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## Effect of Cellulases, Solvent Type and Particle Size Distribution on the Extraction of Chlorogenic Acid and Other Phenols from Spent Coffee Grounds

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**Abstract:** Spent coffee grounds, wastes resulting from the industrial preparation of instant coffee, were subjected to solid-liquid extractions to study the influence of some critical variables on the phenol content of extracts. After grinding, spent coffee grounds were passed through several sieve sizes (125, 250, 500 and 1000  $\mu\text{m}$ ) and classified into four different particle size groups. The highest yields of total phenols were consistently obtained from the smallest particles and an unexpected reduction in the phenol release was observed when extraction was assisted by cellulase treatment. Aqueous ethanol (60% w/w) was the solvent having the highest phenol-extractive capacity, closely followed by aqueous methanol, whose extracts were ~30% more concentrated than when pure water was used as the solvent. Phenol concentration values obtained from the different treatments ranged from 115-400 mg equivalents of chlorogenic acid/l. HPLC analysis confirmed chlorogenic acid as the major phenolic acid being extracted from spent coffee grounds. Chromatograms of extracts obtained after the enzyme treatment showed that cellulases catalyzed the transformation of chlorogenic acid, resulting in a derivative with similar spectrum, but shorter retention time. Results shown in this study are a first step for further research on the extractive conditions maximizing extraction efficiency from spent coffee grounds, confirming the feasibility of upgrading spent coffee grounds as a promising source of chlorogenic acid, which may be used in biofunctional dietary supplements.

**Key words:** Bulk density, chlorogenic acid, particle size distribution, polyphenols, solid-liquid extraction, spent coffee grounds

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### INTRODUCTION

Coffee is a popular beverage which is consumed worldwide, with annual production costs of ~5,000 billion US Dollars (FAOSTAT-FAO Statistical Database, 2005). In the preparation of instant coffee, the processing of raw coffee powder leads to accumulation of unutilized spent coffee grounds (Leifa *et al.*, 2001). The latter are not suitable for compost recycling due to the high levels of phenol compounds present, which have been reported to be responsible for certain plant germination problems (Singh *et al.*, 1999). Coffee beans, including roasted coffee beans are rich in phenolics, particularly chlorogenic acid and other hydroxycinnamate-quinic acid derivatives (Clifford and Ramirez-Martinez, 1991; Clifford, 2000). On this basis we hypothesized that spent coffee grounds might be upgraded by extracting the phenols, with the prospect that the extracted phenols could find use as food preservatives or dietary supplements for disease prevention. Dietary phenolics, including plant food

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derived flavonoids and phenolic acids, have been suggested to act as physiologically beneficial antioxidants and to perhaps have a role in lowering the incidences of coronary heart disease, thrombotic and atherogenic processes in humans (Morton *et al.*, 2000).

Chlorogenic acid (3-caffeoyl-D-quinic acid), a cell wall bound ester formed between caffeic acid and quinic acid, is one of the major phenolic compounds present in coffee as well as in spent coffee grounds (Petracco, 2005). Both chlorogenic acid and caffeic acid are absorbed in humans (Olthof *et al.*, 2001). Chlorogenic acid is a naturally occurring plant growth regulator also present in fruits, berries, potatoes, tea and other plant foods. Chlorogenic acid exerts strong antioxidant activity oxidation of human low-density lipoproteins *in vitro* (Meyer *et al.*, 1998a). It inhibits human DNA methylation *in vitro* (Lee and Zhu, 2006) and retards formation of mutagenic and carcinogenic N-nitroso compounds *in vitro* (Kono *et al.*, 1995).

Several factors affecting the extraction of phenolic compounds need to be considered in order to optimize extraction efficiency of phenols from plant materials (Pinelo *et al.*, 2005a). Focus has been held on the influence of temperature, solvent-solid ratio and time of contact (Pinelo *et al.*, 2005b). But the choice of an appropriate solvent is another important factor to consider, where ethanol has been reported to be a very effective solvent for phenols from e.g., grape pomace (Pinelo *et al.*, 2006b). Enzyme assisted extraction using plant cell wall degrading enzymes has been demonstrated to improve the phenol release from various fruit press residues (Landbo and Meyer, 2001; Meyer *et al.*, 1998). Likewise, we hypothesized that plant cell wall degrading enzymes could help in the degradation of the coffee grounds matrix and in this fashion increase the amount of phenols in extracts. The reduction of the particle size has also been shown to be a positive factor for the recovery of phenols (Landbo and Meyer, 2001). An excessive reduction of the particle size may however promote the formation of packaging phenomena and preferential ways for the solvent, limiting an efficient wetting of the solid and decreasing the extraction efficiency (Pinelo *et al.*, 2006a).

The objective of this study was to evaluate the possibilities for valorization of spent coffee grounds via extraction of the phenolics, notably chlorogenic acid. An assessment of the fitness of selected aqueous solvents (aqueous ethanol, aqueous methanol and pure water) as well as the influence of the particle size distribution on phenols yields were included to obtain clues for maximization of the phenol release and extraction efficiency.

Spent coffee grounds were divided into four different particle size distribution groups and subjected to liquid-solid extraction with the three different solvents. The phenol concentrations of extracts were compared with regard to both the total surface area resulting from the different particle size groups and the employed solvent. In addition, based on the hypothesis that enzymatically catalyzed hydrolysis of the spent coffee ground matrix could enhance porosity of the particles and in turn increase phenols yields, plant cellulase catalyzed degradation of the spent coffee grounds was evaluated as a pretreatment prior to solvent extraction. In addition to measurements of total phenols, profiling of the phenolics by HPLC analysis was included to specifically assess the presence of chlorogenic acid in the spent ground coffee extracts.

## MATERIALS AND METHODS

### Sample Preparation

The raw material was spent coffee grounds from Arabica coffee (*Coffea arabica*), obtained from Cafinco, SA. de C.V. Cordoba, Veracruz, Mexico. The coffee grounds were vacuum packed and sent by air mail to The Technical University of Denmark for the study. The spent coffee grounds were divided in aliquots in air tight containers, flushed with nitrogen and kept frozen (-30°C) until use. The chemicals used were of analytical degree. Gallic acid and Chlorogenic acid were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO). Ethanol, Methanol, Folin-Ciocalteu phenol reagent and sodium

carbonate were obtained from Merck (Darmstadt, Germany). Spent coffee grounds were milled for 90 sec in a water-cooled IKA-Universal mill model H20 (Jahnke and Kunkle, Staufen, Germany) and separated into different particle size groups by use of a four-sieved sieving tower (Endecotts Filters Ltd., London, UK) with aperture size of 1000, 500, 250 and 125  $\mu\text{m}$ , respectively. The sieving tower was hand shaken for 30 sec to obtain the samples of different particle sizes.

#### **Carbohydrate Composition by HPAEC Analysis**

The carbohydrate composition of the spent coffee grounds was assessed by High Performance Anion Exchange Chromatography (HPAEC) after hydrolysis with trifluoro acetic acid according to De Ruiter *et al.* (1992). HPAEC separation and quantification of monosaccharides were performed according to Obro *et al.* (2004) using a BioLC system equipped with a CarboPac™ PA20 (3×150 mm) analytical column and an ED50 electrochemical detector (Dionex Corp., Sunnyvale, CA). The monosaccharides were eluted isocratically with 2.5 mM NaOH for 20 min followed by isocratic elution at high NaOH concentration 500 mM for 10 min to elute any present acidic monosaccharides (galacturonic and glucuronic). Before each injection (10  $\mu\text{L}$ ) a column reequilibration program was run for 5 min with 100 mM NaOH followed by 5 min with 2.5 mM NaOH. Eluent flow-rate was 0.5 mL  $\text{min}^{-1}$ . Data were collected and analyzed on a PC equipped with the Chromeleon 6.60 Sp2 Build 1472 software (Dionex Corporation, USA).

#### **Extraction**

The extraction of phenols was done in duplicate by using three different aqueous solvents: 60% ethanol, 60% methanol and distilled water. For each extraction, 2 g of spent coffee grounds of each particle size were dispersed in 20 mL solvent and poured into 50 mL centrifugal tubes (all extractions were done in triplicate). The centrifugal tubes were shaken for 10 min at room temperature (20°C) in a tube shaker (J.P. Selecta, Barcelona, Spain). The supernatant solutions were obtained after filtering for 6 min under vacuum.

#### **Enzyme Assisted Extraction**

Two grams of spent coffee samples were incubated with a mixture of the following two enzyme preparations: I) A cellulolytic enzyme preparation derived from *Trichoderma reesei*, Celluclast 1.5 L, dosage: 8 Filter Paper Units  $\text{g}^{-1}$  total solids and II) a  $\beta$ -glucosidase enzyme preparation from *Aspergillus niger*, NS 188, dosage: 13 cellobiose units  $\text{g}^{-1}$  solids-the enzyme preparations were obtained from Novozymes Inc., Bagsværd, Denmark. Enzyme hydrolysis experiments were conducted at the conditions of pH and temperature advised by the enzyme manufacturer (pH = 5 and T = 50°C) and reactions were run for 24 h. After the enzymatic treatment, the proper solvent was added and extraction was carried out at the same extraction conditions as explained above.

#### **Total Phenolics**

Total phenolics were determined by the Folin-Ciocalteu method (Singleton and Rossi, 1965). Each sample (0.2 mL) was mixed with 1 mL of ten-fold diluted Folin-Ciocalteu reagent and after shaking for 1 min, 0.8 mL of a 7.5% w/v sodium carbonate solution were then added. After 2 h, the absorbance of the mixture was measured at 765 nm.

The content of phenolics was expressed as chlorogenic acid equivalents (from a standard curve of chlorogenic acid subjected to the Folin-Ciocalteu method), as chlorogenic acid was the major phenolic acid found in the analyzed extracts.

#### **HPLC Analysis**

HPLC analysis was carried out according to the procedure described by Lamuela-Raventós and Waterhouse (1994) using a Hewlett Packard (Houston, TX, USA) 1100 system equipped with a diode

array detector and a Nova-Pak C18 column (150×3.9 mm, Waters, Milford, MA, USA) and controlled by a PC with HPChem station software. Phenolic compounds (hydroxycinnamates) were identified by spectral and retention time analysis at 316 nm.

### **Statistical Analysis**

The results reported in this work are the average of at least three measurements and the coefficients of variation, expressed as the percentage ratio between Standard Deviations (SD) and the mean values, were found to be <10% in all cases. Significant variables were calculated, subjecting results to a linear regression, using the SPSS statistical program version 10.0 (SPSS Inc., Chicago, IL). Only variables with a confidence level superior to 95% ( $p < 0.05$ ) were considered to be significant.

## **RESULTS AND DISCUSSION**

### **Carbohydrate Composition**

The main monosaccharides in the spent coffee grounds were mannose and glucose, indicating a significant presence of mannan and cellulose in the spent coffee grounds (Table 1). The monosaccharides profile also included arabinose, xylose, rhamnose and galacturonic acid signifying presence of low amounts of hemicellulose and pectin (Table 1). The sum of the monosaccharides only made up ~15% by weight of total dry matter of the spent coffee grounds indicating that other substances, presumably lignin, made up a significant portion of the dry matter. The relatively high proportion of glucose, indicating presence of cellulose, provided a basis for evaluating the effect of enzymatic cellulose degradation for enhancing the yields of phenols (treatment with a pectinase enzyme preparation was also evaluated, giving no effect on yields (data not shown).

### **Particle Size**

To study the effect of the particle size, spent coffee grounds were passed through four different sieves (125, 250, 500 and 1000  $\mu\text{m}$ ) after grinding. Figure 1 shows the phenol concentration of the extracts obtained from the different coffee particle sizes by using aqueous ethanol, aqueous methanol and water as solvents. Results obtained by performing the solvent extractions directly were compared with those from the extractions after pretreatment with cellulases. As can be observed, the phenolic content of extracts was enhanced concomitantly with decreasing particles sizes regardless of the use of enzymes, following a logarithmic trend in all cases. As chlorogenic acid was found to be the main phenolic acid present in coffee all the values of phenol concentration have been given in chlorogenic acid equivalents, despite gallic acid being the conventionally used standard in total phenols analyses. Gallic acid and chlorogenic acid differ in the number of hydroxyl groups in the structure-gallic acid has three hydroxyl groups, chlorogenic acid two, which is why the Folin-Ciocalteu results will vary depending on which one of the standards is used. With gallic acid the phenol concentration ranged from 60 (water, 1000  $\mu\text{m}$ ) to 160 mg gallic acid equivalents  $\text{L}^{-1}$  (ethanol, 125  $\mu\text{m}$ ) and the values were from 115 to 400 chlorogenic acid eq.  $\text{L}^{-1}$  at the same conditions. Presumably, when the particle size is

Table 1: Monosaccharide composition, incl. galacturonic acid ( $\text{mg g}^{-1}$ ), of spent coffee grounds as determined by HPLC

Monosaccharide	Level ( $\text{mg g}^{-1}$ )
Rhamnose	0.70
Arabinose	2.81
Galactose	6.60
Glucose	30.70
Xylose	0.70
Mannose	99.60
Galacturonic acid	5.27

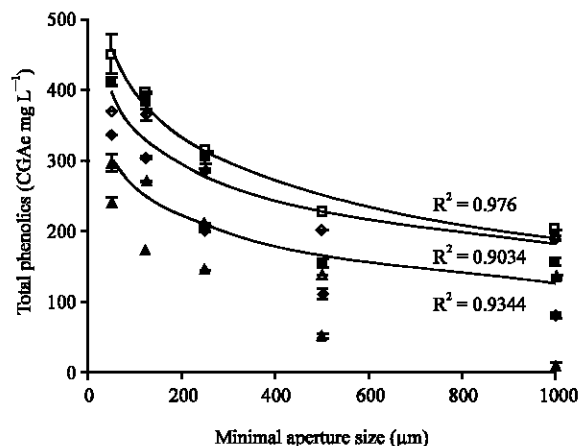


Fig. 1: Phenol concentration of extracts from spent coffee grounds with four different particle size distributions. □: extraction with ethanol, ■: enzymatic hydrolysis followed by extraction with ethanol, ◇: extraction with methanol, ◆: enzymatic hydrolysis followed by extraction with methanol, △: extraction with water, ▲: enzymatic hydrolysis followed by extraction with water. The regression coefficients of the fitted logarithmic curves for the non-enzyme pre-treated samples are also shown

reduced the accessible surface for both the extractive solvent and the enzyme attack is increased, resulting in the observed enhancement of the extraction efficiency. Enzymatic treatment using cellulases did not result in an increase but rather in a reduction of the phenol concentration of extracts. This could be ascribable to spontaneous degradation or oxidation of chlorogenic acid during the 24 h of enzymatic treatment or be due to the presence of some undesirable side activities of the Celluclast 1.5 L or in the NS188 preparation. The enzyme preparation could contain enzymes with oxidase or depsidase activity, promoting the oxidation or the cleavage of the released phenols. Such adverse effects of enzymatic treatments in novel applications have been previously observed with *Aspergillus niger* derived enzyme preparations (Landbo and Meyer, 2001; Wrolstad *et al.*, 1994).

No significant variation of bulk density was observed for particles ranging between 500 and 1000 μm, whilst a dramatic density decrease was observed for those from 500 to 250 and 125 μm (Table 2). This difference indicated the difficulty of the small particles to pack tightly, resulting in an unexpected decrease of the bulk density when particle size decreased. Powder surface area of the different groups of particles was calculated from the values of particle volume and bulk density and is also indicated in Table 2. In complete accordance with the density and particle volume data the particle surface area and total surface area per sample increased significantly with decreased particle size (Table 2).

Figure 2 shows the ascendant trend of the phenol concentration values with total surface area, bearing out the absence of packaging phenomena during the solvent wetting and the importance of the contact area in the mass transfer of the solid-liquid extraction processes. The positive effect of reducing the particle size have been previously reported in other studies about phenolic extraction; it has given very positive results in other plant materials like black currant pomace and black cohosh (Landbo and Meyer, 2001; Mukhopadhyay *et al.*, 2006). However, positive effects of reducing the particle size are not always obvious, as a reduction of the particle size can obstruct the solvent access to all the exposed surface area of the solid and release the phenol compounds (Pinelo *et al.*, 2005a). In this case, however, the low capacity of aggregation of the smaller coffee particles has prevented the eventual packaging

Table 2: Determination of the bulk density and surface area of the different particle size distribution groups

Sieve size ( $\mu\text{m}$ )	Weight measured (g)	Volume measured (mL)	Bulk density ( $\text{kg m}^{-3}$ )	Particle volume ( $\text{m}^3$ )	Particle surface area ( $\text{m}^2$ )	Weight of particle (kg)	No. of particles	Total surface area ( $\text{m}^2$ )
1000	0.203	0.387	524	$5.236 \times 10^{-10}$	$3.142 \times 10^{-6}$	$2.746 \times 10^{-7}$	738543	2.320
500	0.202	0.362	559	$6.545 \times 10^{-11}$	$7.854 \times 10^{-7}$	$3.657 \times 10^{-8}$	5526369	4.340
250	0.205	0.673	304	$8.181 \times 10^{-12}$	$1.964 \times 10^{-7}$	$2.486 \times 10^{-9}$	82298130	16.159
125	0.204	0.910	224	$1.023 \times 10^{-12}$	$4.909 \times 10^{-8}$	$2.291 \times 10^{-10}$	889841653	43.680

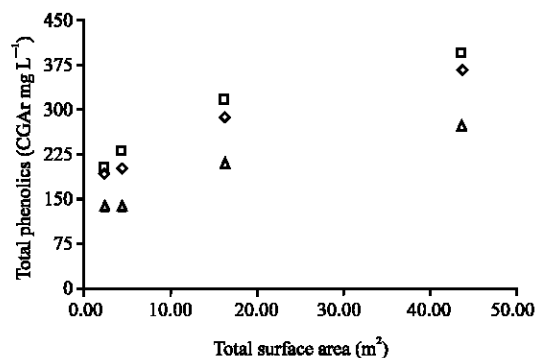


Fig. 2: Phenol concentration of extracts from spent coffee grounds vs the total surface area of four particle size distributions.  $\square$ : extraction with ethanol,  $\diamond$ : extraction with methanol,  $\triangle$ : extraction with water

negative effects. As a consequence, the smaller the particle size of the spent coffee grounds, the higher the extraction efficiency and thus the higher the phenol concentration of the resulting extracts. These results are in complete agreement with our previous data from direct extraction of phenolics from e.g., black currant juice press residues and grape pomace (Landbo and Meyer, 2001; Meyer *et al.*, 1998b).

#### Effect of Solvent

As can be observed in both Fig. 1 and 2, extraction with aqueous ethanol consistently resulted in extraction of higher amounts of phenols from the coffee particles, followed by methanol and water. This trend was independent of the particle size. Slight differences were found between the phenolic concentration in the ethanolic and methanolic extracts, whilst in the aqueous extracts the values were consistently approximately 30% lower than in the alcoholic ones. Alcohols have already been reported to be more efficient than water in extracting phenols from very different natural matrices and many examples can be cited (e.g., citrus peel, black tea, barley) (Turkmen *et al.*, 2006; Zia, 2006). Aqueous extracts with increasing ethanol concentrations were tested in order to find out the capacity to extract phenols and a linear relationship was found between the ethanol percentage in the solvent and the phenol concentration of extracts (data not shown), confirming again the higher extractive capacity of ethanol. Methanol has also provided very positive results in other studies on the phenol extraction from natural matrices, although the tendency in the food industry is progressively to substitute use of methanol.

#### Nature of Extracted Phenolics

An iteration between the chromatographic elution and the spectral analysis revealed that three different chlorogenic acid isomers, but no caffeic acid (or hardly any) was detected in the ethanolic extracts (Fig. 3a and 4); Fig. 3b and c show the ethanol extract chromatogram in which extra chlorogenic acid and extra caffeic acid were added, respectively. Previous studies have shown that

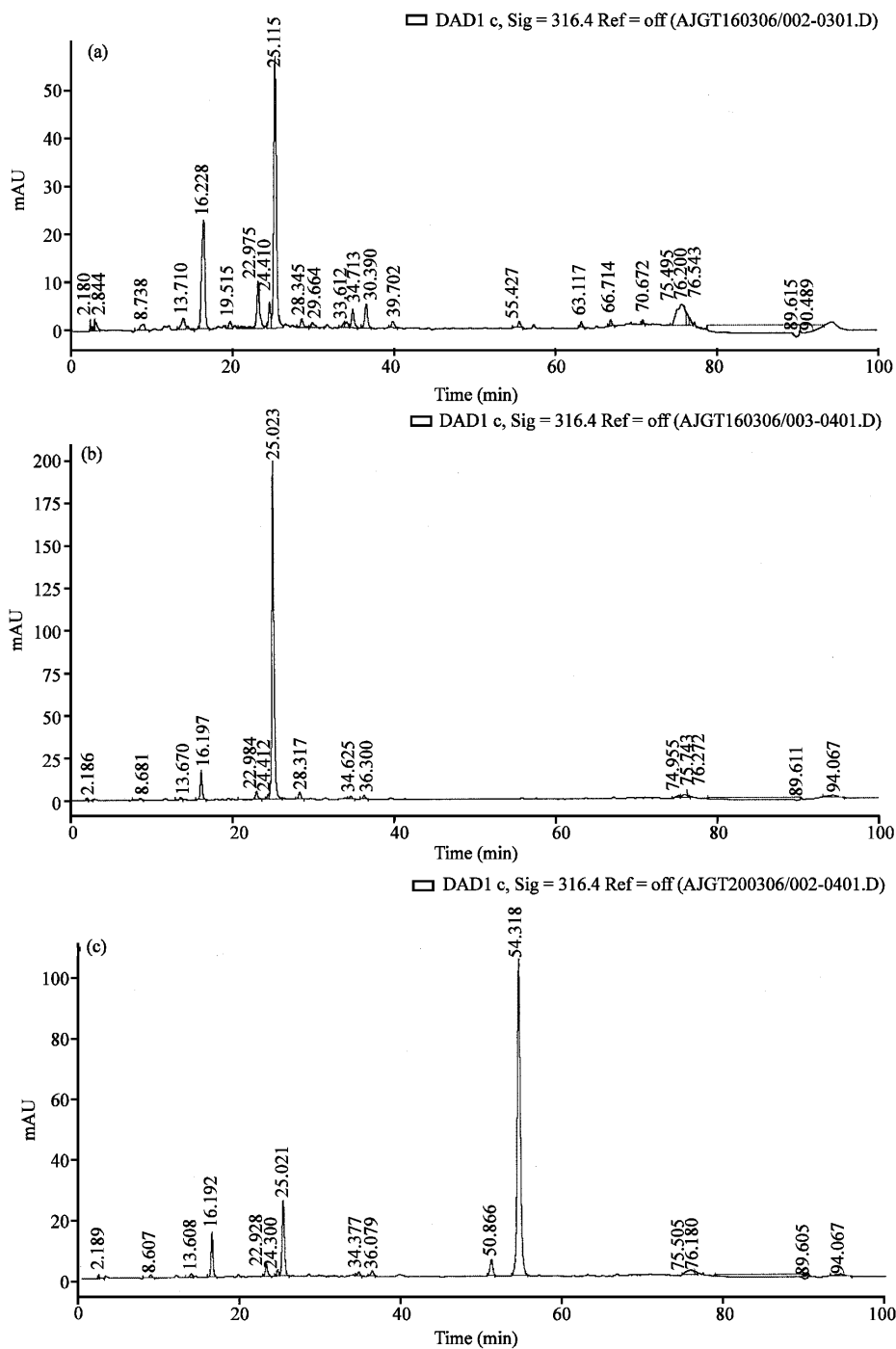


Fig. 3: (a) HPLC chromatogram of the ethanol extract of 0.125  $\mu$ m spent coffee ground particles. (b) Same chromatogram with added chlorogenic acid. (c) Same chromatogram with added caffeic acid



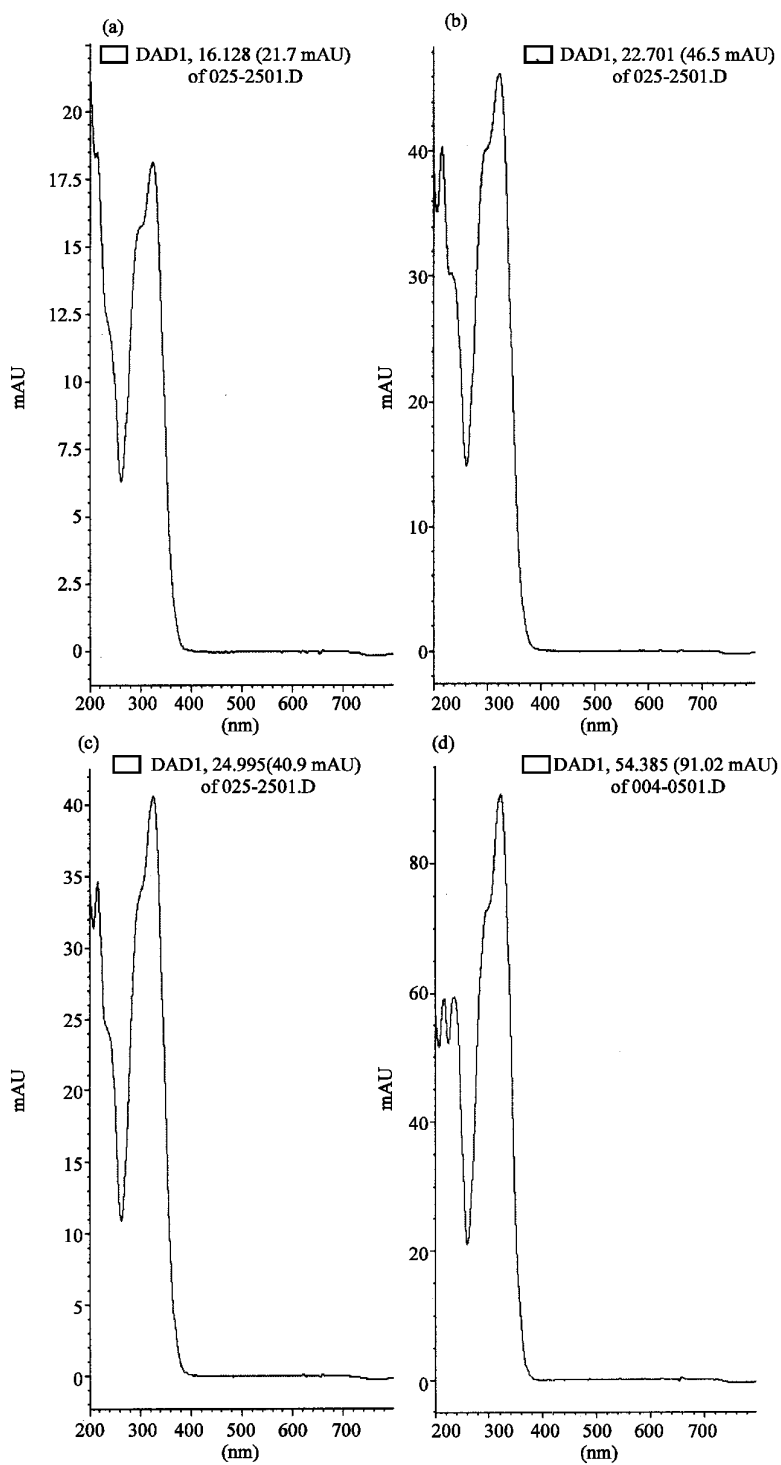


Fig. 4: Spectra of (a), (b) derivatives of chlorogenic acid, (c) chlorogenic acid and (d) caffeic acid

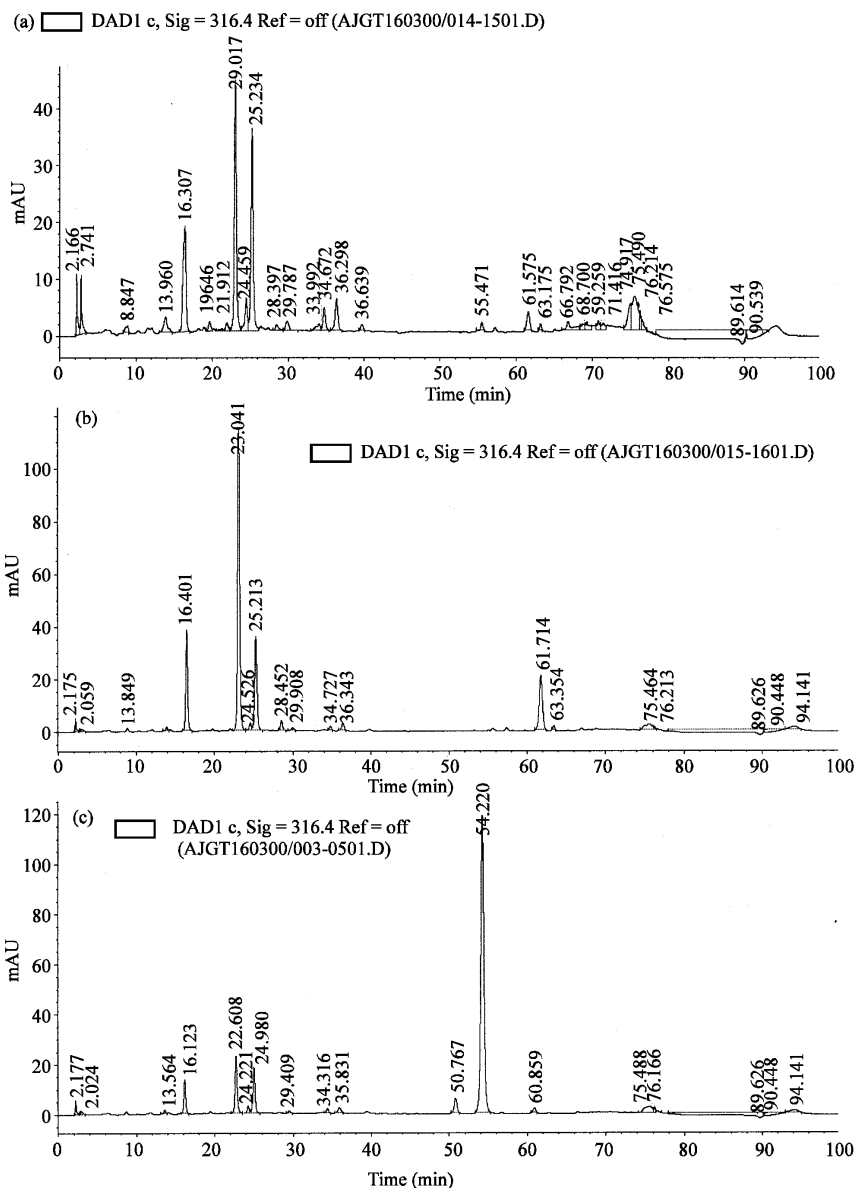


Fig. 5: (a) HPLC chromatogram of the ethanol extract of 0.125  $\mu\text{m}$  spent coffee ground particles subjected to the cellulose treatment. (b) Same chromatogram with added chlorogenic acid and (c) Same chromatogram with added caffeic acid

chlorogenic and caffeic acid are the most abundant phenolic compounds in coffee grounds (Yen *et al.*, 2005; Mattila *et al.*, 2006), but coffee beans contain different chlorogenic acid isomers, notably 3-caffeoyl quinic acid and 4-caffeoyl quinic acid, but also different di-caffeoyl quinic acids and 5-feruloyl quinic acid, in addition to chlorogenic acid itself (5-caffeoyl quinic acid) (Clifford, 2000; Trugo and Macrae, 1984). In the samples (Fig. 3a), a small peak at ~16 min and two major ones at ~23 and ~25 min retention time were detected. When chlorogenic acid was added, the major peak at ~25 min increased (3b). Comparison of our spectral data with previous profiling data of phenolic acids

in coffee (Trugo and Macrae, 1984) led us to propose that the two isomers eluting at ~16 min and at ~23 min, respectively in present chromatogram were 3-caffeoyl quinic acid and 4-caffeoyl quinic acid, respectively-and that the chlorogenic acid itself eluted at ~25 min.

Figure 5a shows the chromatogram of the ethanol extract after the coffee particles having been subjected to enzymatic extraction. In Fig. 5b and c, the chromatograms of the same extracts with extra chlorogenic acid and caffeic acid, respectively, have been plotted. Again, caffeic acid, appearing after 54 min of retention time, was not detected in any of the extracts. In the enzyme-treated samples (Fig. 5), however, it was the peak at 23 min which increased after adding extra chlorogenic acid. It seems, therefore, that there was an effect of the enzyme on both the chlorogenic acid of the extract and on the one added. In contact with the enzyme, chlorogenic acid may undergo structural changes, resulting in a compound with a similar spectrum (Fig. 3a) and retention time, probably an isomer of chlorogenic acid, which elutes simultaneously with the 4-caffeoyl quinic acid. This is an example of how enzymes are able to change the nature of some phenols, which could likely have an influence on their reactivity and properties.

To sum up, the recovery of phenolic compounds is a good alternative to upgrade the spent coffee grounds resulting from industrial obtainment of raw coffee powder. Particle size distribution was found to have a dramatic influence on the phenol release, the latter increasing when smaller particle sizes are employed. Typical shapes of spent coffee grounds avoid the packaging or agglomeration phenomena during extraction, preventing the formation of solvent preferential ways or the formation of off-side zones. This means that a decrease in the particle size always results in major extraction efficiencies. Having found that spent coffee grounds are a good source of phenolic compounds, particularly chlorogenic acid, further research on improving the efficiency of the extractive process could provide important information about the eventual feasibility of the scaling-up of the extraction process at industrial level. Effect of other critical variables like temperature or contact time on the phenol release in fact deserve further investigation, as they have been demonstrated to have a considerable influence in other substrates.

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