**Effects of high intensity ultrasound on disaggregation of a macromolecular
procyanidin-rich fraction from *Vitis vinifera* L. seed extract and evaluation of its
antioxidant activity**

Ana Muñoz-Labrador, Marin Prodanov and Mar Villamiel*

*Instituto de Investigación en Ciencias de la Alimentación CIAL (CSIC-UAM).C/Nicolás
Cabrera 9, Universidad Autónoma de Madrid, 28049 Madrid, España.

*Author to whom correspondence should be addressed:

Tel: +34 910017951

E-mail: m.villamiel@csic.es

Abstract

The impact of high intensity ultrasound (US, 45 and 20 kHz) on a purified macromolecular fraction (more than 85% of polymeric procyanidins) from grape seed extract was investigated. Matrix-Assisted Laser Desorption/Ionisation (MALDI TOF), Reverse Phase High Performance Liquid Chromatography (RP-HPLC) and Fourier-transform infrared spectroscopy (FTIR) revealed a modification in the chemical structure of these macromolecules treated by US and, particularly, bath US produced a considerable increase of up to 49, 41 and 35%, respectively, of catechins and oligomeric and polymeric procyanidin contents of the treated purified fraction. Bath US also produced, an important increase in the number of procyanidins with higher molecular mass (up to decamers) and an overall increase in the mass signal intensities in most of the detected B-type procyanidin series, as well as an important increase of the antioxidant activity of the macromolecular fraction of procyanidins. These results could be ascribed to a certain disaggregation of procyanidins linked to other biopolymers, such as proteins and/or polysaccharides, indicating that US is an efficient technology to modify the chemical structure and hence the bioactivity of tannins.

Key words: ultrasound, procyanidins, grape seeds, disaggregation, antioxidant activity.

1 Introduction

Proanthocyanidins (PCs), also known as polyflavan-3-ols or condensed tannins, are the second most abundant natural phenolics in plants and consist of mixtures of oligomers made of flavan-3-ol monomeric units linked mainly through C-4 and C-8 or C-6 bond [1–4]. Beyond contributing to organoleptic characteristics of foods with astringency, bitterness, sourness, sweetness, salivary viscosity, aroma and color formation, they are responsible for some physiological effects in humans, such as cardiopreventive, anti-inflammatory, antioxidant, antiallergic, antithrombotic, antibacterial and anticarcinogenic activities, among others, supposing benefits to human health [5–9].

Main dietary sources of PCs are beans, cinnamon, nuts, ~~tea tee~~, apples, cocoa, grapes and a wide number of berries [10]. From industrial point of view, grape seeds are the most interesting because of their high content in procyanidins, low cost and abundance as a by-product of the wine industry. This is one of the main reason for the existence of wide varieties of grape seed extracts (GSE) rich in PCs in the world dietetic and supplementary market [11]. Most valued are those known as oligomer procyanidin (OPC) extracts, because of their high bioavailability and low content of highly polymeric procyanidins (PPC), considered also as ~~potent~~ antinutrients. Nevertheless, purification of OPCs generates an important amount of PPC that should be managed in one way. The most proper possibility to reuse PPCs should be their depolymerisation to catechins or OPCs, using preferably physical treatments [12, 13].

Power or high intensity ultrasound (US) are considered an physical emergent and environmentally friendly process that involves reduced times of treatments and energy, constituting an alternative to conventional processes [~~14-16~~~~12-14~~]. The US technology has been developed for processing, conservation and extraction, among other techniques, and is based on the application of acoustic waves of intensity beyond the limit of human hearing

(>16 kHz). Those waves pass through the material in which is spread and the velocity depends on the nature of the wave and the propagation medium. The US propagates by compression and rarefaction series through the medium obtaining the cavitation phenomenon previously mentioned [142]. Due to the concentration of high quantities of energy in different points of the medium treated by US, one of the principal applications is related with the depolymerisation of macromolecules of biological origin. In general, it is considered that whenever there is a decrease in the molecular size, the biological properties can be modified. Hence, there are some reviews [17, 18+5,16] on the principal effects of high intensity US on the disaggregation of proteins and polysaccharides, respectively. However, no studies have been previously reported neither on the US effect on the depolymerisation/disaggregation of tannins nor on the modification of its bioactivity. In general, the main factors that can affect these phenomena are the power, frequency, time, and temperature regarding the working conditions; and the type, molecular mass and concentration in what refer to biomolecules. Thereby, the aim of this work was to study the effect of high intensity ultrasound on procyanidins derived from by-products of wine industry and their antioxidant activity. All of this with the purpose of obtaining potential ingredients with improved functionality in order to raise the value of the raw material and to establish a new approach for reusing PPC by-products.

2 Material and methods

2.1 Samples

Grape seed extract (GSE) with 26% of total soluble substances (TSS) was provided by Output Trade S.L., Villafranca del Penedés (Spain). It was obtained from mature fresh grape seeds from *Vitis vinifera* L. grapes, cv. Airén, cultivated in La Mancha grape vine-growing area (Spain). A high macromolecular fraction was obtained from the above mentioned GSE by cross-flow pressure-driven ultrafiltration (UF) using a membrane of 10

kDa molecular mass cut-off (0.54 m² filtration surface, regenerated cellulose spiral-wound) (model Prep-scale 6) from Millipore (Merck, Darmstadt, Germany), as described in Silván et al. [197]. The PPC fraction was diafiltrated to remove minimal quantity of low molecular weight compounds and freeze-dried. An amount of 106.1 g of dry sample was recovered, which represents about 22% of the dry matter of the clarified GSE (data not shown). The process related to the obtainment of procyanidins fraction is shown in Fig. S1. Samples were stored in dark at 4°C until processing and analysis.

2.2 High intensity ultrasound treatments

Samples obtained in section 2.1 were diluted with distilled water at 0.01, 0.1 and 1 % (w/v) and two different types of assays were performed: i) in ultrasonic bath and ii) in ultrasonic probe. All the treatments were carried out in duplicate.

Ultrasonic bath (Brandson Digital Sonifier 450 full power, 12.7 mm Biogen Científica S.L.) with a frequency of 45 kHz was used to sonicate a volume of 10 mL of each sample dilution in continuous and degas mode for 30 min. Temperature was monitored reaching ranges between 25 and 30 °C.

The ultrasonic probe (~~Brandson~~Branson Digital Sonifier 450 full power, 12.7 mm Biogen Científica S.L.) was applied into 50 mL of each sample dilution through a ~~microtip~~ horn of 12.7 mm diameter at 20 kHz, in pulsed mode (5 s on/5 s off) at 30 and 70% of amplitude whose conditions were digitally set. The temperature was controlled through an ice-water bath to avoid reaching temperatures higher than 60°C in order to maximize the US effect [186]. Samples were finally freeze-dried before analysis.

2.3 ~~Characterisation of procyanidin~~ Characterisation of the isolated GSE macromolecular fraction

The protein content of samples was determined through a colorimetric assay using Bicinchoninic acid (BCA) and bovine serum albumin as standard protein (0.02-2 mg/mL). The product of the reaction is purple and it is formed by the chelation of two molecules of BCA with a cuprous ion. Samples were incubated with the reagent for 15 min at 60 °C and aliquots of 300 µL were analyzed at 560 nm.

Soluble fraction of carbohydrates was analysed by GC-FID previous derivatisation reaction. Trimethylsilylated oximes (TMSO) of the carbohydrates present in samples were determined following a previous method [2048]. A volume of 100 µL of supernatant of PPCs at 10% (w/v) was added to 400 µL phenyl-B-D-glucoside (0.5 mg/mL, internal standard) and evaporated under vacuum with a rotary evaporator. The sugar oximes formation was carried out by adding 250 µL hydroxylamine chloride (2.5 %) in pyridine and heated at 70 °C for 30 min. Afterwards, the oximes were silylated with hexamethyldisilazane (250 µL) and trifluoroacetic acid (25 µL) at 50 °C for 30 min. Reaction mixtures were centrifuged at 10,000 rpm for 2 min and supernatants were injected in GC with the split mode (1:5). Chromatographic analysis was carried out on an Agilent Technologies gas chromatograph (Mod7890A) (Agilent Technologies, Wilmington, DE, USA) equipped with a flame ionisation detector (FID). The TMSO were separated using a 15 m x 0.25 mm x 0.10 µm film, capillary column (SGE HT5, North Harrison Road, Bellefont, USA). Nitrogen was used as carrier gas at flow rate of 1 mL/min. Injector and detector temperatures were 280 and 385 °C, respectively. The oven temperature was programmed from 150 to 380 °C at a heating ratio of 3 °C/min. Data acquisition and integration were performed using Agilent ChemStation software (Wilmington, DE, USA). Quantitative data for carbohydrates were

calculated from FID peak areas relative to phenyl-B-D-glucoside. All analyses were done in duplicate.

Semi-quantitative determination of PPC was done by Normal Phase High Performance Liquid Chromatography (NP-HPLC), according to the method described by Kelm [2149] for analysis of cocoa procyanidins and adapted by Prodanov et al. [2220] for analysis of grape seed procyanidins. Analysis was performed in a ProStar 230 (Varian Instruments, Walnut Creek, California, USA) liquid chromatograph, composed by a tertiary piston pump model 240, automatic autosampler model 410, online degassing system (Althech, Flemington, NJ, USA), Diol (Teknokroma, Tarragona, Spain) stationary phase column (Kromasil 60, 25 x 0.46 cm; particle size 5 µm), column thermostat and photodiode-array detector (PAD) model 335. The mobile phase was composed by: component A: CH₃CN/acetic acid (98/2 v/v), B: CH₃OH/water/acetic acid (95/3/2, v/v) and C: water/acetic acid (98/2, v/v). Column temperature was fixed at 35°C, flow rate was 0.8 mL/min and the detection was carried out at 280 nm. The wavelength of excitation and emission was 273 and 316 nm, respectively.

Qualitative analysis of PPCs was carried by a Matrix-Assisted Laser Desorption/Ionisation Time of Flight Mass Spectrometry (MALDI-TOF MS). Samples of the studied GSE were dissolved in water at a concentration of 2 mg/mL. Aliquots of 5 µL of this solution were mixed with 20 µL of matrix solution (2,5-dihydroxybenzoic acid (DHB) (10 mg of DHB/mL of methanol/water (9/1). Amounts of 0.5 µL of this mixture were placed on stainless-steel plates and dried at open air (without air current). MALDI-TOF measurements were carried in the Ultraflex III TOF/TOF mass spectrometer from Bruker (Billerica, MA, USA) provided with a Nd:YAG laser and operating at 355 nm. All mass spectra were performed in reflector positive mode applying a deflection mass cut off of 450

Da, averaging at least 1000 shots over the 450-5000 Da range. An external calibration was applied using a peptide mixture from Bruker [2324].

2.4. Structural determinations

In order to study possible modifications in the functional groups of the molecules, an analysis through FT-IR (Fourier transform infrared spectroscopy) of control and US treated samples was done using a FTIR Bruker IFS66v with 0.5 mg of samples in a KBr pill. Measurements were done with a spectral of 7000-550 cm⁻¹ (medium IR) and with 4 cm⁻¹ of resolution. The degree of methoxylation (DM) was defined [2422] as (number of esterified carboxylic groups/number of total carboxylic groups) x 100 corresponding to the ratio of the area of the bands presented in the following equation: $DE = A_{1730} / (A_{1730} + A_{1600})$.

2.5. Antioxidant activity

DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was employed to determine the antioxidant potential of the PPCs after the US treatment. Samples (7 µL) corresponding to the different assays carried out by US, were treated with 193 µL of DPPH and incubated in darkness for 30 min at room temperature. The measurements carried out at 517 nm using a spectrometer (Lector KcJunior Biotek) and the antioxidant activity was expressed as percentage of inhibition, corresponding to the quantity of the radical DPPH neutralised by the extract according to the following equation where A_B corresponds to the absorbance of the blank and A_E to the absorbance of the sample at 517 nm: ~~Inhibition percentage = ((Control absorbance - Sample absorbance)/Control absorbance)*100 [2523]:~~

$$DPPH \text{ inhibition (\%)} = \frac{A_B - A_E}{A_E} \times 100$$

2.6. Statistical analysis

The antioxidant behavior of the samples treated by the different sonication modes was studied in triplicate and analyzed twice (n=6) using the one-way analysis of variance (P<0.05) and Tukey's *post hoc* test with SPSS for Window, version 22.0 (IBM Corp., Armonj, NY, USA) in order to determine the significant differences among all the treatments and sample concentrations.

3 Results and discussion

3.1. Characterization of procyanidin-rich macromolecular fraction from a grape seed extract

Samples obtained by UF process were initially characterized; they presented other compounds different from PCs that could be linked to the corresponding oligomers such as carbohydrates or proteins. The previous characterization shows a minimum content of those biomolecules with respect to the major compounds of the sample. The BCA assay showed that PCs sample contains 0.012 g of protein per g of dry extract, and in the analysis by GC-FID soluble carbohydrates as sorbitol, fructose, glucose and *myo*-inositol (1.9, 13.8, 7.3 and 0.2 mg/g of extract, respectively) were also found.

Procyanidins were analyzed by NP-HPLC (Fig. 1) and they eluted in growing order of molecular mass M_w [21,22,19,20]. Numerous peaks were detected in the samples classified in different families of compounds: monomers (catechin, epicatechin), dimers (8 dimeric B-type proanthocyanidins), trimers (64 trimeric B-type proanthocyanidins), etc.) [24–27]. As it can be seen from the chromatogram, more than 85% of its constituents correspond to PPCs and/or procyanidin complexes. The chromatogram also shows that 12% of highly polar compounds and some small amounts (2.5%) of monomeric and oligomeric

flavan-3-ols remained retained in this fraction, due to incomplete purification after the UF treatment.

For the specific identification of procyanidin polymers, MALDI-TOF MS analysis was performed. As one of the main objectives was searching for differences between same species (not for detection of higher molecular masses), reflectron mode was selected. Deflection of the ion current was also set at 450 Da to avoid detector saturation by ions with molecular masses lower than 450 Da and to improve analytical responses of higher molecular mass species. Results for the detected procyanidin ions of the purified fraction are shown in Fig. 2A and Table 1. A global predominance of sodium adduct ions of B-type procyanidins were found, which differ from the corresponding procyanidin ions with 23 Da. No protonated molecular ions were detected. Six B-type procyanidin series were identified, differing among them by one gallic acid (152 Da) and/or one (epi)catechin (288 Da) elemental units, namely nongalloylated (from trimers to octamers), monogalloylatedgalloylated (from trimers to octamers), digalloylatedgalloylated (from trimers to nanomers), trigalloylatedgalloylated (from trimers to octamers), tetragalloylatedgalloylated (from trimers to octamers), pentagalloylatedgalloylated (from trimers to heptamers) and hexagalloylatedgalloylated (from trimers to heptamers) procyanidins. On the other hand, thanks to the applied deflection of low molecular mass ions, the use of positive ionization mode and DHB matrix, detection of procyanidins with degree of galloylation of up to 6 was achieved by the used reflectron mode, which is a noticeable improvement of previous analysis of grape seed extracts [268,279]. Signals corresponding to A-type procyanidins (2 Da less than the predominant B-type procyanidin molecular ions (sodium adducts)) were identified also, belonging to 4 series of procyanidin oligomers (nongalloylated and mono-, di-, and tetragalloylated species) but they will be not discussed here because of their low intensities.

In addition, 3 consecutive series of procyanidin polymers were identified that differ by 16 Da (Fig. 2b). They have been found not only for GSE [268], but also for other proanthocyanidin species from other food sources [28-2930,31]. The results from Table 1 indicate that this is rather a difference between the molecular masses (sodium adducts) between one n-mer nongalloylated (PCCn,G0), one (n-1)-mer digalloylated (PCCn-1,G2) and one (n-2)-mer tetragalloylated procyanidin (PCCn-2,G4), i.e. these differences belong to the already mentioned six procyanidin series. Nevertheless, ions with lower masses (m/z 1497 and 1650) (data not shown) that follow this pattern were also detected. These ions are higher with 152 Da and 2×152 Da than the ion of the trigalloylated procyanidin trimer (m/z 1345 Da) and according to the so established pattern, should correspond to tetra- and pentagalloylated procyanidin trimers, respectively. Such a type of procyanidins has never been described in the literature.

3.2. Effect of high intensity ultrasound on the structural modifications of the procyanidin-rich macromolecular fraction

The study on the effect of US on the structural changes of the procyanidin rich fraction was carried out by HPLC, MALDI and FTIR. 0.1% solutions of purified fraction were treated with US in a bath (continuous and pulsed) and probe under the conditions already described in the section of Material and Methods. Aliquots of these extracts were analyzed by NP-HPLC. The obtained results are shown in Fig. 1. As observed, the main feature is related to a noticeable general increase in the area of peaks corresponding to all groups of flavan-3-ols of the treated with US. Comparison of the areas of the corresponding peaks shows that sonication in US bath under continuous mode was the most effective, among the US systems and conditions used, giving rise to a considerable increase of up to 49, 41 and 35%, respectively, of the peaks corresponding to catechins, oligomeric and polymeric procyanidins.

MALDI-TOF MS analysis of the US treated samples (Table 1 and Fig. 2) shows an important increase in the number of procyanidins with higher molecular mass and number of galloyl esters in most of the detected B-type procyanidin series. In this sense, the series of nongalloylated and mono-, di- and trigalloylated procyanidins rose up to decamers, the tetragalloylated procyanidins rose up to nanomers and the pentagalloylated procyanidins rose up to heptamers. Another relevant difference revealed with by the MALDI-TOF MS analysis was an overall increase of the mass signal intensities in the sonicated procyanidin-rich macromolecular fraction and that this increase was more evident for the non- and monogalloylated species than for these with higher degree of galloylation and higher molecular mass (Table 1, Fig 2). Some exceptions (indicated with * in Table 1) were observed only for the di- and trigalloylated procyanidin dimers, the digalloylated procyanidin trimer, the tri- and pentagalloylated procyanidin heptamers and the tetragalloylated procyanidin octamer, which intensities diminished or did not change. Probably as a consequence of all these changes, sonication produced also an important reduction of the baseline noise of the MALDI-TOF spectrum, which results in a general improvement of selectivity and sensitivity of this technique.

FTIR analyses were carried out to identify any possible changes in the basic structure of the sonicated procyanidin-rich macromolecular fraction. The obtained FTIR spectra (Fig. 3) show the presence of several functional groups such as, hydroxyl groups (belonging to procyanidins or carbohydrates) with their typical band at 3408 cm^{-1} , C-H vibrations of methyl and methylene groups registered at 2923 cm^{-1} , 2852 cm^{-1} and 1613 cm^{-1} , carbonyl groups (from carbohydrates or free and/or esterified gallic acid from procyanidins) with a weak signal at 1750 cm^{-1} , saturate C-C bonds, corresponding to bands at 1370 cm^{-1} , 1285 cm^{-1} , 1242 cm^{-1} , 1105 cm^{-1} and 1061 cm^{-1} , aromatic compounds with their characteristic peaks at 1524 cm^{-1} and 1444 cm^{-1} and benzene rings and their peaks in the interval of 575 cm^{-1} .

864 cm⁻¹. A signal with slight intensity at 1040 cm⁻¹ was also distinguished, which could be attributed to an opening of the structure of cyclic ethers of polyflavan-3-ols [30-32,34]. Nevertheless, these results indicate that ~~there were~~ no appreciable differences in the functional groups ~~were found~~ between the US treated procyanidins and control procyanidins fraction, suggesting that the observed effect could be related, among other factors, to a possible breakdown of some weak PPC bonds with proteins and/or polysaccharides that break free procyanidins.

3.3. Effect of US on the antioxidant activity

Fig. 4 shows the changes in the antioxidant activity of procyanidin rich fraction produced as consequence of the US treatments with respect to the control samples. As observed, in most of the assays, an increase in the antioxidant activity after applying US was found, probably due to the increase of antioxidant compounds, attributed basically to PPCs, in agreement with the results indicated above, since the sonication process could have produced a breakdown of the molecular complexes of PCs with other biomolecules.

Although other mechanisms might be involved, the disaggregation and/or depolymerization of macromolecules can occur by the cavitation effect by means of mechanical degradation during collapse of bubbles (20-100 kHz) and chemical degradation due to the effect of hydroxyl radicals (>100 kHz), the former being the most usual [33,34]. Whereas radical formation or thermal reactions of US can be more effective for compounds with low MW, in the case of macromolecules, the main effects are mechanical which are more pronounced with the increase of the compound size, being the selection of the appropriate frequency very important for the ultrasonic treatment. Portenlänger and

Heusinger [35] treated dextran at 35 kHz-1.6 MHz for 150 min and they found the most effective degradation at the lower frequency value.

Concerning the US bath treatments (45 kHz), the effect of sonication decreased with the decrease of concentration and not significant differences were found between both modes ($P>0.05$). However, in the case of probe assays (20 kHz), the highest effect was observed for the most diluted samples (0.01 %) which values showed significant differences ($P<0.05$) with respect to higher concentrations. As it was indicated in the literature by Soria et al. [18], the concentration of substrate and frequency of US play an important role in the operating conditions of the system. The US assays carried out at 70% amplitude showed a lower effect than the other treatments ($P<0.05$), in concordance with the results afore mentioned for HPLC analysis. This could be ascribed to the fact that the higher power not always is a warranty for higher disaggregation, even if a higher power gives rise more cavitation bubbles, producing a shielding effect of the acoustic wave for what the ~~those~~ bubbles can behave as a barrier against the energy transmission to the system, thus decreasing its efficacy. Moreover, some studies have suggested that higher amplitudes could provoke erosion reducing cavitation and it is also probable that higher amplitude values might exert more agitation than cavitation [36]. In addition, the effect of temperature should be also taken into account, since in the assay at 30% amplitude, this parameter was close to 50°C and at 70% was much lower (25°C) due to the application of an ice-water bath to avoid an excessive increase of temperature. Thus, at temperatures lower than 60°C, an additive or synergic effect could be found between temperature and US on the linkage breakdown and at 30% and 50°C more disaggregation of PCs could have been produced than at 70% and 25°C [18].

4 Conclusions

The results obtained in this work demonstrate that US treatment and, particularly, US bath, produced a considerable increase of up to 49, 41 and 35%, respectively, of the peaks corresponding to catechin and oligomeric and polymeric procyanidin of the purified macromolecular fraction from grape seed extract. MALDI-TOF MS analysis of this fraction showed the existence of 6 B-type procyanidin series and that bath sonication produced an important increase in the number of procyanidins with higher molecular mass (up to decamers) and an overall increase in the mass signal intensities in most of the detected B-type procyanidin series. The increase of mass signal intensities was much more expressed for the non- and monogalloylated species than for these with higher degree of galloylation and higher molecular mass. Sonication produced also an important reduction of the baseline noise of the MALDI-TOF spectrum. In addition, and depending on the operating conditions, US produced an important increase of the antioxidant activity of the treated procyanidin extract, probably due to the release of monomeric, oligomeric and polymeric procyanidins. The most relevant effect in the assays was those carried out in bath US with 1% and probe US with 0.01% of purified macromolecular fraction from grape seed extract.

Although more studies are needed to reveal the fine mechanisms, all these findings suggest that bath US produced certain deliverance of monomeric, oligomeric and polymeric procyanidins, but it seems most probably that they proceed from disaggregation of linkages with other biopolymers, such as proteins and/or polysaccharides. High intensity US constitutes an efficient medium to modify the chemical structure of tannins improving its bioactivity with respect to the original fraction of PCs.

Acknowledgements

341 This work has been funded by MINECO of Spain, Project AGL2014-53445-R; ALIBIRD-
342 CM S-2013/ABI-272, Comunidad de Madrid.

343

344 **References**

- 345 [1] S. Bianchi, A.N. Gloess, I. Krosiakova, I. Mayer, F. Pichelin, Analysis of the structure
346 of condensed tannins in water extracts from bark tissues of Norway spruce (*Picea*
347 *abies* [Karst.]) and Silver fir (*Abies alba* [Mill.]) using MALDI-TOF mass
348 spectrometry, *Industrial Crops and Products*. 61 (2014) 430–437.
- 349 [2] Y.T. Deng, G. Liang, Y. Shi, H.L. Li, J. Zhang, X.M. Mao, Q.R. Fu, W.X. Peng, Q.X.
350 Chen, D.Y. Shen, Condensed tannins from *Ficus altissima* leaves: Structural,
351 antioxidant, and antityrosinase properties, *Process Biochemistry*. 51 (2016) 1092–
352 1099.
- 353 [3] L. Gu, M.A. Kelm, J.F. Hammerstone, G. Beecher, J. Holden, D. Haytowitz, R.L.
354 Prior, Screening of Foods Containing Proanthocyanidins and Their Structural
355 Characterization Using LC-MS / MS and Thiolytic, *J. Agric. Food Chem*. 51 (2003)
356 7513–7521.
- 357 [4] F. Shahidi, M. Naczk, *Phenolics in food and nutraceuticals*, 2006.
- 358 [5] C. Santos-Buelga, A. Scalbert, Proanthocyanidins and tannin like compounds nature,
359 occurrence, dietary intake and effects on nutrition and health, *Journal of the Science*
360 *of Food and Agriculture*. 80 (2000) 1094–1117.
- 361 [6] D. Bagchi, M. Bagchi, S.J. Stohs, D.K. Das, S.D. Ray, C. a Kuszynski, S.S. Joshi,
362 H.G. Pruess, Free radicals and grape seed proanthocyanidin extract: importance in
363 human health and disease prevention., *Toxicology*. 148 (2000) 187–197.

- 364 [7] P.M. Aron, J.A. Kennedy, Flavan-3-ols: Nature, occurrence and biological activity,
365 Molecular Nutrition and Food Research. 52 (2008) 79–104.
- 366 [8] E. Sieniawska, T. Baj, Tannins, Elsevier Inc., 2017.
- 367 [9] E. Middleton, T. Theoharides, C. Kandaswami, The effects of plant flavonoids on
368 mammalian cells: Implications for inflammation, heart disease, and cancer,
369 Pharmacological Reviews. 52 (2000) 673–751.
- 370 [10] R.L. Prior, L. Gu, Occurrence and biological significance of proanthocyanidins in the
371 American diet, Phytochemistry. 66 (2005) 2264–2280.
- 372 [11] Y. Nakamura, S. Tsuji, Y. Tonogai, Analysis of Proanthocyanidins in Grape Seed
373 Extracts, Health Foods and Grape Seed Oils, Journal of Health Science. 49 (2003)
374 45–54.
- 375 [12] Salunkhe DK, Chavan J. Dietary tannins, consequences and remedies. USA: CRC
376 Press; 1989.
- 377 [13] N. Hemmige Natesh, L. Abbey, S.K. Asiedu, An overview of nutritional and anti
378 nutritional factors in green leafy vegetables, Horticultural International Journal, 1 (2017) 58-
379 65.
- 380 [1442]A.C. Soria, M. Villamiel, , Effect of ultrasound on the technological properties and
381 bioactivity of food: a review, Food Science & Technology. 21 (2010) 323–331.
- 382 [1543]Y. Tao, D.-W. Sun, Enhancement of food processes by ultrasound: A review, Critical
383 Reviews in Food Science and Nutrition. 55 (2015) 570–594.
- 384 [1644]F. Chemat, Zill-E-Huma, M.K. Khan, Applications of ultrasound in food technology:
385 Processing, preservation and extraction, Ultrasonics Sonochemistry. 18 (2011) 813–
386 835.
- 387 [1745]F.J. Corzo-Martínez, M., Villamiel, MF.J. Moreno, Impact of High-Intensity

388 Ultrasound on Protein Structure and Functionality during Food Processing In:
389 Ultrasound in Food Processing: Recent Advances, ~~in: J.J.B. (Ed)~~ M. Villamiel, J.V.

390 García-Pérez, A. Montilla, J. Cárcel ,J.J. Benedito. John Wiley & Sons, Chichester,
391 West Sussex, United Kingdom. J. Cárcel (Ed.), Impact of High-Intensity Ultrasound
392 on Protein Structure and Functionality during Food Processing, 2017, pp 418-436.

393 [1846] A.C. Soria, Villamiel, M., Montilla, A. Ultrasound effects on processes and reactions
394 involving carbohydrates. In: Ultrasound in Food Processing: Recent Advances, in:
395 J.J.B. (Ed) M. Villamiel, J.V. García-Pérez, A. Montilla, J. Cárcel ,J.J. Benedito. John
396 Wiley & Sons, Chichester, West Sussex, United Kingdom. 2017, pp 437-463.

397 A. Shanmugam, M. Ashokkumar, Ultrasonic preparation of food emulsions, in: Ultrasound
398 in Food Processing: Recent Advances, 2017: pp. 287-310.

399 [1947] J.M. Silvan, M.A. Pinto-Bustillos, P. Vázquez-Ponce, M. Prodanov, A.J. Martinez-
400 Rodriguez, Olive mill wastewater as a potential source of antibacterial and anti-
401 inflammatory compounds against the food-borne pathogen *Campylobacter*,
402 *Innovative Food Science and Emerging Technologies*. (2018) 4-9.

403 [2048] A. Montilla, J. van de Lagemaat, A. Olano, M. del Castillo, Determination of
404 Oligosaccharides by Conventional High-Resolution Gas Chromatography,
405 *Chromatographia*. 63 (2006) 453–458.

406 [2149] M.A. Kelm, J.C. Johnson, R.J. Robbins, J.F. Hammerstone, H.H. Schmitz, High-
407 performance liquid chromatography separation and purification of cacao (*Theobroma*
408 cacao L.) procyanidins according to degree of polymerization using a diol stationary
409 phase, *Journal of Agricultural and Food Chemistry*. 54 (2006) 1571–1576.

410 [2220] G. Prodanov, M., Vázquez, E., Amador, B. & Reglero, Why Proanthocyanidins elute

Con formato: Español (España)

Con formato: Inglés (Reino Unido)

Con formato: Inglés (Reino Unido)

Con formato: Inglés (Reino Unido)

411 in increasing molecular mass order when analysed by normal phase HPLC,
412 Polyphenols Communications. 2 (2010) 594–595.

413 [2324]C. Kilicariskan Ozkan, H. Ozguna, Ultrasound extraction of valonea tannin part II :
414 Effects on tannin structure and tanning ability, The Journal of the American Leather
415 Chemists Association. 1058 (2013) 63-71.

416 [2422]J. Singthong, S.W. Cui, S. Ningsanond, H. Douglas Goff, Structural characterization,
417 degree of esterification and some gelling properties of Krueo Ma Noy (Cissampelos
418 pareira) pectin, Carbohydrate Polymers. 58 (2004) 391–400.

419 [2523]M. Jiménez Monreal, A. M.; Sánchez Manzanera, M.; Martínez Tomé, Optimization
420 of the DPPH method to evaluate antioxidant activity of coffee brew, Anales de
421 Veterinaria de Murcia. 28 (2012) 67–78.

422 [24]—L. Montero, M. Herrero, M. Prodanov, E. Ibáñez, A. Cifuentes, Characterization of
423 grape seed procyanidins by comprehensive two-dimensional hydrophilic interaction
424 × reversed phase liquid chromatography coupled to diode array detection and tandem
425 mass spectrometry, (2013) 4627–4638.

426 [25]—C. Santos Buelga, Comparative flavan-3-ol composition of seeds from different
427 grape varieties, 53 (1995) 197–201.

428 [26]—M. Prodanov, V. Vacas, T. Hernández, I. Estrella, B. Amador, P. Winterhalter,
429 Chemical characterisation of Malvar grape seeds (Vitis vinifera L.) by ultrafiltration
430 and RP-HPLC-PAD-MS, Journal of Food Composition and Analysis. 31 (2013) 284–
431 292.

432 [27]—F. Tomás-Barberán, J.C. Espín, Phenolic compounds and related enzymes as
433 determinants of quality in fruits and vegetables, Journal of the Science of Food and

434 ~~Agriculture. 876 (2001) 853–876.~~

435 [2628]C.G. Krueger, N.C. Dopke, P.M. Treichel, J. Folts, J.D. Reed, Matrix-assisted laser
 436 desorption/ionization time-of-flight mass spectrometry of polygalloyl polyflavan-3-
 437 ols in grape seed extract, *Journal of Agricultural and Food Chemistry*. 48 (2000)
 438 1663–1667.

439 [2729]M. Monagas, J.E. Quintanilla-López, C. Gómez-Cordovés, B. Bartolomé, R. Lebrón-
 440 Aguilar, MALDI-TOF MS analysis of plant proanthocyanidins, *Journal of*
 441 *Pharmaceutical and Biomedical Analysis*. 51 (2010) 358–372.

442 [2830]J.D. Reed, C.G. Krueger, M.M. Vestling, MALDI-TOF mass spectrometry of
 443 oligomeric food polyphenols, *International Food Research Journal*. 20 (2005) 2023–
 444 2034.

445 [2934]M. Prodanov, I. Garrido, V. Vacas, R. Lebrón-Aguilar, M. Dueñas, C. Gómez-
 446 Cordovés, B. Bartolomé, Ultrafiltration as alternative purification procedure for the
 447 characterization of low and high molecular-mass phenolics from almond skins,
 448 *Analytica Chimica Acta*. 609 (2008) 241–251.

449 [3032]P. L., G.Z. Brosse N., Ping L., Pizzi A., Condensed tannins from grape pomace:
 450 Characterization by FTIR and MALDI TOF and production of environment friendly
 451 wood adhesive, *Industrial Crops and Products*. 40 (2012) 13–20.

452 [3133]H.C.C. Azizi Bharudin M., Zakaria S., Condensed Tannins from Acacia Mangium
 453 Bark: Characterization by Spot Tests and FTIR, Faculty of Science and Technology,
 454 School of Applied Physics, Bangi, Selangor, Malaysia. (2013).

455 [3244]C.B. Chupin L., Motillon C., Charrier-El Bouhtoury F., Pizzi A., Characterisation of
 456 maritime pine (*Pinus pinaster*) bark tannins extracted under different conditions by

spectroscopic methods, FTIR and HPLC, Industrial Crops and Products. 49 (2013) 897–903.

[33] A. Grönroos, P. Pirkonen, O. Rupper, Ultrasonic depolymerisation of aqueous carboxymethylcellulose. Ultrasonics Sonochemistry 11 (2004) 9-12.

[34] L. Zhang, X. Ye, T. Ding, X. Sun, Y. Xu, D. Liu, Ultrasound effects on the degradation kinetics, structure and rheological properties of apple pectin. Ultrasonics Sonochemistry 20 (2013) 222-231.

[35] G. Portenlänger & H. Heusinger, H. The influence of frequency on the mechanical and radical effects for the ultrasonic degradation of dextrans. Ultrasonics Sonochemistry 4 (1997) 127-130.

[36] F. Chemat, N. Rombaut, A.G. Sicaire, A. Meullemiestre, A.S. Fabiano-Tixier, M. Abert-Vian, Ultrasound assisted extraction of food and natural products. Mechanisms, techniques, combinations, protocols and applications. A review, Ultrasonics Sonochemistry, 34 (2017) 540-560.

Con formato: Inglés (Reino Unido)

Con formato: Inglés (Reino Unido)

Con formato: Inglés (Reino Unido)

Con formato: Inglés (Reino Unido)

Con formato: Inglés (Reino Unido)

Con formato: Inglés (Reino Unido)

Con formato: Inglés (Reino Unido)

Con formato: Inglés (Reino Unido)

Con formato: Inglés (Reino Unido)

Con formato: Inglés (Reino Unido)

Con formato: Inglés (Reino Unido)

Con formato: Inglés (Reino Unido)

Con formato: Inglés (Reino Unido)

Con formato: Inglés (Reino Unido)

Con formato: Inglés (Reino Unido)

Con formato: Inglés (Reino Unido)

Legends of the Figures

Fig. 1. Chromatographic profile obtained by HPLC-RID of condensed tannins treated with bath US (Superior chromatogram: control sample in black; continuous mode in red; pulsed mode in green) and treated with probe US (chromatogram below: control sample in black; 30% amplitude in red; 70% amplitude in green). 1: Monomers; 2: Dimers; 3: Trimers; 4: Tetramers; 5: Pentamers; 6: Hexamers; 7: Polymers.

Fig. 2. MALDI-TOF spectrum of (a) PCs control sample and (b) PCs sample treated by bath US.

Fig. 3. FTIR spectrum of PCs samples at 0.1% (w/v): a) control b) treated with bath US.

Fig. 4. Representation of the antioxidant activity increase analyzed by DPPH method at 517 nm of the US treatments with respect to the control samples.

Figure S1. Flowchart of the extraction process of PCs sample from an extract of grape seeds *Vitis vinífera* L. provided by Output Trade.

Table 1. Theoretical and detected positive ions (sodium adducts) of procyanidin polymers (PPC_{C,G}) registered by MALDI-TOF(MS) (reflectron mode) in Control (MMF-GSE_C) and bath US treated (MMF-GSE_{US}) macromolecular fraction from grape seed extract (MMF-GSE).

PPC _{C,G}	G	Theoretical [M+Na] ⁺	Detected [M+Na] ⁺ (signal intensity)			
			MMF-GSE _C B-type PC	A-type PC	MMF-GSE _{US} B-type PC	A-type PC
			(m/z)	(m/z)	(m/z)	(m/z)
PC _{2,2}	2	905	b	b	905.1 (2610)	b
	0	889	888.9 (2720)	887	889.1 (4100)	887
	1	1041	1041.2 (1410)	b	1041.2 (2120)	b
PC _{3,(1-3)}	2	1193	1193.3 (3820)	1191	1193.2 (3620*)	b
	3	1345	1345.3 (2630)	b	1345.3 (1960*)	b
PC _{4,(1-4)}	0	1177	1177.3 (3310)	1175	1177.3 (5030)	1175
	1	1329	1329.3 (1880)	1327	1329.3 (3030)	1327
	2	1481	1481.3 (4690)	1479	1481.4 (3850*)	1479
	3	1633	1633.4 (2400)	b	1633.4 (2820)	1631
	4	1785	1785.4 (1450)	b	1785.4 (2210)	b
PC _{5,(1-5)}	0	1465	1465.4 (2950)	1463	1465.4 (4600)	1463
	1	1617	1617.4 (2030)	b	1617.4 (3400)	1615
	2	1769	1769.4 (3000)	1767	1769.4 (4020)	1767
	3	1922	1921.5 (2560)	b	1921.4 (3000)	1919
	4	2073	2073.5 (1610)	2071	2073.5 (2050)	b
	5	2226	2225.5 (1130)	b	b	b
PC _{6,(1-5)}	0	1754	1753.5 (2190)	1751	1753.4 (3440)	1751
	1	1906	1905.5 (1980)	1903	1905.5 (2640)	1903
	2	2058	2057.5 (2200)	b	2057.5 (3060)	b
	3	2210	2209.5 (1770)	b	2209.5 (2390)	2207
	4	2362	2361.6 (1400)	2359	2361.5 (1510)	2359
	5	2514	b	b	2513.6 (1000)	b
PC _{7,(1-5)}	0	2042	2041.5 (1710)	b	2041.5 (2520)	b
	1	2194	2193.5 (1160)	2191	2193.5 (1680)	b
	2	2346	2345.5 (1670)	b	2345.5 (2230)	b
	3	2498	2498.6 (1540)	b	2497.5 (1390*)	b
	4	2650	2649.6 (1190)	b	2650.6 (1250)	b
	5	2802	2802.5 (940)	b	2802.5 (940*)	b
PC _{8,(1-4)}	0	2330	2330.6 (1200)	b	2329.6 (1380)	b
	1	2482	2482.6 (1150)	b	2481.6 (1190)	b
	2	2635	2634.6 (1280)	2632	2633.6 (1420)	b
	3	2787	2786.6 (1130)	b	2786.6 (1300)	b
	4	2939	2939.6 (1000)	b	2939.6 (960*)	b

PC _{9,(1-4)}	0	2619	b	b	2618.6 (1250)	b
	1	2771	b	b	2770.6 (1120)	b
	2	2923	2923.7 (950)	b	2922.6 (1170)	b
	3	3075	b	b	3074.6 (930)	b
	4	3227	b	b	3225.6 (740)	b
PC _{10,(1-3)}	0	2907	b	b	b	b
	1	3059	b	b	3058.7 (920)	b
	2	3211	b	b	3210.7 (9740)	b
	3	3363	b	b	3363.7 (670)	b

^a Mass calculations were based on the equation $290+288C+152G+23$, where 290 is the molecular mass of an elementary flavan-3-ol unit, C is the number of flavan-3-ol units, G is the number of galloyl esters, and 23 is the molecular mass of sodium (Na), ^bMasses were not detected, * indicates decrease or not change of mass (sodium adducts) signal intensities of pair molecular ions, absence of * indicates increase of mass (sodium adducts) signal intensities of pair molecular ions.

502

Figure 1.

503

504

505

Figure 2.

511 Figure 3.

512

513

515 Figure 4.

516

517

518 Figure S1.

519
520