**Response surface methodology to optimize supercritical carbon dioxide/co-solvent extraction
of brown onion skin by-product as source of nutraceutical compounds.**

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25 **Abstract**

26 Food industry produces a large amount of onion wastes. Due to the high amount of bioactive
27 compounds in onion by-products an idea for their reuse, could be use them as source of high-value
28 functional and health ingredients. In this study, outer dry layers of coppery onion “Ramata di
29 Montoro” were used as source of bioactive compounds. Firstly, the chemical profile of secondary
30 metabolites of exhaustive extract, obtained by ultrasound assisted extraction was established by
31 UHPLC-UV-HRMS/MS analysis. Subsequently, the supercritical fluid extraction was used as
32 alternative and green method to recover flavonoids from onion skin. Main parameters such as
33 pressure, temperature and composition of solvent modifier were optimized in order to improve the
34 extraction efficiency of SFE technique, by using a response surface Box–Behnken design.

35

36 **Keywords:** Onion skin, quercetin derivatives, supercritical fluid extraction, recovery bioactive
37 compounds, experimental design optimization, food by-products, DPPH and ABTS assays.

38

39 **1. Introduction**

40 The term “food waste” is usually described as liquid or solid residues with high organic load, which
41 are produced from raw materials processing of foodstuff (Galanakis, 2012). Since these substances,
42 despite their potential reutilizing inside the food chain, are removed from production process as
43 undesirable materials, most European legislations defines them as “wastes” (Directive, 1975;
44 Directive, 1991). For this reason, among scientists “food by-products” is increasingly used to define
45 “food wastes” which are valuable natural sources of bioactive and functional compounds, to be
46 employed as ingredients in functional foods, supplements, cosmetics and nutraceutical products. In
47 this regard, the possibility to valorise these low-cost food by-products offer economically
48 interesting perspectives for their potentially applications. Nowadays, synthetic food supplements
49 are usually refused by consumers that more often demand for products or functional ingredients
50 preferably originating from natural sources (Ayala-Zavala et al., 2011). This is particularly true for
51 chemicals as phenolic compounds, which unlike other natural chemicals as carotenoids and
52 vitamins, which are not chemically synthesized, and they need to be extracted from plant materials.
53 Thus, an efficient use of food by-products to produce additives or supplements with high nutritional
54 value, have gained increasing interest because, their recovery may generate both economically and
55 environmentally advantages. Onions (*Allium cepa* L.), with a world annual production around 66
56 million tonnes is, after tomatoes, the most important horticultural crop worldwide. In 2016
57 European onion production has been estimated for about 6 million tonnes (FAO 2016)
58 (<http://www.fao.org/faostat/en/#data/QC>) and as result of this vast production, high amounts of
59 wastes are generated. According to literature data (Waldron, 2001) more than 500,000 tonnes of
60 onion wastes are produced annually in the European Union, mainly from Spain, UK and Holland.
61 The major by-products resulting from industrial peeling of onion bulbs are dried skin, the outer two
62 fleshy leaves and the top and bottom bulbs, which are not edible and are removed before processing
63 (Benítez et al., 2011; Ly et al., 2005). Onion wastes, due to a rapid phytopathogens growth and to
64 their strong unique smell, are not suitable for fodder or landfill disposal. Therefore, the valorisation

65 of onion by-products is indispensable and could provide an economic benefit both for the onion
66 producers and processors (Schieber, Stintzing, & Carle, 2001). Onion is one of the most common
67 and the richest natural source of biologically active phytochemicals, including phenolic acids,
68 anthocyanins, cepaenes, thiosulfinates, and flavonoids (Fossen & Andersen, 2003; Slimstad,
69 Fossen, & Vågen, 2007; Suleria, Butt, Anjum, Saeed, & Khalid, 2015). In recent years, several
70 studies have been conducted on the role of phytochemical compounds found in onions. The major
71 bioactive compounds found in dry peel of onion are phenolics as quercetin and quercetin glycoside
72 (Ly et al., 2005). Food-derived phenolics as quercetin are getting great interest due to their
73 antimicrobial and antioxidant activity, strongly related with a role in cancer prevention,
74 inflammatory disorders and cardiovascular diseases (Działo et al., 2016; Flores, Romero-González,
75 Frenich, & Vidal, 2012). Other benefits of quercetin include anti-inflammatory activity,
76 antihistamine effect, allergy medication, and anticancer and antiviral activities. It has also been
77 claimed that quercetin reduces blood pressure in hypertensive subjects (Boots, Haenen, & Bast,
78 2008). However, in order to use onion by-products as source of bioactive compounds, suitable
79 extraction methods must be developed to reduce time, cost and environmental pollution (Galanakis,
80 2012).

81 Many conventional solvent extraction methods (CSE) as maceration, sonication and soxhlet have
82 been extensively used to extract bioactive compounds from plant materials. Indeed, these CSE
83 methods have several drawbacks as: a long extraction time, require large amounts of organic
84 solvents and/or high temperatures, which could lead to extensive degradation of thermo-sensitive
85 molecules, as well as they could leave residues of potentially toxic solvents in the extracts (Abbas,
86 Mohamed, Abdulmir, & Abas, 2008; Ameer, Shahbaz, & Kwon, 2017; Azmir et al., 2013; Calvo,
87 Dado, & Santa-María, 2007; Galanakis, 2012; Rozzi & Singh, 2002). So to overcome these
88 disadvantages, fast, cheap and efficient, “Green extraction techniques” able to reduce /eliminate
89 petrochemical solvents and simultaneously increasing the extraction efficiency (yield and

selectivity toward compounds of interest) are widely used as a valid alternative to conventional extraction methods. For food by-products extraction, microwave-assisted extraction (Kaufmann & Christen, 2002), pulsed electric field assisted extraction (PEF) (Wijngaard, Hossain, Rai, & Brunton, 2012), pressurized liquid extraction (PLE) (Mustafa & Turner, 2011) and supercritical fluid extraction (SFE) represent the most promising green techniques (Ameer et al., 2017; Mustafa & Turner, 2011; Wijngaard et al., 2012). Among all these extraction techniques, SFE is considered an excellent alternative to conventional methods, for the extraction of natural products, since it gives extracts totally free of organic solvents (Herrero, Mendiola, Cifuentes, & Ibáñez, 2010). The SFE is generally performed by using carbon dioxide (CO₂) which is non-toxic, non-flammable, inexpensive, and have low viscosity and high diffusivity (Espinosa, Diaz, & Brignole, 2005). In addition, CO₂ having a low critical temperature and pressure values (31 °C and 74 bar, respectively) represents the perfect solvent for thermo-sensitive molecules extraction (Abbas et al., 2008). The main limitation of SFE use, regards the lipophilic nature of CO₂, which is mostly suitable for the extraction of non-polar and moderately polar compounds (Herrero et al., 2010). Nevertheless, to extend the SFE-CO₂ solvating power and improve its affinity for polar compounds, small volumes of organic solvents (modifier or co-solvents) as water and/or ethanol are often used (Herrero et al., 2010). However, to achieve the extraction of a broader range of compounds polarity, several parameters such as composition of solvent modifier and their concentration within supercritical fluid, extraction conditions (pressure, temperature, time) physical structure of the samples (granulometry, hydration, packing density) should be evaluated and optimized (Fahmy, Paulaitis, Johnson, & McNally, 1993).

To date, only one study has been reported on the application of supercritical fluid extraction for the extraction of flavonoid from onion skin. Namely, Martino, Karina Gorostiaga et al. (Martino & Guyer, 2004) examined the use of SFE to extract quercetin, the main flavonoid found in onion skin.

Unlike previously published manuscript reporting SFE to extract only few compounds from onion,

115 in the present study we aimed to develop a green and innovative extraction technique, the
116 supercritical CO₂ ethanol/water co-solvent to quantitatively extract phenolic compounds from the
117 dry skin of coppery onion. The onion used in this study was an IGP variety, known as “Ramata di
118 Montoro”, a species cultivated in Campania region, in south of Italy. UHPLC-UV-HRMS was
119 employed to characterize the phenolic profile of onion dry skin extracts (exhaustive and SFE) and
120 to optimize the SFE experimental conditions.

121 Main parameters that could influence the extraction efficiency of SFE technique, such as pressure,
122 temperature and composition of solvent modifier were optimized using a response surface Box–
123 Behnken design. Moreover, antioxidant activity of SFE-CO₂ extract, evaluated by DPPH/ABTS
124 assays, were compared with those obtained using ultrasound assisted extraction (USAE). To the
125 best of our knowledge this is the first application of SFE to extract and recovery 18 bioactive
126 compounds from dried onion skin.

127 **2. Experimental**

128 *2.1 Standards and materials*

129 Ultrapure water (18 MΩ) was obtained using a Milli-Q purification system (Millipore, Bedford,
130 USA). MS-grade water (H₂O), methanol (MeOH), and acetonitrile (CH₃CN) were supplied by
131 Romil (Cambridge, UK). Analytical grade MeOH, CH₃CN, Formic acid (HCOOH) and absolute
132 ethanol (EtOH) were supplied by Carlo Erba reagents (Milan, Italy). MS-grade formic acid
133 (HCOOH), quercetin (purity ≥95%), isorhamnetin (purity ≥95% HPLC), kaempferol (purity ≥97%),
134 protocatechuic acid (purity 95%), 2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH) and 2,2' -
135 azinobis (3-ethylben- zothiazoline-6-sulphonic acid) diammonium salt (ABTS) were provided by
136 Sigma-Aldrich (Milan, Italy)

137 *2.2 Onion dry skin samples*

Outer dry layers of brown skin onion bulbs (*Allium cepa* L.) of IGP “Ramata di Montoro” were supplied by the company “Gaia Società Semplice Agricola” (Montoro, Avellino, Italy) located in the production area of onion IGP “Ramata di Montoro”. Samples were collected after the harvest and were dried in the oven at 40°C until constant dry weight. Dry samples were finely blended using a knife mill Grindomix GM-200 (Restek GmbH Germany) operated at 6000 rpm for a short cycles time, in order to avoid the warming of the samples. The ground samples were sieved through a test sieve into a range of 300-600 µm to obtain a powder with a homogeneous particle size distribution. The total amount of sieved sample was stored at -18°C, whereas a small part of this sample used for the extraction, were stored under dry in the dark at 4°C in polyethylene bag for no longer than a week.

148

2.3 Ultrasound assisted solid-liquid extraction for exhaustive extraction of phenolic compounds

The exhaustive extraction of phenolic compounds from dried onion skin was performed by ultrasound assisted solid liquid extraction (USAE). Briefly, 1 g of sample was extracted eight times with 20 mL Ethanol (85% v/v) for 15 min at 25°C and for any extraction cycles a fresh solvent solution was used. The extracts were pooled, filtered (Whatman No. 1 filter) and dried under vacuum at 40 °C in a rotary evaporator (Rotavapor R-200, Buchi Italia s.r.l, Cornaredo, Italy). After drying, the extraction yield (9.3 ± 0.7 g/100g DM, n=3) was calculated gravimetrically using the following formula:

$$\text{yield}_{(\text{g SFE extract} / 100\text{g DM})} = \frac{(\text{weight of flask after evaporation}) - (\text{weight of empty flask})}{\text{Amount of sample loaded into the extraction cell (g)}} \times 100 \quad (1)$$

Código de campo cambiado

158

2.4 UHPLC-DAD-HRMS/MS phenolic profiles of onion skin extracts

Identification and quantification of phenolic compounds in the extracts of dried onion skin were determined using a similar approach and same equipments of our previously method (Pagano et al., 2016). Chromatographic system, Platin Blue UHPLC (Knauer, Labservice Analytica, Bologna,

Italy) was connected to a LTQ OrbiTrap XL high resolution mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). System consists of two UHPLC pumps, an autosampler, a diode array detector and a column oven. The extracts were chromatographed using a UHPLC column Kinetex C18 (2.1x 50 mm, 1.7 μ m; Phenomenex, Torrance, CA, USA) and mobile phases (MS grade) were H₂O (A) and CH₃CN (B), both containing 0.1% formic acid (HCOOH). Following gradient was adopted: 0-3 min, isocratic 2 %B; 3-5 min, linear gradient from 2% to 13% B; 5-9 min, isocratic 13 %B; 9-12 min, linear gradient from 13 to 18% and held for 1 min; 13-17 min, linear gradient from 18 to 30 %B; 17-21 min, linear gradient from 30 to 50 %B; 21-22 min, linear gradient from 50 to 98 %B followed by washing and re-equilibrating of the column both for 5 min. The column temperature (30°C) and flow rate of (600 μ L min⁻¹) was kept constant for all run time analysis and the injection volume was set at 5 μ L. UV spectra were acquired in the range of 200-600 nm, and two wavelengths, 295 and 365 nm were employed for the detection of target analytes on the basis of their maximum absorbance. LTQ OrbiTrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA), equipped with an ESI source, was used in negative and positive ionization mode. Nitrogen (N₂) was used as sheath gas (30 arbitrary units) and auxiliary gas (10 arbitrary units) and helium (He) was used as gas in the collision cell. Mass spectrometer parameters were as follows: source voltage 3.8 kV, capillary voltage 48 V, tube lens voltage 65 V, capillary temperature 300°C. MS spectra were acquired by full range acquisition covering 140–1500 m/z at resolution of 60000. For fragmentation study a data dependent scan was performed by using the collision-induced dissociation (CID). The normalized collision energy of the CID cell was set at 30 eV and the isolation width of precursor ion was set at m/z 2.0 with a mass resolution of 6500. Phenolic compounds were characterized according to the corresponding spectral characteristics: UV and MS spectra, accurate mass, characteristic fragmentation and retention time. EZChrom Elite software (ChromGate[®]) was used to control and acquire data from chromatographic system and detector UV, whereas Xcalibur software (version 2.2) was used for mass spectrometer control and MS data acquisition and processing.

189

190 2.5 Quantitative analysis by UHPLC-UV

191 Analyses were performed using a Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific,
192 San Jose, CA, USA) constituted of an Ultimate 3000 RS Pump, an Ultimate 3000 RS autosampler,
193 an Ultimate 3000 RS column compartment and Ultimate 3000 RS variable wavelength detector.
194 Column and chromatographic conditions were the same used for UHPLC-DAD-HRMS/MS
195 analysis. The UV chromatograms were recorded at two different wavelengths 295 and 365 nm;
196 External standard calibration method was used to quantify the phenolic compounds. Mixtures of 2
197 reference standards (protocatechuic acid and quercetin), at different concentrations were used to
198 obtain calibration curves. Linearity of calibration curves were evaluated in the concentration range
199 of 6.25-100 μ M (6 concentration levels) and triplicate injections were acquired for each level.
200 Regression curves were tested with the analysis of variance (ANOVA) and linear model was found
201 appropriate over the tested concentration range (R^2 values > 0.998). Concerning the precision and
202 intraday repeatability were estimated on all concentration levels (coefficient of variation <5%).
203 Since all compounds found in the onion extract were not commercially available as pure standard .
204 quercetin derivatives were quantified using the calibration curve of quercetin. Quantitative analysis
205 for each analyte were reported as quercetin equivalent (g/100g DM). Extracts exceeding the
206 concentration range of the standard curve were diluted before injection.

207

208 2.6 Experimental design

209 Statgraphic Centurion XVI Version 16.1 (Rockville, USA) was employed for the experimental
210 design analysis, data processing and to optimize SFE parameters. A response surface Box–Behnken
211 design 2-factor interactions was carried out. By using this design, three variables were tested at 3
212 different levels (low, medium and high): extraction temperature (Temp) at 40, 55 and 70 °C,
213 pressure (Press) 100, 200 and 300 bar and composition of solvent modifier (EtOH %) 95, 90 and
214 85%. Selection of ranges within each factor varied was based on preliminary experiments (data not

shown). The UHPLC-UV normalized peak area of each analyte and extraction yield (g/100 g DM) were considered as the response variable. A total of 15 experiments (12 points of the factorial design, 3 center points, 5 freedom degree) were carried out in randomized run. In the Table 1S was reported the experimental matrix design, with the experimental levels of the independent variables (factors) and the results obtained for the analysed response variables (peak area of quercetin-diglycoside, quercetin, quercetin dimer and extraction yield). Data from response surface Box–Behnken design were subjected to regression analysis using least square regression methodology to obtain the parameters of the mathematical models. Analysis of variance (ANOVA) was applied to evaluate the statistical significance of independent variable (A, B and C) contributions and their first order interaction.

225

226 *2.7 Supercritical CO₂ extraction*

SFE extracts were obtained using a Suprex PrepMaster (Suprex, Pittsburgh, PA). The extractor was equipped with a dual piston pump for CO₂ (Carbueros metálicos, Madrid, Spain). Stainless steel extraction cell with a volume approximatively of 20 mL was thermostated in the oven built in our laboratory and backpressure of whole system was regulated used a valve LF-540 Pressure Tech. (Hadfield, United Kingdom). For each extraction 1g of finely ground of sample was packaged in the extraction cell and a small amount of glass wool was placed at the top and bottom of the cell to avoid the clog of system. Solvent modifier, ethanol 85%, was delivered by a Jasco PU2080 HPLC pump (Jasco Inc., Easton, PA) and mixed at high pressure with supercritical CO₂. Compressed mixture of CO₂ and solvent modifier was led into the heater prior to entering the extraction cell. Dynamic extractions were performed using high purity CO₂ at constant flow of 10 mL min⁻¹ mixed with the solvent modifier at flow rate of 0.5 mL min⁻¹. Under the optimum conditions the extraction cell was placed in the oven at temperature and a pressure of 40 °C and 100 bar respectively while EtOH 85% was constantly mixed with SFE before enter into the cell. The extraction time was 120 min for all the experiments.

241

242 2.8 Antioxidant activity assays (DPPH-ABTS)

243 The evaluation of antioxidant activity (AOA) of quercetin standard and of all the extracts were
244 measured using two methods based on a procedure described by Brand-Williams et al. (Brand-
245 Williams, Cuvelier, & Berset, 1995) and Re et al. (Re et al., 1999) the DPPH and ABTS
246 respectively. Assays were carried out according to the literature with little modifications in order to
247 adapt the assays to 96-well plates. Operating conditions of DPPH and ABTS were previously
248 reported by Celano et al. (Celano et al., 2017). Briefly, for DPPH assay, 5 μL of quercetin and
249 extracts (SFE and USAE) in the range of 1-15 μM and 0.25-1 mg mL^{-1} respectively (5 levels), were
250 added to 195 μL of DPPH $^{\bullet}$ at concentration of 120 μM . Due to the coloration of extract it was
251 necessary included a blank (MeOH; control) instead of DPPH into well plates. After 1 and 4 hours
252 for quercetin and extracts respectively, at room temperature and protected from light the reaction
253 was completed and absorbance was read at 515 nm at 25°C with a Multiskan Go microplate
254 spectrophotometer (Thermo Fisher Scientific, San Jose, CA, USA).

255 Regarding ABTS assay, the operating conditions of were as follow: 500 μL of ABTS $^{•+}$ working
256 solution 1 mM was mixed with 5 μL of PBS (control), quercetin and extracts (SFE and USAE) at
257 same concentrations range described above in DPPH assays. Then 300 μL of each mixture were
258 transferred into a 96-well plate and after 60 min of incubation in the dark at 30°C the absorbances
259 were measured at 734 nm, by a microplate spectrophotometer reader Multiskan Go. Antioxidant
260 capacity of both assays were expressed as Trolox equivalent (TEAC) and employed to quantify and
261 compare tested solutions. Trolox, quercetin and curves of each extract were obtained by plotting
262 concentration (mg mL^{-1} for the extracts; μM for trolox and quercetin) against the average inhibition
263 % of radical absorbance ($I\%$)

264

$$265 \quad I\% = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} * 100 \quad (2)$$

266

267 Concentration corresponding at 50% of inhibition (IC_{50}) was extrapolated from curves and the
268 TEAC was calculated using the following formula:

269
270
$$TEAC = \frac{IC_{50\ Trolox}}{IC_{50\ sample}} \quad (3)$$

271

272 3. Results and discussion

273

274 3.1 Qualitative characterization of onion skin exhaustive extract.

275

276 Chemical composition of phenolic compounds in exhaustive extract of onion dried skin, was
277 determined by UHPLC-DAD-HRMS analysis. UHPLC solvents composition and gradient were
278 optimized in order to obtain better chromatographic resolution and the most intense MS signal
279 response. Figure 1 shows the overlay of UV traces (295 and 365 nm) of extract under optimal
280 chromatographic conditions. In the MS analysis, both negative and positive ion modes were
281 employed to establish the molecular formulas, despite the most of compounds exhibited higher
282 responses in negative ion mode. The tentatively identification of main compounds in exhaustive
283 extract was carried out by comparing retention time (tr), UV (λ_{MAX}) spectra and HRMS data of
284 detected analytes with standard compounds, whenever available, or interpreting MS data (accurate
285 masses and MS/MS product ions) combined with chemo-taxonomic data reported in the literature
286 and databases. UHPLC-UV-HRMS/MS analysis of USAE extract allowed to identify 18 main
287 compounds (Table 1). Except for protocatechuic acid, most of detected compounds were flavonols
288 in particular quercetin derivatives as glycosides and oxidized products. Protocatechuic acid (1),
289 quercetin (10), isorhamnetin (13) and keampferol (14) were unambiguous identified by the
290 comparison with reference standards.

291

292 3.1.1 Glycosylated derivatives of quercetin and isorhamnetin

293 According to literature data (Griffiths, Trueman, Crowther, Thomas, & Smith, 2002), onion has a
294 high content of flavonol glycosides, mainly glycosylated derivatives of quercetin and isorhamnetin.
295 In particular, in USAE extract four glycosylated derivatives of quercetin were tentatively identified
296 including two mono-O-hexosides (quercetin glycoside, 7 and 8) and two di-O-hexosides (quercetin
297 di-glycoside, 3 and 4). MS/MS spectra of these compounds (Table 1) showed product ions useful to
298 identify the sugar moieties (neutral losses of hexose units, -162 Da) and aglycone ($C_{15}H_{10}O_7$, < 3.4
299 ppm), for both positive and negative ion modes (Wolfender, Maillard, Marston, & Hostettmann,
300 1992). Likewise, compounds 5 and 9 were tentatively identified as isorhamnetin-O-dihexoside and
301 isorhamnetin-O-hexoside respectively. Their fragmentation pathway resulted in a product ion at m/z
302 315.0511 and m/z 317.0648 ($C_{16}H_{12}O_7$), in NI and PI modes respectively, corresponding to
303 isorhamnetin aglycone derived by the elimination of one or two units of hexose. Therefore, on the
304 basis of these results and according with literature data (Bonaccorsi, Caristi, Gargiulli, & Leuzzi,
305 2005) compounds 3-5 and 7-9 were tentatively identified as quercetin-7,4'-diglycoside, quercetin-
306 3,4'-diglycoside, isorhamnetin-3,4'-diglycoside, quercetin-3-glycoside, quercetin-4'-glycoside and
307 isorhamnetin-4'-glycoside respectively.

308

309 3.1.2 Quercetin oxidized derivatives

310 Several studies showed as quercetin, one of the major phenolic compounds of onion skin (Benítez
311 et al., 2011), can be oxidized with potassium ferricyanide under alkaline conditions or by radical
312 oxidation generating naturally occurring oxidized compounds which are mainly found in the outer
313 dry layers of onion bulbs (Makris & Rossiter, 2002; Ramos et al., 2006). In the USAE extract
314 several oxidized derivatives of quercetin that ranged from oxidized monomer (2), dimers (15, 16,
315 17, 18) and trimer (19) were tentatively identified using UHPLC-HRMS/MS (Table 1). Peak 18
316 corresponded to quercetin dimer previously reported in the onion dried skin by Ly et al. (Ly et al.,
317 2005) and identified as one of the main oxidized derivatives of quercetin (Makris & Rossiter, 2002).
318 The (+)- and (-)-HRMS spectra of 18 exhibited a base peak at m/z 603.0731 $[M+H]^+$ and 601.0617

319 $[M-H]^-$ respectively, corresponding to molecular formula $C_{30}H_{18}O_{14}$. Fragmentation of $[M-H]^-$
 320 resulted in two major product ions, m/z 449.0492 and 299.0186 (Figure 1S). In particular, the ion at
 321 m/z 299.0186 ($C_{15}H_7O_7$, -0.1 ppm), corresponding to quercetin *O*-diquinone ion, generated by a
 322 retro Diels-Alder (rDA) of the dioxane ring of 18 (Figure 1S), whereas the ion at m/z 449.0492
 323 (neutral loss of 152 Da) was produced by the characteristic rDA of the flavonoids C ring with a 5,7-
 324 dihydroxy-substituted ring (Cuyckens & Claeys, 2004). The oxidized compounds 16 and 17
 325 ($C_{36}H_{28}O_{19}$) were tentatively identified as hexoside derivatives of 18 (Table 1) by the diagnostic
 326 product ion at m/z 299.0193 (Figure 2S a) in (-) MS/MS spectra. The MS/MS spectra displayed also
 327 the product ions at 601/600, generated from the elimination of hexoside residue from aglycone and
 328 product ion at m/z 611.1023 produced by rDA of C-ring. A third $C_{36}H_{28}O_{19}$ isomer (15) was
 329 detected in the extract, but on the basis of its fragmentation pathway (Figure 2S b) the structure of
 330 3-(quercetin-8-yl)-2,3-epoxyflavanone was suggested as aglycone. This compound, another
 331 quercetin dimer, was previously reported in onion skin (Ramos et al., 2006). In fact, the (-) MS/MS
 332 spectra of compound 15 not showed the diagnostic product ion at m/z 299.0193 for the dioxane ring
 333 of 16-18, but only the product ion at m/z 611.1028 (Figure 2S b). Finally, a quercetin trimer (19)
 334 was also identified in USAE extract by UHPLC-HRMS analysis of extracts (Table 1). The
 335 fragmentation of $[M-H]^-$ ion m/z 901.0881 ($C_{45}H_{26}O_{21}$) revealed product ions at m/z 599.0452 and
 336 299.0196 corresponding to *o*-dichinone ions generated by rDA of two dioxane rings of the proposed
 337 structure (Figure 3S). Also this oxidized derivative of quercetin was previously isolated into onion
 338 skin extract (Ly et al., 2005). The molecular formula calculated for 11 and 12, $C_{22}H_{14}O_{11}$ (Table 1),
 339 suggested the presence of protocatecoyl-quercetin isomers as reported by Ly et al. (Ly et al., 2005)
 340 but the fragmentation spectra not support these structures.

341

342 3.2 Optimization of SFE

343 3.2.1 Selection of extraction time

344 After the characterization of USA extract, the optimization of SFE conditions was carried out to
345 improve the extraction efficiency, and to avoid degradation phenomena and/or formation of
346 artificial compounds. Firstly, to set extraction time, a kinetic study was performed using central
347 conditions of the experimental design (extraction temperature 55 °C, extraction pressure 200 bar,
348 modifier EtOH 85%). Extract was dried under vacuum using a rotavapor and the solid residue was
349 measured gravimetrically every 30 min for a total extraction time of 240 min (n=3). As shown in
350 Figure 2, extraction yield increases for the first 120 min and then remain almost constant for the rest
351 of time (240 min). Therefore, 120 min were selected as optimum extraction time and used for all the
352 other experiments.

353

354 *3.2.2 Response surface optimization of SFE process*

355 After selection of the extraction time, other parameters of SFE were optimized using a chemometric
356 approach, in order to minimize the number of experiments and evaluate the interaction between
357 each factor. The conventional optimization approach (step by step) could be suboptimal because
358 SFE parameters can influence extraction efficiency directly or by their interaction. For these
359 reasons it is extremely important to evaluate simultaneously the influence of each parameter on
360 response factors using an experimental design. The optimization was carried out considering three
361 of the most important SFE factors (temperature, pressure and solvent modifier composition) that
362 could affect the extraction efficiency. Regarding the composition of solvent modifier, different
363 mixtures of ethanol/water were tested. Ethanol was selected to improve the solubility of analytes
364 with medium polarity (Hamburger, Baumann, & Adler, 2004) whereas addition of small amount of
365 water (5-15%) was used to promote the extraction efficiency of polar compounds (Mustafa &
366 Turner, 2011), thus avoiding great changes in the supercritical conditions of CO₂. On the basis of
367 these considerations, the experimental factors temperature (A), pressure (B) and EtOH% (C) were
368 optimized by a Box–Behnken 2 factor interactions design. Low and high levels of each
369 experimental factor was established on the basis of our experience on SFE technique and by

preliminary experiments. In Table 1S are reported the range of values used (low, medium and high) for each factor, the experimental conditions of design and the response of each variable. Extraction yield and normalized peaks areas of quercetin-di-glycoside, quercetin and quercetin dimer in the SFE extract were considered as response variables. Since the results obtained for most of the compounds were similar, in order to make the discussion of results concise and easily understandable, only data of quercetin-di-glycoside (more polar compound), quercetin (main compound), quercetin dimer (less polar compound) are discussed. Standardized effects on response variables were shown in pareto charts (Figure 3). The R^2 values showed that model as fitted explains 81-95% of the variability. As can be seen in Figure 2 only the composition of solvent modifier (C) affected positive and significantly the response variables (extraction yield and peaks area) whereas the other two independent variables (temperature A and pressure B) and their first-order interactions resulted below the statistically significant threshold (vertical bar in Pareto chart). These results were in agreement with the study conducted by Martino et al. (Martino & Guyer, 2004) which suggest as solvent modifier (EtOH) have an important influence ($p < 0.001$) on free quercetin recovery. As shown in the desirability plot (Figure 4S), the composition of solvent modifier is the parameter mostly influencing on the desired effect, indeed an increase of water percentage from 5 to 15% of solvent modifier (EtOH) determines a remarkable improvement of desirability. Temperature showed a lower remarkable positive effect with a slight increase of desirability. These results highlight that the composition of solvent modifier is main experimental factor that influence the extraction efficiency of phenolic compounds in SFE process. Optimized conditions (best predicted design point) extrapolated from chemometric analysis (temperature, 40 °C; pressure 100 bar and EtOH 85%) showed a desirability levels of 87% with an extraction yield of 16 %. Improvement of SFE extraction efficiency under optimized conditions provided by statistical software, were experimentally evaluated by recovery study.

Recoveries were calculated by comparing the amount of each compound (mg/100 g DM) in SFE extract with those obtained by the exhaustive extraction (USAE). Whereby, considering the

396 recoveries shown in Table 2 is clear that the capability of developed procedure provide an
397 exhaustive extraction efficiency comparable with those obtained by ultrasound extraction technique,
398 confirming that SFE can be used as valid alternative to the conventional extraction techniques for
399 the extraction of bioactive compounds from onion dried skin.

400

401 *3.3 Quantitative analysis and antioxidant activity evaluation*

402 A major challenge in quantification of complex compounds mixture as onion skin by HPLC-UV
403 methods is the unavailability of most standards on a commercial basis. Spectral similarities in the
404 majority of quercetin derivatives (glycoside) makes their determination relatively feasible as
405 “external standard quercetin equivalents”. Unfortunately, this strategy does not provide an accurate
406 absolute quantification of phenolic compounds that are naturally present in the onion extract which
407 present remarkable differences in the chemical structure with the quercetin standard. Therefore,
408 quantitative results reported in Table 2 provide only a relative information about their distribution
409 in the matrix (internal distribution comparison). Quantitative analysis of the main phenolic
410 compounds in SFE extract was performed by UHPLC-UV using external standard method and
411 results were expressed as quercetin equivalent, except for quercetin and protocatechuic acid.
412 Relative compounds concentration which are naturally contained in the onion extract were
413 calculated by the interpolation of each UV peak area in the standard calibration curves, including
414 protocatechuic acid and quercetin in concentration range of 6.25-100 μ M (6 levels). External
415 standard calibration curves for protocatechuic acid and quercetin showed good linearity (ANOVA)
416 within the range of concentrations investigated with correlation coefficients (R^2) of 0.998 and 0.999
417 respectively. The quantitative results shown in Table 2 highlighted that SFE extract can be
418 considered a rich source of phenolic compounds for its high contents of quercetin and quercetin
419 derivatives, therefore is expected to have a good antioxidant capacity. In order to evaluate the
420 antioxidant capacity (AOC) of SFE extract two in vitro antioxidant assays, DPPH and ABTS were
421 carried out. Most assays employed to evaluate the AOC are based on single electron and/or

hydrogen atom transfer reactions (SET/HAT). In our case both assays used (DPPH and ABTS) are suggested for the representative evaluation of antioxidant properties because are based on SET/HAT at the same time (Prior, Wu, & Schaich, 2005).

Before estimation of AOC, the optimization of reaction time (kinetics study) and concentration range for both extracts and standard compounds (quercetin and trolox) were evaluated in order to avoid the underestimation of results (Mishra, Ojha, & Chaudhury, 2012).

Figure 4 showed the AOC expressed as TEAC of quercetin standard and of SFE/USAE extracts for DPPH and ABTS respectively. Results of both assays have similar trend and indicated that pure quercetin shows greater AOC ($p < 0.05$) than both extracts (SFE and USAE), whereas the SFE extract exhibit an AOC (TEAC) slightly above the USAE extract.

Results of antioxidant assay suggested that the amount of antioxidant compounds in SFE extract is similar to extract obtained by USAE and confirm a considerable radical scavenger activity of SFE extract, which can be used as source of food ingredients with good antioxidant activity.

4. Conclusions

In the recent time, the interest in recovering bioactive compounds from food by-products has significantly increased. In this study, a green analytical procedure has been successfully developed for the analysis of phenolic compounds in outer dry layers of brown skin onion. Initially, a conventional extraction, ultrasound assisted solid-liquid extraction techniques, was carried out in order to obtain a reference extract USAE extract which has been further characterized by UHPL high resolution mass spectrometry. After the chemical characterization of phenolic compounds found in onion dried peel, the main extraction parameters of SFE such as pressure, temperature and composition of solvent modifier, were carefully evaluated and optimized using an experimental design, in order to improve extraction efficiency and also the peak area response of phenolic compounds. Compounds found in the SFE extract was quantify by using UHPLC–UV analysis and

the antioxidant capacity was evaluated by the DPPH and ABTS assays. Under the optimized extraction conditions, the developed method was compared with a conventional extraction method (USAE) widely used for the extraction of bioactive substances in food and our results shows similar or greater analytical performances with a good antioxidant capacity. The extraction procedure demonstrated that SFE-CO₂/co-solvent extraction can be a suitable protocol to obtain nutraceutical products with promising antioxidant and free radical scavenging activities from natural and cheap sources as food by-products. In addition, another advantage of SFE process proposed in this work is the possibilities of being used at industrial scale in order to extract large quantities of matrix, obtaining great amount of extract in a single step.

Conflicts of interest

The authors declare no conflict of interest.

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Figure caption

Figure 1

UHPLC-UV profiles 295 nm (red line) and 365 nm (black line) at 2 mg mL⁻¹ of SFE extract under optimized extraction conditions.

Figure 2

Extraction yield vs extraction time at the central point of the response surface design experimental condition pressure 200 bar temperature 55 °C and co-solvent EtOH 90%.

Figure 3

Pareto charts of standardized effects for extraction yield and normalized peaks area of quercetin-diglycoside (Q-dG), quercetin (Q) and quercetin dimer (Q2). (A) Temperature °C; (B) Pressure bar; (C) EtOH %.

Figure 4

Antioxidant capacity of quercetin standard, SFE and USAE extracts by DPPH and ABTS assays.

N°	Compound	Molecular Formula	RT _(UV) (min)	[M-H] ⁻ (m/z)			[M+H] ⁺ (m/z)		
				Measured (m/z)	Error (ppm)	Product ion MS/MS	Measured (m/z)	Error (ppm)	Product ion MS/MS
1	Protocatechuic acid	C ₇ H ₆ O ₄	1.2	153.1235	1.6	/	/	/	/
2	2-(3,4-Dihydroxybenzoyl)-2,4,6-trihydroxy-3(2H)-benzofuranone	C ₁₅ H ₁₀ O ₈	6.0	317.0303	3.3	299; 191; 207; 273	/	/	/
3	Quercetin-7,4'-diglycoside	C ₂₇ H ₃₀ O ₁₇	6.6	625.1405	0.9	463; 301	627.1528		465; 303
4	Quercetin 3,4'-diglycoside	C ₂₇ H ₃₀ O ₁₇	6.9	625.1410	-0.3	463; 301	627.1532	-4.8	465; 303
5	Isorhamnetin-3,4' diglycoside	C ₂₈ H ₃₂ O ₁₇	7.2	639.1571	0.04	477; 315	641.1688	-3.7	317; 479
6	Unknown	/	7.6	497.0351	0.4	299; 453; 361	/	/	/
7	Quercetin-3-glycoside	C ₂₁ H ₂₀ O ₁₂	8.2	463.0874	0.6	301	465.1005	-4.8	303
8	Quercetin-4'-glycoside	C ₂₁ H ₂₀ O ₁₂	10.3	463.0873	0.8	301	465.1007	-4.2	303
9	Isorhamnetin-4'-glycoside	C ₂₂ H ₂₂ O ₁₂	12.0	477.1031	0.7	315	479.116	-4.9	317
10	Quercetin	C ₁₅ H ₁₀ O ₇	13.6	301.0350	0.6	179; 151	303.0487	-3.8	285; 257; 229;
11	Protocatecoyl quercetin	C ₂₂ H ₁₄ O ₁₁	14.1	453.0454	0.4	299	455.588	-4.5	437; 301;
12	Protocatecoyl quercetin	C ₂₂ H ₁₄ O ₁₁	14.4	453.0455	0.6	299	455.0589	-4.4	437; 301;
13	Kaempferol	C ₁₅ H ₁₀ O ₆	16.3	285.0399	2.1	/	287.0538	-4.1	/
14	Isorhamnetin	C ₁₆ H ₁₂ O ₇	16.7	315.0503	1.2	300; 257	317.0642	-4.3	302, 285; 257
15	Quercetin dimer 4'-glycoside	C ₃₆ H ₂₈ O ₁₉	17.2	763.1140	-0.2	611; 449;	765.1258	-5.1	603; 451
16	Quercetin dimer 4'-glycoside	C ₃₆ H ₂₈ O ₁₉	18.3	763.1139	-0.3	611; 600; 299	765.1257	-5.2	603; 585
17	Quercetin dimer hexoside	C ₃₆ H ₂₈ O ₁₉	18.6	763.1139	-0.2	611; 600; 299	765.1263	-4.4	603; 585
18	Quercetin dimer	C ₃₀ H ₁₈ O ₁₄	19.7	601.0617	0.8	449; 299	603.0731	-6.3	585; 313; 303
19	Quercetin trimer	C ₄₅ H ₂₆ O ₂₁	20.8	901.0881	-0.2	299; 449; 599; 601	903.0235	-4.3	885; 751; 585; 613

Table 1 UHPLC-HRMS/MS data of detected compounds in USAE extract.

N°	Compounds	R _t	Rec %	RDS%	mg/100g DM ± (RDS%)
1	Protocatechuic acid	1,2	54	1,8	140,6 ± 6
2	2-(3,4-Dihydroxybenzoyl)-2,4,6-trihydroxy-3(2H)-benzofuranone	6,0	67	3,9	119,9 ± 4
3	Quercetin-7,4'-diglycoside	6,5	98	7,0	66,1 ± 8
4	Quercetin 3,4'-diglycoside	6,9	65	5,5	77,4 ± 12
5	Isorhamnetin-3,4' diglycoside	7,1	112	4,4	nq
6	Unknown	7,9	80	7,2	nq
7	Quercetin-3-glycoside	8,22	80	5,6	nq
8	Quercetin-4'-glycoside	10,3	118	6,4	365,8 ± 8
9	Isorhamnetin-4'-glycoside	12,5	121	6,6	nq
10	Quercetin	13,5	118	7,6	528,4 ± 12
11	Protocatecoyl quercetin	14,0	105	7,8	nq
12	Protocatecoyl quercetin	14,3	104	5,6	nq
13	Kaempferol	16,4	89	2,0	nq
14	Isorhamnetin	16,7	110	2,6	nq
15	Quercetin dimer 4'-glycoside	17,1	98	1,9	45,9 ± 4
16	Quercetin dimer 4'-glycoside	18,2	105	0,8	45,1 ± 6
17	Quercetin dimer hexoside	18,5	103	2,5	nq
18	Quercetin dimer	19,4	92	0,8	114,5 ± 2
19	Quercetin trimer	20,7	115	2,4	65,2 ± 6

nq= not quantified; R_t=retention time; rec%= recovery%; RDS%=relative standard deviation; DM=dry matter

Table 2 Extraction recovery and amount of phenolic compounds of SFE extract

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Con formato: Subíndice

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