# Investigation of Different Extraction Methods on the Content and Biological Activity of the Main Components in *Coffea arabica* L. Extracts

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**Key Words**

**Biological screening**
**Thin-layer chromatography–direct bioautography**
***Coffea arabica* L.**
**Caffeine**
**Chlorogenic acid**

## Summary

**Coffee, due to its common consumption, is one of the main sources of polyphenols in human diet. Coffee species and coffee-related products differ in composition and content of main components, such as chlorogenic acid and caffeine. Chemical and biological fingerprints of various *Coffea arabica* L. extracts were obtained in order to check and compare their antibacterial and antioxidant properties. The antibacterial activity of green and roasted coffee seeds and pomace was evaluated against *Bacillus subtilis* using thin-layer chromatography (TLC)–direct bioautography. TLC–2,2-diphenyl-1-picrylhydrazyl (DPPH) test was used to determine antioxidant properties of the afore-mentioned extracts. Furthermore, different solvents and several extraction methods such as simple maceration, maceration under stirring, and ultrasonic accelerated extraction were tested. The most efficient method of extraction of caffeine and chlorogenic acid was chosen based on quantitative TLC analysis. Additionally, these two main components of coffee were quantitatively determined in commercial products of green coffee.**

## 1 Introduction

Coffee is one of the most commonly consumed beverages in the world and one of the main sources of polyphenols in human diet. At the same time, coffee is, after petroleum, the second most traded commodity [1, 2]. Coffee is an evergreen perennial plant belonging to the family Rubiaceae [3]. There are approximately 60 kinds of coffee cultivars grown throughout the world, of which the most popular include: *Coffea arabica*, *Coffea canephora* (Robusta), and *Coffea liberica* [1].

Green seeds of various coffee species differ in chemical composition. Arabica was found to contain more fats, and Robusta is richer in sucrose, caffeine, and polyphenolic antioxidants such as chlorogenic acid and its derivatives. Seed roasting causes dehydration and degradation of many compounds, including polyphenols. However, rather small variations in the total content of antioxidants related to different degrees of roasting are observed [4–6].

Reasonable coffee consumption improves mood, accelerates heart function, and increases blood pressure. It can also prevent many diseases (including diabetes, cancer, and degenerative and coronary heart diseases) [7, 8]. Coffee consumed in excess dehydrates the body, causes leaching of minerals from the body and irritates the stomach [9, 10]. Due to its properties, coffee is used not only in food industry but also in cosmetics, mainly in personal care products such as lotions, shampoos, and creams, as stimulating blood circulation helps to improve the appearance of human skin. In pharmaceutical industry, coffee is a component of dietary supplements, weight loss agents, pain killers, and medications against hypotension [11].

Many previous studies proved antibacterial properties of coffee extracts against various bacteria strains. The following secondary metabolites: caffeine, chlorogenic acid, ferulic acid, caffeic acid, and protocatechuic acid have been found to be active against *Staphylococcus aureus*, *Streptococcus mutans*, *Enterecoccus faecalis*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella choleraesius*, *Salmonella enteric*, *Citrobacter freundii*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Klebsiella oxytoca*, *Proteus hauser*, and *Serratia marcescen*s [12–16].

The biological activity of infusions depends on the concentration of active compounds, and their content is influenced by factors such as time or type of extraction. Simple maceration is the most commonly applied technique for extraction of secondary metabolites from plants [17]. Other techniques are agitation-assisted extraction and ultrasound-assisted extraction (USAE) [17–19]. These methods allow to significantly reduce the time of extraction.

The biological properties of plant components can be evaluated using thin-layer chromatography hyphenated with direct bioautography (TLC–DB) The principle of the method is very simple: the constituents of a given sample are separated by TLC

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and subjected to biological detection directly on a plate surface. In the case of testing antibacterial properties, a developed TLC plate is dipped in a bacterial broth. During incubation, microorganisms grow directly on a TLC layer excluding places where antimicrobial agents are located. Visualization is mostly carried out using tetrazolium salts, such as MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. Cream zones appearing against purple background – so-called inhibition zones – point to the presence of antimicrobial agents. In the case of searching for antioxidant properties, the developed TLC plate is sprayed with 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) solution. Antioxidants are visible as yellow spots on a purple background [20–22].

The aim of this study was to investigate the influence of different extraction procedures on the content and biological activity of several coffee extracts, prepared from *Coffea arabica* L. TLC–DB was applied to screen the extracts for antibacterial and antioxidant activity.

Antimicrobial activity was evaluated against reference Gram-positive bacteria, *Bacillus subtilis,* for the first time using TLC–DB for this purpose. Antioxidant properties were studied with TLC–DPPH test. Additionally, chemical derivatization was carried out with natural product–polyethylene glycol (NP–PEG) used for detection of polyphenols; anisaldehyde–sulphuric acid reagent (AS) for visualization of terpenoids (general reagent); and iodine–hydrochloric acid reagent (I–HCl) for purine derivatives. The effect of different types of maceration: simple maceration, ultrasound-assisted extraction, and agitation-assisted extraction as well as type of the solvent used for extraction (water or ethanol) on the amount of two main constituents (chlorogenic acid and caffeine) in the tested extracts were evaluated. Based on calibration curves, the content of both chlorogenic acid and caffeine was determined in four commercially available coffee products.

## 2 Experimental

### 2.1 Chemicals and Reagents

Ethyl acetate, methanol, toluene, *n*-heptane, ethanol 96%, sulfuric acid 95%, hydrochloric acid, and iodine were purchased from P.O.Ch. (Gliwice, Poland). Glacial acetic acid was from P.P.H. Standard (Lublin, Poland). 4-Methoxybenzaldehyde (*p*-anisaldehyde) was from Merck Schuchardt (Hohenbrunn, Germany). Mueller-Hinton (M-H) broth, M-H agar, and agarose were purchased from Biocorp (Warsaw, Poland). MTT, Hepes, Triton X-100, natural product (diphenylboryloxyethylamine) reagent, polyethylene glycol-4000, DPPH, chlorogenic acid, and caffeine standards were from Sigma-Aldrich (St. Louis, MO, USA).

### 2.2 Materials and Equipment

Precoated TLC silica gel $F_{254}$ glass-backed plates, and the TLC sprayer were from Merck (Darmstadt, Germany). Hamilton microsyringe was from Bonaduz (Switzerland). The Linomat 5 automatic applicator, TLC Immersion Device, and Visualizer with DigiStore 2 Documentation System and winCATS software, version 1.4.1, were purchased from CAMAG (Muttenz, Switzerland). Ultrasonic bath was from Polsonic (Łódź, Poland), and shaking water bath OLS200 was from Grant Instruments (Cambridgeshire, UK).

#### *2.2.1 Sample Preparation*

Coffee samples (seeds and pomace) were obtained from Cafe Faktoria (Kazimierz Dolny, Poland). The tested samples were: pomace after strong roasting (sample 1), pomace after weak roasting (sample 2), strong Italian roasted seeds (sample 3), fresh green seeds (sample 4), and pomace from green seeds (sample 5). All samples were grounded to powder and directly subjected to maceration procedure.

Coffee samples prepared for biological and chemical screening were macerated with ethanol, while the same samples for quantitative evaluation of caffeine and chlorogenic acid were macerated using both ethanol and water. Commercial coffee products were extracted only with water.

The weighted samples (totally thirty samples, 1 g of each) were covered with 10 mL of ethanol or water and placed in a dark, dry place at room temperature for 24 h. After this time, samples 1–5 macerated in ethanol (five samples) and samples 1–5 macerated in water (five samples) were filtered (simple maceration). The other ten samples (1–5 in ethanol and 1–5 aqueous) were put into the ultrasonic bath for 15 min (maceration with sonification), while the third part of the samples (1–5 in ethanol and 1–5 aqueous) was put into shaking water bath and continuously stirred at 25°C for 1 h.

Four green coffee products were bought in a local shop: P1 – grounded green coffee, P2 – grounded green coffee with marula aroma, and P3, P4 – green coffee capsules from two different producers. The coffee products were macerated with water (1 mg/10 mL).

All samples were filtrated using cotton filters.

#### *2.2.2 Standard Solutions*

Two series of standard solutions of chlorogenic acid at concentrations 0.5, 1.0, 2.0, 5.0, and 10 mg mL$^{-1}$ were prepared: one in ethanol and another in water.

Two series of standard solutions of caffeine at concentrations 0.1, 0.5, 0.75, 1.0, and 1.5 mg mL$^{-1}$ were prepared: one in ethanol and another in water.

#### *2.2.3 Bacterial Strain*

The antibacterial activity of the samples was tested toward *B. subtilis* (ATCC 6633), Gram-positive reference bacteria, which was purchased from the American Type Culture Collections (Manassas, USA).

### 2.3 Thin-Layer Chromatography

#### *2.3.1 Chemical Derivatization*

Chromatography was performed on 10 cm × 20 cm TLC plates. Plant ethanol extracts (5 μL) prepared by simple maceration were applied using Linomat 5 automatic applicator as 10 mm bands, and the TLC plates were developed with mobile phase: ethyl acetate–methanol–water, 77:13:10 (*v*/*v*), to a distance of 8 cm using horizontal sandwich chamber (Chromdes, Lublin). All TLC separations were performed at room temperature

(21°C). After chromatographic separation, the adsorbent layers were dried at room temperature for 1 h to remove the solvent completely. Chemical derivatization was carried out with (1) natural product (1% methanol solution)–polyethylene glycol (5% ethanol solution) (NP–PEG) for detection of polyphenols; (2) anisaldehyde–sulfuric acid reagent (AS) at 110°C/5 min for visualization of terpenoids (general reagent); and (3) iodine–hydrochloric acid reagent (I–HCl) for purine derivatives [23].

*2.3.2 Direct Bioautography*

The plates subjected to biological detection were prepared in the same way as those which were chemically derivatized. The bacterial inoculums were prepared according to the procedures that were optimized earlier in our laboratory [24].

The developed plates were immersed for 8 s in the bacterial suspension using TLC Immersion Device. Then, the plates were placed in a moistened plastic box lined with wetted paper and incubated at 37°C for 17 h. For visualization, the bioautograms were sprayed with 0.2% MTT aqueous solution (to improve intensity of the color, a drop of Triton X-100 was added per 10 mL of aqueous MTT solution). After reincubation at 37°C for 0.5 h, the bioautograms were digitized by Visualizer (CAMAG, Muttenz, Switzerland).

To detect antioxidants, dry TLC plates were sprayed with 0.2% methanol DPPH solution using a TLC sprayer [25, 26]. Antioxidant activities of separation zones were observed almost immediately after spraying as yellow spots against a purple background.

*2.3.3 Quantitative Analysis of the Main Compounds*

The quantitative analysis of the main coffee compounds, chlorogenic acid and caffeine, was carried out both for water and ethanol coffee samples (seeds and pomace). The influence of maceration type on concentration of the main compounds was evaluated. The samples at 5 µL volumes were applied on TLC plates as 10 mm bands. Then, the plates were developed with the mobile phase: ethyl acetate–methanol–water, 77:13:10 (*v*/*v*), to a distance of 8 cm. After solvent evaporation, the plates were documented under 254 nm, using winCATS program (CAMAG). The areas under the peaks were measured using VideoScan software. Calibration curves were constructed as dependences of standard surface areas *vs.* their concentrations. Each standard concentration was prepared in three replicates. The coffee samples and commercial products were analyzed in triplicate at the same chromatographic conditions, as the standards. The amounts of both compounds (chlorogenic acid and caffeine) were determined on the basis of calibration curves.

## 3 Results and Discussion

### 3.1 Chemical and Biological Screening

The chemical derivatization of the separated compounds was carried out by spraying the TLC plate with appropriate reagents. Application of three different derivatization reagents allowed the detection of five (**a**–**e**) compounds in the tested samples: $R_{Fa}$ = 0.14, $R_{Fb}$ = 0.30, $R_{Fc}$ = 0.51, $R_{Fd}$ = 0.68, and $R_{Fe}$ = 0.87 **(Figure 1).** The positive reaction by use of NP–PEG reagent

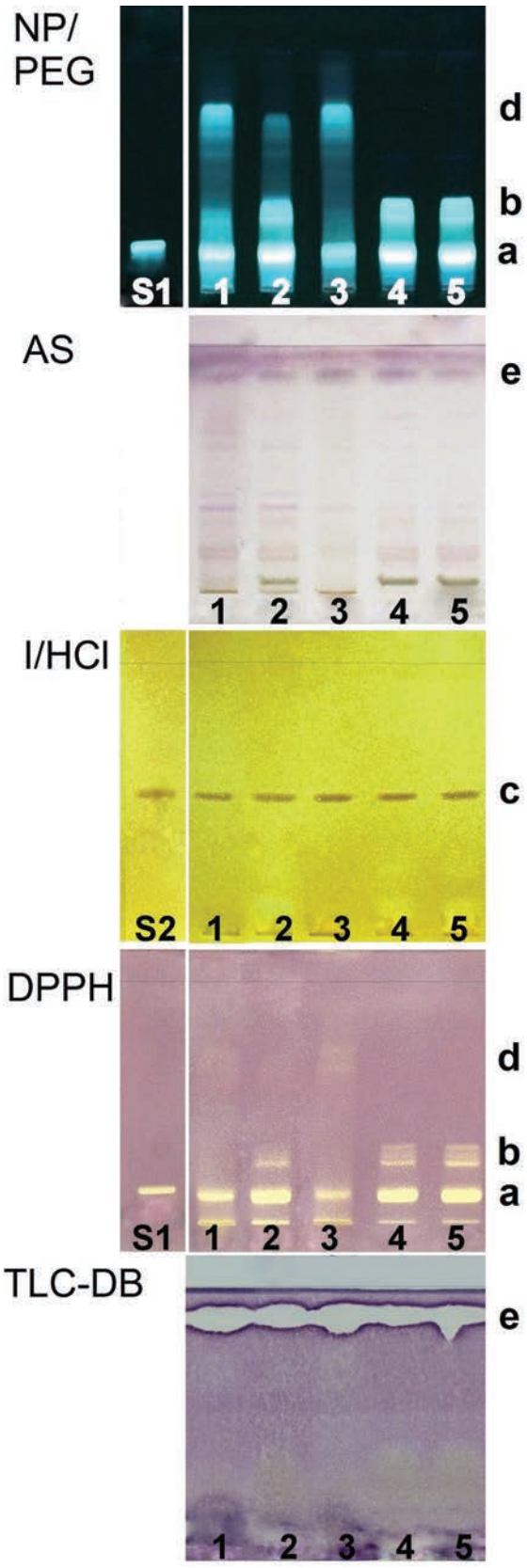


**Figure 1**

**TLC chromatograms and bioautograms of coffee extracts: 1, pomace after strong roasting; 2, pomace after weak roasting; 3, strong Italian roasted seeds; 4, fresh green seeds; 5, pomace from green seeds. NP–PEG, natural product–polyethylene glycol; AS, anisaldehyde–sulfuric acid; I–HCl, iodine–hydrochloric acid; DPPH, antioxidants detection; TLC–DB, antibacterial agents detection. S1, chlorogenic acid; S2, caffeine; mobile phase: ethyl acetate–methanol–water, 77:13:10 (*v*/*v*).**

**Table 1**

**Evaluation of main compounds in ethanol and water coffee samples.**

| Compound–calibration curve | Sample | Type of maceration | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Simple maceration | | | Maceration with stirring | | | Maceration with sonification | | |
| | | Average peak area ± SD | % RSD | $c$ (mg mL$^{-1}$) | Average peak area ± SD | % RSD | $c$ (mg mL$^{-1}$) | Average peak area ± SD | % RSD | $c$ (mg mL$^{-1}$) |
| Ethanol coffee samples | | | | | | | | | | |
| Chlorogenic acid $y = 46{,}929.597 \ln(x) + 52{,}580.607$ $R^2 = 0.999$ | 1 | 68,119.58 ± 2160.13 | 3.17 | 1.39 | 68,127.32 ± 4102.65 | 6.02 | 1.39 | 48,518.20 ± 2498.26 | 5.15 | 0.92 |
| | 2 | 95,941.35 ± 3215.92 | 3.35 | 2.52 | 92,382.22 ± 5517.97 | 5.97 | 2.34 | 62,280.20 ± 3565.83 | 5.73 | 1.23 |
| | 3 | 40,674.79 ± 1409.04 | 3.46 | 0.78 | 41,302.33 ± 2372.13 | 5.74 | 0.79 | 29,211.93 ± 868.67 | 2.97 | 0.61 |
| | 4 | 140,807.25 ± 1890.52 | 1.34 | 6.55 | 138,466.65 ± 4870.54 | 3.52 | 6.23 | 108,120.44 ± 5876.96 | 5.44 | 3.27 |
| | 5 | 145,392.20 ± 1580.24 | 1.09 | 7.23 | 144,624.36 ± 5639.84 | 3.90 | 7.11 | 124,003.95 ± 6024.87 | 4.86 | 4.58 |
| Caffeine $y = 59{,}686.024x + 40{,}298.627$ $R^2 = 0951$ | 1 | 86,456.76 ± 942.39 | 1.09 | 0.77 | 84,265.44 ± 2728.76 | 3.24 | 0.74 | 78,546.68 ± 3221.18 | 4.10 | 0.64 |
| | 2 | 89,187.13 ± 1453.39 | 1.63 | 0.82 | 86,892.40 ± 2932.56 | 3.37 | 0.78 | 78,981.24 ± 2742.97 | 3.47 | 0.65 |
| | 3 | 89,075.64 ± 1317.06 | 1.48 | 0.82 | 85,589.22 ± 5937.03 | 6.94 | 0.76 | 82,475.49 ± 3549.78 | 4.30 | 0.71 |
| | 4 | 90,821.76 ± 1725.28 | 1.90 | 0.85 | 86,120.63 ± 5938.69 | 6.90 | 0.77 | 81,342.25 ± 3252.18 | 4.00 | 0.69 |
| | 5 | 98,212.85 ± 902.13 | 0.92 | 0.97 | 94,566.69 ± 1908.42 | 2.02 | 0.91 | 86,010.41 ± 4192.60 | 4.87 | 0.77 |
| Water coffee samples | | | | | | | | | | |
| Chlorogenic acid $y = 54{,}770.864 \ln(x) + 62{,}662.544$ $R^2 = 0.999$ | 1 | 71,230.72 ± 1392.58 | 1.96 | 1.17 | 75,831.78 ± 2559.45 | 3.38 | 1.27 | 75,085.80 ± 3168.93 | 4.22 | 1.25 |
| | 2 | 96,483.60 ± 1666.81 | 1.73 | 1.85 | 105,630.49 ± 2626.76 | 2.49 | 2.19 | 103,191.08 ± 3338.51 | 3.24 | 2.10 |
| | 3 | 50,828.68 ± 1055.57 | 2.08 | 0.81 | 53,210.68 ± 1903.09 | 3.58 | 0.84 | 56,575.95 ± 1911.46 | 3.38 | 0.89 |
| | 4 | 98,979.31 ± 2372.84 | 2.40 | 1.94 | 115,390.44 ± 2910.57 | 2.52 | 2.62 | 125,036.32 ± 3364.31 | 2.69 | 3.12 |
| | 5 | 166,050.65 ± 2423.39 | 1.46 | 6.60 | 196,598.56 ± 3326.51 | 1.69 | 11.54 | 214,732.36 ± 4725.12 | 2.20 | 16.06 |
| Caffeine $y = 59{,}236.055x + 10{,}329.585$ $R^2 = 0.941$ | 1 | 71,328.64 ± 3342.97 | 4.69 | 1.03 | 72,335.45 ± 1635.92 | 2.26 | 1.05 | 76,485.47 ± 2681.73 | 3.51 | 1.12 |
| | 2 | 70,262.94 ± 2348.82 | 3.34 | 1.01 | 75,153.45 ± 1939.33 | 2.58 | 1.09 | 77,342.38 ± 3899.45 | 5.04 | 1.13 |
| | 3 | 73,739.31 ± 3122.40 | 4.23 | 1.07 | 76,118.54 ± 2470.17 | 3.25 | 1.11 | 80,378.54 ± 3932.21 | 4.89 | 1.18 |
| | 4 | 75,043.21 ± 1808.64 | 2.41 | 1.09 | 79,828.52 ± 2054.60 | 2.57 | 1.17 | 81,984.03 ± 3039.69 | 3.71 | 1.21 |
| | 5 | 80,214.37 ± 3299.59 | 4.11 | 1.18 | 75,517.55 ± 1956.80 | 2.59 | 1.10 | 76,626.74 ± 2035.56 | 2.66 | 1.12 |

proved the presence of three polyphenolic constituents denoted as **a, b,** and **d,** from which one was identified by comparing with standard S1 as chlorogenic acid **(a).** Compound **e** was detected with AS reagent under white light. The I–HCl reagent sprayed onto the plate gave a positive response with compound **c** indicating the presence of caffeine (standard S2). Bioassays coupled with TLC were used to investigate both antibacterial and antioxidant properties. TLC–DB against *B. subtilis* allowed the detection in all tested samples only one band (fraction) showing clear antibacterial activity – **e**, detected also with AS (which can be an indirect prove of the presence of sesquiterpenes). The TLC–DPPH test enabled identification of three polyphenolic compounds **a, b,** and **d** with antioxidant properties including strongly active chlorogenic acid **(a).** Caffeine (**c**) detected in all analyzed samples did not show any biological activity.

Chemical derivatization using AS, I–HCl, and NP–PEG reagents allowed the observation of differences in fingerprints of investigated samples that was especially visible after spraying with NP–PEG reagent.

### 3.2 Quantitative Analysis of Caffeine and Chlorogenic Acid

Next, the influence of sample preparation step (including the use of different extraction solvents) on the content of the main components were investigated. Three types of maceration processes were compared to choose a simple and efficient extraction method for the simultaneous isolation of chlorogenic acid and caffeine (Table 1). All experiments were performed at 25°C.

The quantitative analysis of the ethanol extracts showed that the highest concentrations of caffeine and chlorogenic acid were identified after simple maceration process. The application of maceration under stirring slightly decreased the content of the investigated compounds. The greatest reduction of caffeine and chlorogenic acid concentrations was observed for ultrasonic accelerated extraction. It can be related to the decomposition of these rather thermally stable components caused by ultrasound. For the given maceration, the caffeine content was almost constant in all analyzed samples. The highest content of this alkaloid was detected in pomace from green coffee after simple maceration (sample 5 – 0.97 mg $mL^{-1}$), while the lowest, in pomace after strong roasting (sample 1 – 0.77 mg $mL^{-1}$). The changes in chlorogenic acid content were strongly related to the roasting process. The highest concentration of chlorogenic acid was observed in green coffee pomace (sample 5 – 7.23 mg $mL^{-1}$), and the lowest, in the black seeds (sample 3 – 0.78 mg $mL^{-1}$) (see the data for simple maceration).

The quantitative analysis of the samples extracted with water did not allow choosing the most effective type of maceration. In all samples, similar concentration of caffeine (about 1 mg $mL^{-1}$) was evaluated. The high concentrations of chlorogenic acid in pomace from green coffee seeds (sample 5) were observed after all types of maceration (6.60, 11.54, and 16.06 mg $mL^{-1}$, respectively, for simple maceration and two accelerated macerations). These concentration levels were 5-6 times higher compared to those in the green seeds (sample 4) (1.94, 2.62, and 3.12 mg $mL^{-1}$). In the roasted seeds (sample 3), chlorogenic acid content was almost constant for all maceration types (about 0.8 mg $mL^{-1}$) (Table 1).

Additionally, commercially available coffee products were compared to show differences of the content of bioactive compounds (Figure 2). The highest concentration of chlorogenic acid was identified in the capsules (product 3). A high amount of this acid was also detected in other capsules (product 4). These preparations contained also the highest caffeine amounts **(Table 2)**.

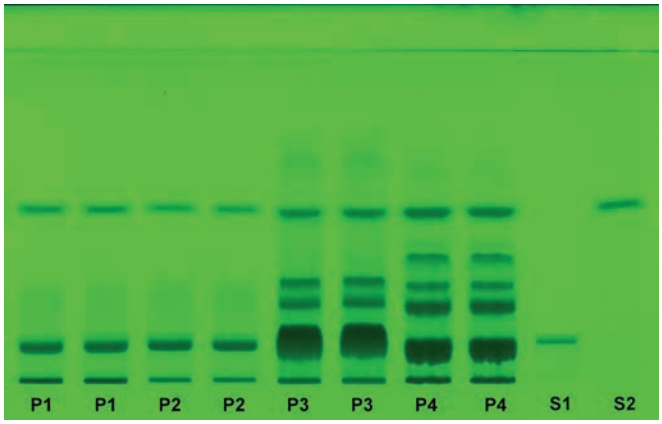


**Figure 2**

**TLC chromatogram (at 254 nm) of commercially available coffee products. Applied volume: 5 µL. P1, grounded green coffee; P2, grounded green coffee with marula aroma; P3 and P4, green coffee capsules from two different producers; S1, chlorogenic acid; S2, caffeine; mobile phase: ethyl acetate–methanol–water, 77:13:10 (*v/v*).**

**Table 2**

**Evaluation of main compounds in water coffee commercial products.**

| Compound | Product | Average peak area ± SD | % RSD | *c* (mg $mL^{-1}$) |
|---|---|---|---|---|
| Chlorogenic acid (S1) | P1 | 102,631.90 ± 518.47 | 0.51 | 2.07 |
| | P2 | 96,371.71 ± 416.23 | 0.43 | 1.85 |
| | P3 | 238,643.13 ± 3709.28 | 1.55 | 24.85 |
| | P4 | 187,229.40 ± 2994.48 | 1.60 | 9.72 |
| Caffeine (S2) | P1 | 59,495.73 ± 271.42 | 0.46 | 2.29 |
| | P2 | 50,479.72 ± 456.86 | 0.91 | 1.97 |
| | P3 | 70,630.07 ± 513.35 | 0.73 | 2.77 |
| | P4 | 94,604.77 ± 262.25 | 0.28 | 4.15 |

## 4 Conclusion

Five coffee samples were extracted with two solvents using three types of maceration. Totally, thirty samples were obtained for which quantitative analysis of the main coffee components, chlorogenic acid and caffeine, were performed. Additionally, five ethanol extracts after simple maceration were screened for their chemical and biological properties. The highest content of chlorogenic acid was found in the green pomace which was obtained from the green seeds as a result of pressing. Oily green seeds possessed much lower content of chlorogenic acid. Furthermore, it was noted that the process of roasting causes degradation of chlorogenic acid. The highest concentrations of chlorogenic acid were obtained as a result of simple maceration in ethanol while

ultrasonic accelerated maceration caused degradation of chlorogenic acid. Caffeine was at constant level in all analyzed samples. Its content was influenced neither by roasting nor by maceration or oil pressing. For the first time, TLC–DB was successfully used to screen antibacterial properties of coffee samples.

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