Optimization of pressurized liquid extraction by response surface methodology of Goji berry (*Lycium barbarum L.*) phenolic bioactive compounds

Giusy Tripodo[1], Elena Ibáñez[2], Alejandro Cifuentes[2], Bienvenida Gilbert-Lopez[2*], Chiara Fanali[1]

[1]Unit of Food Science and Nutrition, Department of Medicine, Università Campus Bio-Medico di Roma, Via Alvaro del Portillo 21, 00128, Rome, Italy

[2]Foodomics Laboratory, Institute of Food Science Research, CIAL, CSIC, Campus de Cantoblanco, Calle Nicolás Cabrera 9, 28049 Madrid, Spain

* **Corresponding author:** Foodomics Laboratory, Institute of Food Science Research, CIAL, CSIC, Campus de Cantoblanco, Calle Nicolás Cabrera 9, 28049 Madrid, Spain. Tel: +34 910 017 900. E-mail: b.gilbert.lopez@csic.es; bgilbert@ujaen.es (present address)

Abbreviations:

ABTS

2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt

ASE

accelerated solvent extraction

DOE

design of experiments

MAE

microwave-assisted solvent extraction

PLE

pressurized liquid extraction

RSM

response surface methodology

SFE

supercritical fluid extraction

SLE

solid-liquid extraction

TEAC

trolox equivalent antioxidant capacity

TF

total flavonoid

TPC

total phenolic content

Keywords: Goji berry, HPLC-MS/MS, Phenolic compounds, Pressurized liquid extraction, Response surface methodology

Abstract

Pressurized liquid extraction (PLE) has been used for the first time in this work to extract phenolic compounds from Goji berries according to a multilevel factorial design using response surface methodology. The global yield (% w/dw, weight/dry-weight), total phenolic content (TPC), total flavonoid (TF) and antioxidant activity (determined via ABTS assay, expressed as TEAC value) were used as response variables to study the effects of temperature (50–180 °C) and green solvent composition (mixtures of ethanol/water). Phenolic compounds characterization was performed by high performance liquid chromatography–diode array detector–tandem mass spectrometry (HPLC-DAD-MS/MS). The optimum PLE conditions predicted by the model were as follows: 180 °C and 86% ethanol in water with a good desirability value of 0.815. The predicted conditions were confirmed experimentally and once the experimental design was validated for commercial fruit samples, the PLE extraction of phenolic compounds from three different varieties of fruit samples (*Selvatico mongolo*, *Bigol* and *Polonia*) was performed. Nine phenolic compounds were tentatively identified in these extracts, including phenolic acids and their derivatives, and flavonols. The optimized PLE conditions were compared to a conventional solid-liquid extraction, demonstrating that PLE is a useful alternative to extract phenolic compounds from Goji berry.

1 Introduction

Lycium barbarum L. is one of the most important traditional Chinese medicinal plants. The consumption of its fruits, called Goji berries, has spread also to other regions from southeastern Asia to Europe and North America. This fruit has recently generated particular interest for its potential beneficial effects on human health, such as antioxidant, anti-inflammatory and antitumor activities [1]. Such beneficial properties are related to the presence of various functional components like polysaccharides, phenolic compounds, and carotenoids [1,2]. The reported phenolic compounds in Goji berries are small amounts of flavonoids and phenolic acids [3]. Several studies have been carried out regarding the phenolic compounds present in Goji berries, reporting different profiles of compounds. Among flavonoids, the most representative are quercetin and kaempferol derivatives, among which rutin is the most frequent [2,4,5,6,7,8]. Myricetin and isorhamnetin-3-O-rutinoside [5,7], commonly named narcissin, have been also reported. Among phenolic acids, isomers of the dicaffeoylquinic acid, chlorogenic acid, caffeoylquinic acid, caffeic acid and p-coumaric acid have been detected [5,9]. Several coumaric acid derivatives, such as the hexose and di-hexose derivatives have been also reported [5,9,10]. Among other detected and quantified phenolic compounds, there are gallic acid, protocatechuic acid, catechin, syringic acid, epicatechin, ferulic acid, sinapinic acid, naringin and naringenin [9].

Moreover, monomers and dimers of phenolic amides containing N-feruloyl tyramine units, called lyciumamides, were discovered and isolated confirming their structure by NMR analysis [2,11].

Although there are reports describing the chemical composition of Goji extracts, very little attention has been paid to the optimization of the extraction method. Extraction

parameters can be optimized in order to obtain the highest yield and selectivity of the compounds of interest. Traditional methods for the extraction of bioactive compounds from plants material require the use of large volumes of organic solvents, so their main disadvantages rely on environmental constraints. Over the last years, modern techniques have been developed with the aim to overcome these problems. Among them, ultrasound assisted extraction (UAE), microwave-assisted solvent extraction (MAE), supercritical fluid extraction (SFE), and pressurized liquid extraction (PLE), are the most promising [12].

Recently, MAE was employed for qualitative and quantitative analysis of bioactive Goji berries' phytochemicals. Experimental conditions of temperature, time and solvent composition were evaluated to study the effect of MAE on the quantitative and qualitative phenolic composition of Goji extracts [9,13].

PLE extraction is another innovative extraction method, also known as accelerated solvent extraction (ASE), which employs organic solvents at high pressure and temperature above their boiling point. Generally, solid sample is packed in an extraction cell with a dispersant and extracted with a suitable solvent under elevated temperature (40–200°C) and pressure (500–3000 psi) for short periods of time (5–20 min) and the sample is collected into a vial by compressed gas. This extraction technique allows obtaining higher yields than those achieved by conventional extraction techniques, in a shorter time and with less solvent consumption. Furthermore, the use of food-grade solvents such as ethanol and water can be proposed as a green approach for the extraction of bioactives [14]. PLE is one of the techniques that have been used for the green extraction of polyphenols from many plant materials and fruits, as reviewed recently by Ameer et al. (2017) [15], however, to the best of our knowledge it has never been used before to investigate Goji berries.

PLE is affected by several factors, such as extraction temperature, extraction time and solvent composition, depending on the target compounds. For this purpose, response surface methodology (RSM) can be applied for the identification of parameters significantly influencing the extraction. RSM has been used to optimize extraction of antioxidant compounds from a variety of plant materials and fruits [16,17]. Recently, RSM has been employed for the optimization of UAE of Goji berries using water as solvent [18]. The purpose of this work was to study for the first time the use of PLE for the extraction of phenolic compounds from Goji berry fruits. To do this, optimization of PLE conditions was carried out using mixtures water-ethanol as green solvent, by a design of experiments (DOE) based on RSM. The impact of green solvent composition and temperature on total yield, total phenolic content (TPC) and total flavonoid (TF) concentration, as well antioxidant activity of the obtained extracts were evaluated. The extraction efficiency of PLE treatment in comparison with conventional solid-liquid extraction method was also studied. Finally, the extracts were analyzed by HPLC-DAD-MS/MS for the characterization of the polyphenols present in the samples.

2 Materials and methods

2.1 Chemicals and reagents

Absolute ethanol for extractions was purchased from VWR International (Leuven, Belgium). ACN with HPLC–MS quality, was purchased from Fisher (Thermo Fisher Scientific, Leicestershire, UK). Ultrapure water with a resistivity value of 18.2 MΩ was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). Folin–Ciocalteu reagent, ABTS (2,2-azinobis (3-ethylbenzothiazoline- 6-sulfonate)), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), potassium persulfate, aluminum chloride and the standard compounds: rutin, p-coumaric acid, kaempferol, 3,4-

dyhydroxybenzoic acid, quercetin, chlorogenic acid and caffeic acid were from Sigma–Aldrich (Steinheim, Germany). Sea sand was from Panreac (Barcelona, Spain).

2.2 Goji berry fruit samples and sample preparation

Commercial samples of Goji berry fruits, produced in Tibet (China), were purchased in herbalist's shop in Spain. Fresh fruits of varieties *Polonia, Bigol* and *Selvatico mongolo* were obtained from an Italian local producer (Lazio). Before extraction, all fruit samples were freeze-dried, ground in a mortar and stored at -20°C in darkness until phenolic compounds extraction.

2.3 Pressurized liquid extraction (PLE) method

PLE extraction of Goji berry fruits was performed using an accelerated solvent extractor (ASE 200, Dionex, Sunnyvale, USA), equipped with a solvent controller. Extractions were performed at different extraction temperatures and green solvent compositions (namely, ethanol/water), according to the experimental design described in the next section. Dried fruit sample (1 g) was mixed with 3 g of sea sand and placed into an 11 mL volume extraction cell. The extraction process was carried out under the following conditions: time, 20 min; pressure, 10 MPa (1500 psi), heat-up time, 5 min; static extraction time, 5 min; flush volume, 60%; purge, N_2 for 60 s; number of cycles, 1. The purged sample extract was collected into a collection vial by compressed gas. The extract was protected from light and stored at -20°C. Samples extracted with 100% ethanol were dried under N_2 stream, samples extracted with ethanol and water mixtures were first dried under N_2 and then freeze-dried in a freeze-dryer (Lyobeta, Telstar, Terrassa, Spain), while those extracted with water were directly freeze-dried.

175 **2.4 Experimental design and statistical analysis**

176 A factorial experimental design 3^2 was employed for PLE optimization, considering
177 extraction yield, total phenolic content (TPC), total flavonoid (TF) and antioxidant
178 capacity (TEAC) as response variables to study the effects of temperature (50, 115,
179 180°C) and percentage of ethanol (0, 50, 100% in water) as independent variables. A
180 total of 12 experiments were conducted in a randomized order for commercial Goji
181 berry samples: nine points of the factorial design and three additional center points to
182 consider the experimental errors. The experimental design and data analysis were
183 carried out using RSM with Statgraphics Centurion XVI® software (Statpoint
184 Technologies, Warrenton, Virginia, USA). The effects of the independent factors on the
185 response variables in the separation process were evaluated at 95% confidence level
186 ($p \leq 0.05$) for all the variables. The significance of the mathematical model was evaluated
187 using ANOVA. A Pareto diagram was used to represent the effect of factors where bar
188 color shadings indicate a positive or negative effect caused in the response variable.
189 Moreover, a response surface plot was built to predict the most favorable PLE
190 conditions to extract phenolic compounds from Goji berry. Optimum PLE extraction
191 conditions were achieved by a multiple response optimization by the combination of
192 experimental factors, aiming to maximize the desirability function for the responses in
193 the extracts. To corroborate the suitability of predicted optimal conditions by the
194 mathematical model, fruits were extracted under optimal conditions, in triplicate.
195 Afterwards, the optimum extraction conditions obtained for commercial fruit were used
196 for the extraction of Goji berry from *Polonia*, *Selvatico mongolo* and *Bigol* varieties.

197 **2.5 Conventional solid-liquid extraction method**

198 A conventional solid-liquid extraction using methanol was used as benchmark method,
199 considering that methanol is the most commonly used solvent for the conventional

solid-liquid extraction of phenolic compounds from fruits and vegetables [19,20], and that it has been used by several authors for the extraction of phenolic compounds from Goji berries [8,9,10]. Briefly, 1 g of dried fruit sample was extracted with 20 mL of methanol under agitation in ultrasonic bath for 5 minutes and then centrifuged at 3500 × *g* for 10 minutes. The extraction was repeated three times and the obtained supernatants were collected together. The solvent was evaporated under vacuum at 40°C. The residue was dissolved in 1 mL of mixture methanol/water (50:50 v/v), centrifuged at 12100 × *g* for 5 minutes and filtered through a 0.45 µm pore size syringe filter. The extraction was carried out in triplicate for each sample.

2.6 Total yield

Glass vials (40-60 mL) were weighed before collecting the extracts and after drying the extracts, to calculate the extract mass. Then the global extraction yields obtained by PLE and conventional methods were calculated as the ratio between the extract mass in dry basis (x) and the mass of initial dry sample fed into the extraction cell (y). The total yield was calculated as following according to equation 1:

extraction yield % (w/dw) = x (extract mass)/y (initial mass) x 100 (1)

2.7 Total phenolic content (TPC)

The total phenolic content was determined according to Folin–Ciocalteu assay [21] using gallic acid as standard. Briefly, 10 µL (concentration 10 mg/mL) of extract (adequately dissolved) were added to 50 µl of Folin reagent. After 1 min, 150 µL of a 20% (w/v) aqueous sodium carbonate solution was added and the volume was made up to 1 mL with water. After 2 h of incubation at room temperature in darkness, 300 µL of the mixture was transferred into a microwellplate. The absorbance of solutions was

measured at 760 nm with a Synergy HT microplate reader, by Bio-Tek Instruments (Winooski, VT, USA). TPC was calculated from a calibration curve using gallic acid as standard (0.031–1.000 mg/L). The results were expressed as mg of gallic acid equivalents (GAE) per g of dry fruit. All the analyses were performed in triplicate.

2.8 Total flavonoids (TF)

The TF content was measured by the aluminum chloride colorimetric assay [22]. Briefly, 100 µL of extract (concentration 2.5 mg/mL) were added to 140 µL of methanol and 60 µL of an 8 mM aqueous solution of $AlCl_3$. After 30 min of incubation in darkness, the absorbance was measured at 425 nm. TF was calculated from a calibration curve using quercetin as standard (from 1 to 14 mg/L). Results were expressed as mg of equivalent of quercetin per gram of dry fruit. All the analyses were performed in triplicate.

2.9 Trolox equivalents antioxidant capacity (TEAC) assay

Antioxidant capacity was measured using the Trolox Equivalents Antioxidant capacity (TEAC) methodology [23] with some modifications. $ABTS^{\bullet+}$ (2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) radical cation was produced by reacting 7 mM ABTS solution and 2.45 mM potassium persulfate solution in the dark at room temperature for 16 h. The aqueous $ABTS^{\bullet+}$ solution was diluted with 5 mM sodium phosphate buffer at pH 7.4 till an absorbance of 0.7 (±0.02) at 734 nm. The extracts were prepared at five different concentrations and 10 µL of each was mixed with 1 mL of $ABTS^{\bullet+}$ solution and 300 µL of the mixture were transferred to a 96-multiwell microplate. After 45 min of incubation in darkness the absorbance was recorded at 734 nm in a Synergy HT Microplate reader, by Bio-Tek Instruments

(Winooski, VT, USA). Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) was used as a reference standard and TEAC values were calculated from the Trolox (from 0.25 to 2 mM) standard curve. The percentage of inhibition of ABTS was calculated for Trolox standard and samples using values of absorbance obtained (absorbance ABTS, absorbance standard (Trolox) and absorbance of sample), as shown in equations 2 and 3 respectively:

$$\%Inhibition = [(A_{ABTScontrol} - A_{ABTSstandard})/A_{ABTScontrol}] * 100 \quad (2)$$

$$\%Inhibition = [(A_{ABTScontrol} - A_{ABTSsample})/A_{ABTScontrol}] * 100 \quad (3)$$

These values were obtained from five different concentrations of each sample tested in the assay giving a linear response between 20 and 80% of the blank absorbance. All the analyses were performed in triplicate. Results were expressed as mmol of equivalent of trolox per gram of dry fruit.

2.10 HPLC-DAD-ESI-IT-MS of Goji berry fruits

High pressure liquid chromatography- diode array detector- tandem mass spectrometry (HPLC-DAD-MS/MS) analysis of the extracts was carried out on an Agilent 1200 series liquid chromatograph (Agilent Technologies, Santa Clara, CA) consisting in a binary pump, an autosampler and a diode-array detector (DAD), directly coupled to an ion trap mass spectrometer (Agilent ion trap 6320) with an electrospray ionization (ESI) interface. HPLC-DAD-MS/MS method was based on previously studies conducted for phenolic compounds analysis in Goji berry [9,10] with minor modifications. HPLC separation was carried out using a C18 reversed-phase column (150 × 4.6 mm i.d., 3 μm particle size, from ACE Ltd, Aberdeen, Scotland) with a Security Guard Cartridge of the same material. Elution was conducted at 30 °C with water/0.1% formic acid (solvent A) and acetonitrile/0.1% formic acid (solvent B) at a constant flow rate of 0.6 mL min.

The gradient program was as follows: 0 min, 0%B; 10 min, 5%B; 15 min, 10%B; 45 min, 60%B; 65 min, 70%B; 70 min, 100%B; 72 min 0%B. The injection volume was 10 μL. Data were acquired using photodiode array detector in the range 200-700 nm and the chromatograms were extracted at 280 and 370 nm. The instrument was controlled by LC ChemStation 3D Software Rev. B.04.03 (Agilent Technologies, Santa Clara, CA, USA).

Chromatograms were acquired in the MS instrument using negative ionization mode with the following parameters: capillary voltage, −3.5 kV; drying temperature, 350 °C; drying gas (N2) flow rate, 9 L min−1; nebulizer gas pressure, 40 psi. Full scan was acquired in the m/z range 50–2200. Automatic MS/MS analyses were also performed, fragmenting the two highest precursor ions (10,000 counts threshold; 1 V Fragmentor amplitude).

3 Results and discussion

3.1 PLE of Goji berry phenolic compounds

PLE conditions were optimized by RSM to test its efficiency towards the extraction of phenolic compounds from Goji berry fruits. An experimental design is proposed in order to study the significance of the main parameters involved in the extraction, i.e., extraction temperature (50–180°C) and green solvent composition (water/ethanol mixtures). Extraction time and pressure were kept constant at 20 min and 1500 psi, respectively, taking into account results obtained in previous published works [24,25]. A factorial experimental design at three levels was set-up as follows: temperature (50, 115, 180 °C) and ethanol percentage in water (0%, 50%, 100%). Employing this design, the influence of extraction conditions on four different response variables was studied.

These response variables were the global extraction yield expressed as g/100 g dry matter, together with the TPC, TF and TEAC values.

Table 1 presents the experimental matrix and results obtained at each extraction condition for the four response variables. As can be seen in Table 1, the response variables showed a different behavior depending on the extraction conditions. Thus, the highest global yields of the PLE extracts were achieved with 50% ethanol in water as extraction solvent, with yields of 41.74 %, 53.46% and 74.84% at 50, 115 and 180 °C, respectively. Moreover, the experimental data showed that an increase in the temperature from 50 to 180 °C led to a substantial increase in the global extraction yield for all the different extraction solvents.

As can be seen in Table 1 the lowest total phenolic content (TPC of 5.72 mg GAE/g) was found in the extract obtained with pure water at 50 °C, and the highest TPC value (74.84 mg GAE/g) was obtained for 100% ethanolic extract at 180 °C. For all employed solvents, TPC increased with temperature. Summarizing, the solvent that extracted more phenolic compounds are, in decreasing order: pure ethanol, ethanol + water (50% v/v), pure water. In a similar manner as TPC, TF values showed a minimum of 0.16 mg QE/g extract when pure water at 50 °C was used and a maximum value of 3.20 mg QE/g extract with 100% ethanol at 180 °C. In addition, TF increased with temperature for all tested solvents. Respect to the antioxidant activity, the highest TEAC value of 1.21 was found in the extract obtained with 50% ethanol solvent at 180 °C, which are the same conditions that gave the highest yield. Also antioxidant activity increased with the temperature except for 100% ethanol as extraction solvent. Thus, based on TPC and TF results, it can be concluded that the higher yields obtained with 50% ethanol as extraction solvent are due to the extraction of other non-phenolic compounds, which have antioxidant activity.

The extraction yields, TPC, TF and TEAC values obtained for different temperatures and solvent compositions were statistically analyzed. Regression analysis was performed on the experimental data and the coefficients of the model were evaluated for significance. Figure 1 shows the standardized Pareto charts for the four response variables studied and their corresponding response surface plot. Different bar color shadings indicate the positive (grey) and negative (black) effects whereas the vertical line tests the significance of the effects at the 95% confidence level (see Figure 1).

Extraction yield was positively influenced by temperature. This behavior can be explained by (i) improvement of mass transfer from the sample to the extraction solvent, (ii) increasing of the solubility of compounds and (iii) reduction in solvent viscosity giving more penetration of the solvent in the matrix [26]. The composition of the solvent also showed a significant effect (see Figure 1), obtaining the highest yield with the mixture of water:ethanol (50:50, v:v). According to the mathematical model, 180 °C and 52.6 % ethanol were the optimum conditions to maximize the extraction yield.

TPC and TF values showed the same behavior. These responses were positively influenced by temperature giving pure ethanol the highest values (see Figure 1). In this case, according to the mathematical model 180 °C and 100% ethanol were the optimum conditions to maximize TPC and TF values. The TEAC values were also positively influenced by temperature, while the increasing amount of EtOH in the solvent showed a significant negative effect on TEAC values as shown in Figure 1. According to the mathematical model, the highest TEAC values can be achieved by using 180 °C and 77% ethanol as optimum conditions.

Since all response variables had equal importance, a multiple response optimization was carried out including extraction yield, TPC, TF and TEAC (see Figure 2). The optimum

PLE conditions predicted by the model were as follows: 180°C and 86% ethanol with a good desirability value of 0.815.

To corroborate the usefulness of our model, three replicate extractions for commercial Goji berry were carried out under the optimum PLE conditions predicted by the model. Table 2 shows the predicted and the experimental values. As can be seen, the values experimentally obtained for yield, TPC, TF and TEAC (77.64, 65.98, 3.02 and 0.80, respectively) were in good agreement with those theoretically predicted (69.95, 62.22, 3.20 and 0.78, respectively), besides, all the RSD values were lower than 8%. Once the experimental design was validated for the commercial Goji berries samples, these parameters were applied for the extraction of phenolic compounds from the other three varieties: *Selvatico mongolo*, *Bigol* and *Polonia*. The results are given in Table 3.

Among the total yield, the results obtained under the PLE optimum conditions for the four fruits samples analyzed in the present study indicate potential differences between commercial (origin: China) and fresh berry varieties (origin: Italy). All samples were freeze-dried before extraction to minimize the effect of humidity. The three fresh fruit samples (*Selvatico mongolo*, *Bigol* and *Polonia*) gave a lower yield values respect to commercial fruit sample (see Tables 2 and 3). Various factors contribute to the phenolic profile of plants: genotype, site location, climatic conditions and year. It is probable that fresh berry fruits, growing in a different habitat with different treatment, have a different phenolic content. In addition, among TPC, TF and TEAC, results obtained were in good agreement with those theoretically predicted (low RSD values). However, interestingly, an appreciable relationship between total phenols, total flavonoids and antioxidant activity was observed.

3.2 Comparison of PLE vs. SLE

374 Solid-liquid extraction (SLE) is commonly used for extraction of phenolic compounds
375 using solvents such as methanol, ethanol and ethyl acetate [25]. This traditional
376 extraction method consists of a direct extraction with an appropriate solvent using an
377 extractor, homogenizer or ultrasonic bath for a given time. Often, traditional methods
378 have some disadvantages like long extraction times and the massive use of solvents. In
379 the present work, PLE is proposed as an alternative method to reduce common
380 disadvantages like extraction time and solvents usage. In this work, a conventional
381 solid-liquid extraction using methanol was used as benchmark method, since methanol
382 is the most widely used solvent for the extraction of phenolic compounds [19,20], and
383 has been previously used for the extraction of phenolic compounds from Goji berries
384 [8,9,10]. Table 4 shows the results obtained for phenolic compounds extraction from
385 commercial Goji berry samples using PLE and SLE. The results presented in Table 4
386 show that PLE is quite more effective than the conventional SLE method for phenolic
387 and flavonoid extraction. The PLE extract also presented higher antioxidant activity
388 than SLE while PLE gave a slightly lower extraction yield than SLE. These results
389 confirm that the combined application of high pressure and temperature in PLE is
390 effective in the recovery of phenolic compounds from Goji berries when compared to a
391 traditional method. By considering economic and practical aspects, PLE can be
392 considered clearly advantageous respect to SLE for Goji berry phenolic compounds
393 extraction. Furthermore, the use of environmentally friendly solvents as ethanol and
394 water can be also proposed as a green approach compared to the use of methanol in
395 conventional SLE method.

396 **3.3 Determination of major phenolic compounds by HPLC-DAD-MS/MS**

397 HPLC-DAD-MS/MS was used to analyze the PLE Goji berry extracts obtained at the
398 aforementioned optimum PLE conditions in order to separate and identify the phenolic

compounds in the extracts. Considering previously studies [9,10], the optimization of analytical method was performed. Taking into account the chemical structure of phenolic compounds, the separation was optimized using a C18 stationary phase. Due to the wide range of polarity of phenolic compounds, a gradient elution was developed. Different mobile phases were tested to optimize the analytical method and acetonitrile (instead of methanol) was found to improve the chromatographic peak resolution of phenolic compounds. In addition, the use of formic acid as modifier was tested in different amounts to improve the peak shape. The best separation was achieved by using water/0.1% formic acid as solvent A and acetonitrile/0.1% formic acid as solvent B. Data on the phenolic compounds found in the different samples are summarized in Table 5. Figure 3 shows representative chromatograms obtained for the four studied Goji berry samples at 280 and 370 nm. As it can be seen, the qualitative profile varied depending on the sample studied. In order to improve compounds' identification, the MS/MS spectrum was recorded in negative ionization mode because of the best performance of phenolic compounds in negative ionization mode compared to positive ionization. Compounds identification was based on DAD and fragmentation mass spectra (MS/MS), comparison of retention time of commercial standards when available and data reported in the literature.

A total of nine phenolic compounds were tentatively identified in the four extracts including hydroxycinnamic acids and their derivatives and flavonols, namely:

Hydroxycinnamic acids derivatives. Peak 1 (Rt: 3.7 min, λ_{max}: 290 nm) was identified as caffeoylhexose on the basis of the fragmentation of the molecular ion ($[M-H]^-$) at m/z 341 showing a loss of 162 amu, $[M-H-hex]^-$ at m/z 179 [28]. Peak 2 (Rt: 8.0 min, λ_{max}: 250 nm) was identified as p-coumaroylquinic acid having a molecular ion ($[M-H]^-$) at m/z 337 and MS/MS fragmentation at m/z 179 as major fragment [29]. Peaks 9 (Rt:

21.0 min, λ_{max}: 280 nm) and 10 were identified as two isomers of the dicaffeoylquinic acid having a molecular ion ($[M-H]^-$) at m/z 515 and 514 respectively. Mass spectrum of these compounds showed fragmentation ions at m/z 353, m/z 191, m/z, m/z 179 and m/z 173 that is the characteristic mass spectrum of this chlorogenic acid [29].

Flavonols. Three flavonols were tentatively identified, quercetin-*O*-rutinoside (rutin), quercetin-3-*O*-glucoside and isorhamnetin 3-*O*-rutinoside. The identity of Peak 18 (Rt: 28.8 min, λ_{max}: 280,360 nm) as rutin was corroborated comparing UV-visible spectrum, retention time and MS/MS data after injecting the standard [28]. Quercetin-3-*O*-glucoside was assigned at peak 20 (Rt: 29.8 min, λ_{max}: 300 nm) [30]. The MS spectrum showed a molecular ion ($[M-H]^-$) at m/z 462.2 and MS^2 fragmentation ion at m/z 301 as quercetin ion. Peak 22 (Rt: 30.3 min, λ_{max}: 240, 290 and 340 nm) was identified as isorhamnetin 3-*O*-rutinoside. This identification was confirmed by the mass spectral data with molecular ion ($[M-H]^-$) at m/z 623 and MS^2 fragment at m/z 315 [4].

Pyroglutamic acid hexose was assigned at peak 3 (Rt: 9.4 min, λ_{max}: 280 nm) and exhibited a deprotonated ion ($[M-H]^-$) at m/z 290. In the MS-MS spectra was observed an ion at m/z 128, after elimination of hexose moiety at m/z 128. Identification of this compound is consistent with a previous study [5].

According to our results, *Lycium barbarum* fruits are mainly rich in flavonoids and phenolic acids derivatives, in good agreement with previously published works [2,5,7] . In this regard, it has already been shown that the phenolic profile of Goji berries from Mongolia, China and Tibet depends on the cultivation area [5]. These berries showed differences in terms of quercetin and isorhamnetin derivatives, rutin, narcissin and kaempferol derivatives, among flavonoids. Furthermore, there was also a significant difference in terms of phenolic acids derivatives. These results are in good agreement

with our study, in which the chemical composition in flavonoids and phenolic acid derivatives is clearly influenced by the different geographic origin, climate, soil and cultivations method. Besides, the four samples studied in the present study showed a different qualitative profile, which it is certainly due to the different cultivars and origins. Among flavonoids, rutin is the most frequent compound [2,4,8,9]. We detected rutin only in the commercial Goji berry sample. Also the other two flavonoids, quercetin 3-o-glu and isorhamnetin 3-o-rut, were detected only in the commercial Goji berry sample and not in the *Polonia, Selvatico mongolo* and *Bigol* varieties. Among phenolic acid derivatives, some compounds as caffeoylhexose, p-coumaroylquinic acid and dycaffeoylquinic acid were identified in all varieties of Goji berry fruits. Caffeic acid derivative was identified in all samples except in variety *Selvatico mongolo*. Dycaffeoylquinic acid was identified in varieties *Polonia* and *Bigol*.

4 Concluding remarks

A green extraction method based on pressurized liquid extraction has been developed for the first time to obtain phenolic bioactive compounds from Goji berries. Optimum extraction conditions were obtained using RSM. An experimental design was applied to optimize the extraction conditions in order to maximize the selected response variables (extraction yield, total phenols, total flavonoid and antioxidant activity of the extracts). The optimal PLE conditions were achieved at 180°C and 86% ethanol in water as solvent, showing better figures of merit than the conventional SLE method using methanol. At optimal conditions, experimental values obtained coincided with the predicted theoretical values by RSM. The chemical characterization of those extracts, carried out by HPLC-DAD-MS/MS, allowed the tentative identification of nine phenolic compounds in four different Goji berry varieties. Major fragments of the non-

elucidated compounds included in the present work may help future comparisons about phenolic composition of Goji berry fruits. The qualitative differences found among the Italian varieties and the commercial sample native from China indicate that an in-depth characterization of Goji berry phenolic compounds from different varieties may be of potential interest for future works addressing biomarkers for geographical and authentication studies.

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The authors declare that they have no conflict of interests.

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Figures

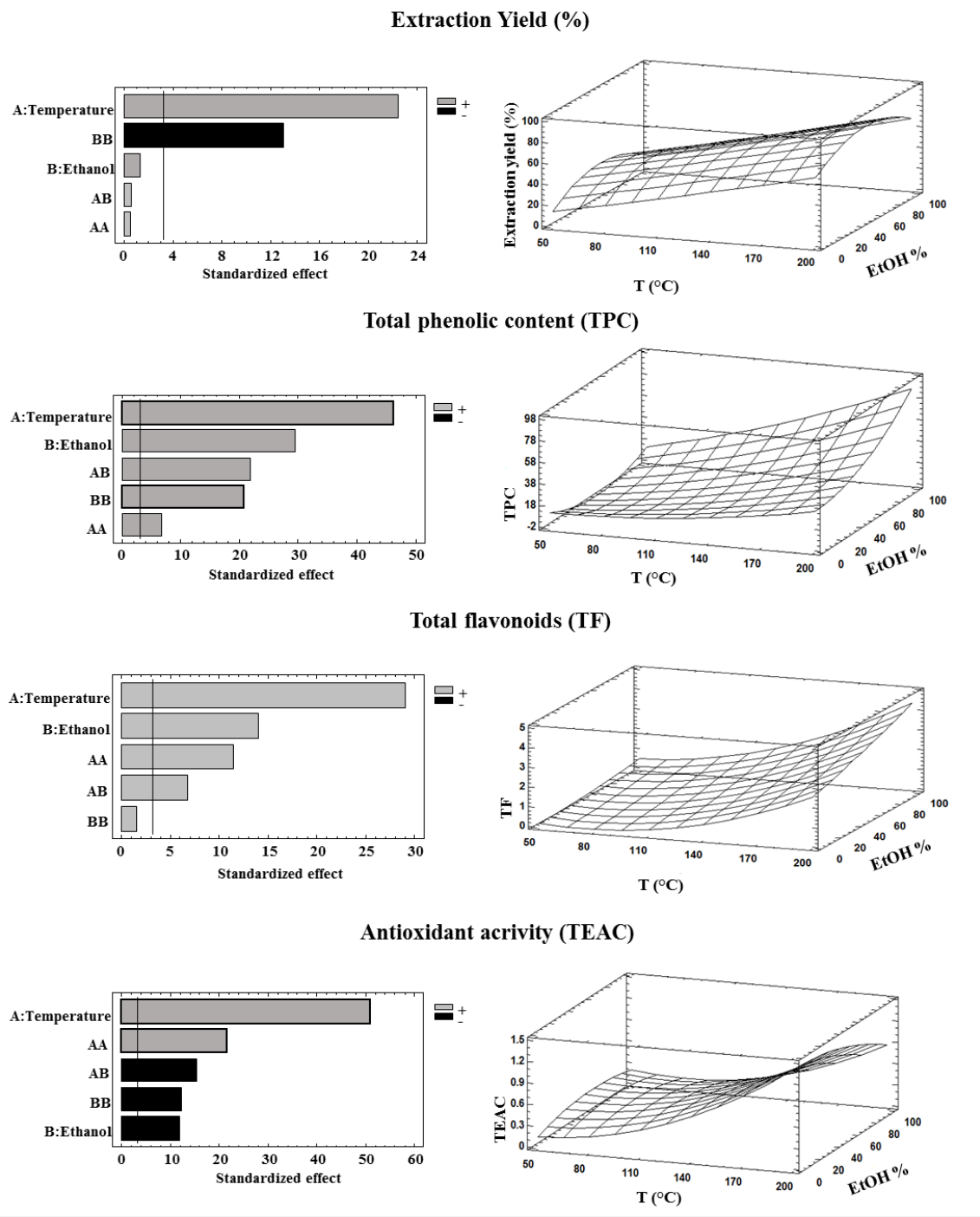


Figure 1. Standardized Pareto charts for: A, extraction yield, B, TPC, C, TF and D, TEAC according to the experimental factors temperature and ethanol percentage (grey and blue bars show negative and positive effects, respectively); and their corresponding response surface plot obtained from the analyses of variance.

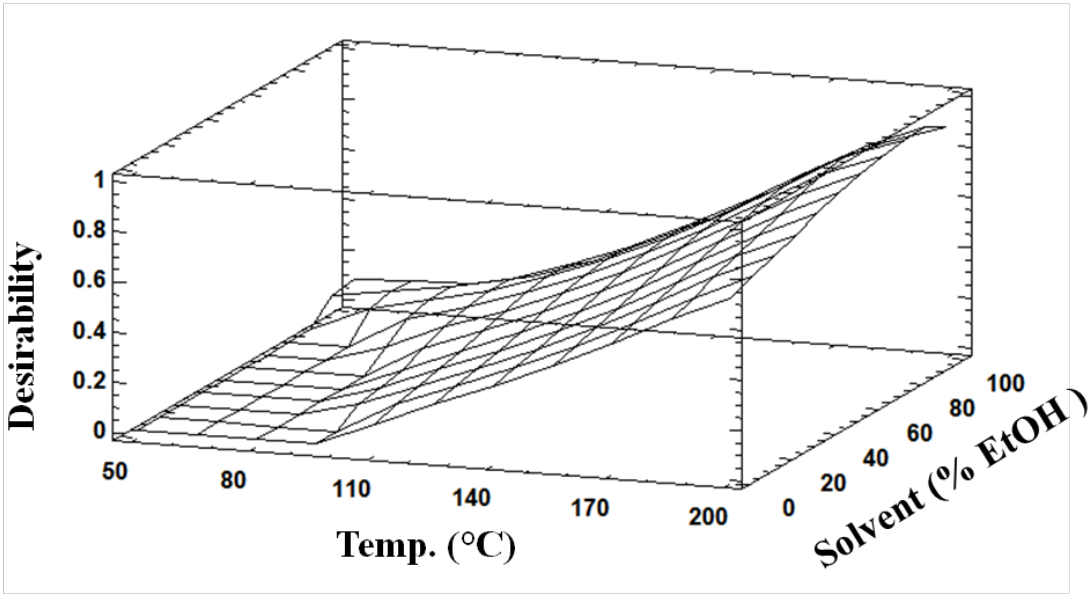
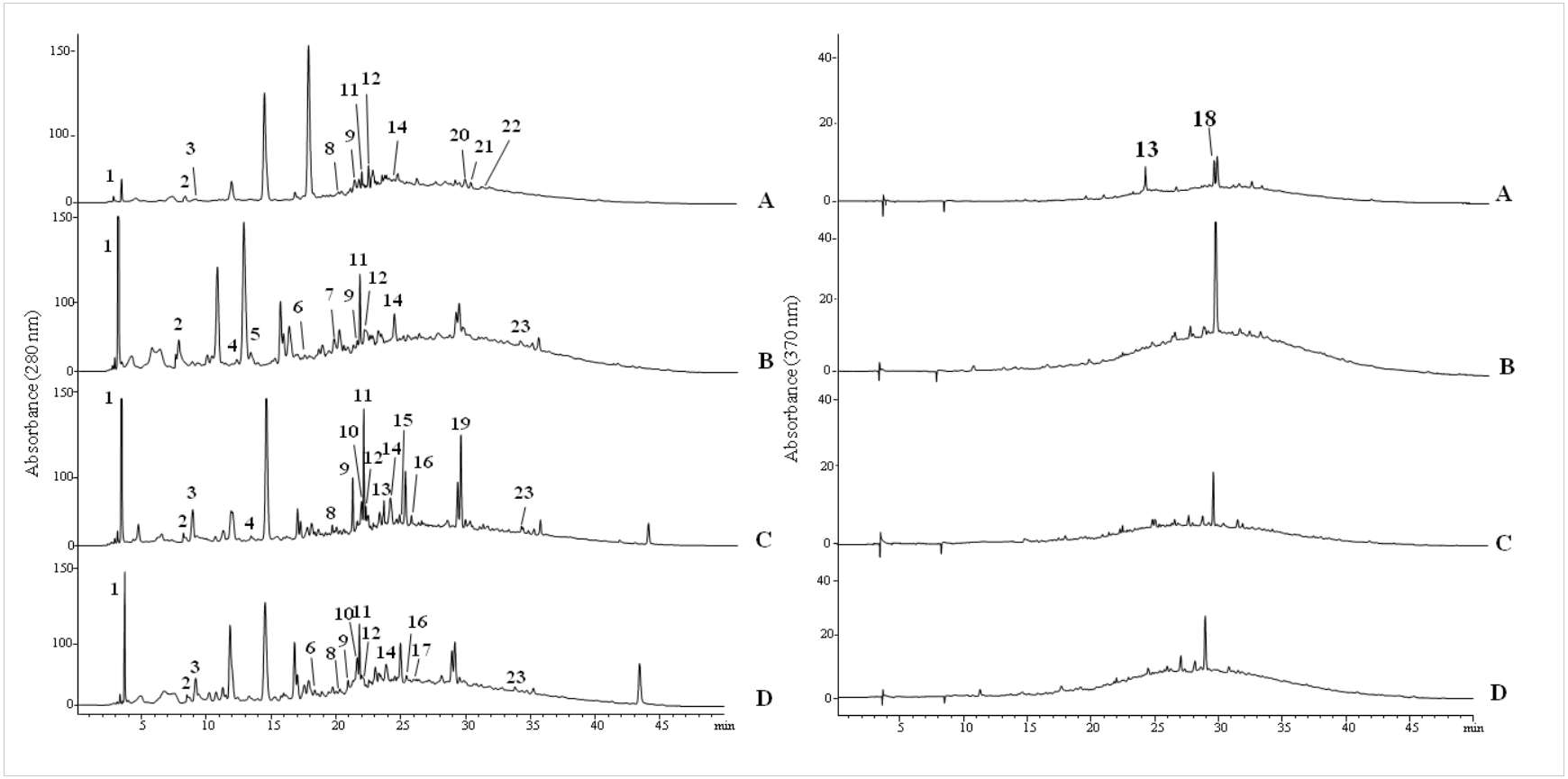


Figure 2. Response surface plot for the effects of solvent (percentage of ethanol in water) and temperature on the overall desirability in PLE extractions.



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566 **Figure 3.** HPLC-DAD chromatograms (λ=280 and 370 nm) obtained under the optimal PLE conditions (180°C and 86% ethanol in water)

567 corresponding to: A) Commercial fruit, B) *Selvatico mongolo*, C) *Bigol* and D) *Polonia* Goji berry extracts.

Table 1. Results observed in the studied response variables (i.e., yield, TPC, TF and TEAC) for commercial Goji berries extraction using PLE and a three-level two-factor experimental design (3^2).

Run	Factors		Response Variables			
	T (°C)	**Solvent (%)[1]**	**Yield (%)**	**TPC (mg GAE/g)**	**TF mg (Eq quercetin/g)**	**TEAC (mmol Eq Trolox/g)**
1	50	0	12,74	5,72	0,16	0,22
2	115	0	28,81	8,31	0,25	0,30
3	180	0	62,70	28,80	1,68	1,05
4	50	50	41,74	8,35	0,37	0,27
5	115	50	56,11	10,15	0,58	0,36
6	115	50	51,26	11,74	0,51	0,40
7	115	50	53,46	12,54	0,73	0,37
8	115	50	56,58	12,14	0,55	0,38
9	180	50	74,84	35,60	2,86	1,21
10	50	100	6,43	6,97	0,38	0,15
11	115	100	46,74	44,83	1,82	0,49
12	180	100	59,22	75,84	3,20	0,47
Individual Optimum	180	52,6	**78,84**			
	180	100		**75,30**	**3,48**	
	180	77,2				**1,05**

[1] Refers to % ethanol in water.

Table 2. Predicted and observed values of each individual response variable (i.e., yield, TPC, TF and TEAC) for commercial Goji berries PLE extraction.

Sample	Response	Predicted	Observed	SD	RSD
Commercial	Yield %	69,95	77,64	5,44	7,37
	TPC, mg GAE/g	62,22	65,98	2,66	4,15
	TF, mg QE/g	3,20	3,02	0,13	4,16
	TEAC, mmol TE/g	0,78	0,80	0,02	2,25

Table 3. Observed values of each individual response variable (i.e., yield, TPC, TF and TEAC) after PLE extraction of different Goji berries varieties (*Polonia*, *Selvatico mongolo* and *Bigol)*.

Sample	Response	Observed	SD	RSD
Polonia	**Yield %**	32,85	0,85	2,58
	TPC mg GAE/g	66,02	3,07	4,64
	TF mg QE/g	2,73	0,25	9,33
	TEAC mmol TE/g	0,83	0,02	2,60
Selvatico mongolo	**Yield %**	26,92	3,31	12,29
	TPC mg GAE/g	75,15	3,03	4,04
	TF mg QE/g	3,15	0,20	6,34
	TEAC mmol TE/g	1,06	0,07	6,48
Bigol	**Yield %**	26,57	1,75	6,60
	TPC mg GAE/g	59,18	1,13	1,91
	TF mg QE/g	1,66	0,07	4,48
	TEAC mmol TE/g	0,86	0,05	5,33

Table 4. Comparison of the four response variables (yield, TPC, TF and TEAC) obtained after using PLE or conventional solid-liquid extraction (SLE) of Goji berries.

Extraction method	Time	Solvent	Yield %	TPC mg GAE/g	TF mg QE/g	TEAC mmol TE/g
SLE	45	Methanol	81,63	40,82	0,61	0,69
PLE	20	Ethanol/Water	77,64	65,98	3,02	0,80

Table 5. Retention time (R_t), wavelengths of maximum absorbance in the UV-Vis region, molecular ions ($[M-H]^-$), major fragment ions, and tentative identification of phenolic compounds in four different varieties of Goji berries.

Peak number	R_t (min)	$[M-H]^-$	Main fragments	UV-Vis (nm)	Tentative identification	Goji berry variety			
						Commercial (A)	*Selvatico mongolo* (B)	*Bigol* (C)	*Polonia* (D)
1	3.7	341	191,179	290	caffeoylhexose	x	x	x	x
2	8.0	337	191,179,173	250	p-coumaroylquinic acid	x	x	x	x
3	9.4	290	200,128	280	pyroglutamic acid hexose	x		x	x
4	12.2	439	393,351,321,115	280	n.i.		x	x	
5	13.4	283	211,151	280	n.i.		x		
6	18.6	453	291	280	n.i.		x		x
7	19.6	629	347,275	280	n.i.		x		
8	20.3	283	179,151	280	caffeic acid derivate	x		x	x
9	21.0	515	353,191,173	280	dycaffeoylquinic acid	x	x	x	x
10	21.4	514	353,179	280	dycaffeoylquinic acid			x	x
11	21.9	796	634,472,308	280	n.i.	x	x	x	x
12	22.8	634	472,308	280,320	n.i.	x	x	x	x
13	23.6	632	470,334,217	320,370	n.i.	x		x	

14	24.0	355	309,211,151	280	n.i.	x	x	x	x
15	24.7	497	335,305,292	280	n.i.			x	
16	25.6	543	381,179,135	280	n.i.			x	x
17	27.0	625	300,271,179	280	n.i.				x
18	28.8	609	301	280,360	rutin*	x			
19	29	717	681,519,357	260,300	n.i.			x	
20	29.8	462.2	418, 301, 151	300	quercetin 3-o-glu	x			
21	30.0	517	471,399,345,247,157	280	n.i.	x			
22	30.3	623	315, 299, 271	240,290,340	isorhamnetin 3-o-rut	x			
23	33.6	916			n.i.		x	x	x

4 * identification corroborated by co-injection of the standard compound

5 n.i.: not identified

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