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Abstract

Wet spent coffee grounds (SCG) from espresso capsules, a post-consumer organic solid residue produced worldwide, were analysed to determine their chemical and microbiological stability during storage. In particular, the changes in the total phenolic content and antioxidant capacity (based on two free radical scavenging assays and one oxygen radical absorbance assay) were determined on espresso SCG stored in capsules for up to one month at room temperature in a container open to the air. Phenolic compounds were also identified and quantified using high performance liquid chromatography coupled with diode array and mass detectors. Microbiological analysis was performed in parallel on the same stored SCG to determine the total counts and quantify the main microbial groups present during the storage. The total phenolic content, antioxidant capacity and the most important bioactive compounds, such as the total caffeoylquinic acids, were significantly stable during storage for up to one month, while overall microbial stability was observed for up to two weeks of storage. Overall, the recovery of espresso coffee capsules within 15 days could guarantee the maintenance of microbiological stability as well as the content of valuable antioxidant compounds.

Keywords: spent coffee grounds; espresso capsules; antioxidant assays; bioactive compounds; microbiological stability; storage.

1. Introduction

The need to reduce waste production as well as to minimise its economic and environmental impacts has prompted researchers to optimise extraction techniques with the goal of obtaining bioactive compounds from plant-derived residues (Wijngaard, Hossain, Rai, & Brunton, 2012). New and promising studies with the aim to chemically characterise fruit and vegetable by-products and waste biomass have been performed, highlighting their potential use as valuable source of bioactive components such as polyphenols, dietary fibre (Lozano-Sánchez et al., 2011; O'Shea, Arendt, & Gallagher, 2012) and other molecules (Pfaltzgraff, De Bruyn, Cooper, Budarin, & Clark, 2013).

Among food manufacturing sectors, the coffee industry produces high quantities of solid wastes and by-products, which recent studies describe to be rich in phytochemicals and bioactive molecules that have potential in the formulation of functional foods (Esquivel & Jiménez, 2012; Franca & Oliveira, 2009; Mussatto, Machado, Martins, & Teixeira, 2011). Spent coffee grounds (SCG) obtained in large quantities from coffee brewing are one of the most interesting organic post-consumer coffee residues. A total of 50% of SCG come from the industrial preparation of instant soluble coffee (Esquivel & Jiménez, 2012) and the remaining 50% come from the worldwide production of different coffee brews in cafeterias, restaurants and homes. Currently, disposable espresso capsules are among the most popular ways to consume coffee brew and are an interesting and widely method adopted in homes or at offices (Parenti, Guerrini, Masella, Spinelli, Calamai, & Spugnoli, 2014). Spent coffee grounds have recently been characterized showing high quantities of water-soluble organic bioactive antioxidant compounds, such as caffeine, chlorogenic acids and melanoidins (Bravo et al., 2012; Bravo, Monente, Juániz, Paz De Peña, & Cid, 2013; Panusa, Zuorro, Lavecchia, Marrosu, & Petrucci, 2013; Ramalakshmi, Rao, Takano-Ishikawa, & Goto, 2009; Zuorro, & Lavecchia, 2012) and inorganic components, such as

minerals (Cruz et al., 2012). Nevertheless, the studies mentioned above have been carried out on fresh spent coffee grounds analysed after stages of preparation such as drying, defatting and/or freeze-drying to preserve the original organic sample. While SCG produced from soluble coffee production can be immediately treated at the industrial level (Bravo et al. 2012), SCG from capsules can be stored in the place where capsules are consumed. Therefore, because one half of the SCG production originates from the consumption of espresso capsules, it would be interesting to know how storage can affect the composition of the bioactive of polyphenolic compounds present in this solid residue and its potential for industrial reuse.

Therefore the aim of this study was to define the stability of antioxidant activity, polyphenolic compounds, bacteria and fungi present in Arabica spent coffee capsules during storage for up to one month in air at room temperature to reflect real storage conditions at home or in the workplace before the industrial reuse of these residues.

2. Materials and methods

2.1 Chemicals and reagents

n-Hexane, acetone, ethanol, methanol, formic acid, *trans*-5-*O*-caffeoylquinic acid (*trans*-5-CQA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis-(3-ethylbenzothiazolin-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2’-azobis(2-amidinopropane) dihydrochloride (AAPH) and 3’,6’-dihydroxyspiro[isobenzofuran-1[3H],9’[9H]-xanthen]-3-one (fluorescein), Folin-Ciocalteu’s phenol reagent, sodium nitrite, aluminium chloride, potassium persulphate, hydrochloric acid and sodium hydroxide were purchased from Sigma-Aldrich (Milan, Italy). All chemicals were of reagent- or HPLC grade level. Ultra-pure water was produced with a Milli-Q System (Millipore,

Milan, Italy). Plate Count Agar (PCA), Malt Extract Agar (MTA) and Ringer's solution were supplied by Oxoid (Milan, Italy).

2.2 SCG samples preparation and storage

Commercial aluminium coffee capsules (Lavazza Blue Tierra 2 Intenso, 100% Arabica) from the same batch (one hundred capsules) were provided by Lavazza S.p.A. (Turin, Italy) and used to produce a typical Italian espresso coffee brew with an automatic espresso machine (Lavazza Blue LB 1000, Lavazza, Italy). In particular, one espresso coffee was obtained from each capsule (8 g of ground coffee), using oligomineral water (electrical conductivity (20 °C) 69.5 μS/cm) by stopping the espresso machine to obtain an espresso with a final volume of 20 mL. The extraction parameters for espresso production are as follows: water temperature of approximately 90 °C, water pressure of 9 bar, percolation time of 10 s for 20 mL of espresso. All used capsules were collected and stored in air at room temperature (22 °C ± 3) in a polyethylene terephthalate container. Spent capsules had twenty-two small holes (each of 2 mm diameter) in the aluminium cover. After 0, 7, 15 and 28 days of storage, ten capsules were randomly opened, the SCG were collected, mixed and immediately used to perform all chemical and microbiological analyses. Unused ground coffee from capsules were analysed as a reference for chemical analyses. Three replicates were carried out for each analysis and each step of storage.

2.3 Moisture and pH determination

The moisture content was determined using an electronic moisture balance (Eurotherm, Gibertini Elettronica, Milan, Italy) with 5 g of sample. For the determination of pH, samples of coffee or SCG were mixed with ultrapure water 1:25 (w/v) with continuous shaking (Asal srl, Stirrer 711,

Cernusco sul Naviglio, Milan, Italy) for 15 min at room temperature. The pH of the decanted liquid phase was measured for 5 min using a pH-meter (Micro pH 2002, Crison, Italy).

2.4 Extraction of phenolic compounds

Extraction of phenolic compounds was carried as reported by Pinelo, Tress, Pedersen, Arnous and Meyer (2007), in agreement with the recent literature (Panusa et al., 2013). Briefly, two grams of coffee or SCG were added to 20 mL of a mixture of ethanol/water 60:40 (v/v), shaken on an orbital shaker for 30 min at a constant oscillation (100 oscillations per min) in the dark and at room temperature and then centrifuged (15 min, 4 °C, 16800 *g*) (Heraeus Megafuge 11R, Thermo Electron, LED GmbH, Germany). The supernatant was filtered (0.45 µm), diluted to 25 mL with ethanol/water 60:40 (v/v) and immediately analysed. The extractions were performed in triplicate for each sample. Extracts were used for the determination of the total phenolic content, antioxidant capacity, HPLC-photo diode array detector (PDA) and MS/MS analyses.

2.5 Total Phenolic Content (TPC) assay

TPC was spectrophotometrically assayed by means of the modified Folin-Ciocalteu method (Singleton & Rossi, 1965; Singleton, Orthofer & Lamuela-Raventos, 1999). Briefly, 0.5 mL of phenolic extract was appropriately diluted and mixed with 2.5 mL of Folin-Ciocalteu reagent that had been diluted with water 1:10 (v/v). The mixture was incubated at room temperature for 3 min, and 2 mL of 7.5% (w/v) aqueous sodium carbonate solution was added. The mixture was incubated at 45 °C for 15 min and finally cooled in a water-ice bath to stop the reaction. The specific absorbance at 765 nm was immediately measured at room temperature with a UV-visible spectrophotometer (UV-1700 PharmaSpec, Shimadzu, Milan, Italy). A mixture of solvent and reagents was used as blank. The total phenolic content was expressed as mg gallic acid

equivalents (GAE) per gram of sample on a dry basis, through a calibration curve of gallic acid. The linearity range of the calibration curve was 0-250 mg/L (r^2 = 0.998).

2.6 In vitro Antioxidant Capacity (AC) assays

2.6.1 Trolox Equivalent Antioxidant Capacity (TEAC) assay

The TEAC values of phenolic extracts were estimated according to the original analytical procedure described by Re et al. (1999), with slight modifications. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulphate (final concentration). The mixture was allowed to stand in the dark at room temperature for 12-16 h before use. Immediately before the analysis, the $ABTS^{\bullet+}$ stock solution was diluted with ethanol to reach an absorbance of 0.70 (± 0.02) at 734 nm, and equilibrated at 30 °C. Sample solutions (or standard) (30 µL) were mixed with $ABTS^{\bullet+}$ solution (3 mL). Absorbance readings were taken at 30 °C exactly 6 min after the initial mixing. An appropriate solvent blank was obtained by mixing 60% ethanol (30 µL) with $ABTS^{\bullet+}$ solution (3 mL), while absolute ethanol was used as a control. The $ABTS^{\bullet+}$ scavenging effect (% Inhibition) was calculated using the equation:

$$\% \text{ Inhibition} = [(A_{734\text{blank}} - A_{734\text{sample}})/A_{734\text{blank}}] \times 100$$

where $A_{734\text{blank}}$ and $A_{734\text{sample}}$ are the absorbances of $ABTS^{\bullet+}$ solution at 734 nm before and after sample addition. Results are expressed as µmol Trolox equivalent (TE) per gram of sample on a dry basis, by means of a dose-response curve for Trolox (0-350 µM).

2.6.2 DPPH Radical Scavenging Capacity (DPPH RSC) assay

The DPPH RSCs of the phenolic extracts were measured based on the discolouration of the purple colored methanol solution of the 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$). The spectrophotometric assay was conducted according to the method reported by von Gadow, Joubert & Hansmann (1997). Briefly, 75 µL of sample extract was added to 3 mL of 6.1 x 10^{-5} M

DPPH$^{\bullet}$ solution in methanol. The decrease in absorbance at 515 nm was recorded at room temperature condition until stable values (1 h) using methanol as control and methanol solution of DPPH$^{\bullet}$ as blank. All operations were performed in the dark or dim light (Sharma & Bhat, 2009). The inhibition percentage (IP) of the DPPH$^{\bullet}$ by phenolic extracts was calculated according to the formula

$$IP = [(A_{0min} - A_{60min})/A_{0min}] \times 100$$

where A_{0min} is the absorbance of the blank at t = 0 min, and A_{60min} is the absorbance of samples at 60 min. Results were expressed as µmol Trolox equivalent (TE) per gram of sample on a dry basis, by means of a dose-response curve for Trolox (0-350 µM).

2.6.3 Oxygen Radical Absorbance Capacity (ORAC) assay

The ORAC assay was carried out in a PerkinElmer 2030 Multilabel Reader with 96-well black plates. The reaction was carried out with 75 mM potassium phosphate buffer (pH 7.4) used as a reagent blank and different Trolox solutions, ranging from 0.25 to 6 µM, were used as standards (Ou, Hampsch-Woodill, & Prior, 2001). The sample solutions were prepared by diluting phenolic extracts with phosphate buffer. To start the incubation, aliquots of fluorescein (3',6'-dihydroxyspiro[isobenzofuran-1[3H],9'[9H]-xanthen]-3-one) solution (150 µL of a 48 nM solution in potassium phosphate buffer) were dispensed into each well, followed by 20 µL of either buffer standard or sample solutions added in duplicate. The plate was covered and incubated in the preheated (37 °C) microplate reader for 10 min, which included shaking for 3 min. At the end 30 µL of AAPH solution (133 mM in phosphate buffer) were added and the reaction started when the plate was reinserted into the reader at 37 °C. All fluorescence measurements were expressed relative to the initial reading of the fluorescence signal. Readings were repeated every minute for 35 min at the emission wavelength of 535 nm with excitation at

485 nm. The net area under the curve (AUC) was calculated by subtracting the AUC of the blank from the AUC of either the standard or the sample. The Trolox equivalent molar concentrations of the samples were calculated using a linear regression equation between the Trolox concentration and the corresponding net AUC. To compare the antioxidant activity of the extracts, it was decided to calculate the relative ORAC values as μmol of Trolox equivalents present in 1 g of extract on a dry basis.

2.7 HPLC-PDA-MS/MS analysis

A Thermo-Finnigan SpectraSystem HPLC (Thermo-Finnigan, Waltham, USA), equipped with a P2000 binary gradient pump, a SCM 1000 degasser, an AS 3000 automatic injector and a Finnigan Surveyor PDA Plus detector (PDA) coupled in tandem with a API 3200 QTRAP (Applied Biosystem Sciex, Foster City, CA, USA) with a Turbo V source (Applied Biosystem Sciex) was used. The ChromQuest software (version 5.0) was used for instrument control and UV-data collection and processing, while Analyst software (version 1.6) was used for MS/MS analysis. Separation was achieved on a Luna C18 column (15 x 2 mm, 5 μm, 100 Å, Phenomenex, Castel Maggiore, Italy) equipped with a SecurityGuard™ analytical guard cartridge system (Phenomenex). The mobile phase was composed of solvent A (formic acid 0.1% in ultrapure water) and solvent B (methanol). The flow rate was set at 0.25 mL/min and the injection volume was 10 μL. The elution program was as follows: A 90% kept in isocratic for 1 min, A 83% in 29 min, kept in isocratic for 15 min, A 65% in 10 min, kept in isocratic for 12 min, A 0% in 8 min, kept in isocratic for 2 min, A 90% in 15 min. PDA spectra were recorded in full scan modality over the wavelength (λ) range of 220 to 600 nm, and quantification was performed using PDA chromatograms extracted at 325 nm according to a calibration curve obtained for the *trans*-5-CQA analytical standard and expressed as mg/kg dry weight (dw). MS/MS conditions for the

identification of caffeoylquinic acids (CQAs) analysis were optimised using the *trans*-5-CQA standard. The ion source was operated in negative ion mode using the following conditions: ion spray voltage -4500 V; turbo spray temperature 500 °C; curtain gas 2.07 x 10^5 Pa; interface heater on; nebuliser gas 2.4 x 10^5 Pa; heater gas 10 x 10^5 Pa. Nitrogen was used as the nebuliser, heater, curtain and collision gas. Masses were recorded in the range of *m/z* 100-700 amu using an enhanced mass spectrum (EMS) scan experiment with a declustering potential (DP) of -20 V and an entrance potential (EP) of -10 V. Product ions (MS/MS) were generated according to the information depend acquisition (IDA) mode, with a threshold of 50000 cps and a collision energy (CE) of -30 eV and were collected in enhanced product ions (EPI) mode.

2.8 Microbiological analysis

For each sampling point, 10 g of unused or spent subsample of coffee ground were mixed with 40 mL of Ringer's solution (Oxoid, Milan, Italy) for 2 min with a stomacher (Interscience, Turin, Italy). Subsequently, serial decimal dilutions were performed and plated, in triplicate, on Plate Count Agar (PCA, Oxoid, Milan, Italy) for enumeration of the total bacterial count and on Malt Extract Agar (MTA, Oxoid, Milan, Italy) for filamentous fungi and yeasts. PCA plates were incubated for 48 h at 30 °C while MTA plates at 30 °C for 72 h. To enumerate thermoresistant bacteria, the homogenate was subjected to a thermal treatment at 80 °C for 10 minutes and then plate count was performed on PCA. Filamentous fungi grown on MTA were identified by sequencing of the D1/D2 region of the gene encoding the 26S rRNA (Kurtzman & Robnett, 1998), as described by Cocolin, Bisson, and Mills (2000).

2.9 Statistical and mathematical analysis

A one-way analysis of variance (ANOVA) using Tukey's test for mean comparison was used to highlight significant differences among SCG samples. All calculations were performed with the STATISTICA for Windows statistical software (Release 7.0; StatSoft Inc., Tulsa, OK, USA). An overall antioxidant potency composite index (APCI) was determined (Seeram et al., 2008). An equal weight was assigned to all assays and an index value of 100 was assigned to the best score for each test, and the corresponding index score was then calculated for each sample as follows:

antioxidant index score = (sample score/best score) × 100.

The average of the index scores obtained in all tests of a specific extract was defined as its APCI.

3. Results and discussion

3.1 Moisture and pH of samples

The initial moisture content of SCG was approximately 59% (Table 1), in agreement with values reported for other wet residues (Cruz et al., 2012; Kondamudi, Mohapatra, & Misra, 2008; Zuorro & Lavecchia, 2012). This value remained significantly stable for a week. After one month of storage in capsules, a significant decrease of approximately 17% was observed.

Roasted coffee presented a pH of 5.46, similar to that reported in other Arabica roasted coffee (Bicho, Leitão, Ramalho, De Alvarenga, & Lidon, 2011). After espresso production, fresh SCG exhibited a pH value higher than coffee, in agreement with Cruz et al. (2012). During storage, a slight significant decrease in pH values of approximately 8% was observed after one week of storage. However, this observation is probably not correlated with microbial development.

3.2 Qualitative analysis of phenolic compounds

A total of 16 phenolic compounds were found in coffee and SCG samples. A representative PDA chromatogram is displayed in Figure 1. Table 2 displays the retention times (R_t), the UV maxima

(λ_{max}), the negative pseudomolecular ions ($[M-H]^-$), MS/MS and the identification method of the detected compounds. All compounds exhibited λ_{max} between 322 and 327 nm, which are characteristic of CQAs, di-CQAs, caffeoylquinolactones (CQL) and feruloylquinolactones (FQL) (Ramalakshmi et al., 2009). A peak at approximately 6th min had the following characteristics, λ_{max}=323 and $[M-H]^-$=353. Despite the fact that no MS/MS typical of CQAs was detected (probably due to the low peak intensity) the compound can be tentatively identified as a CQA isomer on the basis of the findings of Panusa et al. (2013). In addition, this peak was detected only in the SCG sample at time 0 of storage. Peaks 1, 3 and 5 ($[M-H]^-$ at *m/z* 353) were identified as 3-CQA, 4-CQA and 5-CQA by comparison with analytical standards and/or on the basis of data reported in literature, whereas peak 2 was annotated as a CQAs isomer on the basis of data reported in literature (Panusa et al., 2013). CQAs have been reported to be the most abundant compounds in green coffee beans and SCG, with 5-CQA as the major component (Farah & Donangelo, 2006; Bravo et al., 2012). Peaks 4, 9 and 11 ($[M-H]^-$ at *m/z* 367) can be assigned to feruloylquinic acids (FQA) (Panusa et al., 2013). The comparison of MS/MS fragments (obtained in this work from a single chromatographic run by applying IDA experiments) with those obtained in analogous studies also allows us to tentatively annotate peak 4 as 5-FQA (Clifford, Johnston, Knight, & Kuhnert, 2003; Narvaez-Cuenca, Vincken, Zheng, & Gruppen, 2013), whereas peaks 6-8 and 10 all have $[M-H]^-$ at *m/z* 335. As reported by Jaiswal, Matei, Ullrich, & Kuhnert (2011), these isobaric molecules can be assigned both to CQL and caffeoylshikimic acids (CSA). In particular, the assignment of peak 6 to CSA and peaks 7, 8 and 10 to CQL may be possible on the basis of the comparison of their MS/MS fragments with literature data (Jaiswal et al., 2011). Peaks 12-15 ($[M-H]^-$ at *m/z* 349) can be tentatively identified as FQL or feruloylshikimic acids (Jaiswal et al.). However, the assignment of peaks identification cannot be done in this case due to the lack of a significant numbers of MS/MS fragments, probably due to

the low peak intensities (data not shown). CQL and FQL also have been previously reported in SCG (Farah & Donangelo, 2006; Panusa et al., 2013). The only peak (16) with $[M-H]^-$ at *m/z* 515 may be attributed to a dicaffeoylquinic acid (di-CQAs) isomer among those previously detected in green coffee beans and SCG (Bravo et al., 2012; Perrone, Farah, Donangelo, de Paulis, & Martin, 2008). Differently from Bravo et al., in this work only one of the dicaffeoylquinic acid isomers was detected, probably for the different extraction conditions used.

3.3 Phenolic content, antioxidant capacity and amounts of CQAs in SCG

The mean values of the total phenolic content (TPC) and the antioxidant capacity (AC) assessed during the storage of SCG samples and on untreated grounds coffee are summarised in Table 1. The total phenolic content of intact roasted coffee was similar to those reported for espresso coffee brew (55 mg of GAE/g of coffee) by Pérez-Martínez, Caemmerer, Paz De Peña, Cid, & Kroh (2010). Also Brezová, Šlebodová, and Staško (2009) reported values of total phenolics ranging from 41 to 58 mg GAE/g of different commercial high quality coffees. As expected, after coffee brewing the amount of total phenolics in fresh SCG decreased by approximately 44.6% with respect to untreated coffee grounds. Panusa et al. reported a TPC in extracts of SCG recovered from used capsules that was two-fold lower than those observed in our fresh SCG samples. This discrepancy could be due to different blends of coffee (not only the species but also the provenience), the type of roasting and the technological extraction parameters. Antioxidants activities data detected using all assays were equally characterised by a decrease in fresh SCG of greater than 40% with respect to untreated coffee. Because of the use of various extraction techniques for different SCG (e.g. SCG from filter, espresso, plunger or mocha), it is difficult to compare our AC results with those in the literature. For espresso SCG, which would be assumed to be more similar to SCG capsules, Bravo et al. (2012) reported ABTS and DPPH values of

131.83 and 74.57 μmol TE/g dw, respectively, while Páscoa, Magalhães, & Lopes (2013) assessed an ABTS value of 126 μmol TE/g dw, which is more similar to our results. ORAC data of SCG extracts are limited in scientific literature (Ramalakshmi et al., 2009), and therefore not comparable with our results, due to the fundamental differences in extraction methods used. The stability of TPC for up to 15 days of storage is noteworthy. Only after 28 days of storage, TPC significantly decreased by approximately 26%, suggesting a minimal degradation of phytochemical compounds. The same trend was observed in the AC assays, with a slight decline of antioxidant capacity during storage, which was significant only for DPPH RSC results after 28 days of storage. In particular, on SCG stored for one month, a decrease of approximately 30% of DPPH RSC with respect to fresh SCG, and decreases of approximately 26% and 14% for TEAC and ORAC values, respectively, were observed. While DPPH RSC and TEAC values decreased linearly as well as TPC, after a strong decrease from untreated coffee to fresh SCG, ORAC levels were substantially preserved for the first 15 days of storage to finally decreased again on the 28th day. The differences in the responses of TPC, DPPH RSC and TEAC and ORAC could be explained by the different reaction mechanisms and/or different affinities for particular antioxidants of these assays (Stratil, Klejdus, & Kubáň, 2006). Moreover, foods may interfere by having different magnitudes of effect on different assays (Craft, Kerrihard, Amarowicz, & Pegg, 2012). It is well known that the Folin-Ciocalteu, ABTS and DPPH assays, based on similar electron-transfer redox reactions, are able to assess not only the phenolic compounds but also the antiradical or antioxidant capacity of non-phenolic compounds, such as Maillard reaction products, including melanoidins formed during roasting (Pérez-Martínez et al., 2010). On the contrary, the ORAC assay is typically a hydrogen transfer-based assay that involves a reaction schema in which antioxidants and substrate compete kinetically for generated peroxyl radicals. For this reason, many authors suggest running multiple antioxidant methods to obtain a better

estimation of the antioxidant capacity of food extracts (Alvarez-Suarez, Tulipani, Romandini, Vidal, & Battino, 2009; Prior, Wu, & Schaich, 2005; Seeram et al., 2008). The APCI values were calculated to give an equal weight to all methods used to quantify the antioxidant capacity of the SCG extracts (Table 3). When all methods were combined into a single index of antioxidant activity, the rank order was related to the storage time. The APCI value decreased by approximately 24 points on the 28th day (one third lower antioxidant potency in comparison to the fresh SCG), demonstrating that after two weeks the antioxidant capacity dropped by only approximately 11%.

The total CQAs content has been calculated as the sum of the contents of 3-CQA, CQA, 5-CQA and 4-CQA. CQAs have been reported to be the most abundant compounds in the green coffee beans and SCG (Farah & Donangelo, 2006; Bravo et al., 2012). According to the literature (Farah, De Paulis, Trugo & Martis, 2005), the total CQAs of *C. arabica* greatly decreased from green coffee to roasted coffee, due to the increasing degree of roasting and roasting time (from 52.87 to 2.09 mg/g dw), with lower values assigned to very dark coffee, which is roasted for a longer time. On the basis of these observations, the value of total CQAs obtained in this work for coffee (8.29 ± 0.29 mg/g dw) (Table 1) was similar to those reported for a dark medium coffee roasted for 8 min. The total CQAs of SCG was found to be about four times lower than that of coffee, ranging from 1.95 to 2.31 mg/g dw. In particular, no significant differences among the total CQAs of SCG at 0, 7 and 28 days of storage were detected. Instead, a significant increase was observed at the 15th day of storage. Values of the total CQAs found in this work are very similar to those obtained by Panusa et al. (2013) (2.26 ± 0.06 mg/g dw) who used a mixture of ethanol/water (60:40 v/v) for the extraction as that was used in this study and differently, but a single step of extraction coupled with a higher temperature (60 °C). Instead, the different

extraction technique used by Bravo et al. is probably the reason for the higher values of CQAs found in their espresso SCG (6.16 mg/g dw).

3.4 Microbiological aspects of SCG samples

The results of the microbiological analysis of SCG before storage showed negligible microbial contamination (Table 4). On both PCA and MTA media the microbial count was less than 10 colony forming units (cfu/g). In the subsequent sampling points, a progressive increase in the counts on both PCA and MTA media was observed. More specifically, for the first 15 days of storage, the total bacteria detected were on the order of 10^2-10^3 cfu/g, while at 28 days the count reached was above 10^6 cfu/g. Similarly, the count on MTA was constant, in the order of 10^2 cfu/g up to 15 days of storage, thus a characterisation of moulds during this phase was not considered worthwhile, and it reached a final value of 2.2×10^6 cfu/g on the 28th day. No thermoresistant spores were detected by the microbiological analysis performed after the thermal treatment of the spent coffee homogenate. Based on the morphology of the fungal colonies, two main populations could be discriminated. To obtain a definitive identification, two representative isolates of each morphology were isolated in pure culture, and the total DNA was extracted. A fragment of the gene encoding the 26S rRNA was PCR amplified, sequenced and identified using the sequence database (http://www.ncbi.nlm.nih.gov) by BLAST comparison (Altschul, Gish, Miller, Myers, & Lipman, 1990). The two main populations belonged to the species *Penicillium expansum* and *Penicillium toxicarium*. In cumulatively assessing the results, it can be concluded that a satisfactory microbial stability for spent coffee stored under uncontrolled conditions would be expected for up to 15 days. Extension of the storage period is not recommended because extensive microbial growth developed after 15 days.

4. Conclusions

Wet exhausted espresso SCG stored in capsules provided, on the basis of this preliminary data, satisfactory microbiological stability for up to approximately 15 days of storage performed at room temperature in air. Moreover, a generally insignificant degradation of more important bioactive compounds such as CQAs and, consequently, stability of antioxidant capacity was observed during storage. The exploitation of this post-consumer stored coffee residue as a renewable source to recover functionalizing molecules could be important, even after a simple and inexpensive aseptic method of capsule storage (containers sprayed with ethanol and/or air removal).

Acknowledgements

This study was funded by the project 'Ricerca & Innovazione per il Miglioramento della Sostenibilità della Filiera Agro-alimentare ECOFOOD' – European Fund for Regional Development F.E.S.R. 2007/2013 – European Union, Italian Economy and Finance Ministry, Piedmont Region (Italy). The authors thank Lavazza S.p.A. for kindly providing the roasted coffee capsule samples.

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Figure Captions

Figure 1. Typical HPLC-PDA chromatogram of bioactive compounds of SCG extracted at λ=325 nm. Peaks 1-16 correspond to, 3-*O*-CQA (1); CQA (2); 5-*O*-CQA (3); FQA (4); 4-*O*-CQA (5); CQL (6); CQL (7); CQL (8); FQA (9); CQL (10); FQA (11); FQL (12); FQL (13); FQL (14); FQL (15); di-CQA (16).

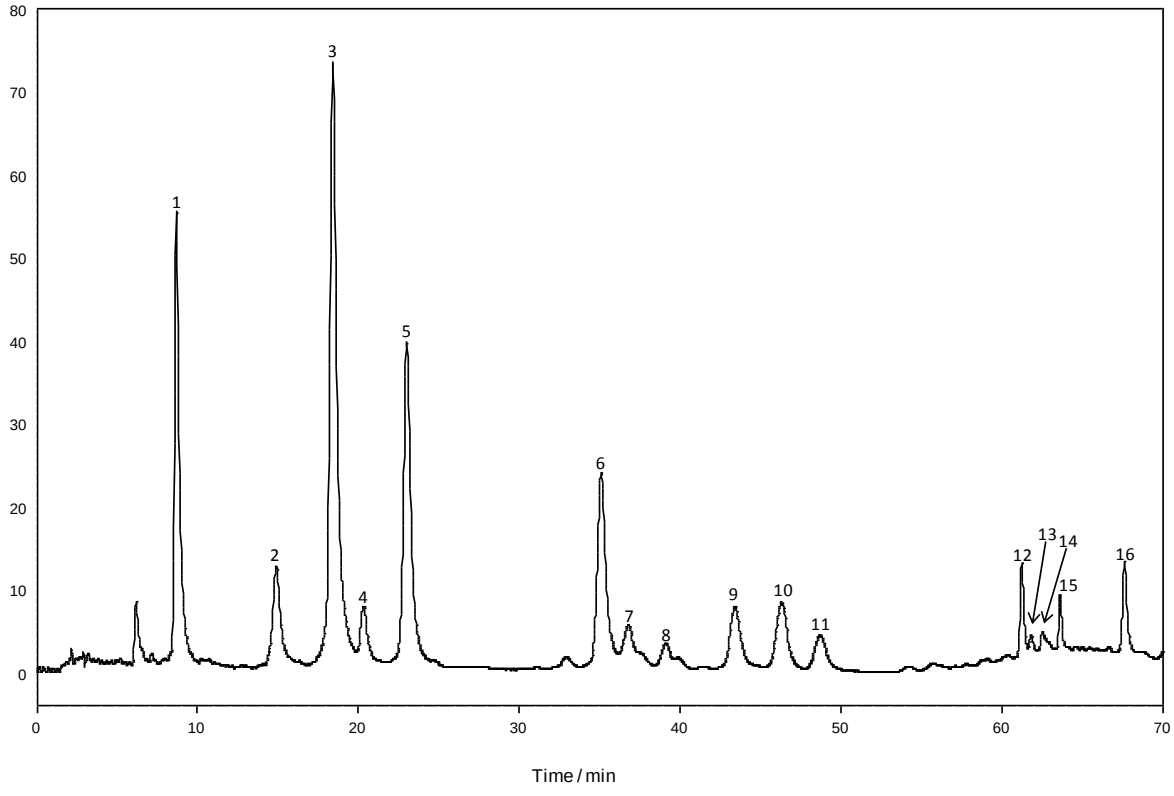

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Table 1. Values of pH, moisture, phenolic content (TPC), antioxidant capacity (RSC, TEAC and ORAC) and total caffeoylquinic acids (CQAs) on roasted coffee and spent coffee grounds during storage.

	Coffee	Spent Coffee Grounds				
		Storage days				*p*
		0	7	15	28	
pH	5.46 ± 0.31	5.92 ± 0.05[c]	5.44 ± 0.06[b]	5.17 ± 0.05[a]	5.24 ± 0.07[a]	***
Moisture (% w/w)	17.6 ± 0.02	59. 08 ± 0.02[c]	57.56 ± 0.0[c]	53.91 ± 0.0[b]	49.17 ± 0.0[a]	***
TPC (mg GAE/g dw)	45.48 ± 6.59	25.17 ± 2.14[b]	24.30 ± 0.07[b]	21.89 ± 2.35[ab]	18.60 ± 1.50[a]	**
DPPH RSC (µmol TE/g dw)	227.96 ± 33.70	132.48 ± 22.83[b]	119.56 ± 2.21[ab]	109.13 ± 16.23[ab]	92.59 ± 7.10[a]	*
TEAC (µmol TE/g dw)	201.85 ± 28.64	119.48 ± 27.54	105.93 ± 3.64	100.07 ± 5.38	88.57 ± 11.00	ns
ORAC (µmol TE/g dw)	889.39 ± 49.13	477.65 ± 20.11	487.35 ± 52.90	483.94 ± 23.08	411.74 ± 27.91	ns
tot CQAs (mg/g dw)	8.29 ± 0.29	1.95 ± 0.06[a]	1.94 ± 0.05[a]	2.31 ± 0.17[b]	2.01 ± 0.14[a]	*

Data are expressed as mean ± SD (n = 3). Values in each row having different lowercase letters are significantly different at $p<0.05$; *: significance at $p< 0.05$, **: significance at $p<0.01$; ***: significance at $p<0.001$; ns = not significant; dw: dry weight; tot CQAs: total caffeoylquinic acids.

Table 2. LC-MS/MS characteristics of bioactive compounds detected in negative ion mode in SCG samples.

Peak	R_t(min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS/MS (*m/z*)	Identification[§]
1	8.7	324	353	191, 135, 179	3-*O*-CQA[b]
2	14.9	324	353	191, 179	CQA[b]
3	18.4	325	353	191, 179, 135	5-*O*-CQA[a]
4	20.3	322	367	193, 134	FQA[b]
5	23	325	353	173, 179, 191, 135	4-*O*-CQA[b]
6	35.1	326	335	173, 161, 135, 179	CSA[b]
7	36.8	324	335	161, 135, 133	CQL[b]
8	39.1	324	335	161, 173, 135	CQL[b]
9	43.4	324	367	191, 173	FQA[b]
10	46.3	324	335	161, 135	CQL[b]
11	48.7	325	367	191	FQA[b]
12	61.2	326	349	175	FQL[b]
13	61.8	325	349	-	FQL[b]
14	62.6	325	349	-	FQL[b]
15	63.6	327	349	-	FQL[b]
16	67.65	326	515	353, 335, 173, 179, 191, 135	di-CQA[b]

[§]:Identification method - [a]: comparison with reference standards; [b]: tentative identification with literature data. CQA: caffeoylquinic acids; FQA: feruloylquinic acids; CQL: chlorogenic acid lactones; CSA: caffeoylshikimic acids; FQA: feruloylquinic acids; FQL: feruloylquinolactones; di-CQA: dicaffeoylquinic acid; -: not present.

Table 3. Antioxidant Potency Composite Index (APCI) of spent coffee grounds extracts stored for different days.

Storage days	TPC index[a]	RSC index	TEAC index	ORAC index	APCI[b]
0	100.0	100.0	100.0	98.0	99.5
7	96.5	90.3	88.7	100.0	93.9
15	87.0	82.4	83.8	99.3	88.1
28	73.9	69.9	74.1	84.5	75.6

[a]Antioxidant index score = [(sample score/best score) × 100]
[b]Average of all four tests for each storage day.

Table 4. Results of microbiological analysis on wet spent coffee grounds in capsules during storage.

Storage days	Total bacterial count ($\log_{10}$ cfu/g)	Yeasts and moulds ($\log_{10}$ cfu/g)
0	$<1^{a\S}$	$<1^{a}$
7	3.73 ± 0.32^{c}	2.44 ± 0.20^{b}
15	2.70 ± 0.40^{b}	2.69 ± 0.61^{c}
28	$> 6^{d}$	6.35 ± 0.50^{d}

Values are expressed as means of $\log_{10}$ colony forming units per g ± standard deviation.

[§]Values in each column having different letters are significantly different at $p<0.05$.