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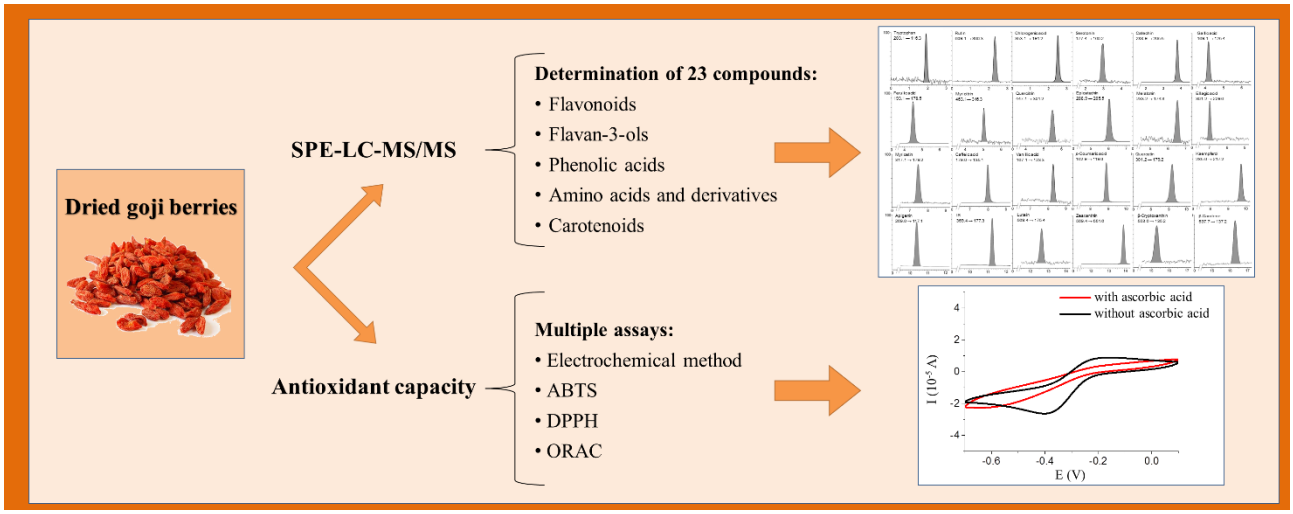

**ABSTRACT**

Goji berries and derived products represent a relevant source of micronutrients, most of which are natural antioxidants and contribute to the high nutritional quality of these fruits. Three brands of dried goji berries have been analysed by a multidisciplinary approach to get an insight into both their content of selected antioxidants and their antioxidant capacity (AC).

The former goal has been achieved by developing a liquid chromatographic method coupled to mass spectrometry and combined to a fast solid phase extraction. Several significant representative antioxidant compounds belonging to the following classes: flavonoids, flavan-3-ols, phenolic acids, amino acids and derivatives, and carotenoids have been taken into account. Quercetin and rutin were found to be the predominant flavonoids; chlorogenic acid was the most abundant phenolic acid and zeaxanthin was the major carotenoid.

The AC of the goji berries has been evaluated by four analytical methods in order to estimate the contributions of different reactions involved in radicals scavenging. In particular, AC has been determined using 3 standardised methods (DPPH, ABTS, ORAC) and a recently proposed electrochemical method, which measures the scavenging activity of antioxidants towards OH radicals generated both by hydrogen peroxide photolysis and the Fenton reaction.

The results obtained from chemical composition and antioxidant capacity assays confirm the high nutritional and commercial value of goji berries and highlight that the three brands do not exhibit significant differences.

**KEYWORDS:** Goji berries; phenolic compounds; SPE-LC-MS/MS; analytical profiling, antioxidant capacity; Fenton reaction; OH radical electrochemical measurement.

## 1. INTRODUCTION

Many governmental and international Agencies have recently begun to implement new concepts in the effort to provide a longer, healthier life expectancy to the world's citizens. In particular, the focus is being slowly re-directed from "curing diseases" to "preventing people from getting ill" and "maintaining people's health and well-being", thus shifting everyone's attention toward healthier lifestyles, healthy foods and beneficial supplements. In this reference frame, "nutraceutical" is one of the key words, coined as a portmanteau from "nutrition" and "pharmaceutical" in 1989 by Stephen DeFelice. According to him, "a nutraceutical is any substance that is a food or a part of food and provides medical or health benefits, including the prevention and treatment of disease" [1], thus indicating foods, beverages and supplements containing useful amounts of bioactive compounds with health-promoting effects. Among nutraceutical compounds, antioxidants are currently one of the most studied classes. In fact, oxidative stress has been linked to aging and to several pathologies, which are frequent causes of death or invalidity: from inflammation and cancerogenesis to neurodegenerative and cardiovascular diseases [2]. According to this hypothesis, compounds able to lower body oxidative stress levels, or to avoid their excessive increase, could also delay or prevent numerous negative aging effects [3]. This has led to the study of the nutraceutical, antioxidant potential of some promising traditional foodstuffs, like the goji or wolfberry tree (*Lycium barbarum* L., *Lycium* spp.), which is being cultivated in several Chinese regions for hundreds of years. These fruits are commercialised and consumed fresh as such in the cultivation zones; however, in the rest of the world, they are mostly available as dried fruits and/or transformed into alimentary products or dietary supplements having different formulations (dried

berries, berry pieces, juices, herbal teas, yogurt products, granola). In recent times, dried goji berries and derived products have known an increasing diffusion and consumption in Western countries, due to their supposed beneficial effects on human health, particularly related to their antioxidant properties. However, none of these claims is currently supported by clinical studies [4].

In this framework, an analytical multidisciplinary approach is herein proposed to carefully evaluate goji antioxidant properties.

Goji berries contain many different classes and types of antioxidants such as polyphenols, carotenoids and polysaccharides [5]. Although some already published methods analyse bioactive compounds in *Lycium spp.* berries, most of them just focus on a single or a few chemical classes [6-11]; other methods have been applied to different parts of the plant, such as flowers [12], leaves [13,14], seed oil [15] or root bark [16]. Moreover, evaluations were made on geographically localised subspecies or on variants not marketed for human consumption [17,18].

The present study describes the development of an analytical method to estimate the content of a wide range of compound classes for a complete quali-quantitative investigation on Goji nutraceutical profile, integrated with the evaluation of the total antioxidant power of such berries.

A liquid chromatographic method coupled to mass spectrometry (LC-MS/MS) and combined to a fast and feasible solid phase extraction (SPE) has been developed and validated to simultaneously determine several significant, representative antioxidant compounds belonging to the following classes: flavonoids (quercetin, myricetin, apigenin, kaempferol, rutin, quercitrin, myricitrin), flavan-3-ols (catechin, epicatechin), phenolic acids (caffeic, ferulic, coumaric, vanillic, ellagic, gallic and chlorogenic acid), amino acids and derivatives (tryptophan, serotonin, melatonin) and carotenoids (zeaxanthin, β-cryptoxanthin, β-carotene, lutein).

As for the antioxidant capacity (AC), an analytical multidisciplinary approach is herein proposed, since the antioxidant constituents of Goji berries are so chemically different that more than one assay must be employed in order to estimate the contributions of the different reactions involved in radicals scavenging [4].

For this reason, AC has been evaluated using four assays, DPPH, ABTS, ORAC and a recently proposed electrochemical method, exploiting an innovative electrochemical sensor able to detect OH radicals by means of induced degradation on an insulating polyphenol film deposited on a glassy carbon electrode [19].

Finally, a relationship between the antioxidant capacity resulting from the four assays and the levels of the selected classes of the antioxidants has been attempted.

## 2. EXPERIMENTAL

### 2.1. Chemicals

All chemicals and reagents were of analytical grade and used as received. L-Ascorbic acid, hexaaminoruthenium(III) chloride, hydrogen peroxide solution (30%, w/w), 2,2'-azino-bis-(3-ethylbenzothiazolin-6-sulfonic acid) (ABTS), 2,2'-azobis-2-methyl propanimidamide dihydrochloride (AAPH), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), potassium mono- and di-hydrogen phosphate, acetonitrile (HPLC-grade), methanol (HPLC-grade), formic acid 99% (V/V), quercetin, myricetin, apigenin, kaempferol, rutin, quercitrin, myricitrin, catechin, epicatechin, caffeic acid, ferulic acid, *p*-coumaric acid, chlorogenic acid, vanillic acid, ellagic acid, gallic acid, tryptophan, serotonin, melatonin, zeaxanthin, β-cryptoxanthin, β-carotene and lutein, as well as curcumin (used as the internal standard, IS), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium acetate, phenol, sodium persulfate, sodium and potassium hydroxides, acetic acid and sulfuric acid were bought from Carlo Erba (Milan, Italy), sodium fluoresceinate and ethanol from Fluka Italia (Milan, Italy). All solutions for antioxidant power assays were prepared using doubly distilled water (DD); ultrapure water (18.2 M cm) for LC-MS/MS assays was obtained by means of a Millipore (Milford, MA, USA) MilliQ system.

### 2.2. LC-MS/MS method

Separations were obtained on a Waters XTerra MS-C18 column (100 x 2.1 mm I.D., 3.5 µm), equipped with a guard column. The mobile phase was a mixture of 0.25% formic acid in water (A) and 0.25% formic acid in acetonitrile (B), flowing at a constant rate of 400 L/min. The gradient program of the mobile phase started with A:B (92:8) for the first 3 min, then ramped up to A:B

(60:40) over 2 min; this ratio was maintained for 5 min, then ramped up to A:B (15:85) over 2 min; this ratio was maintained for 5 min then ramped down to A:B (92:8) over 5 min and maintained for 2 min. Total time for a chromatographic run was 24 minutes, including column reconditioning. The two components of the mobile phase were filtered through Sartorius (Göttingen, Germany) membrane filters (47 mm diameter, polyamide, 0.2 µm pore size) and degassed in an ultrasonic bath for 10 min. The injection volume was 10 µL.

Mass spectrometry acquisition was carried out in multiple reaction monitoring (MRM) scan mode, using an electrospray ionisation source in both positive ($ESI^+$) and negative ($ESI^-$) ionisation mode. The parameters used for the assays were: ion source voltage, 3 kV; ion source temperature, 130°C; desolvation temperature, 350°C; desolvation gas flow, 600 L/h; cone voltage: 15 V; capillary voltage: -1.8 kV; extractor potential, 3 V; collision exit potential, 1 V; scan duration: 0.3 s. Nitrogen was used as the desolvation gas, while argon was used as the collision gas. A complete list of parent ions, quantitative and qualitative MS/MS fragments and collision energies for each analyte and IS are reported in Table 1.

**Table 1.** Mass spectrometry parameters

| Analyte | MW | Ion mode | Precursor ion *m/z* | Product ion [a] *m/z* | Collision energy V |
|---|---|---|---|---|---|
| Quercetin | 302.2 | - | 301.2 | 179.2 (*151.1*) | 15 |
| Myricetin | 318.2 | - | 317.1 | 179.2 (*151.1*) | 22 |
| Apigenin | 270.2 | - | 269.0 | 117.1 (*151.1*) | 20 |
| Kaempferol | 286.2 | - | 285.0 | 217.2 (*199.2*) | 25 |
| Rutin | 610.5 | - | 609.1 | 300.3 (*271.3*) | 35 |
| Quercitrin | 448.4 | - | 447.1 | 301.2 (*179.4*) | 20 |
| Myricitrin | 464.4 | - | 463.1 | 316.3 (*179.4*) | 25 |
| Catechin | 290.3 | - | 288.9 | 205.5 (*109.4*) | 10 |
| Epicatechin | 290.3 | - | 288.9 | 205.5 (*109.4*) | 20 |
| Caffeic acid | 180.2 | - | 179.0 | 135.1 (*89.3*) | 15 |
| Ferulic acid | 194.2 | - | 193.1 | 178.5 (*134.1*) | 12 |
| *p*-Coumaric acid | 164.2 | - | 162.9 | 119.0 (*93.4*) | 15 |
| Vanillic acid | 168.1 | - | 167.1 | 123.3 (*152.6*) | 15 |
| Ellagic acid | 302.2 | - | 301.2 | 229.6 (*185.1*) | 10 |
| Gallic acid | 170.1 | - | 169.1 | 125.4 (*79.0*) | 10 |
| Chlorogenic acid | 354.3 | - | 353.1 | 191.2 (*179.4*) | 17 |
| Tryptophan | 204.2 | - | 203.1 | 116.3 (*301.4*) | 12 |
| Serotonin | 176.2 | + | 177.4 | 160.2 (*130.3*) | 10 |
| Melatonin | 232.3 | + | 233.2 | 174.3 (*141.2*) | 25 |
| Zeaxanthin | 568.9 | + | 569.4 | 551.6 (*135.2*) | 20 |
| β-Cryptoxanthin | 552.9 | + | 553.6 | 135.2 (*119.0*) | 28 |
| β-Carotene | 536.9 | + | 537.7 | 137.2 (*177.1*) | 25 |
| Lutein | 568.9 | + | 569.4 | 175.4 (*459.1*) | 25 |
| IS | 368.4 | + | 369.4 | 177.3 (*285.0*) | 29 |

[a] in brackets, confirmatory product ion

### *2.2.1. Sample extraction and clean-up*

In order to be analysed by LC-MS/MS, goji berries were treated as follows: ten whole berries were dried in an oven at 40°C to constant weight and finally finely ground in a ball mill. Two grams of the resulting paste were accurately weighed and extracted 3 times with 5 mL of a methanol/water (80/20, V/V) mixture under vortex mixer agitation for 30 min, at room temperature and protected from light sources. The extracts were merged, filtered, reduced to a final volume of 5 mL and subjected to solid-phase extraction (SPE).

The SPE procedure was carried out in a controlled-vacuum Agilent (Santa Clara, CA, USA) VacElut 12 manifold by means of Waters (Milford, MA, USA) Oasis HLB cartridges (50 mg, 1 mL). Cartridges were conditioned by passing 3 mL of methanol through the cartridge and then conditioned with 3 mL of ultrapure water. An aliquot of 1 mL of water and 50 µL of IS working solution were added to 500 µL of goji berry extract and the resulting mixture was loaded onto a previously conditioned SPE cartridge. The cartridge was washed 3 times with 1 mL of water/methanol (80/10, V/V) mixture and dried (full vacuum for 30 s). The analytes were then eluted with 2 mL of methanol and the cartridge dried again (full vacuum for 1 min). The eluate was dried under a nitrogen stream, redissolved in 0.25% aqueous formic acid/acetonitrile mixture (50/50, V/V) and processed by LC-MS/MS.

### *2.2.2. Validation*

#### *2.2.2.1. Linearity*

Linearity assays were carried out on analyte standard solutions at six different concentrations, containing IS at a constant concentration. Each concentration was prepared and injected in triplicate.

The analyte/IS peak area ratios (dimensionless numbers) were plotted against the corresponding analyte concentrations (expressed as ng/mL) and the calibration curves set up by means of the least-squares method. Limit of quantitation (LOQ) and limit of detection (LOD) were calculated according to international guidelines [20] as the analyte concentrations giving rise to peaks whose heights were respectively 10 and 3 times the baseline noise.

*2.2.2.2. Extraction yield*

Extraction yields were evaluated by a combination of multiple extractions of goji berries for the first pretreatment step and of standard solution extraction by SPE for the second one. The solutions analysed were 6 replicates of 3 different concentrations, corresponding to the lower limit, a middle point and the upper limit of each calibration curve. The analyte peak areas were compared to those obtained from standard solutions prepared at the same concentrations.

*2.2.2.3. Precision*

The same berry extraction and SPE assays described above to test the extraction yield, were repeated 6 times within the same day to calculate intraday precision and 6 times over six different days to obtain interday precision, in both cases expressed as percentage relative standard deviation (RSD%).

*2.2.2.4. Matrix Effect*

Matrix effect, expressed as percentage relative error (RE%) has been examined during the development of the analytical method by comparing the MS/MS response (peak areas) of analytes

in spiked sample extracts with the MS/MS response of standard solutions at 3 concentration levels. To investigate the matrix effect influence on the determination of the analytes as assessed by post-extraction method, a matrix-spiking step with suitable analyte concentrations was employed. First, methanol/water extracts of goji berry samples were prepared, then the extracts were passed through the SPE cartridge, resulting in almost complete analyte adsorption; the remaining isolated matrix depleted from the compounds was then spiked with known analyte concentrations.

*2.2.2.5. Carryover*

Carryover was determined by injecting a solvent blank immediately after an extracted sample spiked with the analytes at a concentration equivalent to the upper limit of the linearity range. The acceptance criterion was: no analyte peak higher than the LOD level.

*2.2.2.6. Accuracy*

Method accuracy was evaluated by adding standard solutions of the analytes and IS to goji berry samples whose content had been already determined. Results were expressed as percentage recovery, repeating the assays 3 times during the same day to obtain also SD data.

**2.3. Equipment**

Electrochemical experiments were carried out in a single-compartment three-electrode cell using a CH Instruments (Austin, TX, USA) Mod. 660C electrochemical analyser/workstation (potentiostat/galvanostat), controlled by a personal computer via CH Instruments software. All potentials were measured with respect to an aqueous saturated calomel electrode (SCE) and a Pt

wire was used as the counter electrode. A 3-mm glassy carbon disk was used as the working electrode (GCE).

An Agilent 8453 UV-Visible diode array instrument (Palo Alto, CA, USA) and an Edinburgh Instruments (Livingstone, UK) FLSP90 fluorimeter were used to perform the spectrophotometric and fluorimetric measurements, respectively.

The LC-MS/MS assay of the analytes was carried out on a Waters (Milford, MA, USA) Micromass Quattro Micro triple-quadrupole mass spectrometer coupled to a Waters Alliance e2695 chromatographic pump with autosampler. Data processing was performed using Waters MassLynx 4.1 software, while statistical analysis was carried out using Data Analysis ToolPak for Microsoft Excel: one-way analysis of variance (ANOVA) tests were used to determine the significance of the difference among samples, with a significance level of $p < 0.05$.

A Crison (Barcelona, Spain) Basic 20 pHmeter, a Hettich (Tuttlingen, Germany) Universal 32R centrifuge, a Velp (Usnate, Italy) RX3 vortex mixer, a Memmert (Schwabach, Germany) ventilated oven, a Sigma-Aldrich Mini-Vap Evaporator and an Elma (Singen, Germany) Sonic T310 Trans ultrasonic bath were also used.

**2.4. Antioxidant capacity measurements**

AC has been evaluated using four assays, namely DPPH, ABTS, ORAC and a recently proposed electrochemical method. DPPH [21] and ABTS [22] are based on the measurement of the scavenging capacity of antioxidants towards stable free radicals, namely α, α-diphenyl-β-picrylhydrazyl (DPPH) and 2,2'-azino-bis-(3 ethylbenzo-thiazolin-6-sulfonic acid) radical cation ($ABTS^{\cdot+}$), respectively. Both assays evaluate the ability of antioxidants in the deactivation of radical species through electron

transfer reactions. ORAC method [23] is based on the competitive reaction between antioxidant compounds and fluorescein for the scavenging of peroxyl radicals, generated *in situ* by the decomposition of azo-compounds. Such assay estimates the ability of the antioxidant compounds in removing peroxyl radicals through hydrogen transfer reactions. The electrochemical test evaluates the ability of antioxidant compounds to protect oxidisable substrates from the action of OH radicals, currently considered the most harmful species among ROS (reactive oxygen species) [24]. The method exploits an innovative electrochemical sensor to detect OH radicals by means of induced degradation on an insulating polyphenol film deposited on a glassy carbon electrode [25].

### *2.4.1. ABTS method*

ABTS was dissolved in 0.05 M acetate buffer (pH 4.6) at a $5\times10^{-4}$ M concentration and the radical cation was produced by the reaction with $2.5\times10^{-4}$ M potassium persulfate. The mixture was kept in the dark at room temperature for 18 h to ensure the reaction completely occurs. ABTS radical solution was diluted with acetate buffer in a 3/10 (V/V) ratio and 2 mL of this solution were placed into a spectrophotometric cuvette. 100 µL of the sample solution were added and absorbance values were recorded at 732 nm at the end point of 20 min. The data were elaborated using the percentage inhibition according to:

$\%\Delta A = (1 - A_{sample}/A_{blank})*100$

The results of this assay as well as those of all the others are expressed as µmoles of vitamin C equivalents $g^{-1}$ (VCE $g^{-1}$) by comparison with the calibration line obtained for the chosen standard.

### *2.4.2. DPPH method*

The assay measures the scavenging capacity of antioxidants towards the stable free 2,2-diphenyl-1-picrylhydrazyl radical. 80 µL of the sample to be tested were added to 3.0 mL of a $6.5\times10^{-5}$ M DPPH solution in ethanol while continuously recording the absorbance decrease at 516 nm. Also in this case, the spectrophotometric data were elaborated using the percentage inhibition i.e., plotting the remaining % DPPH (after 30 min).

### *2.4.3. ORAC method*

ORAC method is a typical hydrogen transfer-based assay with a competitive reaction between antioxidant compounds and fluorescein which compete for the scavenging of peroxyl radicals, generated *in situ* by thermal decomposition of azo compounds (e.g., AAPH).

Samples were suitably diluted with phosphate buffer (0.075 M, pH 7.4). 200 µL of either the diluted sample and 1500 µL of $9.6\times10^{-8}$M fluorescein solution (phosphate buffer) were put in a cuvette thermostatted at 37 °C. 300 µL of AAPH (0.133 M, in phosphate buffer) were added in order to activate radical production. The emission intensity was recorded in the 495-700 nm range for 45 min ($\lambda_{exc}$=485 nm). The calibration lines were constructed by plotting the net area under the curve (after 45 min) *vs.* the concentration of the standard antioxidant.

### *2.4.4. OH radical scavenger capacity method (OH-RSC)*

The glassy carbon electrode was cleaned first with sandpaper and then with aqueous alumina (0.05 mm) slurry on a wet polishing cloth to obtain a mirror surface. Then a cyclic voltammetry (CV) of bare glassy carbon electrode was recorded into a 0.5 M, pH 4.6 acetate buffer, containing $5\times10^{-3}$ M

$Ru(NH_3)_6^{3+}$. The polyphenol film was deposited on the GCE by performing a potentiostatic electropolymerisation (E = + 1.0 V for 60 s) of phenol (0.05 M) in 1 M sulfuric acid.

Then, the modified electrode was put into the acetate buffer solution and submitted to five cycles of potential between 0 and +0.8 V in order to stabilise the polyphenol film.

A CV in the $Ru(NH_3)_6^{3+}$ solution was again recorded (blank signal) to make sure the deposition of a completely-insulating film had been deposited as suggested by the absence of redox signals. The antioxidant capacity (AC) of goji berries was evaluated by studying the degradation kinetics of the polyphenol film in two different configurations.

In the first experimental setup, hydrogen peroxide photolysis was used as source of OH radicals (PhOH-RSC). To this end, a 0.01 M $H_2O_2$ solution, which also contained a proper amount of sample, was poured into the photolysis reactor in which the modified electrode was placed, near a lamp operating at 254 nm (UV18F, Italquarz, with emission power of 17 W) using a homemade holder that ensured the reproducibility of the sensor position during irradiation. The light intensity of the lamp was daily measured to verify its stability. After a defined irradiation time, the electrode was taken out from the reactor, rinsed with DD water and put in acetate buffer where the CV signal related to the $Ru(NH_3)_6^{3+}$ redox system was again recorded. The intensity of redox signal is inversely proportional to the antioxidant power that was estimated as percentage of inhibition.

In the second configuration, OH radicals were generated by the Fenton reaction (FeOH-RSC). 20 mL of a 0.05 M $FeSO_4$ solution containing a proper amount of antioxidant were poured in an electrochemical cell containing the modified electrode. 800 µL of 10 M $H_2O_2$ solution were added to get the reaction started. After 10 min, the electrode was taken out from the solution, rinsed with DD water, and put in acetate buffer where the CV signal related to the $Ru(NH_3)_6^{3+}$ redox system was

recorded. The intensity of redox signal is inversely proportional to the antioxidant power that was estimated as percentage of inhibition. Figure 1 shows the redox signals recorded by CV for $Ru(NH_3)_6^{3+}$ at the bare GC, at the modified electrode (blank signal) and after a certain extent of the polyphenol film degradation due to the OH· attack, both in presence and in absence of an antioxidant (L-ascorbic acid).

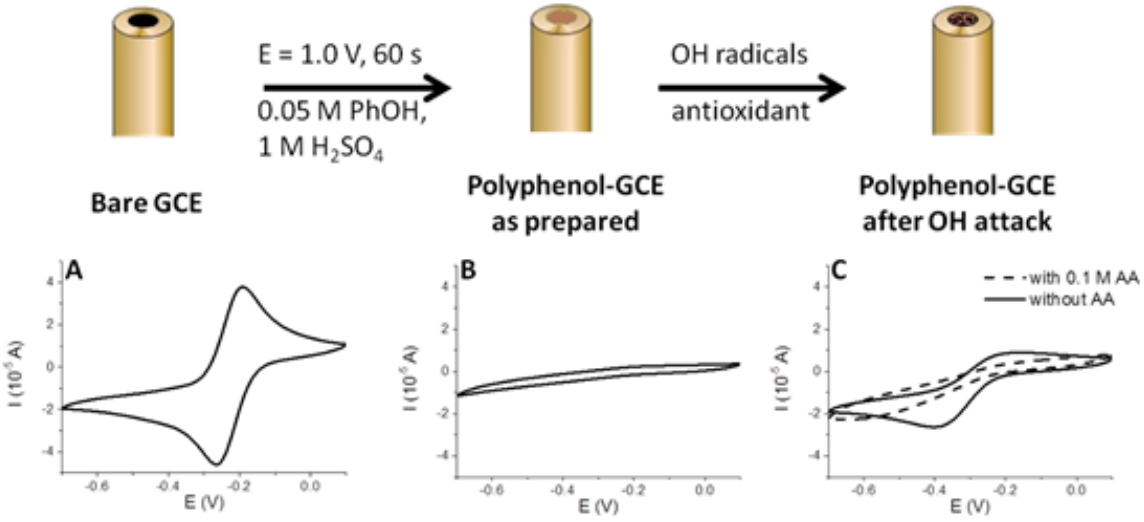


**Figure 1.** CVs recorded at the bare and modified GCE for $5\times10^{-3}$ M $Ru(NH_3)_6^{3+}$ in acetate buffer.

## 3. RESULTS AND DISCUSSION

### 3.1 LC-MS/MS method development

For this study, several stationary phases for reverse phase chromatography were evaluated for their ability to adequately separate the considered antioxidant compounds within relatively short run times. Initially, C8, C18 and pentafluorophenyl (PFP) sorbents were considered: in this initial screening, medium bore (4.6-3.0 mm) and resolution (5-µm diameter) columns were tested. The C8 column yielded inadequate resolution, the PFP column resulted in poor peak shapes, while C18 sorbent type had good peak shape and resolution between all compounds, but still with relatively long run times (> 40 min). Due to the relatively large number of analytes and the wish to reduce analysis times as much as possible, narrow bore (2.1 mm) and high-resolution (3.5 µm particles) C18 columns were tested. Within this refining screening process, the best overall results were obtained using a XTerra MS C18 column, a silica-polymeric hybrid particle sorbent coupled to low residual silanols designed to be highly compatible with mass spectrometry applications. Together with this sorbent, two different volatile acidic additives at different concentrations were tested: formic acid (0.1%, 0.25%, 0.5%, V/V) and acetic acid (0.1%, 0.25%, 0.5%, V/V), both with acetonitrile and methanol as the organic modifiers. The use of 0.25% formic acid and acetonitrile, flowing under an optimised composition gradient, produced sharp and symmetric peaks for all the compounds. Although complete resolution for all peak couples was not obtained, all 23 analytes are eluted in less than 24 min. MS/MS detection allows the reliable and selective determination of all analytes even when they are not completely separated; it should be noted that the only structural isomers, not discriminated by MS/MS (catechin and epicatechin), are well-resolved by the chromatographic method.

MS and MS/MS spectra of the analytes and IS were first acquired in full-scan mode (50-600 *m/z*) by infusion of 1 µg/mL solutions, using a 0.25% aqueous formic/acetonitrile (50/50, V/V) mixture as the solvent. All spectra were acquired using both $ESI^+$ and $ESI^-$ ionisation modes. The MS/MS conditions were first automatically optimised using the corresponding instrument software function; then, *m/z* ratios for quantitative and confirmatory purpose, as well as cone voltage, were adjusted manually for each analyte in order to obtain the best possible sensitivity and selectivity. A complete table of the MS/MS transitions used and the respective variable MS/MS conditions for the analytes and the IS are reported in Table 1. To further increase sensitivity and repeatability, due to the high number of analytes, each MRM transition was monitored only within a 3-min time window centered on the respective retention time of each analyte.

**3.2 Extraction procedure from goji berries**

Due to the high selectivity of MS/MS detection, initial efforts were aimed at keeping extraction procedures from goji berries as simple as possible. Several extraction solvents were tested (methanol, acetonitrile, diethyl ether, organic solvent/water mixtures at different ratios). Finally, a methanol/water (80/20, V/V) mixture proved to grant the best compromise between sufficiently high extraction yields for all analytes and reasonable matrix purification [26,27,28]. However, a strong matrix effect was detected (>20%). To further eliminate undesired matrix components, the samples were subjected to SPE after extraction. Several kinds of sorbents were tested such as C1, C2, C8, C18, phenyl (PH), hydrophilic/lipophilic balance (HLB), with best results always provided by HLB cartridges. Keeping the SPE procedure as simple as possible (1 single washing with water, 1 single elution with methanol), the matrix effect was efficiently eliminated (<5%).

### 3.3. LC-MS/MS validation results

The extraction and quantitation method thus developed was validated in terms of linearity, LOQ, LOD, extraction yield, repeatability and intermediate precision, matrix effect, carryover and accuracy.

The respective validation assays were carried out as described in the Experimental section and the resulting values are reported in Table 2 for linearity, LOQ, LOD and related parameters and in Table 3 for extraction yield and precision.

As can be seen, all parameters fall within the usual acceptability ranges. In particular, method sensitivity is very satisfactory (LOQ ≤ 3 ng/mL, LOD ≤ 1 ng/mL) and coupled to fairly wide linearity ranges (up to 2 µg/mL); precision is remarkable, with RSD% values within the 3-10% range even at LOQ levels; extraction yields are always higher than 80% and accuracy is higher than 92%. Matrix effect and carryover are both minimal, and do not significantly contribute to the method imprecision or inaccuracy.

**Table 2.** LC-MS/MS linearity data

| Analyte | Linearity range ng/mL | $r^2$ | LOQ ng/mL | LOD ng/mL |
|---|---|---|---|---|
| Quercetin | 1.0-1000 | 0.9997 | 1.0 | 0.3 |
| Myricetin | 2.0-2000 | 0.9994 | 2.0 | 0.6 |
| Apigenin | 2.5-2000 | 0.9994 | 1.0 | 0.7 |
| Kaempferol | 0.5-1000 | 0.9995 | 0.5 | 0.2 |
| Rutin | 2.0-2000 | 0.9991 | 2.0 | 0.6 |
| Quercitrin | 3.0-1500 | 0.9993 | 3.0 | 1.0 |
| Myricitrin | 3.0-2000 | 0.9989 | 3.0 | 1.0 |
| Catechin | 0.5-1000 | 0.9999 | 0.5 | 0.2 |
| Epicatechin | 0.5-1000 | 0.9999 | 0.5 | 0.2 |
| Caffeic acid | 2.5-2000 | 0.9998 | 2.5 | 0.7 |
| Ferulic acid | 1.0-1000 | 0.9998 | 1.0 | 0.3 |
| *p*-Coumaric acid | 1.0-1000 | 0.9993 | 1.0 | 0.3 |
| Vanillic acid | 1.0-1000 | 0.9996 | 1.0 | 0.3 |
| Ellagic acid | 1.0-1000 | 0.9996 | 1.0 | 0.3 |
| Gallic acid | 1.5-1500 | 0.9997 | 1.5 | 0.5 |
| Chlorogenic acid | 1.0-1000 | 0.9995 | 1.0 | 0.3 |
| Tryptophan | 0.2-1000 | 0.9992 | 0.2 | 0.06 |
| Serotonin | 0.1-1000 | 0.9993 | 0.2 | 0.06 |
| Melatonin | 0.01-500 | 0.9991 | 0.1 | 0.03 |
| Zeaxanthin | 0.5-1000 | 0.9996 | 0.5 | 0.2 |
| β-Cryptoxanthin | 1.0-1000 | 0.9992 | 1.0 | 0.3 |
| β-Carotene | 0.5-1000 | 0.9994 | 0.5 | 0.2 |
| Lutein | 2.0-1500 | 0.9989 | 2.0 | 0.6 |

**Table 3.** LC-MS/MS extraction yield and precision data

| Analyte | Concentration ng/mL | Extraction yield % | Intraday precision RSD% [a] | Interday precision RSD% [a] |
|---|---|---|---|---|
| Quercetin | 1.0 | 90 | 5.5 | 5.7 |
| | 100 | 92 | 4.0 | 4.4 |
| | 500 | 98 | 3.5 | 3.9 |
| Myricetin | 2.0 | 93 | 7.9 | 9.0 |
| | 500 | 97 | 7.0 | 8.0 |
| | 1000 | 91 | 6.5 | 6.9 |
| Apigenin | 2.5 | 86 | 7.7 | 7.9 |
| | 500 | 92 | 6.9 | 7.3 |
| | 1000 | 87 | 6.8 | 6.9 |
| Kaempferol | 0.5 | 84 | 6.6 | 7.2 |
| | 100 | 85 | 6.0 | 6.1 |
| | 500 | 91 | 5.9 | 5.9 |
| Rutin | 2.0 | 88 | 6.5 | 7.4 |
| | 500 | 90 | 6.0 | 6.1 |
| | 1000 | 96 | 5.4 | 5.9 |
| Quercitrin | 3.0 | 83 | 7.8 | 8.9 |
| | 500 | 85 | 6.0 | 6.8 |
| | 1000 | 85 | 5.9 | 6.3 |
| Myricitrin | 5.0 | 82 | 8.8 | 9.5 |
| | 500 | 88 | 7.4 | 8.3 |
| | 1000 | 81 | 7.1 | 8.2 |
| Catechin | 0.5 | 95 | 5.8 | 6.5 |
| | 100 | 95 | 4.6 | 4.1 |
| | 500 | 94 | 3.0 | 3.4 |
| Epicatechin | 0.5 | 90 | 5.8 | 6.4 |
| | 100 | 96 | 4.3 | 5.0 |
| | 500 | 95 | 3.0 | 3.9 |
| Caffeic acid | 2.5 | 91 | 6.2 | 6.7 |
| | 500 | 93 | 5.6 | 6.1 |
| | 1000 | 91 | 5.3 | 6.0 |
| Ferulic acid | 1.0 | 92 | 7.6 | 8.2 |
| | 100 | 92 | 7.4 | 8.5 |
| | 500 | 96 | 6.8 | 8.8 |
| *p*-Coumaric acid | 1.0 | 89 | 5.4 | 6.7 |
| | 100 | 95 | 4.3 | 5.1 |
| | 500 | 95 | 3.9 | 4.6 |
| Vanillic acid | 1.0 | 89 | 6.6 | 7.6 |
| | 100 | 90 | 5.1 | 5.8 |
| | 500 | 93 | 4.8 | 4.9 |

**Table 3.** *(continued)*

| | | | | |
|---|---|---|---|---|
| Ellagic acid | 1.0 | 91 | 8.1 | 8.3 |
| | 100 | 94 | 7.6 | 7.9 |
| | 500 | 93 | 7.4 | 7.6 |
| Gallic acid | 1.5 | 91 | 5.2 | 5.9 |
| | 500 | 93 | 4.2 | 5.1 |
| | 1000 | 99 | 4.0 | 4.7 |
| Chlorogenic acid | 1.0 | 92 | 5.5 | 5.8 |
| | 100 | 95 | 4.7 | 5.7 |
| | 500 | 90 | 4.7 | 5.0 |
| Tryptophan | 0.2 | 83 | 8.8 | 9.4 |
| | 100 | 87 | 7.3 | 8.0 |
| | 500 | 88 | 7.0 | 7.1 |
| Serotonin | 0.1 | 84 | 8.7 | 9.4 |
| | 100 | 86 | 7.9 | 8.9 |
| | 500 | 86 | 7.2 | 8.0 |
| Melatonin | 0.01 | 83 | 8.6 | 9.7 |
| | 1.0 | 83 | 7.7 | 8.7 |
| | 100 | 80 | 7.1 | 7.9 |
| Zeaxanthin | 0.5 | 81 | 8.5 | 9.1 |
| | 100 | 83 | 7.0 | 7.7 |
| | 500 | 83 | 6.7 | 6.8 |
| β-Cryptoxanthin | 1.0 | 83 | 9.2 | 9.6 |
| | 100 | 86 | 8.2 | 8.6 |
| | 500 | 84 | 7.0 | 7.7 |
| β-Carotene | 0.5 | 85 | 8.3 | 8.7 |
| | 100 | 84 | 7.3 | 8.1 |
| | 500 | 85 | 6.5 | 7.1 |
| Lutein | 2.0 | 82 | 8.9 | 9.3 |
| | 500 | 86 | 7.4 | 7.9 |
| | 1000 | 85 | 7.1 | 7.7 |

[a] n=6

### 3.4 LC-MS/MS quantitative data

After method validation, three kinds of dried goji berries commercially available in Italy were analysed: “Viva il goji berry” by Noberasco (Brand 1), “Goji premium tibetano” by Gaia Superfood (Brand 2) and “Bacche di goji” by Vitamore (Brand 3). One example of a goji sample MRM chromatogram is shown in Figure 2: as one can see, all compounds were simultaneously detected and unambiguously identified by their retention time and quali-quantitative *m/z* transitions, when compared with standard reference compounds.

The assays were repeated 3 times for each sample and the results of the analyses are reported in Table 4, expressed as average concentrations ± SD. The investigated antioxidant compounds can be categorised into the following classes: flavonoids, flavan-3-ols, phenolic acids, amino acids and derivatives, and carotenoids.

As one can see, the concentration ratios of the analytes were similar for the three supplements. In particular, phenolic acids and flavonoids were always more concentrated than carotenoids, while all 3 samples contained significant levels of zeaxanthin when compared to other carotenoids. Among flavonoids, the most abundant compound was always rutin, while chlorogenic acid was the most prevalent among organic acids; between catechin and epicatechin, the latter was the most abundant isomer in all analysed samples, whereas the phytohormone melatonin was always found in much lower concentrations when compared to its precursors tryptophan and serotonin. The observable variability, both in individual compounds levels and in total content of each class, does not appear significantly in favor of any of the three considered brands ($p > 0.05$); in fact, such differences range from ~ 20% to 10% as to the individual compounds and the classes, respectively.

Consequently, none of these three supplements can claim better “antioxidant quality” than the others.

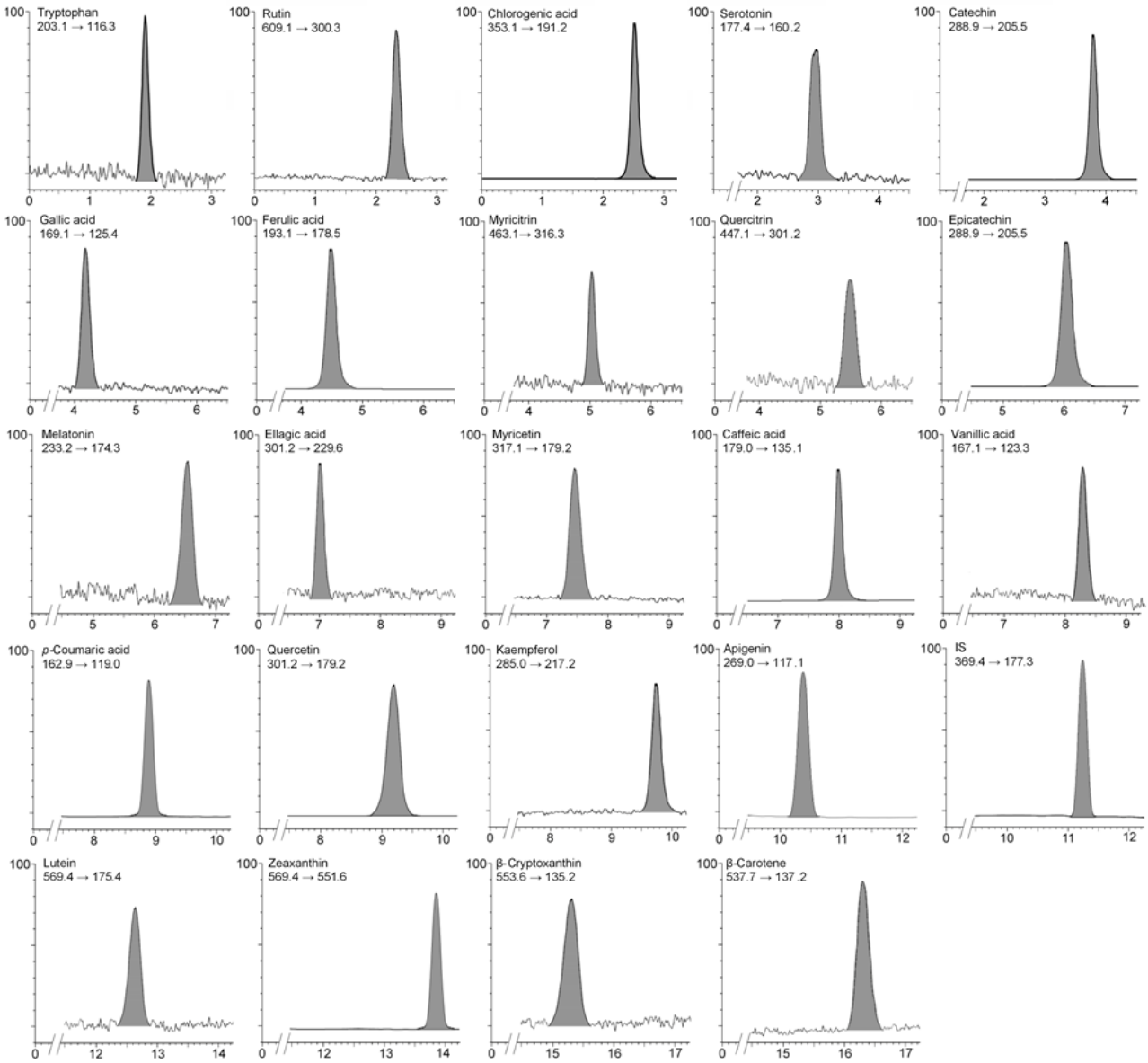


**Figure 2.** Extracted MRM chromatograms from a dried goji berry sample (Brand 1).

**Table 4.** LC-MS/MS quantitative data from commercial dried goji berry samples

| Class | Analyte | Concentration (µg/g, dry weight) ± SD [a] | | |
|---|---|---|---|---|
| | | Brand 1 (Noberasco) | Brand 2 (Goji Premium Tibetano) | Brand 3 (Vitamore) |
| Flavonoids | Quercetin | 941.3 ± 3.3 | 1023.3 ± 5.0 | 977.1 ± 4.8 |
| | Myricetin | 104.1 ± 3.2 | 129.8 ± 2.9 | 98.4 ± 3.3 |
| | Apigenin | 492.4 ± 5.2 | 521.2 ± 6.0 | 506 ± 5.7 |
| | Kaempferol | 76.3 ± 1.1 | 83.3 ± 1.8 | 91.4 ±3.0 |
| | Rutin | 1128.3 ± 9.5 | 1283.5 ± 8.3 | 1099.4 ± 4.8 |
| | Quercitrin | 11.5 ±0.8 | 12.7 ± 1.0 | 10.8 ± 1.8 |
| | Myricitrin | 13.9 ± 1.6 | 10.1 ± 0.9 | 12.4 ± 1.2 |
| Flavan-3-ols | Catechin | 1341.7 ± 3.9 | 1294.4 ± 5.2 | 1129 ± 5.8 |
| | Epicatechin | 2180.4 ± 7.4 | 2003.5 ± 5.9 | 1984 ± 6.3 |
| Phenolic acids | Caffeic acid | 899.2 ± 3.2 | 931.2 ± 3.9 | 926 ± 3.6 |
| | Ferulic acid | 931.2 ± 7.1 | 978.4 ± 6.9 | 927 ± 7.7 |
| | *p*-Coumaric acid | 985.6 ± 4.3 | 943.9 ± 3.7 | 994.7 ± 5.6 |
| | Vanillic acid | 18.6 ± 1.7 | 15.8 ± 0.6 | 17.1 ± 3.1 |
| | Ellagic acid | 17.0 ± 1.2 | 14.5 ±1.4 | 17.5 ± 2.1 |
| | Gallic acid | 130.0 ± 2.1 | 129.6 ± 5.1 | 116 ± 2.3 |
| | Chlorogenic acid | 10740.1 ± 8.3 | 11039.0 ± 8.1 | 11931 ± 9.4 |
| Amino acids and derivatives | Tryptophan | 198.8 ± 2.5 | 233.6 ± 1.3 | 229.2 ± 1.5 |
| | Serotonin | 121.9 ± 2.0 | 139.4 ± 3.0 | 114.6 ± 2.0 |
| | Melatonin | 65.0 [b] ± 1.0 | 55.0 [b] ± 2.0 | 63.2 [b] ± 1.0 |
| Carotenoids | Zeaxanthin | 750.7 ± 5.6 | 832.6 ± 8.1 | 793.6 ± 6.4 |
| | β-Cryptoxanthin | 65.1 ± 9.2 | 61.2 ± 7.8 | 54.9 ± 6.6 |
| | β-Carotene | 34.3 ± 3.3 | 40.3 ± 2.9 | 29.5 ± 2.0 |
| | Lutein | 45.7 ± 2.0 | 57.7 ± 3.1 | 33.8 ± 2.1 |

[a] n = 3

[b] (ng/g)

### 3.5 Antioxidant capacity of goji berries

The AC of the goji berries has been evaluated using four different assays (DPPH, ABTS, ORAC and OH-RSC) in order to have an estimation of the mechanisms involving the molecular species that act as radical scavengers. The values of AC obtained are reported in Table 5. DPPH and ABTS are assays that measure the sample ability in deactivating radical species through electron transfer reactions. The DPPH free radical-scavenging capacities ranged from 13.9 to 18.5 µmol VCE $g^{-1}$ and such values are slightly lower of the ones reported by Zhang et al. [5] who expressed the AC as µmol trolox equivalent $g^{-1}$. Since the values of AC for AA and trolox were found to be almost the same in one of our previous papers [24], the comparison of the data here presented with those by Zhang et al. can be properly made.

The AC values obtained by the ABTS assay ranged from 54 to 61 µmol VCE $g^{-1}$, and are in good agreement with those reported by Zhang et al.

The ORAC assay estimates the ability of removing peroxyl radicals through hydrogen transfer reactions. The results coming from this assay ranged between 180-260 µmol VCE $g^{-1}$.

The PhOH-RSC assay gives values, which are strictly related to the ability of the samples in removing OH radicals. In this case, the AC values resulted in the 470-630 µmol VCE $g^{-1}$ range, i.e., very close to the one obtained from ORAC assays, so suggesting that the antioxidant capacities estimated by ORAC and PhOH-RSC are related to all the reactive oxygen species. It has to be pointed out that the ACs displayed by the three goji berries samples are very similar among them when determined by DPPH, ABTS, ORAC, and PhOH-RSC assays ($p > 0.05$). These results highlight that these fruits do not exhibit big differences when tested for their AC, as also suggested by chemical analysis. Moreover, independently from the assay that is used for the determination, goji berries show high AC values,

which are comparable or even higher than the ones reported for those fruits generally considered as antioxidant-rich (such as strawberry and blueberry) [29]. These data confirm the high nutritional and commercial value of goji berries.

It is worthy to mention that the PhOH-RSC method evaluates the amount of just those OH radicals that are blocked by scavengers. However, the OH radical is a very reactive species and the best way to avoid its harmful effects is to slow down or block its production. In order to evaluate this factor and, therefore, to determine the protective effect of a sample, we also produced OH radicals by the Fenton reaction that can be considered their main source in a biological environment. The FeOH-RSC method was applied to the same samples, which had been already analysed with the PhOH-RSC. The antioxidant power resulted obviously much greater than the one obtained from PhOH-RSC and was in the 1.25 - 1.28 mol VCE $g^{-1}$ range. Such very high values can be explained by considering the different phenomena at the basis of the assay. Firstly, the FeOH-RSC method evaluates the ability of blocking the OH radical formation; therefore, the antioxidants are not degraded if no radical is produced. Consequently, if an antioxidant would completely block the ·OH production, its antioxidant power should be theoretically infinite. Moreover, ascorbic acid is a very weak antioxidant in the experimental conditions where the Fenton reaction takes place, as suggested by Ou *et al.* during the development of the OH-ORAC assay based on a Fenton-like reaction [30]. Finally, the scavenger effects can be apparently increased when the antioxidant acts as a good ligand for iron because, in such a case, it is placed very close to the ·OH source. Since the rate of any reaction involving OH radicals is close to the rate of diffusion, the proximity to the site where ·OH is generated plays a key role in order to obtain an efficient deactivation of the radicals. The values deriving from the FeOH-RSC method are also shown in Table 5. For this assay as well, the three goji berry samples

result similar in their capacity to block the OH radical production ($p > 0.05$). Therefore, we can conclude that the chemical composition of the samples should be very similar also as far as the compounds able to block the Fe (II) pro-oxidant effect are concerned.

**Table 5**. Antioxidant capacity (± SD)[a] expressed as VCE $g^{-1}$

| | ABTS ($\mu mol\ g^{-1}$) | DPPH ($\mu mol\ g^{-1}$) | ORAC ($\mu mol\ g^{-1}$) | FeOH-RSC ($mol\ g^{-1}$) | PhOH-RSC ($\mu mol\ g^{-1}$) |
|---|---|---|---|---|---|
| Brand 2 | 54 ± 5 | 14.3 ± 0.1 | 180 ± 10 | 1.3 ± 0.1 | 470 ± 50 |
| Brand 3 | 60 ± 5 | 18.5 ± 0.1 | 260 ± 10 | 1.2 ± 0.1 | 590 ± 70 |
| Brand 1 | 61 ± 5 | 13.9 ± 0.1 | 230 ± 10 | 1.6 ± 0.1 | 630 ± 70 |

[a] n = 3

## 4. CONCLUSION

An array of different assays has been developed and applied to different goji berry supplements for the evaluation of their overall ACs and of the concentration of single significant antioxidants.

The LC-MS/MS method allows the determination of 23 different analytes with well-known antioxidant properties; although the analytes belong to widely different chemical classes, they are all quantifiable within a single chromatographic run. A feasible, straightforward sample pretreatment procedure based on SPE with HLB cartridges provided good extraction yields and sample purification without excessively increasing analysis times. The method has been validated according to current international guidelines and has provided reliable results for all analysed samples.

The AC of the goji berry samples has been tested by three conventional assays (ORAC, ABTS and DPPH) and by a recently developed electrochemical method which is able to evaluate the ability to remove OH radicals if these species are produced by $H_2O_2$ photolysis (PhOH-RSC) or the ability to block OH radical formation based on the Fenton reaction (FeOH-RSC). The results confirm the high antioxidant capacity of goji berries and the substantial equivalence of the investigated samples, thus supporting the results obtained with the LC-MS/MS method.

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