JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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ARANTZAZU VALDÉS GARCÍA, Lorena Vidal, Ana Beltran, Antonio Canals, and María Carmen Garrigós

J. Agric. Food Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.5b01011 • Publication Date (Web): 25 May 2015

Just Accepted

Microwave Assisted Extraction of Phenolic Compounds from Almond Skin By-products (*Prunus amygdalus):* A Multivariate Analysis Approach

Arantzazu Valdés*, Lorena Vidal, Ana Beltrán, Antonio Canals, María Carmen Garrigós.

Analytical Chemistry, Nutrition & Food Sciences Department, University of Alicante, 03080, Alicante, Spain.

*corresponding author:

Tel: +34 965903400. Ext 1187. Fax: +34 965903527.

E-mail: arancha.valdes@ua.es

ABSTRACT:

A microwave-assisted extraction (MAE) procedure to isolate phenolic compounds from almond skin by-products was optimized. A three-level, three-factor Box–Behnken design was used to evaluate the effect of almond skin weight, microwave power and irradiation time on total phenolic content (TPC) and antioxidant activity (DPPH). Almond skin weight was the most important parameter on the studied responses. The best extraction was achieved using 4 g, 60 s, 100 W and 60 mL of 70% (v/v) ethanol. TPC, antioxidant activity (DPPH, FRAP) and chemical composition (HPLC-DAD-ESI-MS/MS) were determined by using the optimized method from 7 different almond cultivars. Successful discrimination was obtained for all cultivars by using multivariate linear discriminant analysis (LDA) suggesting the influence of cultivar type on polyphenols content and antioxidant activity. The results showed the potential of almond skin as a natural source of phenolics and the effectiveness of MAE for the reutilization of these by-products.

KEYWORDS: Microwave-assisted extraction, Phenolic compounds, Almond skin, HPLC-DAD-ESI-MS/MS, Antioxidant activity, Linear discriminant analysis.

INTRODUCTION

Almond (*Prunus amygdalus*) production has increased significantly in the last years, with a worldwide production of about 1.9 million tonnes in 2012.[1] Food applications of almonds such as confectionary items and bakery, snack formulations, cereals and marzipan, require the almonds without the seed coats.[2] The external coating of almonds is industrially removed from hot water blanching process, with the brown skin contributing to around 6.0-8.4% of the seed weight.[3] Almond skin agricultural by-products are produced upon almonds processing in large amounts. Industries are forced to consider ways of treating or using these residues, since most of them are just incinerated or dumped without control causing several environmental problems or used as animal feed.[4]

Almond skin contains 50-75% of the total phenols present in the nut, such as aldehydes and hydroxybenzoic acids, flavanones, isoflavones, flavonol glycosides, phenolic acids, flavonol aglycones, flavan-3-ols, flavonone aglycones, flavonone glycosides and lignans. Flavanol and flavonol glycosides are the most abundant phenolic compounds, and particularly epicatechin, catechin, isorhamnetin-3-*O*-glucoside, isorhamnetin-3-*O*-rutinoside, kaempferol-3-*O*-rutinoside and naringenin-7-*O*-glucoside.[5-6] Polyphenols have been found to decrease the risk of coronary heart disease and function as anti-inflammatory agents due to their high antioxidant capacity.[6] These compounds can function as natural preservatives for meat products, reducing their lipid oxidation.[7-8] Therefore, almond skin by-products rich in antioxidant compounds could be reused by food industries as natural additives to control the oxidative process, adding value to this residue. It is of economical and ecological significance to find and efficient method to isolate phenolic compounds from these by-products. However, cultivar differences may affect almond flavonoid concentration.[3]

Extraction of phenolic compounds from food is one of the most important steps prior to their determination by HPLC.[9] Recently, some novel extraction methods of flavonoids such as MAE were developed showing several advantages over the conventional extraction techniques such as the reduction of solvent used for extraction and energy consumption, moderately high recoveries, good reproducibility, shortened extraction time and minimal sample manipulation for extraction process.[10-12] This technique has been successfully used with effectively improved flavonoids yield for the extraction of different food matrices; such as honey, peanut skins, sweet potato and maize.[13-17]

Regarding the extraction of almond skin antioxidants, conventional extraction is usually performed at reflux by using high temperatures for several hours or maceration with solvent for days at room temperature.[18-20] To our knowledge, no MAE application for the extraction of phenolic compounds from almond skin has been found in bibliography. Therefore, the objectives of this study were: (1) to optimize a new extraction procedure for the extraction of phenolic compounds in almond skin by MAE using an experimental design in terms of highest total phenolic content (TPC) and antioxidant activity (DPPH), (2) to increase the potential added-value of almond agricultural by-products, reducing costs for the food industry, and (3) to select the almond cultivar with higher antioxidant capacity as a potential antioxidant source. For this purposes, the determination of TPC, flavonoids (HPLC-UV-ESI-MS/MS) and antioxidant activity (DPPH, FRAP) were performed on seven different almond cultivars; and the presence of different categories within almond skin samples was studied using stepwise linear discriminant analysis (LDA). This characterization is an essential step for the re-utilization of these almond skin by-products.

MATERIALS AND METHODS

Chemicals and Reagents. Water (ultrapure grade) and ethanol (HPLC grade) were acquired from Merck (Madrid, Spain). Quercetin, sodium carbonate, Folin–Ciocalteu reagent (2 N), 2,2-diphenyl-1-picrylhydrazyl (DPPH), (±) 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), HPLC grade acetonitrile and methanol were supplied by Sigma-Aldrich (Madrid, Spain). All other reagents used were of analytical or chromatographic grade and were purchased from Panreac (Barcelona, Spain). Standard compounds such as (+)-catechin, (−)-epicatechin, quercetin-3-*O*-rutinoside, kaempferol-3-*O*-rutinoside, isorhamnetin-3-*O*-glucoside, isorhamnetin-3-*O*-rutinoside, naringenin-7-*O*-glucoside, naringenin and daidzein (internal standard) were purchased from Extrasynthese (Genay, France).

Sample preparation. Seven almond cultivars from the 2011 harvest were selected for this study and were supplied by "Almendras Llopis" (Alicante, Spain): three Spanish (Marcona, Guara and Planeta) and four American (Butte, Colony, Carmel and Padre). The blanching process of almonds (100 g) was carried out at 95 ºC for 3 min using 150 mL deionised water, to remove the skins from the kernels by hand.[6] Prior to MAE extraction, the obtained skins were oven-dried for 12 h and ground with a ZM 200 high speed rotary mill (Retsch, Haan, Germany) in order to increase the extraction efficiency.[12] Particles passing through a 0.5 mm sieve were used to ensure the homogeneity of the residue powder. The almond skin fraction obtained was dried in an oven at 40 °C for 24 h to reduce its moisture content.

Extraction procedure. Microwave-assisted extraction was carried out using a modified M1711N domestic microwave oven (Samsung M1711N, Taiwan), with a hole (18 mm diameter) in the top of the oven, at a frequency of 2,450 MHz and 800 W maximum power.[21] The sample was stirred at 300 rpm during extraction using a

118 microwave stirrer (Bel-Art Products, Wayne, NJ). The appropriate weight of
119 homogenized almond skin powder was placed in a 100-mL quartz flask which was
120 connected to a vapour condenser. The system operated as an open vessel extraction
121 system, where the solvent is heated and refluxed through the sample allowing a very
122 efficient heating.[22] Ethanol was selected as an effective extraction solvent for phenolic
123 compounds in food samples.[13, 23] Ethanol is also recommended by the US Food and
124 Drug Administration as an environmentally non-toxic food grade organic solvent.[24]
125 MAE was carried out at different extraction time and microwave power using 60
126 mL of 70% (v/v) ethanol. The obtained extracts were centrifuged at 4500 rpm for 5 min,
127 filtered through a 0.45 μm PVDF filter (Teknokroma, Barcelona, Spain), made up to 50
128 mL and kept at −20 °C until analysis.
129 **Experimental Design.** The extraction of phenolic compounds from almond skin
130 was performed under different extraction conditions according to the experimental
131 design shown in Table 1. The parameters considered during MAE optimization were
132 almond skin weight (0.5, 2.0, 3.5 g), microwave power (100, 200, 300 W) and
133 irradiation time (20, 40, 60 s). Butte cultivar was selected for the optimization of MAE
134 conditions. The range of studied variables was selected based on results obtained in
135 preliminary experiments. In this sense, at increased microwave power level of 300 W in
136 the screening experiment, rapid heating of the extraction medium and bubbling of the
137 substance occurred due to high cavitation; leading to the entry of the extraction medium
138 into the condenser. A Box-Behnken design (BBD), comprising 16 experimental runs,
139 was used and experiments were carried out in randomized order.[25]. The responses
140 obtained from the experimental design were evaluated in terms of TPC and antioxidant
141 activity (DPPH).

Regression analysis was used for the experimental data and fitted into the following empirical second-order polynomial model:

$$Y = \beta_0 + \Sigma\, \beta_i X_i + \Sigma\, \beta_{ii} X_i^2 + \Sigma\Sigma\, \beta_{ij} X_i X_j$$

where Y is the predicted response, X represents the variables of the system, *i* and *j* are design variables, β_0 a constant, β_i the linear coefficients, β_{ii} the quadratic coefficients and β_{ij} the interaction coefficients of variables i and j.

HPLC analysis of flavonoid compounds. HPLC-DAD-ESI-MS/MS analysis was performed, in triplicate, using a 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) coupled to a photodiode array UV/Vis detector and an LC/MSD Trap SL ion trap mass spectrometer (Agilent, Stuttgart, Germany) via an electrospray ionization (ESI) source. Mass spectra were recorded in the negative ionization mode (*m/z* 50-900).[26] The electrospray chamber was operated at 3.5 kV with a drying gas temperature of 350 °C, N_2 pressure and flow-rate on the nebulizer at 50 psi and 10 L/min, respectively; and MS/MS collision energies set at 20 V.

The column used for flavonoids separation (10 µL) was a 4.6 mm x 150 mm i.d., 5 µm, Eclipse XDB-C18 (Agilent Technologies, Waldbronn, Germany) at 25 °C. The mobile phase consisted of 2% acetic acid in ultrapure water (solvent A) and 2% acetic acid in ultrapure water:acetonitrile (73:25, v/v, solvent B) at a flow rate of 1 mL/min. The linear gradient started with 0 min, 40% B; 25 min, 70% B (hold 5 min); 32 min, 100% B (hold 4 min); back to 40% B (hold 5 min). UV detection of flavonoid compounds was carried out at 290 nm. The identification of the most abundant phenolic compounds was made by comparison of retention times, and UV/Vis and MS/MS spectra with those of commercially standard compounds and available literature.[26] Quantitation was carried out using MS/MS detector and daidzein as internal standard

(20 mg/kg). Final concentrations of flavonoids were expressed in μg/g of dry almond skin.

Total Phenolic Content (TPC). The TPC of almond skin extracts was determined, in triplicate, by the Folin-Ciocalteu colorimetric method, as reported by Singleton and Rossi[27], using a Biomate-3 UV/Vis spectrophotometer (Thermospectronic, Mobile, AL). Deionised water (30 mL) and Folin–Ciocalteu reagent (2.5 mL) were added to 500 μL of almond skin extract. The mixture was vortexed and incubated for 5 min. Then, 7.5 mL of 20% aqueous Na_2CO_3 and 10 mL of deionised water were added and mixed. The absorbance was measured at 760 nm after 90 min of incubation against deionised water as a blank. Quercetin was used as the reference standard (25–1000 mg/kg) and TPC was expressed as mg quercetin equivalent (QE)/g of dry almond skin.

Determination of Antioxidant Activity. The DPPH assay was used to determine the free radical scavenging activity of almond skin extracts as described by Assimopoulou et al.[29] 100 μL of the almond skin ethanolic extract were mixed with 2.7 mL of DPPH solution (10^{-4} M in ethanol). The percentage of free radicals scavenged by DPPH radical was determined at steady state (60 min) at 517 nm.

The capacity of almond skin extracts to reduce ferric ions was assessed by the FRAP method.[32] 250 mL of acetic acid buffer (300 mM, pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) made up in 10 mL of 40 mM HCl and 10 mL of 20 mM ferric chloride solution were mixed at 10:1:1 ratio, to make the FRAP reagent. 100 μL of the almond skin ethanolic extract were added to 4.9 mL of FRAP reagent. Measurements were performed at 593 nm after 30 min incubation at 37 ºC in darkness. Trolox was used as the reference standard (85–365 mg/kg) and the antioxidant capacity was expressed as μmol Trolox equivalent (TE)/ gram of dry almond skin.

Both methods were performed in triplicate using a Biomate-3 spectrophotometer (Thermospectronic, Mobile, AL).

Statistical analysis. Statgraphics-Plus software 5.1 (Statistical Graphics, Rockville, MD) was employed to generate and analyse the results of the BBD. Graphic analysis of the principal effects and interactions between variables was used for interpretation of results. Response surface methodology (RSM) was used to determine the optimal extraction conditions. Least squares regression analysis was performed to obtain the coefficients of the quadratic polynomial model previously described. The adequacy of the fitted model was determined by evaluating the lack of fit, the coefficient of determination (R^2), and F-test obtained from the analysis of variance (ANOVA). Statistical significance of model parameters was determined at the 5% probability level ($\alpha = 0.05$).

SPSS commercial software, ver. 15.0 (Chicago, IL) was used for statistical analysis of almond cultivars results by means of ANOVA. The Tukey test was used to determine differences between means at a $p \leq 0.05$ significance level. In chemometrics data analysis, pattern recognition methods are a powerful tool in context of food quality assessment and food composition analysis.[33] The presence of different categories within almond skin cultivars was studied using stepwise LDA as a multidisciplinary approach.[34]

RESULTS AND DISCUSSION

Optimization of extraction conditions. A preliminary study was performed to determine the effect of solvent volume and solvent ratio (ethanol in water) on the recovery of total phenolic content (TPC) and antioxidant activity from almond skin. It was found that 60 mL of 70% (v/v) ethanol provided the maximum TPC and DPPH

scavenging activity (data not shown). These conditions were then fixed for further optimization of extraction conditions. The selection of 70% (v/v) ethanol in water was in concordance with results obtained by other authors from the determination of natural phenols in different samples, since extraction efficiency depends on the solubility of the analytes in the extraction solvent.[12, 14, 22, 25] Hughey et al.[5] studied the distribution of polyphenols from almond skin in blanch water as a function of time and temperature, the intrinsic solubility of each polyphenol in water being different depending on its structure. In general, major phenolic compounds present in almond skin are sparingly soluble in hot water. As a consequence, a highest yield extraction of these compounds as the ethanol portion increases can be expected. However, the use of high ethanol contents as solvent extraction could lead to polyphenols degradation and overpressure inside the vessel due to overheating of the sample.[11] In this sense, dipolar rotation and ionic conduction are simultaneously produced during MAE.[26] As ethanol concentration increases, higher dielectric loss is obtained.[27] As a consequence, the higher capacity of the solvent to absorb microwave energy can lead to a faster rate of solvent heating with respect to the plant material.[12]

Regarding solvent volume, it was found that 60 mL was the more effective volume to be used with a maximum quantity of sample of 3.5 g without the formation of almond skin aggregates in the quartz flask during extraction. In this sense, extraction solvent efficiently absorbs microwave energy and leads to enhance swelling of food material, which is favourable to increase the contact surface area between phases.[12] However, a high volume of solvent could decrease the microwave adsorption of material, because more energy was absorbed by the solvent. In this case, the breaking of cell wall material and mass transmission might negatively influence and decrease the phenolics extraction and antioxidant capacity.[28]

There are different factors which can affect the extraction efficiency of MAE; such as microwave power, type and composition of solvent, extraction time, particle size of sample, solvent to solid ratio, soaking time, and extraction cycles.[35] The present study evaluates some of these variables by using a Box–Behnken experimental design. Butte almond skin was used for the experimental design and method optimization assays. The experimental data obtained in terms of TPC and DPPH scavenging activity are presented in Table 1. A statistical analysis of results was performed to estimate the statistical significance of the factors and interactions between them that had the greatest effect on obtaining extracts with high yield of TPC and antioxidant capacity (Figure 1). Among the studied factors, the almond skin weight had the greatest influence on the studied responses, showing a positive effect.

The rest of investigated parameters had no significant impact on the studied responses. Regarding magnetron power, the increasing microwave energy can favour the penetration of solvent into the food matrix and offer a rapid transfer of energy to the solvent and matrix, increasing temperature and allowing the dissolution of compounds to be extracted with an increase in the phenolics extraction yield.[26] However, a negative effect with increasing microwave irradiation energy could be observed by thermal degradation of antioxidant compounds and overpressure inside the vessel due to an increase in the extraction temperature.[12] Higher temperatures can also reduce extraction selectivity as matrix materials and non-desired compounds can also be extracted. Microwave power is strongly dependant on time and extraction temperature. In this sense, the increase in microwave power and extraction temperature causes the rapid cell rupture increasing the amount of impurities in extracts which can affect antioxidant activity. Furthermore, longer exposure with low or moderate power is considered a wiser choice since it results in better purity of the obtained extracts.[11] Regarding

266 irradiation time, this parameter generally has a positive influence on the TPC
267 response.[12] This behaviour can be explained by considering that the thermal
268 accumulation within extraction solution due to the absorption of microwave energy
269 promotes the dissolution process of phenolic compounds into the solution.[26] In the
270 present work, the extraction temperature rose to 47 ± 4 ºC by using the lower studied
271 power (100 W) and maximum time (60 s). Then, the combination of a low microwave
272 power and short extraction times could lead to moderate high temperatures which could
273 be considered able to extract almond skin antioxidant compounds with high antioxidant
274 activity.

275 The mathematical models obtained for both studied responses by applying
276 multiple regression analysis on the experimental data were expressed by the following
277 equations:

278 $$TPC = 45.10 + 32.70\ A - 1.73\ B + 1.55\ C - 4.41\ AB - 1.16\ AC + 0.07\ BC - 0.69\ A^2 +$$

279 $$1.99\ B^2 + 3.65\ C^2$$

280 $$DPPH = 48.57 + 30.045\ A + 1.87\ B + 0.34\ C - 1.84\ AB + 1.63\ BC - 2.92\ AC - 6.35\ A^2$$

281 $$+ 1.71\ B^2 + 0.36\ C^2$$

282 where A, B and C are the coded variables for almond skin weight, microwave power
283 and irradiation time, respectively.

284 The computing program showed that the two fitted models were considered
285 satisfactory as the lack of fit was not significant ($p > 0.05$). R^2 is defined as the ratio of
286 the explained variation to the total variation and is a measurement of the degree of
287 fitness.[14] The model can fit well with the actual data when R^2 approaches unity. The R^2
288 values obtained for TPC and DPPH were 0.9835 and 0.9691, respectively. These values
289 indicated a relatively high degree of correlation between the actual data and predicted
290 values; indicating that both models could be used to predict the studied responses.

Optimal conditions found by prediction of computing program to obtain highest TPC and DPPH values of 89.2 mg CE and 78.4%, respectively; were determined as follows: Almond skin weight, 3.5 g; microwave power, 100 W; extraction time, 60 s. As the AS weight clearly had a strong significant positive effect, this value was increased until 4 g. The values obtained for TPC and DPPH scavenging after extraction of almond skin under these optimal conditions, in triplicate, were 54 ± 2 mg QE/g almond skin and 90 ± 1%, respectively. As a result, for the extraction of phenolic compounds from almond skin, the best selected extraction conditions were: 100 W, 60 s, 4 g and 60 mL of 70% ethanol.

MAE can be considered a rapid technique showing several advantages compared to conventional extraction methods which are time-consuming, eventually lead to thermal degradation of antioxidant compounds and usually require higher quantity of organic solvents, raising process costs and reducing the environmental sustainability. For example, almond skin was extracted during 24 h at 40 ºC by using 70% of aqueous organic solvent (methanol, acetone or acetonitrile) by Tsujita et al.[36]. Also, Hughey et al.[5] extracted almond skin antioxidant compounds during 24 h at 37 ºC by using 50% water:methanol with 3.5% (v/v) acetic acid. Finally, Monagas et al.[20] extracted antioxidant compounds from almond skin with 80% (v/v) acetone at a solid to solvent ratio of 1:10 (w/v) during 30 min at 50 ºC.

Analysis of flavonoid compounds in almond skin cultivars. An adequate separation and good resolution of compounds were obtained for identification and quantitation (Figure 2). According to the unsaturation and oxidation degrees of the three-carbon segment, various families of flavonoids can be distinguished such as flavanones, flavonols, flavones, isoflavones and anthocyanidins.[9] Peaks 1 and 2 showed a $[M-H]^-$ at *m/z* 289 and characteristic MS/MS ions at *m/z* at 245, 205, and 179,

316 respectively; and they were identified as two flavan-3-ols: (+)-catechin and (−)-
317 epicatechin, respectively. Peaks 3, 4, 5, 6 and 7 were identified as five glycosidic
318 compounds: quercetin-3-*O*-rutinoside (*m/z* 609 with MS/MS fragment at *m/z* 300),
319 kaempferol-3-*O*-rutinoside (*m/z* 593 with MS/MS fragment at *m/z* 285), naringenin-7-
320 *O*-glucoside (*m/z* 433) with MS/MS fragment at *m/z* 271, isorhamnetin-3-*O*-rutinoside
321 (*m/z* 623 with MS/MS fragment at *m/z* 315) and isorhamnetin-3-*O*-glucoside *(m/z* 477
322 with MS/MS fragment at *m/z* 315); respectively. The isoflavone daidzein at *m/z* 253,
323 with MS/MS fragments at *m/z* 224 and 135, which was used as internal standard, was
324 identified at peak 8. Finally, peak 9 was identified as the flavanone aglycone naringenin
325 (*m/z* 271) with MS/MS fragments at *m/z* 177 and 151. The compounds identified in this
326 work are in agreement with published literature determining the phenolic profile of
327 almond skin.[5, 18]

328 Table 2 summarizes the individual flavonoids content found in the studied
329 almond skin cultivar extracts. Significant differences were obtained among almond skin
330 cultivars regarding their flavonoid profiles. Flavonoids are products of the shikimate
331 pathway from acetate and phenylalanine in plants. The genetic variation in the
332 shikimate pathway of almond cultivars is likely responsible for the different flavonoid
333 profiles between cultivars.[18] For epicatechin, isorhamnetin-3-*O*-glucoside, kaempferol-
334 3-*O*-rutinoside and isorhamnetin-3-*O*-rutinoside, Guara skin showed the highest content
335 compared to the rest of the studied cultivars. On the other hand, catechin, naringenin-7-
336 *O*-glucoside, quercetin-3-*O*-rutinoside and naringenin were quantified at higher
337 amounts in Planeta skin.

338 As it has been previously reported, during blanching process the blanch water will
339 increase in polyphenols, while blanched almond skins will decrease in phenolic
340 content.[5, 13] The results obtained in this work from the quantitation of flavonoid

compounds are in agreement with those found by Hughey et al.[5] As the number of –OH functional groups decreases in the molecule a higher hydrophobic character and lower solubility in boiling water of the phenolic compound are obtained. As a result, these compounds may present more affinity for an organic phase such as ethanol during MAE extraction.[28] Thus, isorhamnetin-3-*O*-rutinoside, kaempferol-3-*O*-rutinose, isorhamnetin-3-*O*-glucoside and quercetin-3-O-rutinoside, with more than seven –OH groups present in their molecules, were quantified in higher amounts in almond skin cultivars; followed by catechin, epicatechin and naringenin-7-*O*-glucoside with only five –OH groups. Finally, naringenin was quantified at the lower amount with only three –OH groups in its molecule. Hughey et al.[5] also reported a lower concentration of naringenin in almond skins after blanching. Bolling et al.[37] found the highest and lowest quantities for isorhamnetin-3-*O*-rutinoside and quercetin-3-*O*-rutinoside, respectively, in different almond skin cultivars. Similar results were reported also by Mandalari et al.[4] after lipid removal by almond skin extraction with *n*-hexane and further extraction by sonication. Finally, Garrido et al.[3] reported similar results for the flavonoids quantified in the present work when analyzing almond skin mixtures of Spanish and American cultivars subjected to blanching.

Analysis of total flavonoids, TPC and antioxidant activity in almond skin cultivars. The total flavonoids content quantified by HPLC-ESI-MS/MS, TPC results and radical scavenging activity by DPPH and reducing power by FRAP are shown in Table 3 for all almond skin cultivar extracts. Guara skin showed the highest total flavonoids content (1162 µg/g almond skin), TPC (119 mg QE/g almond skin) and FRAP (556 µmol TE/g almond skin) values. Padre, Butte and Colony cultivars followed Guara for total flavonoids content, and, finally, Planeta, Carmel, and Marcona. Regarding TPC results, Guara was followed by Planeta, Colony and Carmel and,

finally, Marcona, Padre and Butte. For FRAP, Guara and Marcona did not show statistical differences, with highest antioxidant activity, followed by Padre, Planeta, Carmel, Butte and, finally, Colony. These results are in accordance with those found in a previous work in which an exhaustive study of the phenolic composition of almond skin was carried out to evaluate their potential application as a functional food ingredient.[38] In this study, TPC and radical scavenging activity results were significantly higher for the almond skin mixture of Spanish varieties than for the American ones. Similar results were also obtained in a previous work in which the polyphenol content and antioxidant activity of seven different almond skin cultivars harvested over three seasons in California were studied.[18] From this work, it was concluded that cultivar had a differential impact on individual polyphenol synthesis, flavonoid content and antioxidant activity of almonds. Regarding DPPH results, a high radical scavenging activity (> 90%) was obtained for all studied cultivars, although no significant differences were obtained among them ($p>0.05$).

The total flavonoids content found in the present study, which ranged from 46-116.2 mg/100g of almond skin, is higher than the mean value reported for almonds in the USDA flavonoid content database (15.24 mg/100g almonds), which is based on data from the Food Composition Nutrient Data Laboratory of the USDA.[39] These data were obtained using extracts from whole almonds, whereas in the present study the skin was analysed, which accounts for 78–98% of the flavonoid content from whole almonds.[19, 39, 40] On the other hand, differences found in the absolute amount of recovered polyphenols, flavonoid content, and TPC and FRAP results obtained from almond skins between studies may arise from the use of different methods of extraction and analysis.[18, 40]

The obtained results clearly showed the efficiency of the optimized MAE method and the high antioxidant potential of almond skin extracts as natural antioxidant sources; Guara skin showing the highest TPC and flavonoids content. Therefore, it could be concluded that MAE could be considered a potential alternative to conventional extraction methods for the isolation of phenolic compounds from almond skin.

Multivariate analysis. LDA was applied as a multidisciplinary approach by inserting together all parameters obtained from the determination of TPC, antioxidant activity by FRAP and individual flavonoid contents quantified by HPLC-ESI-MS/MS as predictors; evaluating the capability of the complete model to discriminate samples according to the cultivar. The results obtained for DPPH from almond skin cultivars were not included in the LDA since no significant differences were obtained among samples (Table 3). As a result, 100% of samples were correctly classified obtaining a λ_w =0.175, with a good resolution among categories.

Six discriminant functions were obtained, using the variable selection rule for minimizing Wilk's lambda, which account the 61.5, 26.6, 8.1, 3.1, 0.5 and 0.6% of the total variance, respectively. Projections of cultivars scores on the first two determined discriminant functions are shown in Figure 3, where cultivars appear associated, suggesting seven groups. The first discriminant function showed differences in the discriminant space among the almond skin cultivars. This function was positively affected by TPC; isorhmanetin-3-O-rutinoside, kaempferol-3-O-rutinoside and isorhamnetin-3-O-glucoside contents. On the other hand, FRAP; quercetin-3-O-rutinoside, naringenin-7-O-glucoside, naringenin, catechin and epicatechin predictors negatively affected function 1. Regarding the second discriminant function, it was more affected by TPC; naringenin-7-O-glucoside and isorhamnetin-3-O-glucoside contents

whereas the rest of predictors had a negative influence on it. As a result, the application of the multidisciplinary approach revealed the potential of the obtained model for the discrimination and classification of almond skin cultivars according to the results obtained for TPC, antioxidant capacity (FRAP) and individual flavonoids content (HPLC-ESI-MS/MS). Similarly, Bolling et al.[18] found that canonical discriminant analysis of polyphenols content and antioxidant activity (FRAP) could distinguish almonds from different cultivars harvested in different seasons with 80% confidence. Also, in a previous work, we found that a multidisciplinary LDA approach of structural (FTIR) and thermal parameters (DSC, TGA) could successfully classify and discriminate three different almond cultivars.[34] The obtained results revealed the suitability of the studied techniques combined with LDA for a fast discrimination among different almond skin cultivar residues in food processing.

ACKNOWLEDGEMENTS

Authors would like to acknowledge "Almendras Llopis S.A" for kindly providing the almond skin by-products as well as to Spanish Ministry of Economy and Competitiveness for financial support (MAT-2011-28468-C02-01). A. Valdés would like to thank Conselleria de Educación (Spain) for ACIF/2010/172 Predoctoral Research Training Grant.

SUPPORTING INFORMATION

The ANOVA obtained for TPC and DPPH responses is summarized in Table S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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FIGURE CAPTIONS

Figure 1. Pareto charts of factors and interactions obtained from the Box-Behnken design for each response: TPC (mg QE/g almond skin) and DPPH scavenging (%). The vertical line indicates the statistical significance at 5% of the effects.

Figure 2. Extracted ion chromatograms of flavonoid standards obtained from $[M-H]^-$ ion by HPLC-ESI-MS/MS analysis (50 mg/Kg).

Figure 3. Projections of almond skincultivar scores on the space determined by the two first discriminant functions obtained by the multidisciplinary approach.

Table 1. Box–Behnken Experimental Design and MAE results.

Run	Almond skin weight (g)	Microwave power (W)	Irradiation time (s)	TPC (mg QE)	DPPH (% inhibition)
1	0.5	100	40	15.3	13.4
2	3.5	100	40	86.4	77.3
3	0.5	300	40	15.3	14.2
4	3.5	300	40	68.7	70.7
5	0.5	200	20	12.7	11.5
6	3.5	200	20	83.5	77.3
7	0.5	200	60	15.0	13.7
8	3.5	200	60	81.2	67.8
8	2.0	100	20	46.8	44.6
10	2.0	300	20	48.5	51.7
11	2.0	100	60	52.8	46.3
12	2.0	300	60	54.9	60.0
13	2.0	200	40	49.7	53.1
14	2.0	200	40	42.7	43.6
15	2.0	200	40	40.7	43.1
16	2.0	200	40	47.3	54.5

Table 2. Flavonoids content (μg/g almond skin) of the studied cultivars by HPLC-ESI-MS/MS analysis.

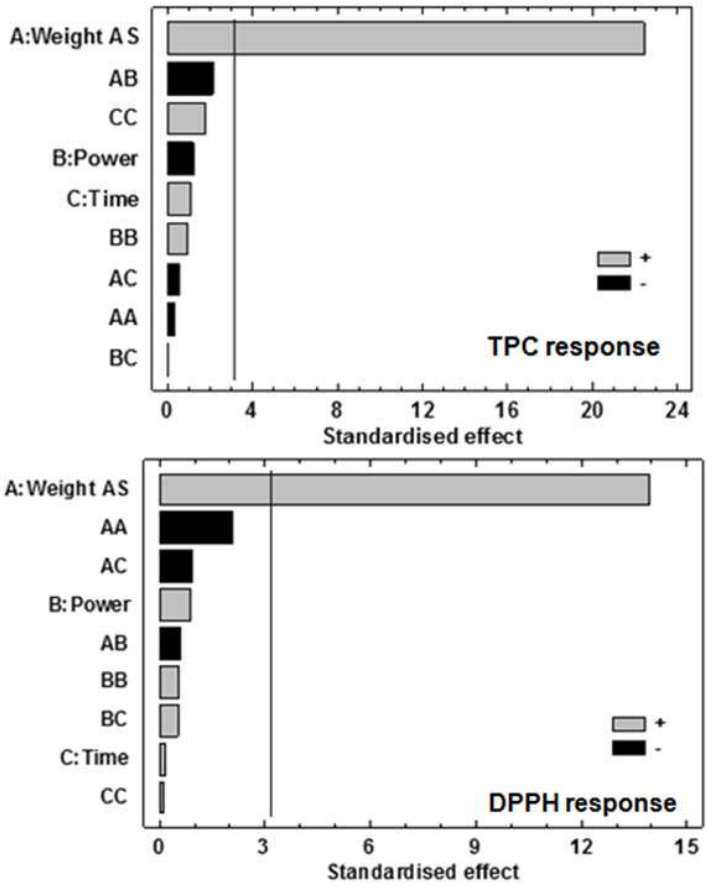
Almond cultivar	(+)-catechin	(−)-epicatechin	kaempferol-3-*O*-rutinoside	isorhamnetin-3-*O*-glucoside	isorhamnetin-3-*O*-rutinoside	quercetin-3-*O*-rutinoside	naringenin-7-*O*-glucoside	Naringenin
Marcona	13.0 ± 1.1^{ab}	5.8 ± 0.9^{a}	1.6 ± 0.7^{a}	14.1 ± 2.5^{a}	383.8 ± 69.8^{a}	nd	32.8 ± 0.9^{a}	8.5 ± 1.2^{a}
Guara	18.5 ± 4.2^{ad}	23.1 ± 10.4^{b}	238.7 ± 49.6^{b}	32.5 ± 9.0^{b}	752.1 ± 110.9^{b}	58.2 ± 15.6^{a}	33.6 ± 2.0^{a}	5.5 ± 1.8^{b}
Planeta	35.4 ± 5.8^{c}	5.1 ± 3.2^{a}	174.9 ± 32.9^{c}	2.0 ± 0.5^{c}	140.5 ± 30.7^{c}	411.9 ± 99.0^{b}	43.3 ± 3.1^{b}	46.1 ± 6.4^{c}
Butte	14.6 ± 2.5^{ab}	10.3 ± 5.2^{ab}	1.0 ± 0.5^{a}	19.1 ± 9.3^{a}	555.8 ± 12.7^{a}	80.2 ± 18.6^{a}	30.9 ± 1.5^{a}	14.2 ± 4.5^{a}
Colony	7.3 ± 0.4^{b}	9.2 ± 2.4^{a}	32.9 ± 7.6^{d}	27.2 ± 5.4^{ab}	756.5 ± 53.7^{b}	nd	38.4 ± 2.8^{b}	7.9 ± 0.6^{a}
Carmel	25.5 ± 3.2^{d}	1.3 ± 0.4^{a}	95.3 ± 11.4^{c}	2.2 ± 0.1^{c}	495.6 ± 53.7^{a}	nd	32.2 ± 0.6^{a}	0.4 ± 0.2^{e}
Padre	17.6 ± 0.7^{ad}	6.1 ± 0.9^{a}	205.1 ± 10.1^{b}	14.6 ± 2.0^{a}	671.6 ± 6.8^{b}	nd	35.2 ± 1.8^{ab}	15.8 ± 3.1^{a}

Mean ± SD (n = 3). Different superscripts within the same column indicate statistically significant different values ($p < 0.05$) as obtained by Tukey´s test application.

Table 3. Total Flavonoids, TPC and Antioxidant activity of almond skin cultivars.

Almond cultivar	Flavonoids (μg/g skin)	TPC (mg QE/g skin)	DPPH (% inhibition)	FRAP (μmol TE/g skin)
Marcona	460 ± 31^{a}	66 ± 1^{a}	93 ± 4^{a}	553 ± 8^{a}
Guara	1162 ± 22^{b}	119 ± 7^{b}	92 ± 1^{a}	556 ± 12^{a}
Planeta	688 ± 20^{ac}	95 ± 3^{c}	93 ± 4^{a}	416 ± 38^{bc}
Butte	900 ± 21^{bc}	54 ± 2^{d}	90 ± 1^{a}	382 ± 35^{c}
Colony	879 ± 32^{bc}	84 ± 8^{e}	93 ± 2^{a}	369 ± 50^{c}
Carmel	653 ± 28^{ac}	80 ± 3^{e}	93 ± 3^{a}	390 ± 7^{c}
Padre	966 ± 25^{bc}	62 ± 1^{a}	92 ± 5^{a}	480 ± 14^{ab}

Mean ± SD, n = 3. Different superscripts within the same column indicate statistically significant different values ($p < 0.05$) as obtained by Tukey's test application.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TPC response
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Standardised effect
A:Weight AS
AA
AC
B:Power
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C:Time
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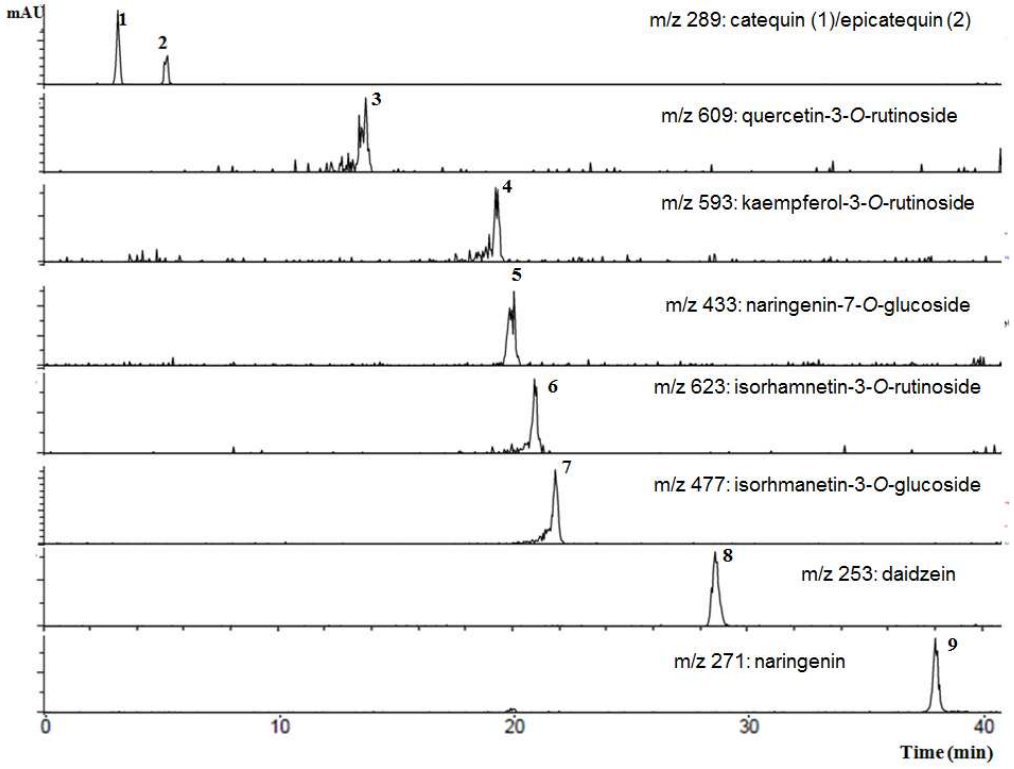
Standardised effect

Figure 1.


mAU
1
2
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3
m/z 609: quercetin-3-O-rutinoside
4
m/z 593: kaempferol-3-O-rutinoside
5
m/z 433: naringenin-7-O-glucoside
6
m/z 623: isorhamnetin-3-O-rutinoside
7
m/z 477: isorhmanetin-3-O-glucoside
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9
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0
10
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30
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Time (min)

Figure 2.

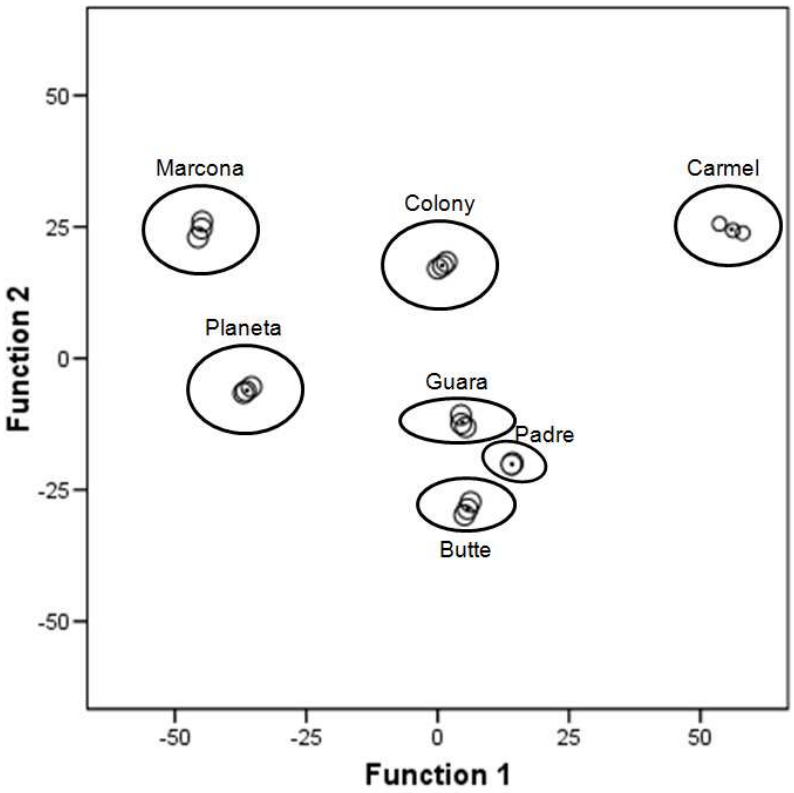
Marcona
Colony
Carmel
Planeta
Guara
Padre
Butte
Function 2
Function 1
50
25
0
-25
-50
-50
-25
0
25
50

Figure 3.

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