TITLE: Influence of the brewing method and acidity regulators on the antioxidant capacity of coffee brews

AUTHORS: Mónica Pérez-Martínez\textsuperscript{a}, Bettina Caemmerer\textsuperscript{b}, M. Paz De Peña\textsuperscript{a}, Concepción Cid\textsuperscript{a} and Lothar W. Kroh\textsuperscript{b}

SHORT TITLE: Antioxidant capacity of coffee brews: brewing method and additives

\textsuperscript{a} Department of Nutrition, Food Science, Physiology, and Toxicology, School of Pharmacy (CIFA), University of Navarra, E-31080-Pamplona, Spain

\textsuperscript{b} Institut für Lebensmittelchemie, Technische Universität Berlin, Gustav-Meyer-Allee 25, D-13355 Berlin, Germany

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*Corresponding author: M. Paz de Peña. Tel: +34 948 425600 (6580); Fax: +34 948 425649.
E-mail address: mpdepena@unav.es
ABSTRACT

Antioxidant capacity of the coffee brews prepared with different coffeemakers (filter, plunger, mocha and espresso) was measured by colorimetric (Total Phenolic compounds and ABTS) and Electro Spin Resonance (ESR) spectroscopy techniques (Fremy’s salt and TEMPO). Mocha coffeemaker had the highest yield in coffee antioxidant extraction per g of ground roasted coffee, but espresso coffee was the richest in terms of antioxidant intake (per mL of coffee brew) followed by mocha, plunger and filter. Both Folin-Ciocalteau (Total Phenolic compounds) and ABTS assays reacted with standard solutions of chlorogenic acids (CGA) and melanoidins (MO-Ala and MO-Gly). However, Fremy’s salt was mainly scavenged by chlorogenic acids, whereas the stabilized radical TEMPO was effectively scavenged by melanoidins, but not by chlorogenic acids. Thus, electron spin resonance (ESR) spectroscopy allows distinguishing between phenolic and nonphenolic antioxidants. Moreover, the addition of pH-regulator agents to coffee, such as sodium carbonate (75ppm) and bicarbonate (75ppm), to extend its shelf-life, slightly increases the pH, modifying the antioxidant capacity in those coffee brews with the highest capacity (mocha and espresso).

KEYWORDS: Coffee, antioxidants, phenolics, chlorogenic acids, melanoidins, additives, coffee brews.
INTRODUCTION

Coffee is the second most consumed brew in the world, not only due to its pleasant taste and aroma, but also because of its stimulating qualities. During the past few years, evidence of the healthy benefits (1) and the important contribution of the coffee brew to the intake of the antioxidants in the diet (2-4) help to increase coffee consumption. Several coffee compounds have been proposed as antioxidants. Some of them are phenolic compounds, such as chlorogenic and hydroxycinnamic acids, originally present in green coffee, which remain in smaller amounts in roasted coffee (5). Others, such as melanoidins and other Maillard Reaction Products (MRP), are developed during roasting (6-9). Other coffee components, such as caffeine (10) and volatile compounds (11-13), are also proposed as antioxidants in model systems, but their antioxidant activity is unclear in coffee matrix (14). Moreover, during the past few years, some works proposed that coffee melanoidins incorporate chlorogenic and hydroxycinnamic acids, contributing to the antioxidant capacity of coffee brews (15). At the same time, although the antioxidant capacity usually prevails in foods, it should be taken into account that some antioxidants and their decomposition products might also act as pro-oxidants depending upon the conditions (16).

The antioxidant activity of coffee has been studied using different methods. Traditionally, the methodologies were searching for the evaluation of the overall antioxidant capacity, such as the Folin-Ciocalteau colorimetric method to routinely measure the Total phenolic compounds. Other colorimetric reactions were developed in order to evaluate the ability of a food compound or product to quench and/or reduce radicals or metals using DPPH or ABTS radicals or the FRAP assay, respectively. The antioxidant capacity is normally compared to that of a very well-known antioxidant, such as vitamin E, using Trolox (a water-soluble vitamin E analogue). For this reason, these methodologies are usually called Trolox Equivalent Antioxidant Capacity (TEAC) assays. These colorimetric methodologies are
currently the most widespread, but all of them are based on similar electron-transfer redox reactions and evaluate more than phenolic compounds (17). During the past few years, a new methodology, the electron spin resonance (ESR) spectroscopy, has been proposed as a much more specific technique, capable of distinguishing the contribution of phenolic and nonphenolic antioxidants in coffee by means of different stabilized radicals (18). The basis of the ESR spectroscopy is the absorption of microwave energy by unpaired electrons, such as those of free radicals, when they are in a magnetic field. This technique has been frequently applied in order to measure the antioxidant capacity of a substance to scavenge a stabilized radical, such as Fremy’s salt and TEMPO (19).

The antioxidant capacity of coffee can be influenced by several factors, such as the variety and origin of coffee (20), the roasting degree (6), the type of roast (natural or torrefacto) and their blends (14, 21), and the technological parameters applied for coffee brew extraction. The preparation of a cup of coffee can be made by several extraction methods that extract the potential antioxidants of coffee in different amounts (22). The most popular coffee brew preparation is the filter one, but during the past few decades the consumption of espresso coffee has increased. Moreover, in Southern European countries such as Italy and Spain, the use of the mocha coffeemaker is much extended at the domestic level, and the plunger coffeemaker is being used more often for coffee aroma lovers (23). In each case, the technical conditions applied, such as the coffee/water ratio, water temperature, water pressure, etc., also contribute to the different chemical composition of coffee brews (24-27).

In ready-to-drink coffee beverages, the preservation conditions (temperature, oxygen, etc.) could influence the degradation of phenolics and other antioxidants (28) and the antioxidant capacity (29). However, because of the sensory quality decrease and the limited shelf-life of the coffee brew, other ingredients, such as milk derivatives or additives, are usually added. In a previous work carried out by our research group, the addition of certain pH-regulator agents
to a black coffee has been proposed in order to extend shelf life \((30)\), but the influence of these additives on the antioxidant capacity of ready-to-drink coffee brews has not yet been evaluated.

For all these reasons, the aims of this work were (1) to compare the antioxidant capacity, measured by colorimetric and ESR techniques, of the coffee brews prepared with different coffeemakers (filter, plunger, mocha and espresso) and to investigate the main responsible groups of antioxidants, and (2) to study the effect of the addition of some acidity correctors on the antioxidant capacity of each coffee brew.

**MATERIALS AND METHODS**

**Coffee.** Vacuum-packed Colombian Arabica ground roasted coffee (2.25% water content, \(L^* 19.57 \pm 0.09\)) was provided by a local factory and stored in darkness and at room temperature until the coffee brew preparation, less than one month after roasting. The \(L^*\) value was analyzed by means of a tristimulus colorimeter (Chromameter-2 CR-200, Minolta, Osaka, Japan) using the D65 illuminant. The instrument was standardized against a white tile before sample measurements. Ground roasted coffee was spread out in an 1 cm Petri plate, and the \(L^*\) value was measured in triplicate and on the CIELab scale.

**Chemicals and reagents.** The methanol used was of spectrophotometric grade from Panreac (Barcelona, Spain). Pure reference standards of chlorogenic acids (CGAs) were obtained from Roth (Karlsruhe, Germany), gallic acid from Serva (Heidelberg, Germany), and TEMPO (2,2,6,6-tetramethyl-1-piperidin-1-oxyl), Fremy’s salt (potassium nitrosodisulfonate) and Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) were purchased from Sigma-Aldrich (Steinheim, Germany). 2,2’-Azino-bi(3-ethylbenzo-thiazonile-6-sulfonylic acid) diammonium salt (ABTS) was obtained from Sigma-Aldrich (Steinheim, Germany). Sodium carbonate and sodium bicarbonate were purchased from Panreac (Barcelona, Spain).
Preparation of Melanoidins. Melanoidins were prepared according to Caemmerer & Kroh (31). As a carbonyl compound a maltooligosaccharide mixture DP 3-10 (MO) (Merck) was used, along with the amino acids D,L-alanine (Ala) (Fluka) or glycine (Gly) (Serva). For preparation of the melanoidins, 50 g of carbohydrate and 5 g of the amino acids were mixed thoroughly in solid state, and aliquots of 5g were heated at 170 °C for 6h (MO-Ala) and 10 h (MO-Gly), respectively. MO-Ala was dialyzed and freeze-dried according to Caemmerer et al. (32). Finally, melanoidins were dissolved at 3.6 g/L with distilled water.

Coffee brew preparation. Four coffee-brewing procedures were selected: filter coffee machine, plunger coffeemaker, mocha coffeemaker, and espresso machine. The coffee brews were prepared as described by Lopez-Galilea et al. (22). The ground coffee packages were opened immediately before the preparation of the coffee brews in order to avoid oxidative damage.

The Filter Coffee Brew was prepared from 24 g of ground roasted coffee for a volume of 400 mL, using a filter coffee machine (model K108, AKA). Extraction took 7 min at 90 °C.

The Plunger Coffee Brew was prepared from 40 g of ground roasted coffee, which was extracted with 500 mL of water at 98°C, using a plunger coffeemaker (model Bistro Nouveau Coffee Maker, Bodum, 1L capacity). The water and the coffee powder were kept in contact for 5 min before the plunger was slowly pushed down.

The Mocha Coffee Brew was prepared from 40 g of ground roasted coffee for a volume of 500 mL, using a mocha coffeemaker (Model Vitro-Fulgor, Varila, Spain). The heating temperature and extraction time were approximately 10 min at 93 °C.

The Espresso Coffee Brew was prepared from 7g of ground roasted coffee, for a volume of 40 mL, using an espresso machine (model Saeco Aroma, Italy). The water pump pressure was 15 bar, and the extraction time and temperature were approximately 30s at 90°C.
For the study of the influence of the additives on the antioxidant capacity of coffee, three aliquots of each coffee brew were separated: one as reference, the second with the addition of 75 ppm of sodium carbonate and the third with the addition of 75 ppm of sodium bicarbonate. The selection of the additives and their concentrations were reported in a previous paper (33).

**pH and Total Solids.** Coffee brew samples were rapidly cooled at 20ºC and the pH was measured with a pH-meter (Orion 420A Benchtop pH meter). The total solids were determined by oven drying 40 mL of coffee brew to a constant weight (14h, 102 ± 3ºC).

**Total phenolic compounds.** Total phenolic compounds were measured using the Folin-Ciocalteau reagent according to the Singleton’s method (34), and then calculated using gallic acid as standard. For every coffee sample, 1:10 dilutions with demineralized water were prepared and 2.5 mL of Folin-Ciocalteau reagent (Merck, Darmstadt, Germany) were added to 500 μL of the coffee sample solution. Next, waiting 2 min, and 2mL of a 7.5% sodium carbonate solution was added. Next, the sample was incubated at 50ºC for 15 min, cooled for 4 min in ice and tempered at 25ºC during 5 min. The absorbance of the sample was measured at 760 nm in a spectrophotometer (Pharmacia LKB Biochrom, Germany). Gallic acid (GA) was used as reference and the results were expressed as mg GA per mL of coffee brew or per g of ground coffee.

**Determination of antioxidant capacity by ABTS assay.** The 2,2’-azinobis (3ethylbenzothiazoline-6-sulfonic acid) (ABTS) method for coffee brews was performed according to Rohn et al. (35). The radicals ABTS⁺ were generated by the addition of potassium persulfate to an ABTS solution prepared in phosphate buffered saline (pH 7.4). 0.1mL of each coffee brew sample diluted with demineralized water (1:200) was added to 0.5mL of 0.5mM ABTS solution. After the addition of 0.2mL of potassium persulfate, the absorbance was then measured spectrophotometrically at 734 nm in a spectrophotometer (Analytic Jena, Jena, Germany), after exactly 6 min. Calibration was performed with Trolox
solution (a water-soluble vitamin E analogue) and Total antioxidant capacity was expressed as \( \mu \text{mol Trolox per mL of coffee brew or per g of ground coffee.} \)

**Determination of antioxidant capacity by Electro Spin Resonance (ESR) spectroscopy.**

The ESR spectroscopy measurements were performed with Fremy’s salt and TEMPO as stabilized radicals with the same procedure described by Roesch et al. (36) and modified by Caemmerer & Kroh (18). When investigating with Fremy’s salt, 100 \( \mu \text{L} \) of every coffee brew sample diluted 100-fold with demineralized water were allowed to react with an equal volume of an aqueous 1 mM Fremy’s salt solution prepared in 50 mM phosphate buffer (pH 7.4). ESR spectra were recorded every 35 s for 30 min. When investigating with TEMPO, aliquots of 200 \( \mu \text{L} \) of coffee brew sample were allowed to react with 100 \( \mu \text{L} \) of 1 mM TEMPO solution. ESR spectra were obtained after 30 min, by which time the reaction was complete. Microwave power was set at 10 dB. Modulation amplitude, center field, and sweep width were set at 1.5, 3397, and 71 G, respectively. Both Fremy’s salt and TEMPO antioxidant activity were calculated as Trolox equivalents and expressed as \( \mu \text{mol Trolox per mL of coffee brew or per g of ground coffee.} \)

**Statistical analysis.** Each parameter was analyzed in triplicate. Results are shown as means ± standard deviations. A two-way analysis of variance (ANOVA) was performed to establish the impact of both the extraction method and the additive (sodium carbonate and bicarbonate) on antioxidant capacity parameters of coffee brew samples (Table 1). When interactions were significant, a one-way ANOVA was applied. A T-Tukey test was applied as a test *a posteriori* with a level of significance of 95%. Correlations among variables were assessed by means of the Pearson correlation test. All statistical analyses were performed using the SPSS v.15.0 software package.

**RESULTS AND DISCUSSION**
Influence of the coffee extraction method

The effects of the application of different extraction methods for preparing a coffee brew on the antioxidant capacity measured by colorimetric assays (Folin-Ciocalteau and ABTS) and ESR (Fremy’s Salt and TEMPO) are shown in Figures 1 and 2, respectively. In order to determine the efficiency of each brewing method for extracting the potential antioxidants of coffee, the results were expressed per g of ground roasted coffee.

The Folin-Ciocalteau assay is traditionally used for quantifying the Total Phenolic compounds in foods. Although the exact chemical nature of the Folin-Ciocalteau reagent is not known, it is believed that the molybdenum in the complex can be easily reduced by an electron-transfer reaction. Thus, the Folin-Ciocalteau reagent can be reduced by many electron-donors compounds (17). Total Phenolic compounds of the coffee brews were in the range of 37-55 mg GA per g of coffee (Figure 1a), similar to those results reported by other authors (37, 38).

The radical monocation of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS⁺) generated by oxidation of ABTS with potassium persulfate is a stable free radical with strong absorption in the range of 600-750nm. The radical cation reacts energetically with a Hydrogen-donor, such as phenolic compounds, being converted into a noncolored form of ABTS. The decay of the radical cation caused by the presence of antioxidants in a sample is monitored by the decolorization at 734nm in a spectrophotometer and compared to those of a Trolox solution. Figure 1b shows the results of the ABTS assay of the coffee brews. The results were in the range of 0.296-0.445 mmol Trolox per g of coffee, slightly higher than those reported by Brezova et al. (37), but 3-4 folds lower than those observed by Sanchez-Gonzalez et al. (38). This discrepancy could probably be due to the different coffee/water ratios and other technological parameters, but also due to the influence of the coffee type or blend. Thus, as reported by Lopez-Galilea et al. (21), blends with Torrefacto roasted coffee
(roasted with sugar) showed higher antioxidant capacity than conventional roasted coffee. In this work, the coffee used was Colombian Arabica conventional roasted coffee, whereas the coffee selected by Sanchez-Gonzalez et al. \cite{38} was a blend with 30% of Torrefacto coffee.

As already stated, the basis of the ESR spectroscopy is the absorption of microwave energy by unpaired electrons when they are in a magnetic field. This technique has been used for detecting and identifying free radicals generated by chemical reactions, including in vivo, but it is a rapid technique that is highly suitable for measuring the antioxidant capacity of a substance or product to scavenge a stabilized radical. The usage of different radicals permits us to distinguish between antioxidants with different structural properties \cite{19}. A wide range of antioxidants, mainly phenolic compounds, can be detected when Fremy’s salt is used as a stabilized radical, whereas TEMPO could be mainly scavenged by Maillard reaction products, such as melanoidins \cite{18,39}. The scavenging of two different synthetic stabilized radicals, Fremy’s salt (potassium nitrosodisulfonate) and TEMPO (2,2,6,6-tetramethyl-1-piperidin-1-oxyl), in coffee brews prepared with different extraction methods, and measured by the electron spin resonance (ESR) spectroscopy is shown in Figure 2. The results were in the range of 1.6-2.6 mmol Trolox and 5.7-9.5 μmol Trolox per g of ground roasted coffee in Fremy’s salt and TEMPO antioxidant capacity. The TEMPO antioxidant capacity level was much lower than the antioxidant capacity level of Fremy’s salt. This coincides with Bekedam et al. \cite{39} who reported that the contribution of the roasting-induced antioxidants to the overall antioxidant capacity is rather limited and that phenolic antioxidants evaluated by Fremy’s salt dominate the overall antioxidant capacity of coffee brews.

Several technological factors play an important role in the extraction of the antioxidant compounds. In previous works, Lopez-Galilea et al. \cite{22} and Andueza et al. \cite{24,27} observed that the concentration of 5-caffeoylquinic acid, the most abundant phenolic compound in coffee, and melanoidins, measured by the absorbance at 420nm, increased with pressure and
coffee/water ratio. Espresso coffeemaker had the highest water pressure of the espresso coffeemaker (15 bar maximum pump power) and the highest coffee/water ratio (7g per 40mL). Plunger and mocha are also pressure coffee extraction methods that work at 0.01-0.5 and 0.5 relative atmospheres, respectively (23), with the same coffee/water ratio (40g per 500ml). Filter coffee is an infusion method obtained by gravity at atmospheric pressure where hot water flows through medium ground roasted coffee (24g per 400mL), allowing a longer contact between water and coffee particles. The extraction time, ranking from the fastest one, which is espresso (30 seconds), to the slowest one, which is mocha (10 minutes), through plunger (5 minutes) and filter (7 minutes), could also influence the extraction of soluble potential antioxidants, together with other technological factors (water temperature, etc.).

It was observed that the mocha coffeemaker extracted the highest amount of antioxidants per g of ground roasted coffee, followed by the filter, espresso and then plunger coffeemakers, in all antioxidant capacity parameters, except Total Phenolic compounds. Other authors also reported higher ABTS antioxidant capacity in filter and mocha coffees than in espresso (20, 38). However, there was apparently a discrepancy with the results of DPPH antioxidant capacity obtained by Lopez-Galilea et al. (22) that proposed the following order: Filter<Plunger≤Mocha≤Espresso. Despite the fact that DPPH and ABTS assays might show different results due to the use of different radicals, when results of the previous work (22) were expressed per g of ground roasted coffee instead of per mL of coffee brew, DPPH antioxidant capacity maintained the same order and proportion as ABTS in this present work, showing that mocha coffeemaker extracted the highest DPPH reducing antioxidants. Consequently, although the highest water pressure in espresso coffeemaker favors antioxidant extraction, the shortest water-coffee contact time and the highest coffee/water ratio decrease the extraction efficiency (per g of ground roasted coffee) versus the other coffee brew techniques. In addition to the technological aspects of the coffee brew preparation, some
considerations regarding techniques for the measurement of antioxidant capacity should be
made in order to find possible explanations for the different order of coffee extraction
methods obtained with the Total Phenolic compound results measured by the Folin Ciocalteau
technique (Figure 1a) versus the other antioxidant capacity parameters.

Elucidating the antioxidants extracted in coffee brews

In order to elucidate the main responsible coffee antioxidants evaluated by the colorimetric
assays (Total Phenolic compounds and ABTS) and the ESR (Fremy’s salt and TEMPO) and
to attempt to clarify the discrepant results previously observed, three standard solutions (3.6
g/L) of chlorogenic acids (CGA), one dialyzed and one non-dialyzed melanoidins (MO-Ala
and MO-Gly, respectively) were analyzed. Both melanoidins were selected in order to
compare the antioxidant capacity of the high molecular weight melanoidins (dialyzed MO-
Ala) with the antioxidant capacity of a mixture of low, medium and high molecular weight
Maillard Reaction products (non-dialyzed MO-Gly) Figures 3 and 4 show the results
obtained.

As shown in Figure 3a, although the colorimetric reaction was much higher in the CGA
solution (0.42mg GA/mL), the melanoidin solutions also produced colorimetric reaction,
acting as electron-donors. Caemmerer & Kroh (18) and Bekedam et al. (39) proposed that the
antioxidant capacity of coffee melanoidins measured by the Folin-Ciocalteau method could be
due to both melanoidins and to the phenolic compounds incorporated into the melanoidin
skeleton. However, in the present experiment, the melanoidins were synthesized using only
Maillard reaction precursors (Maltooligosaccharides and Alanine or Glycine aminoacids).
Thus, the colorimetric reaction in the melanoidin model systems could only be due to the
Maillard reaction products, not to the presence of phenolic compounds. Consequently, it is
clear that the Folin-Ciocalteau assay evaluates not only the phenolic compounds, but also the
reducing or antioxidant capacity of other non-phenolic chemical compounds, such as Maillard reaction products like melanoidins.

**Figure 3b** shows the antioxidant capacity measured by the ABTS assay of the chlorogenic acids and melanoidin solutions. All of the solutions reacted with the ABTS$^{+}$, but chlorogenic acids exhibited three times more antioxidant capacity than melanoidins. This coincides with the contribution of the melanoidins to the total antioxidant capacity in coffee measured by ABTS reported by other authors (9), showing their lower effectiveness as Hydrogen-donors vs phenolic compounds. It is well known that the radical ABTS$^{+}$ reacts with any hydroxylated aromatic compound regardless of its antioxidant potential *in vitro* or *in vivo*. Even though at least 45 chlorogenic acids have been identified in coffee brews, caffeoylquinic acids (CQA), and mainly 5-CQA, are the most abundant phenolic compounds (around 86%) (5, 40). The antioxidant activity of these chlorogenic acids is due to the electron donating effects on the ring of both the COOH-CH=CH- and the dihydroxylation in the 3,4 position in caffeic acid (3,4-dihydroxy-cinnamic acid) that enhance the availability as hydrogen donors (41).

Consequently, both Folin-Ciocalteau and ABTS assays which are based on similar electron-transfer redox reactions, can be used to evaluate the overall antioxidant capacity in a simple way but they can not distinguish antioxidant capacity due to phenolic and non-phenolic chemical compounds, such as Maillard reaction products. Moreover, the different Folin-Ciocalteau results obtained for both dialyzed and non-dialyzed melanoidin model systems might partially explain the different order of coffee extraction methods in Total Phenolic compounds results because each method could favor the extraction of low, medium or high molecular weight Maillard reaction products with different antioxidant activity (39).

The electron spin resonance (ESR) spectroscopy may be used to measure the degree of antioxidant effectiveness of a substance by the ability to scavenge a synthetic stabilized free
radical, such as Fremy’s salt, TEMPO or others. In comparison to the more often used colorimetric assays, the ESR is much more specific due to the formation of the characteristic signals. Moreover, ESR is a direct measurement of the unpaired electrons, avoiding the use of the indirect measurement of the discoloration of radicals. Although standardization is needed, it is less subjected to the influence of the medium conditions (pH, solvents, etc.). Figure 4a shows that Fremy’s salt was mainly scavenged by chlorogenic acids, whereas only small traces were observed with melanoids. Bekedam et al. (39) also observed that other antioxidants, such as Trolox and ascorbic acids, can scavenge this stabilized radical with reaction velocities similar to those of chlorogenic acid, in a nonspecific manner. However, Figure 4b shows that the stabilized radical TEMPO was effectively scavenged by both melanoids, but not by chlorogenic acid. Therefore, by using both Fremy’s salt and TEMPO as a stabilized radical, a distinction could be made between phenolic and nonphenolic antioxidants (18, 39), resulting in a more precise assessment of the antioxidant activity of the sample. Moreover, TEMPO results also show that melanoids have different antioxidant capacities, depending on the structure and composition. MO-Gly was a non-dialyzed melanoidin, and consequently, it had several low molecular weight Maillard Reaction products that could interfere with the antioxidant capacity of the pure melanoidin, but this model system was more similar to the coffee matrix than the MO-Ala which was a dialyzed melanoidin and contains mainly high molecular weight structures.

Taking into account the results from the solutions, and in comparison with the other coffee makers, it could be said that it appears to be quite clear that mocha coffee maker extracted more antioxidant capacity per g of ground roasted coffee due to both the chlorogenic acids (Fremy’s salt) (Figure 2a) and the melanoids (TEMPO) (Figure 2b). In fact, if results of the previous work of Lopez-Galilea et al. (22) are expressed in terms of the extraction of 5-CQA, i.e. mg per g of coffee, it could be observed that filter and mocha
coffeemakers extracted the highest amount of this chlorogenic acid (7.0 and 6.8 mg/g in conventional coffee, respectively) followed by plunger (5.5 mg/g) and espresso (3.9 mg/g), showing a similar behavior of the Fremy’s salt antioxidant capacity (Figure 2a) that was mainly scavenged by chlorogenic acids. Moreover, in the same previous work of Lopez-Galilea et al. (22), when browned compound concentration were corrected in terms of the extraction of browned compounds, i.e. per g of ground roasted coffee, it could also be observed that mocha extracted the highest amount of browned compounds followed by the other coffeemakers (filter, plunger and espresso), such as in the TEMPO antioxidant capacity (Figure 2b). Thus, it seems clear that TEMPO is positively related to the extraction of browned compounds in coffee, mainly melanoidins.

**Influence of the addition of acidity correctors to coffee brew**

Ready-to-drink coffee beverage consumption has been increased in last few decades (23). In this type of products, other ingredients or additives, mainly milk derivatives, are usually added to preserve the coffee brew quality, but no studies regarding their influence on the antioxidant capacity have been found. In a previous work carried out by our research group, the addition of sodium carbonate or sodium bicarbonate, as pH-regulator agents, to black coffee brews has been proposed to extend the shelf life (33).

For the study of the influence of the additives on the antioxidant capacity of coffee, three aliquots of each coffee brew were separated: one as reference, the second with the addition of 75 ppm of sodium carbonate and the third with the addition of 75 ppm of sodium bicarbonate. The selection of the additives and their concentrations were mainly based on sensorial parameters and reported in a previous paper (33). Sodium carbonate (75 ppm) and sodium bicarbonate (75 ppm) water solutions were used as blanks and no reactions in any of the antioxidant capacity parameters were observed. Thus, the differences in the antioxidant capacity among coffee brews prepared with the same coffeemaker, but with different
additives, were only due to the influence of the additives on the antioxidant compounds involved in each technique.

A two-way ANOVA was performed to establish the impact of the additive addition and the brewing extraction method on the antioxidant parameters (Table 1). In most cases, significant interaction between the additive and the extraction method has been observed. TEMPO antioxidant capacity was significantly affected by both factors without interaction between them. Moreover, F values corresponding to extraction method were higher than the F values of the additives for all the antioxidant capacity parameters, showing greater importance of the extraction method effect that has been previously discussed.

The effects of the acidity regulator addition on the antioxidant capacity measured by colorimetric assays (Folin-Ciocalteau and ABTS) and ESR (Fremy’s salt and TEMPO) are shown in Figures 5 and 6, respectively. Results are expressed per mL of coffee in order to facilitate the knowledge of the antioxidant intake. In all parameters, coffee extraction methods were ranking in the same order, showing the highest antioxidant capacity per mL in espresso coffee, following by mocha, plunger and filter. As discussed above, these results coincide with those reported by Lopez-Galilea et al. (22), using DPPH and redox potential antioxidant parameters, and by Sanchez-Gonzalez et al. (38) using the Folin-Ciocalteau technique.

Total Phenolic compounds, with the exception of the filter and plunger coffee brews, were significantly different in coffee brews, with or without additives (Figure 5a). Taking into account the fact that, the antioxidant quantities had to be the same in each extraction method because of the experimental design, it could be said that the slight increase of the pH in coffee brews with both pH-regulator agents (Table 2) could change the H-donating ability of the antioxidant compounds, or induce some other phenolic or non-phenolic compounds into becoming in potential reducers or oxidants.
When antioxidant capacity was measured by ABTS method, no significant differences were found between reference coffee brew and the coffee brews with additives (sodium carbonate and bicarbonate) in filter and plunger coffee brews. However, the addition of the pH-regulator agents significantly decreased the ABTS scavenging activity of coffee brews extracted with mocha and espresso coffee machines, possibly because the addition of these additives might influence the Hydrogen-donor capacity of the antioxidants when ABTS antioxidant capacity is higher and acidity is also slightly higher. The application of Pearson Correlation statistical test showed a highly significant excellent correlation between the Total Phenolic compounds and the radical scavenging ABTS activity (0.914, p<0.001). This might be because both techniques are based on similar electron-transfer redox reactions, but also because both assays evaluate the antioxidant capacity of both phenolic and non-phenolic compounds as shown in Figure 3.

Figure 6a shows a similar behavior of the Fremy’s salt antioxidant capacity of coffee brews in comparison to ABTS. Mocha and espresso coffee brews, with the highest antioxidant capacity, were also more affected by the addition of sodium carbonate and bicarbonate than the others. As Fremy’s Salt mainly evaluates the antioxidant capacity due to chlorogenic acids, this similar behavior between Fremy’s Salt and ABTS appears to point out that the acidity regulators influence the antioxidants electron distribution and, consequently, their Hydrogen-donor capacity. Excellent correlations between Fremy’s Salt antioxidant capacity and Total Phenolic compounds (0.900, p<0.001) and ABTS (0.819, p<0.01) parameters in coffee brews were found. This could be because chlorogenic acids showed the highest antioxidant activity in these three assays.

The antioxidant capacity measured by the radical scavenging of TEMPO (Figure 6b) evaluates the antioxidant capacity due to the Maillard reaction products (Figure 4b). As no statistical interaction between both factors, the extraction method and the additive, was found
in Two-way ANOVA, it is possible to study both variables separately. A gradual, but significantly, increase in the TEMPO antioxidant capacity was observed while at the same time the browned compounds increased (22). When the effect of the additive was studied, it could be said that sodium bicarbonate increased the TEMPO antioxidant capacity, but no significant differences (p<0.05) in sodium carbonate coffee brews were found. Although the change of pH might be a factor that influences the antioxidant activity of melanoidins or other Maillard reaction products, it could be said that it is not the most relevant. For this reason, further investigations of the effects of pH-regulator agents on the different coffee melanoidins or other Maillard reaction products should be carried out.

In conclusion, while mocha coffeemaker seems to have the highest yield per g of ground roasted coffee in coffee antioxidants extraction, espresso coffee is the richest in terms of antioxidant intake (per mL of coffee brew). Moreover, the measurement of the coffee antioxidant ability to scavenge synthetic stabilized free radicals, such as Fremy’s salt and TEMPO, by electron spin resonance (ESR) spectroscopy allows us to distinguish between phenolic and nonphenolic antioxidants, such as Maillard reaction products and melanoidins. The addition of pH-regulator agents to coffee, such as sodium carbonate and bicarbonate, to extend its shelf-life slightly changes the pH, modifying the antioxidant capacity in those coffee brews with the highest capacity (mocha and espresso). In addition to the importance of clarifying which antioxidant compounds are evaluated by each colorimetric or spectroscopic assays, the application of ESR may contribute to the knowledge of whether the antioxidant capacity is due to the presence of phenolic compounds that can be bioavailable (42) or to the melanoidins or other Maillard reaction products whose bioavailability is still unclear but may act as prebiotic compounds (43).

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Table 1. Two-Way ANOVA results of coffee antioxidant capacity parameters

<table>
<thead>
<tr>
<th></th>
<th>Additive effect</th>
<th>Coffee extraction method effect</th>
<th>Interaction effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>p</td>
<td>F</td>
</tr>
<tr>
<td>Total Phenolic compounds</td>
<td>19.097</td>
<td>&lt;0.001</td>
<td>2910.130</td>
</tr>
<tr>
<td>ABTS</td>
<td>13.867</td>
<td>&lt;0.001</td>
<td>248.237</td>
</tr>
<tr>
<td>Fremy’s Salt</td>
<td>34.127</td>
<td>&lt;0.001</td>
<td>322.160</td>
</tr>
<tr>
<td>TEMPO</td>
<td>24.777</td>
<td>&lt;0.001</td>
<td>184.074</td>
</tr>
</tbody>
</table>
Table 2. pH and Total Solids results of the coffee brews with additives

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>Total Solids (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filter</td>
<td>Reference</td>
<td>5.2 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Na₂CO₃</td>
<td>5.4 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>NaHCO₃</td>
<td>5.3 ± 0.0</td>
</tr>
<tr>
<td>Plunger</td>
<td>Reference</td>
<td>5.2 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Na₂CO₃</td>
<td>5.4 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>NaHCO₃</td>
<td>5.3 ± 0.0</td>
</tr>
<tr>
<td>Mocha</td>
<td>Reference</td>
<td>5.1 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Na₂CO₃</td>
<td>5.3 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>NaHCO₃</td>
<td>5.2 ± 0.0</td>
</tr>
<tr>
<td>Espresso</td>
<td>Reference</td>
<td>5.1 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Na₂CO₃</td>
<td>5.3 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>NaHCO₃</td>
<td>5.2 ± 0.0</td>
</tr>
</tbody>
</table>
FIGURE CAPTIONS

Figure 1. Total phenolic compounds (a) and ABTS antioxidant capacity (b) extracted from ground coffee by filter, plunger, mocha and espresso methods. Bars with different letters are significantly different (p<0.05).

Figure 2. Fremy’s Salt (a) and TEMPO (b) antioxidant capacity extracted from ground coffee by filter, plunger, mocha and espresso methods. Bars with different letters are significantly different (p<0.05).

Figure 3. Total phenolic compounds (a) and ABTS antioxidant capacity (b) of the chlorogenic acid (CGA), the maltooligosaccharide-Alanine (MO-Ala) and the maltooligosaccharide-Glycine (MO-Gly) solutions (3.6 g/L).

Figure 4. Fremy’s Salt (a) and TEMPO (b) antioxidant capacity of the chlorogenic acid (CGA), the maltooligosaccharide-Alanine (MO-Ala) and the maltooligosaccharide-Glycine (MO-Gly) solutions (3.6 g/L).

Figure 5. Effect of the extraction method and the sodium carbonate and bicarbonate addition on the Total Phenolic compounds (a) and ABTS antioxidant capacity (b) of coffee brews. Bars with different letters are significantly different (p<0.05).

Figure 6. Effect of the extraction method and the sodium carbonate and bicarbonate addition on the a) Fremy’s salt and b) TEMPO Antioxidant Capacity of coffee brews. Bars with different letters are significantly different (p<0.05).
Figure 1. Total phenolic compounds (a) and ABTS antioxidant capacity (b) extracted from ground coffee by filter, plunger, mocha and espresso methods. Bars with different letters are significantly different (p<0.05).

a) Total Phenolic compounds

![Graph showing total phenolic compounds extracted from ground coffee by different methods.](image)

b) ABTS antioxidant capacity

![Graph showing ABTS antioxidant capacity extracted from ground coffee by different methods.](image)
Figure 2. Fremy’s Salt (a) and TEMPO (b) antioxidant capacity extracted from ground coffee by filter, plunger, mocha and espresso methods. Bars with different letters are significantly different (p<0.05).

a) Fremy’s Salt

b) TEMPO
Figure 3. Total phenolic compounds (a) and ABTS antioxidant capacity (b) of the chlorogenic acid (CGA), the maltooligosaccharide-Alanine (MO-Ala) and the maltooligosaccharide-Glycine (MO-Gly) solutions (3.6 g/L).

a) Total phenolic compounds

![Graph showing total phenolic compounds for CGA, MO-Ala, and MO-Gly](image)

b) ABTS antioxidant capacity

![Graph showing ABTS antioxidant capacity for CGA, MO-Ala, and MO-Gly](image)
**Figure 4.** Fremy’s Salt (a) and TEMPO (b) antioxidant capacity of the chlorogenic acid (CGA), the maltooligosaccharide-Alanine (MO-Ala) and the maltooligosaccharide-Glycine (MO-Gly) solutions (3.6 g/L).

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b) TEMPO
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a) Total Phenolic compounds

b) ABTS antioxidant capacity
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a) Fremy’s Salt

b) TEMPO