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

Keywords: Onion skin, Quercetin derivatives, Supercritical fluid extraction, Recovery bioactive compounds, Experimental design optimization, Food by-products, DPPH and ABTS assays

1. Introduction

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

bulbs, which are not edible and are removed before processing (Benítez et al., 2011, Ly et al., 2005). Onion wastes, due to a rapid phytopathogens growth and to their strong unique smell, are not suitable for fodder or landfill disposal. Therefore, the valorisation of onion by-products is indispensable and could provide an economic benefit both for the onion producers and processors (Schieber, Stintzing, & Carle, 2001). Onion is one of the most common and the richest natural source of biologically active phytomolecules, including phenolic acids, anthocyanins, cepaenes, thiosulfinates, and flavonoids (Fossen and Andersen, 2003, Slimestad et al., 2007, Suleria et al., 2015). In recent years, several studies have been conducted on the role of phytochemical compounds found in onions. The major bioactive compounds found in dry peel of onion are phenolics as quercetin and quercetin glycoside (Ly et al., 2005). Food-derived phenolics as quercetin are getting great interest due to their antimicrobial and antioxidant activity, strongly related with a role in cancer prevention, inflammatory disorders and cardiovascular diseases (Działo et al., 2016, Flores et al., 2012). Other benefits of quercetin include anti-inflammatory activity, antihistamine effect, allergy medication, and anticancer and antivirus activities. It has also been claimed that quercetin reduces blood pressure in hypertensive subjects (Boots, Haenen, & Bast, 2008). However, in order to use onion by-products as source of bioactive compounds, suitable extraction methods must be developed to reduce time, cost and environmental pollution (Galanakis, 2012).

Many conventional solvent extraction methods (CSE) as maceration, sonication and soxhlet have been extensively used to extract bioactive compounds from plant materials. Indeed, these CSE methods have several drawbacks as: a long extraction time, require large amounts of organic solvents and/or high temperatures, which could lead to extensive degradation of thermo-sensible molecules, as well as they could leave residues of potentially toxic solvents in the extracts (Abbas et al., 2008, Ameer et al., 2017, Azmir et al., 2013, Calvo et al., 2007, Galanakis, 2012, Rozzi and Singh, 2002). So to overcome these disadvantages, fast, cheap and efficient, ‘‘Green extraction techniques” able to reduce/eliminate 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 and 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 (CO_2) which is non-toxic, non-flammable, inexpensive, and have low viscosity and high diffusivity (Espinosa, Diaz, & Brignole, 2005). In addition, CO_2 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 CO_2, which is mostly suitable for the extraction of non-polar and moderately polar compounds (Herrero et al., 2010). Nevertheless, to extend the SFE-CO_2 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, in the present study we aimed to develop a green and innovative extraction technique, the supercritical

CO_2 ethanol/water co-solvent to quantitatively extract phenolic compounds from the dry skin of coppery onion. The onion used in this study was an IGP variety, known as "Ramata di Montoro", a species cultivated in Campania region, in south of Italy. UHPLC-UV-HRMS was employed to characterize the phenolic profile of onion dry skin extracts (exhaustive and SFE) and to optimize the SFE experimental conditions.
Main parameters that could influence the extraction efficiency of SFE technique, such as pressure, temperature and composition of solvent modifier were optimized using a response surface Box–Behnken design. Moreover, antioxidant activity of SFE-CO_2 extract, evaluated by DPPH/ABTS assays, were compared with those obtained using ultrasound assisted extraction (USAE). To the best of our knowledge this is the first application of SFE to extract and recovery 18 bioactive compounds from dried onion skin.

2. Experimental

2.1. Standards and materials

Ultrapure water (18 MΩ) was obtained using a Milli-Q purification system (Millipore, Bedford, USA). MS-grade water (H_2O), methanol (MeOH), and acetonitrile (CH_3CN) were supplied by Romil (Cambridge, UK). Analytical grade MeOH, CH_3CN, Formic acid (HCOOH) and absolute ethanol (EtOH) were supplied by Carlo Erba reagents (Milan, Italy). MS-grade formic acid (HCOOH), quercetin (purity ≥ 95%), isorhamnetin (purity ≥ 95% HPLC), kaempferol (purity ≥ 97%), protocatechuic acid (purity 95%), 2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH) and 2,2′-azinobis (3-ethylben- zothiazoline-6-sulphonic acid) diammonium salt (ABTS) were provided by Sigma-Aldrich (Milan, Italy).

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.

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/100 g DM, n = 3) was calculated gravimetrically using the following formula:(1)

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 (2.1 × 50 mm, 1.7 μm; Phenomenex, Torrance, CA, USA) and mobile phases (MS grade) were H_2O

(A) and CH_3CN (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 μL min^{-1}) was kept constant for all run time analysis and the injection volume was set at 5 μL. 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.

2.5. Quantitative analysis by UHPLC-UV

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

2.6. Experimental design

Statgraphic Centurion XVI Version 16.1 (Rockville, USA) was employed for the experimental design analysis, data processing and to optimize SFE parameters. A response surface Box–Behnken design 2-factor interactions was carried out. By using this design, three variables were tested at 3 different levels (low, medium and high): extraction temperature (Temp) at 40, 55 and 70 °C, pressure (Press) 100, 200 and 300 bar and composition of solvent modifier (EtOH %) 95, 90 and 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-di-glycoside, 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.

2.7. Supercritical CO_2 extraction

SFE extracts were obtained using a Suprex PrepMaster (Suprex, Pittsburgh, PA). The extractor was equipped with a dual piston pump for CO_2 (Carburos 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 1 g 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 CO_2. Compressed mixture of CO_2 and solvent modifier was led into the heater prior to entering the extraction cell. Dynamic extractions were performed using high purity CO_2 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.

2.8. Antioxidant activity assays (DPPH-ABTS)

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

Regarding ABTS assay, the operating conditions of were as follow: 500 µL of $ABTS^{\bullet+}$ working solution 1 mM was mixed with 5 µL of PBS (control), quercetin and extracts (SFE and USAE) at same concentrations range described above in DPPH assays. Then 300 µL of each mixture were transferred into a 96-well plate and after 60 min of incubation in the dark at 30 °C the absorbances were measured at 734 nm, by a microplate spectrophotometer reader Multiskan Go. Antioxidant capacity of both assays were expressed as Trolox equivalent (TEAC) and employed to quantify and compare tested solutions. Trolox, quercetin and curves of each extract were obtained by plotting concentration (mg mL^{-1} for the extracts; µM for trolox and quercetin) against the average inhibition % of radical absorbance (*I*%).(2)

Concentration corresponding at 50% of inhibition (IC_{50}) was extrapolated from curves and the TEAC was calculated using the following formula:(3)

3. Results and discussion

3.1. Qualitative characterization of onion skin exhaustive extract

Chemical composition of phenolic compounds in exhaustive extract of onion dried skin, was determined by UHPLC-DAD-HRMS analysis. UHPLC solvents composition and gradient were

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

3.1.1. Glycosylated derivatives of quercetin and isorhamnetin

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

3.1.2. Quercetin oxidized derivatives

Several studies showed as quercetin, one of the major phenolic compounds of onion skin (Benítez et al., 2011), can be oxidized with potassium ferricyanide under alkaline conditions or by radical oxidation generating naturally occurring oxidized compounds which are mainly found in the outer dry layers of onion bulbs (Makris and Rossiter, 2002, Ramos et al., 2006). In the USAE extract several oxidized derivatives of quercetin that ranged from oxidized monomer (2), dimers (15, 16, 17, 18) and trimer (19) were tentatively identified using UHPLC-HRMS/MS (Table 1). Peak 18 corresponded to quercetin dimer previously reported in the onion dried skin by Ly et al. (2005) and identified as one of the main oxidized derivatives of quercetin (Makris & Rossiter, 2002). The (+)- and (−)-HRMS spectra of 18 exhibited a base peak at *m/z* 603.0731 $[M+H]^+$ and 601.0617 $[M\text{-}H]^-$ respectively, corresponding to molecular formula $C_{30}H_{18}O_{14}$. Fragmentation of $[M\text{-}H]^-$ resulted in two major product ions, *m/z* 449.0492 and 299.0186 (Fig. 1S). In particular, the ion at *m/z* 299.0186 ($C_{15}H_7O_7$, −0.1 ppm), corresponding to quercetin *O*-diquinone ion, generated by a retro Diels-Alder (rDA) of the dioxane ring of 18 (Fig. 1S), whereas the ion at *m/z* 449.0492 (neutral loss of 152 Da) was produced by the characteristic rDA of the flavonoids C ring with a 5,7-dihydroxy-substituted ring (Cuyckens & Claeys, 2004). The oxidized compounds 16 and 17 ($C_{36}H_{28}O_{19}$) were tentatively identified as hexoside derivatives of 18 (Table 1) by the diagnostic product ion at *m/z* 299.0193 (Fig. 2S a) in (−) MS/MS spectra. The MS/MS spectra displayed also the product ions at 601/600, generated from the elimination of hexoside residue from aglycone and product ion at *m/z* 611.1023 produced by rDA of C-ring. A third $C_{36}H_{28}O_{19}$ isomer (15) was detected in the extract, but on the

basis of its fragmentation pathway (Fig. 2S b) the structure of 3-(quercetin-8-yl)-2,3-epoxyflavanone was suggested as aglycone. This compound, another quercetin dimer, was previously reported in onion skin (Ramos et al., 2006). In fact, the (-) MS/MS spectra of compound 15 not showed the diagnostic product ion at *m*/*z* 299.0193 for the dioxane ring of 16–18, but only the product ion at *m*/*z* 611.1028 (Fig. 2S b). Finally, a quercetin trimer (19) was also identified in USAE extract by UHPLC-HRMS analysis of extracts (Table 1). The fragmentation of $[M-H]^-$ ion *m*/*z* 901.0881 ($C_{45}H_{26}O_{21}$) revealed product ions at *m*/*z* 599.0452 and 299.0196 corresponding to o-dichinone ions generated by rDA of two dioxane rings of the proposed structure (Fig. 3S). Also this oxidized derivative of quercetin was previously isolated into onion skin extract (Ly et al., 2005). The molecular formula calculated for 11 and 12, $C_{22}H_{14}O_{11}$ (Table 1), suggested the presence of protocatecoyl-quercetin isomers as reported by Ly et al. (2005) but the fragmentation spectra not support these structures.

3.2. Optimization of SFE

3.2.1. Selection of extraction time

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

3.2.2. Response surface optimization of SFE process

After selection of the extraction time, other parameters of SFE were optimized using a chemometric approach, in order to minimize the number of experiments and evaluate the interaction between each factor. The conventional optimization approach (step by step) could be suboptimal because SFE parameters can influence extraction efficiency directly or by their interaction. For these reasons it is extremely important to evaluate simultaneously the influence of each parameter on response factors using an experimental design. The optimization was carried out considering three of the most important SFE factors (temperature, pressure and solvent modifier composition) that could affect the extraction efficiency. Regarding the composition of solvent modifier, different mixtures of ethanol/water were tested. Ethanol was selected to improve the solubility of analytes with medium polarity (Hamburger, Baumann, & Adler, 2004) whereas addition of small amount of water (5–15%) was used to promote the extraction efficiency of polar compounds (Mustafa & Turner, 2011), thus avoiding great changes in the supercritical conditions of CO_2. On the basis of these considerations, the experimental factors temperature (A), pressure (B) and EtOH% (C) were optimized by a Box–Behnken 2 factor interactions design. Low and high levels of each 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 (Fig. 3). The R2 values showed that model as fitted explains 81–95% of the variability. As can be seen in Fig. 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 (Fig. 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 recoveries shown in Table 2 is clear that the capability of developed procedure provide an exhaustive extraction efficiency comparable with those obtained by ultrasound extraction technique, confirming that SFE can be used as valid alternative to the conventional extraction techniques for the extraction of bioactive compounds from onion dried skin.

3.3. Quantitative analysis and antioxidant activity evaluation

A major challenge in quantification of complex compounds mixture as onion skin by HPLC-UV methods is the unavailability of most standards on a commercial basis. Spectral similarities in the majority of quercetin derivatives (glycoside) makes their determination relatively feasible as "external standard quercetin equivalents". Unfortunately, this strategy does not provide an accurate absolute quantification of phenolic compounds that are naturally present in the onion extract which present remarkable differences in the chemical structure with the quercetin standard. Therefore, quantitative results reported in Table 2 provide only a relative information about their distribution in the matrix (internal distribution comparison). Quantitative analysis of the main phenolic compounds in SFE extract was performed by UHPLC-UV using external standard method and results were expressed as quercetin equivalent, except for quercetin and protocatechuic acid. Relative compounds concentration which are naturally contained in the onion extract were calculated by the interpolation of each UV peak area in the standard calibration curves, including protocatechuic acid and quercetin in concentration range of 6.25–100 μM (6 levels). External standard calibration curves for protocatechuic acid and quercetin showed good linearity (ANOVA) within the range of concentrations investigated with correlation coefficients (R2) of 0.998 and 0.999 respectively. The quantitative results shown in Table 2 highlighted that SFE extract can be considered a rich source of phenolic compounds for its high contents of quercetin and quercetin derivatives, therefore is expected to have a good antioxidant capacity. In order to evaluate the antioxidant capacity (AOC) of SFE extract two in vitro antioxidant assays, DPPH and ABTS were 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).

Fig. 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-CO_2/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.

Appendix A. Supplementary data

The following are the Supplementary data to this article: Download : Download Acrobat PDF file (210KB) Supplementary data 1.

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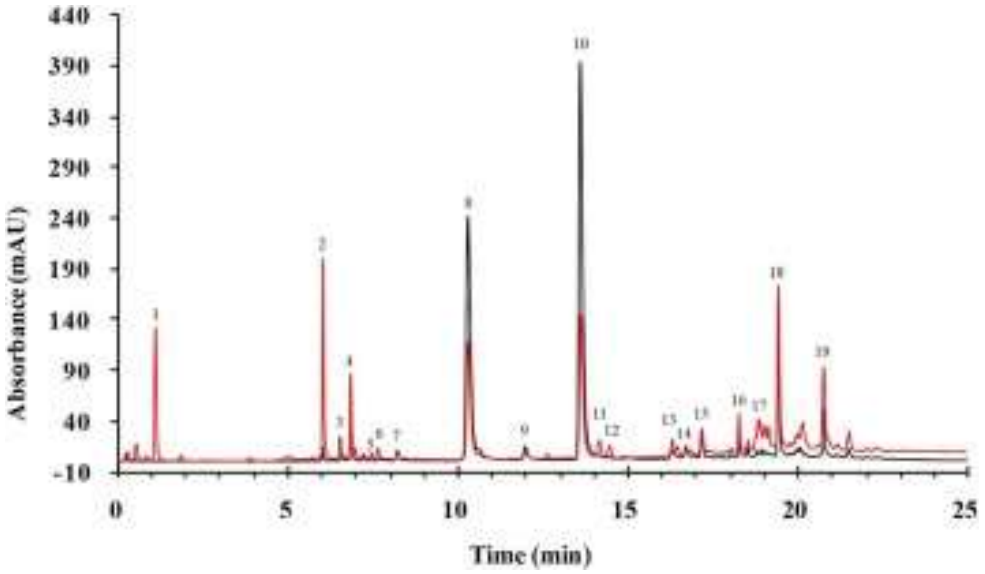

Fig. 1. UHPLC-UV profiles 295 nm (red line) and 365 nm (black line) at 2 mg mL^{-1} of SFE extract under optimized extraction conditions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

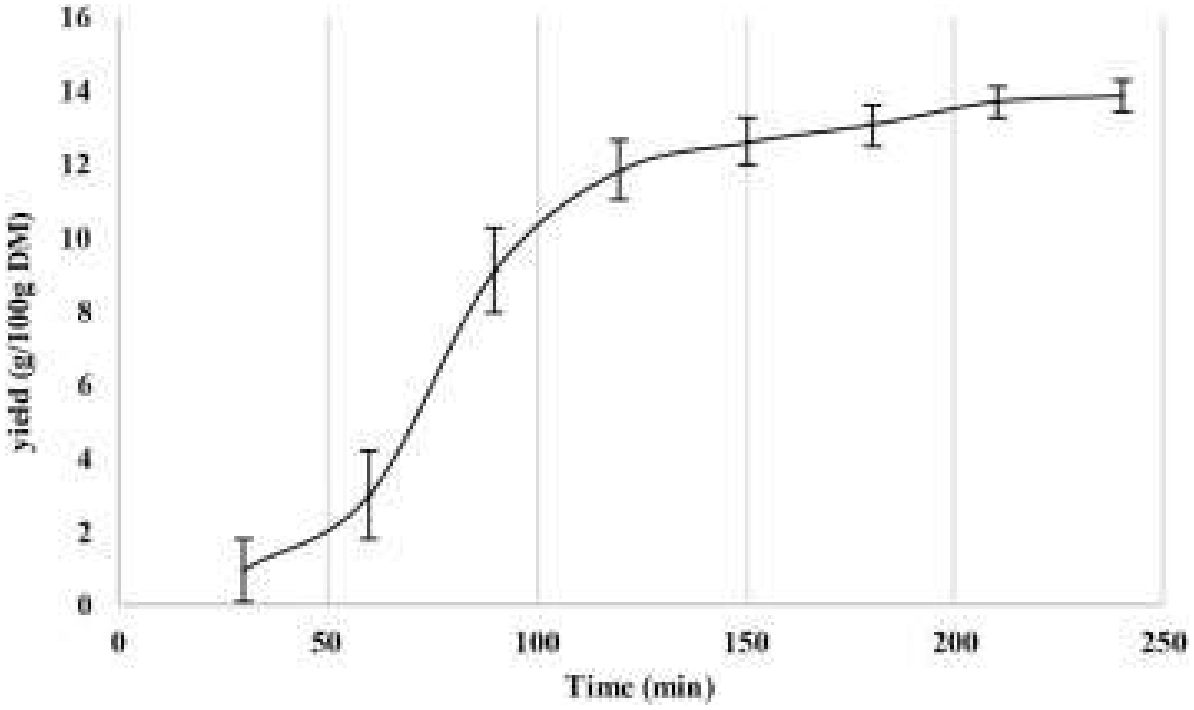

Fig. 2. Extraction yield vs extraction time at the central point of the response surface design experimental condition pressure 200 bar temperature 55 °C and co-solvent EtOH 90%.

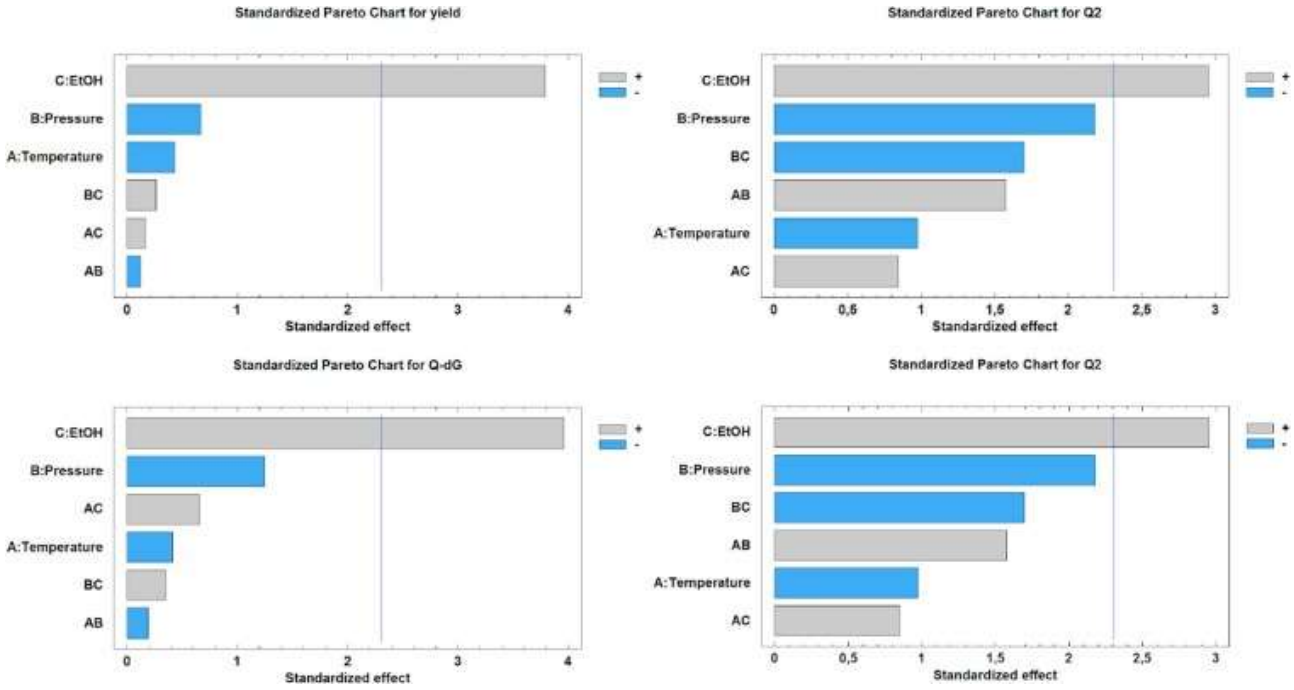

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Fig. 3. Pareto charts of standardized effects for extraction yield and normalized peaks area of quercetin-di-glycoside (Q-dG), quercetin (Q) and quercetin dimer (Q2). (A) Temperature °C; (B) Pressure bar; (C) EtOH %.

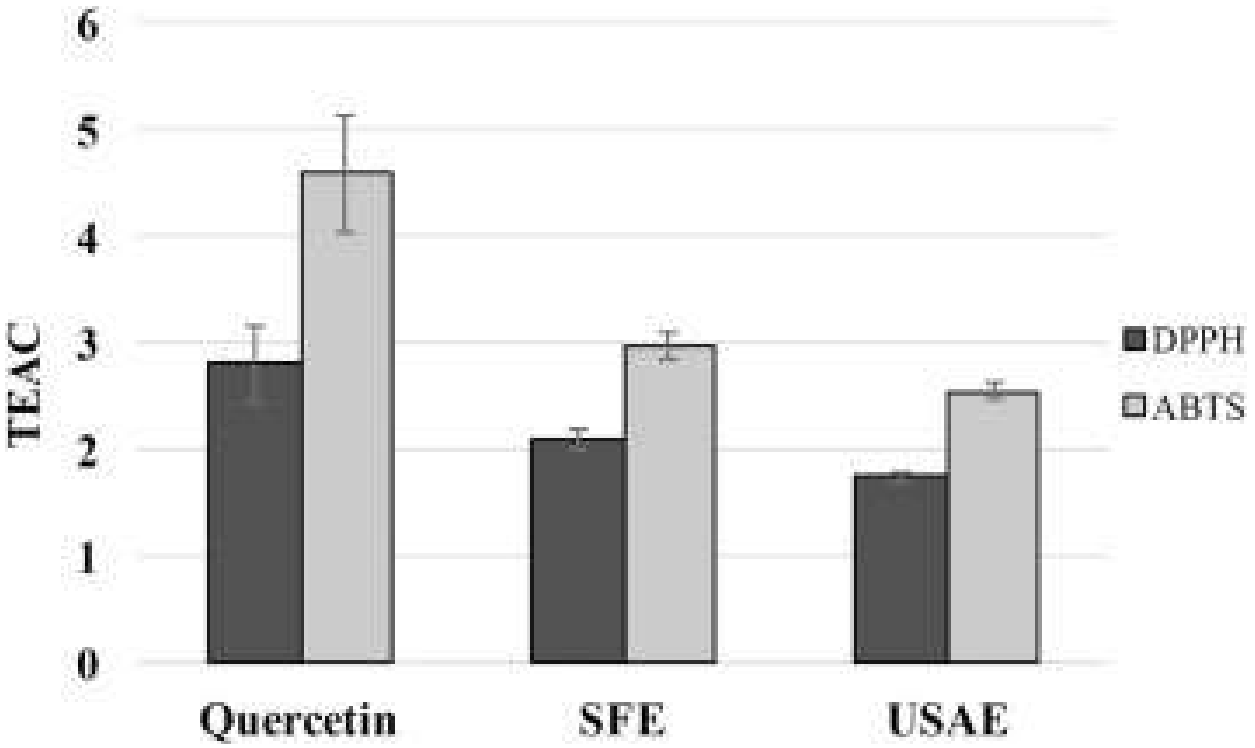

Fig. 4. Antioxidant capacity of quercetin standard, SFE and USAE extracts by DPPH and ABTS assays.

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

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	$C_7H_6O_4$	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 2. Extraction recovery and amount of phenolic compounds of SFE extract.

N°	Compounds	$R_{(t)}$	Rec %	RDS%	mg/100 g 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

N°	Compounds	$R_{(t)}$	Rec %	RDS%	mg/100 g DM ± (RDS%)
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	1,3	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.