin C, TP, minerals (except Cu, P and S), amino acids (with the exception of Trp and Ala) and the RAC.

The highest RC of carotenoids and Chls was reached at the strongest HIPEF conditions (35 kV/cm for 2000 μs in bipolar mode). In contrast, at this HIPEF combination, the lowest RC of Chls degradation compounds was detected. The maximum content of vitamin C, TP, minerals, amino acids and AC was observed at different HIPEF conditions.

The content of Chls (Chl *a* and Chl *b*) and carotenoids (lutein and β-carotene) increased as electric field strength and treatment time augmented in both monopolar and bipolar mode. At the highest electric field strength (35 kV/cm) and treatment time (2000 μs) in bipolar mode, the RC of Chl *a* (RC of 116%), Chl *b* (RC of 120.7%), lutein (RC of 121.2%) and β-carotene (RC of 130.5%) increased. While at 15 kV/cm for 500 μs at the same polarity, their RC decreased up to 81.84, 79.29, 71.23 and 58.4%, respectively. In line with the results reported in this research, Odriozola-Serrano et al. (2008a) observed that the concentration of lycopene in tomato juice rose as the treatment time (from 100 to 2000 μs) and electric field strength (from 20 to 35 kV/cm) increased, compared to the fresh tomato juice. Also, HIPEF-processed tomato juice (35 kV/cm for 1000 μs using bipolar pulses) exhibited higher concentration of total carotenoids than unprocessed juice (Odriozola-Serrano et al. 2009a). Similarly, Torregrosa et al. (2005) observed that the concentration of carotenoids in orange-carrot juice increased after applying pulses at 25-30 kV/cm for 60-340 μs, respect to untreated juice.

Other studies applying different non-thermal technologies, such as high hydrostatic pressure (150 MPa) in combination to heat (75-80 °C), reported a slight increment in the Chls degradation products (Weemaes et al. 1999). The same authors observed that as the pressure level rose, the Chls loss augmented. In contrast to high-hydrostatic pressure treatment, when the HIPEF parameters augmented, an increment in Chls content as well as reduction of Chlide, Phe and Phb content were observed.

The augment in the content of Chls and carotenoids could be explained by the fact that an activation of specific enzymes related to Chl and carotenoids biosynthesis pathway could take place by the influence of HIPEF processing. For instance, the Mg-chelatase is an enzyme that catalyzes the insertion of Mg^{+2} into protoporphyrin IX to yield Mg-protoporphyrin IX, which is then further metabolized into chlorophyll (Mochizuki et al. 2010, Reinbothe et al. 2010). Moreover, broccoli is a vegetable with an elevated content of magnesium (18.1-27.3 mg/100g) (Kmiecik et al. 2007, López-Berenguer et al. 2007) and it has been demonstrated that the synthesis of Chls is favored at elevated Mg^{+2} concentration (Nakayama et al. 1998). In the case of carotenoids, ζ-carotene desaturase and carotene isomerase participate in the formation of lycopene and the lycopene ε-cyclase and lycopene β-cyclase catalyzes the formation of carotenes and/or xanthophylls (Cazzonelli 2011, Hannoufa & Hossain 2012, Joyard et al. 2009). In this context, several studies have also shown an enzyme activation when was exposed to HIPEF. For example, the activity of watermelon, tomato and strawberry juice LOX (Aguiló-Aguayo et al. 2008b, Aguiló-Aguayo et al. 2009b, 2010b), tomato juice hidroperoxide lyase (Aguiló-Aguayo et

al. 2009b) and strawberry juice β-glucosidase (Aguiló-Aguayo et al. 2008b) augmented after the treatment by HIPEF. The pathway of carotenoid biosynthesis is tightly influenced by environmental stimuli (Howitt & Pogson 2006). Hence, the rate of lycopene biosynthesis depended on temperature, which takes place between 12 and 32 °C. However, in tomatoes stored at 34 and 37 °C, the lycopene levels increased 5 and 37% respectively (Dumas et al. 2003). HIPEF treatments were conducted at temperatures that never exceeded 35 °C, which could favor the biosynthesis of lycopene and subsequent formation of carotenoids.

On the other hand, a significant reduction in the content of Chls (RC of 81.84% and 74.67% for Chl *a* and Chl *b*, in that order) and carotenoids (RC of 65.5% for lutein and RC of 50.7% for β-carotene) from broccoli juice was observed at the lowest HIPEF treatment conditions (15 kV/cm for 500 μs) applied. These results could be justified by the fact that the elimination of microorganisms and oxidative enzymes that could degradate these pigments, was not completely achieved at 15 kV/cm for 500 μs, as was reported by other researchers. That is, the lower electric field strength, the lower enzymes (Elez-Martínez et al., 2006) and microorganisms (Elez-Martínez et al., 2005) elimination. As a result, bioactive compounds such as Chls and carotenoids are reduced by the action of remaining microorganisms and enzymes. This hypothesis is supported by the literature where it has been demonstrated that the lower the electric field strength and the treatment time, the lower the microbial and enzyme inhibition by HIPEF processing (Barsotti & Cheftel, 1999; Pedro Elez-Martínez, Martín-Belloso, Rodrigo, & Sampedro, 2007; Espachs-Barroso, Barbosa-Cánovas, & Martín-Belloso, 2003; Martín-Belloso & Elez-Martínez, 2005; Min, Evrendilek, & Zhang, 2007). Indeed, an inverse correlation among degradative enzymes (Chlase and LOX) and Chls (Chl *a* and Chl *b*) and carotenoids content (lutein and β-carotene) was observed (Table 1), indicating that, when the enzyme activities increased, the RC of Chls and carotenoids decreased. Reductions in the RC of Chls and carotenoids during HIPEF treatments could be due mainly to two factors; the first is the residual activity of oxidative enzymes that are able to catalyse the degradation of bioactive compounds; and finally the oxidation reactions that could occur on the electrode surface (Morren et al. 2003).

The RC of Chlide, Phe and Phb diminished as *E* and *t* decreased. For instance, applying 35 kV/cm for 2000 μs in bipolar mode the RC of Chlide, Phe and Phb were 116.9, 91.2 and 95.2%, respectively; whereas the use of the weakest HIPEF conditions (15 kV/cm for 500 μs in bipolar mode) led to RC values of 135.1 (Chlide), 120 (Phe) and 116% (Phb). It could be related to incomplete inactivation of degradative enzymes of Chls, such as Chlase and LOX. According to Pearson analysis, Chlase and Chls had a negative correlation; therefore, the reduction of this pigment is in part due to the residual Chalse and LOX activity. Similarly, Kohata et al. (1998) reported an increment in the pheophorbide content which was associated to the remaining activity of Chlase even after being heated during the processing of tea. Whereas, Yin et al. (2007) associated the preservation of spinach puree Chls and its color to the microorganism and enzyme elimination.

Both electric field strength and treatment time influenced the RC of vitamin C. Indeed, HIPEF treatments carried out at 35 kV/cm and any treatment time in monopolar mode, led to the lowest reduction of vitamin C content in broccoli juice. On the contrary,

P. Elez-Martínez and Martín-Belloso (2007), Odriozola-Serrano, Aguiló-Aguayo, Soliva-Fortuny, Gimeno-Añó, and Martín-Belloso (2007), Odriozola-Serrano, Soliva-Fortuny, and Martín-Belloso (2009) and Oms-Oliu, Odriozola-Serrano, Soliva-Fortuny, and Martín-Belloso (2009) observed an inverse trend in comparison with that observed in the present study. These authors reported that the loss of vitamin C content in "gazpacho", tomato, orange, strawberry and watermelon juices augmented as electric field strength and treatment time increased. However, it is important to take into consideration that each food contains specific quality related enzymes, which could be more or less resistant to HIPEF treatment, and bioactive compounds, that could exert synergistic/antagonistic interactions among themselves or with other food constituents.

A reduction of vitamin C RC was observed when HIPEF treatments at 35 kV/cm from 500 μs to 2000 μs were applied. This trend was not followed after applying bipolar mode pulses at 15 kV/cm, where vitamin C RC diminished as treatment time decreased. Different combinations of electric field strength and treatment time allow obtaining the same RC of vitamin C, either in monopolar or bipolar mode. For example, 70% of vitamin C RC can be reached by processing broccoli juice at 18 kV/cm for 556 μs or at 34 kV/cm for 1270 μs in bipolar mode, as well as at 25 kV/cm and 1800 μs in monopolar mode. The degradation of vitamin C observed in this study could be justified by the electrochemical reactions due to the electric current flowing between electrodes during HIPEF processing (Morren, Roodenburg, & de Haan, 2003). In this sense, Ottaway, Ottaway, and Associates Ltd (2010) reported that the contact of food with traces of heavy metal ions such as Fe and Zn causes the degradation of vitamin C. Additionally, an incomplete inactivation of oxidative enzymes could catalyze the vitamin C losses. Although HIPEF is able to reduce the enzymatic activity in diverse fruit and vegetable juices (Aguiló-Aguayo, Soliva-Fortuny, & Martín-Belloso, 2009; Quitão-Teixeira, Aguiló-Aguayo, Ramos, & Martín-Belloso, 2008) there are HIPEF resistant enzymes, as has been demonstrated in other studies (Aguiló-Aguayo, Oms-Oliu, Soliva-Fortuny, & Martín-Belloso, 2009; Van Loey, Verachtert, & Hendrickx, 2001).

The RC of TP in broccoli juice depended on the HIPEF treatment conditions applied. Broccoli juice processed by HIPEF at 25 kV/cm for 1250 μs retained the highest content of TP (96.1%). In agreement with the present study, Odriozola-Serrano, Soliva-Fortuny, and Martín-Belloso (2008b) and Aguilar-Rosas, Ballinas-Casarrubias, Nevarez-Moorillon, Martín-Belloso, and Ortega-Rivas (2007) reported reductions in TP content after HIPEF processing of strawberry and apple juices, respectively. An incomplete inactivation of polyphenol oxidase, among other enzymes, can catalyse the polyphenol degradation (Giner, Ortega, Mesegué, Gimeno, Barbosa-Cánovas, & Martín, 2002). Hence, residual polyphenol oxidase activity might reduce the content of phenolic compounds in HIPEF-processed broccoli juice.

In the case of minerals and amino acids, their content was influenced by electric field strength and treatment time, except for the minerals Cu, P and S and the amino acids Trp and Ala. The changes in minerals and amino acids depended on the combinations of electric field strength and treatment time applied. In general, the RC of the minerals increased or was kept unchanged when broccoli juice was processed by HIPEF. Indeed, Fe, Mn and Zn exhibited the highest raises. HIPEF treatments set up at 35 kV/cm for 2000 μs

in monopolar mode led to augments in the content of Fe (114) and Zn (48%). In monopolar mode pulses the RC of selenium increased as treatment time and electric field strength rose. However, in bipolar mode, this condition took place only at 15 and 25 kV/cm. On the contrary, the Mg content in HIPEF-treated broccoli juice did not exhibited significant changes respect to fresh juice. Each mineral evaluated in HIPEF-treated broccoli juice exhibited different tendency. In accordance with the present research, some reports observed variable pattern. In the same line, Morales-de la Peña et al. (2011) observed that there were significant differences between the treatment time conditions tested (800 and 1400 μs) on Cu, Mn, Zn, Ca and Mg of a fruit juice-soymilk beverage. In contrast, Akin and Evrendilek (2009) and Altuntas et al. (2010) reported that electric field strength and treatment time did not change significantly the mineral content of a formulated carrot juice-based beverage and sour cherry juice. Likewise, Pifarré et al. (2006) did not found differences in the content of Na, K, Ca, Mg and Zn among HIPEF-processed (59 kV/cm for 59 μs), heat (75 ^{o}C for 15 min) and untreated beverage based on orange juice and whey powder.

The content of almost all amino acids decreased as treatment time rose from 500 to 1250 μs; nevertheless, from 1250 to 2000 μs a noticeable increment in their content occurred at 15 kV/cm in monopolar mode. For instance, the content of Arg rose 17.7%, respect to untreated broccoli juice, but increasing the treatment time (1250 μs), its content diminished until 97.7% and augmented 39.6% when 2000 μs of treatment time were applied. Likewise, shifting the electric field strength from 15 to 25 kV/cm for 1250 μs in bipolar mode, the Arg content varied from 99.3 to 114.7%, and increasing the electric field from 25 to 35 kV/cm, the Arg content changed from 114.7% to 105.6%. Similarly, Zhao et al. (2009) reported that there was a significant increment in the total free amino acids concentration of green tea infusions as electric field strength increased from 20 to 40 kV/cm. However, amino acids as Asp, Glu, Ser, Gly, and Thr showed reductions in their concentration at 30 and/or 40 kV/cm. Increments in the content of some free amino acids have been also reported by other authors. In this context, Garde-Cerdán et al. (2007) proposed that during HIPEF processing the formation of pores through the plasmatic membrane and organelle disruption took place. As a consequence, amino acid, as well as proteins and proteases were released, which react among them leading to the protein degradation in smaller peptides and amino acids. Otherwise, reductions in the amino acids content of HIPEF processed broccoli juice could be due to reactions of oxidation that inevitably occur on the electrode. In fact, the highest losses in the amino acid content at the strongest HIPEF conditions in monopolar mode were observed. At this polarity the electrochemical reactions and generation of free radicals are more common.

Respect to AC, it was significantly affected by electric field strength and treatment time. In fact, it was observed a reduction in the RAC of broccoli juice, irrespectively of the HIPEF treatment applied. Maximum RAC (95.9%) was achieved at 35 kV/cm for 1250 μs, although no significant differences were observed when broccoli juice was treated by HIPEF at 35 or 25 kV/cm for 1250 μs (Table 2). In this sense, Odriozola-Serrano, Soliva-Fortuny, Gimeno-Añó, and Martín-Belloso (2008) reported reductions in the AC of HIPEF-treated tomato juice. The authors showed that the highest AC was reached at 20 kV/cm in tomato juice, followed by that when processing at 35 kV/cm.

Broccoli is a vegetable with a high AC (Kurilich, Jeffery, Juvik, Wallig, & Klein, 2002), and it has been suggested that the AC in broccoli and other vegetables depends on the scavenger substances found at high levels in foods (Sies & Stahl, 1995). In this context, among the bioactive compounds determined in the present work, vitamin C was the most abundant, followed by the phenolic compounds. However, it has been reported that the contribution of vitamin C to the total AC of broccoli was only 12% (Chu, Sun, Wu, & Liu, 2002). Other authors observed that the AC of broccoli juice was mainly ascribed to the phenolic compounds (Sun, Powers, & Tang, 2007). Likewise, the HIPEF conditions (25 kV/cm for 1250 μs in bipolar mode) where the lowest RC of vitamin C (67%) was observed coincided with a high RAC (95.1%). In the same way, the greatest RC of TP (96.1%) corresponded to the most elevated RAC values (95.1%). Therefore, the reductions of AC in HIPEF-treated broccoli juice could be mainly attributed to the extent to which this treatment may modify the retention or losses of TP. In the same way, a positive and significant correlation between AC and TP (R^2 = 0.7377) was observed, meaning that as the RC of TP decreased, the RAC diminished (Table 1). In general and according to correlation analysis, the RAC of HIPEF-processed broccoli juice is mainly attributed to TP (R^2=0.73377), lutein (R^2=0.6786), β-carotene (R^2=0.5996), selenium (R^2=0.5919), chlorophyll *b* (R^2=0.5780), amino acids (R^2=0.5592), chlorophyll *a* (R^2=0.5277) and to a lesser extent to the vitamin C (R^2=0.4932). In contrast, the relative AC was negatively correlated with the enzymatic activity of Chlase, LOX and PPO, meaning that as their activity augmented the antioxidant capacity decreased, as product of the Chls and carotenoids degradation (Table 1).

In general, increments in the content of bioactive compounds such as Chls, carotenoids and amino acids, could be associated to biochemical processes stimulated by HIPEF processing. Also, intact cells exposed to HIPEF could cause a cell membrane breakdown and the releasing of cytoplasmic content. This fact, may justify the mentioned augments. In contrast, reductions in the content of some bioactive compounds could be related to oxidation reactions that could occur in broccoli juice as consequence of emitted metal ions during HIPEF processing (Morren et al. 2003). Moreover, residual enzyme activity could catalyse the degradation of bioactive compounds.

Influence of HIPEF processing parameters on some enzymes of broccoli juice.

Residual activity (RA) of the enzymes evaluated (chlorophyllase, lipoxygenase and polyphenol oxidase) was significantly affected by the HIPEF parameters applied (electric field strength, treatment time and polarity). The lowest RA of chlorophyllase (Chlase) (26.3%), lipoxygenase (LOX) (68.7%) and polyphenol oxidase (PPO) (36.1%) was reached at the same HIPEF conditions (35 kV/cm for 2000 μs in bipolar mode).

A second order model fit properly the experimental data of Chlase, PPO and LOX residual activity. The determination coefficient (R^2) ranged between 0.89 and 0.91 and the lack of fit was not significant, indicating that the models were adequate for predicting the responses across the design space.

Polarity

RA of Chlase, LOX and PPO were influenced by the polarity. HIPEF treatments in bipolar mode induced the highest enzyme inactivation. For example, HIPEF treatments set up at 35 kV/cm for 500 μs led to Chlase, LOX and PPO RA values of 43.5%, 85.28% and 50.78, respectively. However, applying electric pulses at the same HIPEF conditions in monopolar mode, higher residual activity was detected (54.5%, 96.18% and 67.54%). In agreement with these results, studies about the effects of HIPEF on PPO from strawberry, apple and peach juices (Aguiló-Aguayo et al. 2010a, Giner et al. 2001, Giner et al. 2002), LOX from soymilk and tomato juice (Li et al. 2008, Min et al. 2003a), as well as POD carrot juice (Quitão-Teixeira et al. 2008) demonstrated that treatments in bipolar mode induced lower RA than those applied in monopolar mode.

The difference in the effectiveness of polarity during HIPEF processing on the enzyme inactivation could be justified by the fact that successive monopolar pulses separate particles with electric charge and the formation of shielding layer in the surface of electrodes take place, inducing a non-uniform treatment and therefore, less enzyme inactivation occurs (Min et al. 2007). On the contrary, bipolar pulses minimize polar deposit of charged molecules, avoiding the shielding layer development and thus, making the HIPEF treatment more uniform (Barbosa-Cánovas et al. 1998, Min et al. 2007, Qin et al. 1994). Moreover, bipolar pulses have simultaneous positive and negative amplitude per pulse, which could cause changes in the direction of charged molecules (Barbosa-Cánovas et al. 1999, Barsotti & Cheftel 1999).

The findings observed in this study are in contrast with those reported by Aguiló-Aguayo et al. (2009b) and Elez-Martínez et al. (2006) in tomato juice LOX and orange juice POD, where these oxidative enzymes were more sensitive to monopolar than bipolar mode pulses. These discrepancies in polarity may be due to differences in enzyme structure, such as its quaternary arrangement (Elez-Martínez et al. 2006) and the food where it is present (Giner et al. 2001).

Electric field strength and treatment time

The RA of Chlase, LOX and PPO was also affected by the electric field strength and treatment time. Also, the interaction electric field strength – treatment time significantly influenced the Chlase, LOX and PPO RA. In fact, increasing both variables the RA of the enzymes was reduced. In all cases, the lowest RA was reached at the highest treatment time (2000 μs) and electric field strength (35 kV/cm) in bipolar mode. In agreement with these results, a great number of studies in vegetable (Min et al. 2003b), and fruit juices (Elez-Martínez et al. 2006, Elez-Martínez et al. 2007b, Giner et al. 2001, Riener et al. 2008a, Aguiló-Aguayo et al. 2010a), and model solutions (Luo et al. 2010, Zhong et al. 2005) have reported a major enzyme inactivation as electric field strength and treatment time increased.

Nowadays, the precise mechanisms concerned in enzyme inactivation by HIPEF are not fully understood; however, different theories have been postulated to explain the enzyme behaviour when a food is subjected to an electric field.

HIPEF processing could change the conformational state of enzymes at different structural levels leading to their inactivation. Barsotti and Cheftel (1999) proposed that HIPEF treatment destabilizes the covalent or non-covalent interactions, protein unfolding and denaturation. In the same way, some authors have suggested that enzyme inactivation by HIPEF treatment are the result of changes in secondary and tertiary structures that modify certain molecular linkages in the active centres and entire globular configuration (Góngora-Nieto et al. 2002, Zhong et al. 2005). In addition, a high correlation between the loss of secondary and tertiary structures and the diminution of peroxidase, PPO, lysozyme and LOX activity submitted to HIPEF treatment was reported (Luo et al. 2010, Yeom et al. 1999, Zhao & Yang 2008a, b, Zhong et al. 2005, Zhong et al. 2007). Furthermore, Zhao and Yang (2008b) and Zhong et al. (2007) reported an elevated correspondence between enzyme inactivation and the loss of α-helical fraction in secondary structure induced by HIPEF treatment. It could be due to HIPEF polarize the protein molecule, changing the protein conformation and subsequently their inactivation (Perez & Pilosof 2004). Hence, these findings could explain the Chlase, LOX and PPO inactivation observed when broccoli juice was treated by HIPEF.

In general, the enzyme activity exposed to HIPEF treatment depends of the treatment conditions and the enzyme itself. When the duration of the electric pulses is long enough, the influence of HIPEF could lead to conformational alterations of the enzymes, resulting in its inactivation (Elez-Martínez et al. 2007a, Zhao et al. 2007, Zhong et al. 2005).

On the other hand, reductions in the activity of Chlase, PPO and LOX were well correlated with the maintenance of bioactive compounds (Table 1). That is, a non-inactivation of oxidative enzymes means the loss of Chls, carotenoids and TP.

Enzymes exposed to similar HIPEF conditions led to different values of inactivation. At the highest HIPEF conditions (35 kV/cm for 2000 μs in bipolar mode) the Chlase was the most sensitive enzyme, followed by PPO. In contrast, LOX was the most resistant.

The incomplete reduction of Chlase activity by HIPEF in broccoli juice could be associated to the fact that thylakoid, chloroplast and cell membranes protected the Chlase against HIPEF. This hypothesis is supported by Hornero-Méndez and Mínguez-Mosquera (2001) and Matile et al. (1997) who demonstrated that Chlase is an enzyme bounded to chloroplast membrane. A notable resistance of broccoli juice LOX and PPO to HIPEF processing was observed. In fact, there was not PPO and LOX inactivation when broccoli juice was HIPEF-treated at 15 kV/cm and 500 μs in monopolar mode. The incomplete LOX and PPO inactivation by HIPEF in broccoli juice may be due to the presence of isoenzymes, which have different resistance to HIPEF treatment (Aguiló-Aguayo, Sobrino-López, Soliva-Fortuny, & Martín-Belloso, 2008; Min, Jin, & Zhang, 2003). In these sense, Indrawati, Van Loey, Ludikhuyze, and Hendrickx (1999) showed a heat-labile and a heat-stable fraction of green beans juice LOX. Similarly, LOX of tomato juice showed low sensitivity (RA_{LOX}= 80.26%) to HIPEF when pulses were applied at 35 kV/cm, 150 Hz for 1000 μs and 1 μs of pulse width (Aguiló-Aguayo, Soliva-Fortuny, & Martín-Belloso, 2009). Green pea LOX was

not inactivated when pulses at 20 kV/cm for 400 µs were applied (Van Loey, Verachtert, & Hendrickx, 2001).

On the other hand, the variation between LOX and PPO inactivation could be explained by the differences in the molecular weight among enzymes, which could be a determinant factor in the enzyme inactivation. Moreover, the diverse sensitivity of enzymes to HIPEF could be based on the differences in the secondary or tertiary structure among enzymes (Min et al. 2007).

Influence of HIPEF processing parameters on color of broccoli juice

Color difference (ΔE) was significantly affected by the HIPEF parameters electric field strength and treatment time but not by polarity. A second order model fit properly the experimental data ΔE. The determination coefficient (R^2) was 0.91 and the lack of fit was not significant, indicating that the model was adequate for predicting the response across the design space.

The lowest ΔE value (0.29 units) was observed at 15 kV/cm for 500 µs in bipolar mode. Cortés, Esteve, Rodrigo, Torregrosa, and Frígola (2006) observed ΔE values of up to 7 units when orange juice was processed by HIPEF at 25 kV/cm for 340 µs. Yin, Han, and Liu (2007) described an increment in a^* parameter as electric field strength increased from 20 to 60 kV/cm, when spinach puree was HIPEF treated. On the contrary, Akin and Evrendilek (2009) reported that HIPEF treatments between 13-27 kV/cm from 82 to 262 µs did not display significant differences in L^*, a^* and b^* parameters of a formulated carrot juice, regarding to the unprocessed juice. Similarly, Quitão-Teixeira, Aguiló-Aguayo, Ramos, and Martín-Belloso (2008) reported that there were not differences in the color coordinates between HIPEF processed (shifting frequency from 50 to 250 Hz and pulse width from 1 to 7 µs, in bipolar or monopolar mode) and untreated carrot juice.

Color changes in HIPEF-processed broccoli juice could be justified by the fact that metal ions released during HIPEF processing could replace the magnesium atom of chlorophyll or its derivative compounds, changing their optical properties and thereby generating a color change in broccoli juice. This hypothesis is supported by the fact that the formation of metallocomplexes of chlorophyll derivatives during food processing has been observed in green vegetables (LaBorde & Von Elbe, 1994).

From an industrial point of view, ΔE values between 1.1–2.8 and 2.8–5.6, correspond to rigorous and normal color tolerances, respectively. Values higher than 5.6 units are easily distinguished (Lozano, 1978). In this research ΔE of broccoli juice displayed less than 3 units; therefore, HIPEF processing could be considered that did not have a significant influence on visual color appearance.

Comparison between HIPEF and thermal treatment

There were noticeable differences in the retention of bioactive compounds and enzyme inactivation, as well as in the color difference (ΔE) between HIPEF and thermal treatment. In general, HIPEF-processed broccoli juice exhibited higher RC of bioactive compounds and lower (ΔE) than that treated by heat. After thermal treatment a complete enzyme inactivation was achieved.

At any HIPEF condition, the RC of Chls, carotenoids, and TP was greater than those obtained in thermally treated broccoli juice. RC of Chl *a* and Chl *b* in heat treated broccoli juice diminished 81.72% and 75.77%, respectively. Whereas after HIPEF processing better retention and even some increments in the Chls content was achieved. Therefore, the formation of Phe (RC= 186.31%) and Phb (RC= 142%) in heat-processed broccoli juice was greater than that treated by HIPEF. In contrast, no significant differences in the RC of Chlide among treatments were observed. It could be attributed to the formation of Chlide by the Chlase enzyme action (Schwartz & Lorenzo 1990), but also by a high temperature effect (Canjura & Schwartz 1991). Traditional pasteurization of broccoli juice inactivated Chlase but Chlide formation was observed due to the high temperature applied (90 °C). HIPEF treatment was performed at temperature below to 35 °C; therefore, the residual activity of Chlase could catalyze the formation of Chlide.

The Phe and Phb formation during thermal treatment are the most harmful reactions (Coultate 2009, Simpson 1985). In spite of thermal processed broccoli juice exhibited the highest formation of Phe and Phb, their values of luminosity (L^*) were significantly greater than that treated by HIPEF. It could be associated to the fact that broccoli juice was heat treated through a copper tube. Hence, regreening may occur when copper is released from the tube to the juice substituting the magnesium atom of tetrapyrrole ring (Simpson 1985). Consistently, during thermal processing the copper content rose 23.7% in comparison to fresh and HIPEF-treated broccoli juice.

The RC of lutein and β-carotene after thermal processing was 51.17 and 30.57%, respectively. Hence, greater retention of carotenoids in HIPEF-treated broccoli juice in comparison with that heat treated. In agreement with the results reported in this research, Odriozola-Serrano et al. (2009a) observed a better preservation of tomato juice carotenoids after HIPEF processing than thermal treatment.

In the case of vitamin C, similar content between thermally and HIPEF treated broccoli juice was observed only at 25 kV/cm for 1250 μs in bipolar mode. However, the vitamin C content of broccoli juice was greater in the rest of HIPEF conditions than that thermally treated juice. In the same line, Min et al. (2003a), Sánchez-Moreno et al. (2005), Elez-Martínez and Martín-Belloso (2007) and Zulueta et al. (2010) reported higher vitamin C losses in thermally processed tomato, and orange juice, "gazpacho" and orange juice-milk beverage, respectively, than those products treated by HIPEF.

The RC of TP in HIPEF-treated broccoli juice ranged from 79.6 to 96.1%. In comparison to fresh and samples processed by HIPEF, significant losses were observed when broccoli juice was heated (28.16%). In accordance with the present research, studies carried out in tomato and apple juices, demonstrated that TP content was lower after heat treatment than that of HIPEF (Aguilar-Rosas et al. 2007, Odriozola-Serrano et al.

2008b). On the contrary, Odriozola-Serrano et al. (2009a) and Quitão-Teixeira et al. (2009) did not find significant differences in the content of TP between untreated and HIPEF-processed tomato and carrot juice, respectively.

Minerals such as, K, Mg, P and S in broccoli juice were not significantly affected by the thermal treatment applied. In contrast, the content of Fe, Ca, Mn, Na, Zn and Se diminished in comparison to untreated broccoli juice. The trend observed in these elements suggests changes in their solubility due to thermal treatment as was reported by Koplík et al. (2004), whom observed a diminution in the mineral content of peas after been cooked. These authors also proposed that thermal treatment could lead to denaturation of metallobiomolecules or liberation of the metal ions and formation of insoluble inorganic compounds. However, the extent of change in the content of minerals depends on the mineral stability. In contrast, the RC of Mg, Cu and S incremented in thermally processed broccoli juice, being Cu the mineral with the highest increment. The noticeable rise in Cu observed in this study could be associated to the release of this mineral from the tubular heat exchanger (made of copper) towards broccoli juice during this treatment. In line with these results, Gutzeit et al. (2008) observed losses and increments in mineral content in heat-processed sea buckthorn juice, suggesting as causes the processing steps. Morales-de la Peña et al. (2011) observed significant changes in the Mn and Fe content in a fruit juice-soymilk beverage processed by heat. However, these authors did not find differences in the content of Cu, Ca and Mg between heat treatment, HIPEF (800 μs) and unprocessed beverages.

Amino acids were well preserved after HIPEF processing, excluding those conditions at 35 kV/cm for 2000 μs in monopolar mode, which noticeable reductions in amino acids took place. On the contrary, significant losses in the amino acids content (excepting Asp) were observed after applying heat processing. In agreement with the results showed in this research, Murcia et al. (2001) reported that the amino acids concentration was reduced when the whole broccoli was treated by heat at different times. In contrast, Garde-Cerdán et al. (2007) did not found differences in the free amino acids concentration between thermally treated and untreated grape juice.

There were no differences in the relative AC between thermally treated broccoli juice (72.4%) and that HIPEF processed at 15 kV/cm for 500 μs in monopolar mode (71.9%). Nonetheless, in the rest of HIPEF conditions, the relative AC was always higher than thermal processing. Cortés et al. (2008) compared the AC between orange juices treated at 30 kV/cm for 100 μs and heat processed, observing that the highest antioxidant capacity occurred in HIPEF-treated orange juice. The losses of heat-sensitive nutrients such as vitamin C, polyphenols, and carotenoids during juice processing are directly related to AC depletion (Odriozola-Serrano et al. 2008c, Plaza et al. 2006). In contrast, Odriozola-Serrano et al. (2008b) reported that heat treatment had a similar AC respect to fresh tomato juice. This fact could be linked to the formation of Maillard reaction products, which has antioxidant properties (Odriozola-Serrano et al. 2008b). Differences in AC reported in literature could be associated to different severities of the heat treatment (Gliszczyńska-Świgło et al. 2006).

The depletion of bioactive compounds has been associated to the effect of heat and oxidation during thermal processing (Shoji 2007). Nonetheless, the difference between treatments could be attributed to the susceptibility of bioactive compounds to high temperature applied during thermal processing. In this respect, when broccoli juice is heated a wide variety of chemical reactions on the bioactive compounds can happen, such as oxidation, carboxylation, dehydrogenation, isomerization, Maillard reaction (Belitz et al. 2004, Feeney et al. 1985, Simpson 1985). Indeed, it is important to highlight that throughout HIPEF treatments the temperature never exceeded 35 ^{o}C. Therefore, bioactive compounds are less affected by HIPEF than heat treatment.

Within the range of assayed conditions, HIPEF-treated broccoli juice attained lower ΔE values than that of thermally treated, which could be associated to higher retention of pigments, such as chlorophylls, phenols, and carotenoids during HIPEF processing. The ΔE observed in HIPEF processed broccoli juice could be associated to the apparition of degradation compounds of chlorophylls, such as pheophytin and pheophorbide. On the contrary, thermal processed broccoli juice exhibited higher brightness (L^*) than those treated by HIPEF. This pattern could be explained by the fact that broccoli juice was passed through a copper tube during thermal treatment. Therefore, regreening may happen when copper substitutes the magnesium atom of tetrapyrrole ring from chlorophyll (Simpson, 1985). This trend was not observed in juices that not contain chlorophylls. For instance, orange juice treated by HIPEF at 40 kV/cm for 97 μs, showed higher L and hue angles values than that thermally processed (Min, Min, & Zhang, 2003).

Chlase, LOX and PPO were completely inactivated when broccoli juice was heated at 90 ^{o}C for 60 s. Enzymes are globular proteins sensitive to denaturation by heat. However, uncomplete broccoli juice LOX and PPO inactivation was reached when HIPEF treatments were applied. Notwithstanding, HIPEF processing has shown higher retention of bioactive compounds and sensorial characteristics than those juices treated by heat.

Optimization of HIPEF process

An optimization was obtained through Design Expert software in order to find the HIPEF conditions at which the greatest enzyme inactivation and bioactive compounds retention, as well as the lowest formation of products of chlorophyll degradation and color difference could be obtained. The combination of these conditions is shown in Table 2.

The optimal conditions for HIPEF processing were fixed at 27.81 kV/cm for 2000 μs in bipolar mode.

Table 1. Overall correlation among bioactive compounds in HIPEF-processed broccoli juice

	Chlase	Chl a	Chl b	Lut	β-Carot	AC	TP	Vit C	LOX	PPO	ΔE	Chlide	Phe	Phb	AA	Se
Chlase		-0.7389[a]	-0.8209[a]	-0,2104	-0,1937	-0.5525[a]	0.1657	0.3257	0.2586	0.3120	-0,4442[a]	0.8925[a]	0.4185[a]	0.6351[a]	0,2269	0,3537
Chl a	-0.7389[a]		0,9709[a]	0,8519[a]	0,8435[a]	0,5277[a]	0.3671	0,1277	-0.5527[a]	0.2947	0,6515[a]	-0.8733[a]	-0,8269[a]	-0.8367[a]	0,0253	0.3421
Chl b	-0.8209[a]	0,9709[a]		0,8879[a]	0,8836[a]	0,5780[a]	0,3877[a]	0,1664	-0.6956[a]	0,1644	0,6955[a]	-0.8649[a]	-0,8279[a]	-0.3584	-0,0325	0.5384
Lut	-0,2104	0,8519[a]	0,8879[a]		0,9778[a]	0,6786[a]	0.0212	-0,0326	-0.7882[a]	-0,2382	0,5998[a]	0.1783	0.1689	0.1133	0.3445	0.5345
β-Carot	-0,1937	0,8435[a]	0,8836[a]	0,9778[a]		0,5996[a]	0,2069	0,1259	-0.7351[a]	-0,2177	0.6708[a]	-0.5202	-0.5642	-0.4447	-0,1695	0.6221
AC	-0.5525[a]	0,5277[a]	0,5780[a]	0,6786[a]	0,5996[a]		0,7377[a]	0.4932[a]	-0.6388[a]	-0,3162	0,1917	0.3951[a]	0.3560[a]	0.2312	0.5592[a]	0,5919[a]
TP	0.1657	0.3671	0,3877[a]	0.0212	0,2069	0,7377[a]		-0,2111	0,4054[a]	-0.7412[a]	-0,6121[a]	0.2718	0.1589	0.0731	0.3574	0,3518
Vit C	0.3257	0,1277	0,1664	-0,0326	0,1259	0.4932[a]	-0,2111		0,2934	0,2461	0.0948	0,3246	0,0098	0,2295	-0,3783	0,0769
LOX	0.2586	-0.5527[a]	-0.6956[a]	-0.7882[a]	-0.7351[a]	-0.6388[a]	0,4054[a]	0,2934		0.2585	-0,6829[a]	0.3786	0.3940	0.3848	0,2401	0,5573[a]
PPO	0.3120	0.2947	0,1644	-0,2382	-0,2177	-0,3162	-0.7412[a]	0,2461	0.2585		-0,4940[a]	0.1598	0.1041	0,1634	0,2331	0,1672
ΔE	-0,4442[a]	0,6515[a]	0,6955[a]	0,5998[a]	0.6708[a]	0,1917	-0,6121[a]	0.0948	-0,6829[a]	-0,4940[a]		-0,5103[a]	-0,5935[a]	-0.5018[a]	0.2435	0.1534
Chlide	0.8925[a]	-0.8733[a]	-0.8649[a]	0.1783	-0.5202	0.3951[a]	0,2718	0,3246	0.3786	0.1598	-0,5103[a]		0,1012	0.3910	0,2036	-0,0762
Phe	0.4185[a]	-0,8269	-0,8279	0.1689	-0.5642	0.3560[a]	0.1589	0,0098	0.3940	0.1041	-0,5935	0,1012		0.5811	0.2581	0.1221
Phb	0.6351[a]	-0.8367[a]	-0.3584	0.1133	-0.4447	0.2312	0.0731	0,2295	0.3848	0,1634	-0.5018[a]	0.3910	0.5811		0.2072	0.1938
AA	0,2269	0,0253	-0,0325	0.3445	-0,1695	0.5592[a]	0,3574	-0,3783	0,2401	0,2331	0.2435	0,2036	0.2581	0.2072		0,0690
Se	0,3537	0.3421	0.5384	0.5345	0.6221	0,5919[a]	0,3518	0,0769	0,5573[a]	0,1672	0.1534	-0,0762	0.1221	0.1938	0,0690	

Table 2. Optimization of HIPEF processing of broccoli juice

E	t	Polarity	Chlase[a]	Chl a[b]	Chl b[b]	Lut[b]	β-Carot[b]	AC[b]	TP[b]	Vit C[b]	LOX[a]	PPO[a]	ΔE	Chlide[b]	Phe[b]	Phb[b]	Desirability
27.81	2000	Bipolar	33.49	107.84	111.03	113.46	113.26	87.34	92.47	67.74	73.87	42.75	1.18	117.08	93.30	96.73	0.70

E = Electric field strength (kV/cm)
t = Treatment time (µs)
[a] = Variables expressed as residual enzyme activity (%)
[b] = Parameters expresed as relative content (%)

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VI CONCLUSIONS

CONCLUSIONS

From the studies developed in the present work and taking into account the fixed objectives, it can be settled that high-intensity pulsed electric fields (HIPEF) processing is able to preserve the bioactive compounds, color, as well as inactivate deteriorative enzymes presents in broccoli juice.

Particularly it was observed that:

- The effect of HIPEF parameters on bioactive compounds and deteriorative enzymes was adequately modelled using equations of second order, with coefficients of determination (R^2) between 0.74 and 0.99, with the exception for minerals and amino acids.

A) Bioactive compounds

- The HIPEF parameters electric field strength and treatment time significantly influenced the relative content (RC) of the bioactive compounds lutein, β-carotene, chlorophylls, vitamin C, total phenolic compounds, minerals, amino acids, as well as on the relative antioxidant capacity (RAC) of broccoli juice.

- The RC of Chl *a*, Chl *b*, lutein and β-carotene increased when broccoli juice was HIPEF-processed. That is, when electric field strength and treatment time increased, the content of chlorophylls and carotenoids augmented, while the RC of degradation compounds of Chls (Chlide, Phe and Phb) diminished.

- The application of pulses in bipolar mode led to higher RC of Chls, carotenoids total phenolic and RAC than those applied in monopolar mode. In contrast, RC of vitamin C, Chlide, Phe and Phb was greater with pulses in monopolar mode in comparison with those applied in bipolar mode.

- HIPEF treatments applied in bipolar mode led to higher relative content of Chl *a*, Chl *b*, lutein, β-carotene, total phenolic and RAC than those applied in monopolar mode. In contrast, RC of vitamin C was greater when broccoli juice was treated by HIPEF in monopolar mode in comparison with bipolar mode.

- Broccoli juice treated by heat exhibited the highest losses of bioactive compounds and RAC, in comparison with those HIPEF-treated and unprocessed broccoli juice.

B) Enzymes and color

- HIPEF was able to reduce the enzyme activity of chlorophyllase; however, HIPEF resistance was observed in the enzymes PPO and LOX. The enzyme that displayed the lower susceptibility was LOX, followed by PPO.

- In general, enzyme inactivation was higher as processing conditions increased. That is, the higher electric field strength and treatment time, the higher enzyme inactivation.

- HIPEF treatments applied in bipolar mode demonstrated to be more efficient in the enzyme inactivation than those in monopolar mode.

- HIPEF-processed broccoli juice showed the lowest changes of color (ΔE), in comparison with that treated by heat.

General conclusion

- The results obtained in this study demonstrated that HIPEF technology may represent a good alternative to traditional thermal treatments for obtaining broccoli juice enzymatically stable with high nutritional value and visual quality.