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Reducing Acrylamide in Roasted Coffee Beans by L-Asparaginase Using Ultrasound

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ABSTRACT

Coffee is a popular drink in many countries in the world. However, long exposure to acrylamide, a chemical generated via Maillard reaction in the coffee roasting process, has been associated with health impacts. In our work, *Coffea robusta* beans were treated with L-asparaginase under ultrasound to investigate the effects of L-asparaginase concentration, pH, temperature and immersion time on the acrylamide mitigation in roasted coffee. The effects of parameters were evaluated by measuring the UV-Vis spectra of sample solutions containing acrylamide. Treatment of green coffee beans with a solution of 3.0 IU/mL asparaginase at 37 °C, pH 7.3 for 30 min led to a 42% and 14% reduction of acrylamide in the final product compared to reference samples R7 and C0, respectively. Furthermore, material with initial moisture content of 5.5% resulted in an acrylamide reduction of 47.9% and 22.7% compared to R7 and C0. Enzyme-treated ground coffee gave no difference in sensory evaluation compared to the regular roasted product.

Keywords: Acrylamide; L-asparaginase; Roasted coffee; Robusta; Ultrasound

1. Introduction

Acrylamide is a by-product produced when asparagine reacts with reducing sugar in heat-processed foods [1-4]. It was found that asparagine is a crucial precursor in the acrylamide formation by this pathway. Acrylamide was shown to be a neurotoxin, carcinogen and reproductive toxin in animal species. The neurotoxic effects of acrylamide in humans have been documented [5]. Therefore, the methods for reducing the acrylamide levels in thermally processed foods have been developed [1, 3, 4, 6-9]. Acrylamide production is proportional to asparagine content. Hence, precursor reduction, e.g., asparagine, was a practical strategy to significantly reduce levels of acrylamide in cooked foods. The enzymatic approach to interfere with the reaction pathway was proposed. L-asparaginase has been considered to be effective since it selectively consumes precursor by catalyzing the conversion of

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asparagine into aspartic acid and ammonia [9, 10].

The percentage of acrylamide that humans absorb from foods mainly comes from heated products such as chips (16-30%), potato snacks (60-64%), coffee (13-39 %), baked goods/biscuits (10-20%), and toast (10-30%) [1]. Obviously, the amount of acrylamide that humans absorb from coffee (13-39 %) is significant. Therefore, the efforts were made to reduce exposure to this substance. Porto *et al* investigated acrylamide reduction using L-asparagine combined with steam vapor. The efficiencies of asparagine removal in green coffee *C. arabica* and *C. canephora* var *robusta* were 60% and 35%, respectively [3]. Treating coffee with enzymes to reduce the acrylamide content formed in roasted coffee *C. arabica* in combination with batch, spray and shaking methods was reported [6].

Ultrasound has been known as one of the methods that can be used to improve mass transfer processes, which attributed to the cavitation phenomenon [11]. Furthermore, mass transfer can be accelerated by increasing the temperature of medium. In this respect, the ultrasonic liquid-solid interaction can be favorable for consuming asparagine by asparaginase. However, the efficiency of the application of asparaginase under ultrasound conditions for acrylamide reduction in *Robusta* has not been found yet.

In the present work, we investigated the effect of asparaginase on formation level of acrylamide in *Coffea robusta* using ultrasound. The work was conducted to: a) determine the appropriate roasting time at 220°C; b) evaluate effects of factors of enzyme concentration, pH, temperature and immersing time on the acrylamide level in roasted ground coffee; c) examine the dependence of the enzyme efficiency on initial moisture content of green coffee beans; and d) assess the chemical components and sensory evaluation of the roasted ground coffee samples.

2. Materials and Methods

2.1 Materials

Green coffee *Robusta* was collected from the farm in Gia Lai province, Vietnam. Coffee beans were cleaned and stored in zip-lock packages at room temperature to avoid exposure to the sunlight.

L-Asparaginase (freeze-dried powder, 2500 IU, extracted from *Escherichia coli* ASI.357 and greater than 96.0% in purity) was purchased from Prospec-Tany TechnoGene Ltd., Israel, and stored at -18 °C (Protec-Tany TechnoGene Ltd).

2.2 Methods

2.2.1 Effects of roasting time on acrylamide mitigation and coffee beans color

Green coffee beans (20 g) were roasted at 220 ºC for 2, 4, 6, 7, 8 min (named R2, R4, R6, R7, R8 samples, respectively) without immersion and enzyme treatment. Using the UV-Vis spectra to determine the acrylamide levels in roasted coffee solutions. The color space measurements were evaluated from roasted samples.

2.2.2 Enzyme treatment combined ultrasound

a) Enzyme concentration: A stock solution (500 IU/mL) was prepared by mixing L-asparaginase (2500 IU) with distilled water (5.0 mL). In order to investigate the effects of enzyme concentrations on the acrylamide reduction, mixtures containing green coffee beans and enzyme in the ratio of 1:2 [w (g):v (mL)] were prepared by adding coffee beans to enzyme solutions at various concentrations: 0.0 (C0); 3.0 (C3); 5.0 (C5) and 7.0 (C7) IU/mL. The mixtures were subjected to ultrasound (37 kHz) at 37 ºC, pH 7.3 for 40 min.

b) Temperature: The mixtures containing coffee beans and enzyme (3.0 IU/mL) were subjected to the ultrasound batch at 30 (T30), 37 (T37), 45 (T45) and 50 (T50) °C, pH 7.3 for 40 min.

c) *pH*: Coffee beans were immersed in the enzyme solutions (3.0 IU/mL) at various pH values of 5.0 (P5); 6.0 (P6); 7.3 (P7.3) and 8.6 (P8.6) at 37 °C for 40 min. pH values were prepared by using a phosphate buffer mixed from 0.1 M potassium phosphate monobasic (KH_2PO_4) and 0.1 M potassium phosphate dibasic (K_2HPO_4) solutions. The desired pH values were obtained using a sensitive pH meter.

d) Immersion time: The enzyme concentration (3.0 IU/mL), temperature (37 °C) and pH 7.3 were fixed while the immersion time was changed: 0 (Ti0); 20 (Ti); 30 (Ti30); 40 (Ti40); 50 (Ti50); 60 (Ti60) min. For the above experiments, R7 (without enzyme and ultrasound treatment) and C0 (non-treated with enzyme, under ultrasound treatment) were used as reference samples.

2.2.3 Coffee samples preparation

To enable good access of asparaginase to the inside of the coffee beans, the enzyme penetration was assisted by ultrasound conditions (37 kHz) for 1.0 min, taking a 4-minute rest after each interval to control temperature. The Erlenmeyer flasks containing green coffee beans (10 g) and enzyme solutions (20 mL) at various enzyme concentrations, pH values and immersing time were placed in the ultrasonic bath. After enzyme treatment, the coffee beans were dried at 50 °C for approximately 3 hrs until sample weight reached initial value. The beans were then roasted at 220 °C for 7 min. The roasted beans were cooled down to room temperature and ground to fine powder using a coffee grinder Tiross TS530/TS532 (Fig. 1).

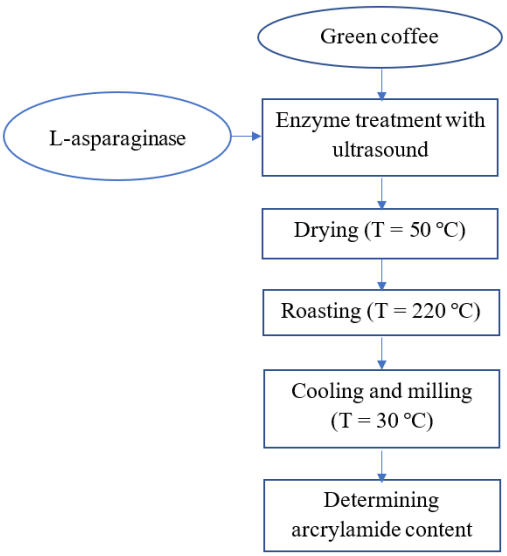


Fig. 1. Flowchart of coffee sample preparation.

2.2.4 Effects of moisture content on acrylamide mitigation

Green coffee beans were dried at 45 °C for 0, 1, 5, 13, 24 hr(s) to obtain the materials having moisture contents of 12.5, 10.6, 8.4, 6.6 and 5.5%, respectively. To evaluate the effect of moisture content on the acrylamide level in roasted coffee, the green coffee beans were treated with and without enzyme solution (3 IU/mL) at 37 °C, pH 7.3 for 30 min.

2.2.5 UV-Vis measurements

a) Calibration curve and asparagine measurement

In a volumetric flask, asparagine (> 99%, Acros Organics, Belgium, 10 mg) was dissolved in 100 mL HCl 1N solution. The asparagine solution (2.5 mL) was diluted with HCl 1N solution to obtain the stock asparagine solution (2.5 µg/mL). A series of asparagine solutions (0, 0.25, 0.5, 0.75, 1, 1.25, 1.5 µg/mL) was scanned with UV-Vis spectrophotometer (UH5300 UV-Vis Spectrophotometer, Hitachi, Japan) in a wavelength range from 200 to 230 nm. The absorbance values at 208 nm versus concentrations were plotted to afford a calibration curve.

Asparagine in coffee samples was extracted according to Porto's method with a modification [3]. Coffee beans were ground into fine powder. The powder was added to HCl 1N solution with a ratio of 1:20 (w:w) and the mixture was homogenized by magnetic stirring at 60±2 °C for 12 hrs. The supernatant was then filtered (Ø = 20 μm) to afford the sample solution. The asparagine solution was diluted by 1N HCl and measured the absorbance value at 208 nm to determine concentration from a calibration curve.

b) Calibration curve of acrylamide

Acrylamide (> 99%, Acros Organics, Belgium, 10 mg) was dissolved in distilled water (1.0 L). An acrylamide solution of 0.5 mL was transferred to a NaOH 1M solution (1.0 L) to give a stock acrylamide solution of 5 ng/mL. The UV-Vis spectra of acrylamide solutions (from 0 to 2.25 ng/mL were measured in a wavelength range from 215 to 245 nm.

Acrylamide in coffee samples was extracted according to Dange's method [8]. Roasted coffee beans were ground into fine powder. A mixture of coffee sample and distilled water with a ratio of 1:20 (w:w) was centrifuged for three repetitions (4000 rpm for 20 min). The solid was removed by filtration to afford the acrylamide solution. The solution was diluted with 1 M NaOH solution and the absorbance value measured at 224 nm to determine acrylamide concentration from a calibration curve.

c) Determination of reducing sugars

Reducing sugar content was determined by the DNS method [12]. Yellow-orange complexes of D-glucose and DNS reagent (1 g dinitrosalicylic acid $C_7H_4N_2O_7$, 1,6 g NaOH, 30 g natrikali tartrate $KNaC_4H_4O_6.4H_2O$) were prepared by adding DNS reagent to a series of D-glucose solutions (0; 0.2; 0.4; 0.6; 0.8; 1.0 mg/mL) in a ratio of 3:1 (v:v). The mixtures were boiled for 5 min then quickly cooled down to room temperature. The complexes had their UV-Vis spectra scanned in a wavelength range from 500 to 570 nm.

Coffee beans were ground into fine powder. Coffee powder (1 g) was extracted from fat using the Soxhlet method with a mixture of petroleum:diethyl ether (1:1, v:v). The solvent was removed by drying beans at 60 °C. Reducing sugar from beans was extracted with ethanol 80% (60 mL) by refluxing system for 2 hrs. The extract was obtained by removing the solvent under reduced pressure. The extract was diluted with distilled water and measured optical density following the above description.

2.2.6 Colour space measurements

The color of the ground coffee samples was determined by a CR-400 chroma meter (Minolta, Japan) in comparison with one of the samples without roasting. The L value represents white/black from 0 to 100 [13].

2.2.7 Sensory evaluation

Sensory properties of ground coffee samples were evaluated by a panel of 9 trained assessors consisting of 4 females and 5 males with the ages of 22-24. The sensory test was taken according to Kreuml's method [14]. The quantitative descriptive analysis was performed to evaluate the intensity of the attributes using a 5-unit scale (0 = imperceptible and 5 = very intense descriptive). Evaluated attributes were color, fragrance/aroma, body and taste. The taste sensations include the balance of tastes (bitter, sweet, salty, sour) and aftertaste [15]. Sensory assessment sessions were conducted in individual booths under fluorescent light. The samples were randomly coded with three-digit numbers. The testing room was cleaned without strange odor.

All experiments were repeated three times. Experimental data were statistically analyzed by one-way ANOVA.

3. Results and Discussion

3.1. Calibration curves

The absorption spectra of acrylamide, asparagine and D-glucose solutions were shown in Figs. 2A, C, E. The maximum absorbance value of acrylamide was obtained at 224 nm while the values of asparagine and D-glucose appeared at 208 and 540 nm, respectively. The unknown concentrations of solutions can be found from calibration equations, which built by plotting the calibration graphs of absorbance values versus concentrations (Figs. 2B, D, F).

3.2. Effects of roasting time on acrylamide mitigation and appearance

The effects of roasting time at 220°C on acrylamide contents of coffee samples are given in Fig. 3A. The results illustrate that the acrylamide contents increased from 2028 µg/kg to 2928 µg/kg when the roasting time varied in a range from 0 to 7 min, i.e., a 44.4% increase of acrylamide. However, the acrylamide content decreased by approximately 18.8% when the roasting time was longer than 1 min (2928 µg/kg for 7 min and 2379 µg/kg for 8 min, respectively). The reduction of acrylamide concentration was attributable to the decomposition rate of material, e.g., reducing sugar during roasting being greater than the formation rate of acrylamide at 220 °C [16].

The changes in brightness presented by L value and appearance of the final products are shown in Fig. 3B and Fig. 4. The L value decreased with increased roasting time, i.e., the color of the product became darker. The appearance of coffee also reflected the change in color between samples. When roasting time reached 2 min (R2), coffee beans gave a brighter color compared to green coffee (G). The observation can be explained by the fact that the beans were dehydrated during roasting stage and thus, the outer shell became dry and flaked off to reveal the light inner part. Coffee beans roasted for 6 min (R6) were light brown while the beans for 7 min (R7) gave a smooth surface. Furthermore, when the green coffee was roasted for 7 min, there were no oil pouring phenomenon, characteristic aroma, and dark brown in color for the final product. The desired coffee color was obtained when the L value was in the range of 10-25, which was dependent on the roasting regimes [6]. The product of sample R8 contains a phenomenon of pouring oil. The appearance becomes black and the smell of burning appeared. The color of the roasted coffee was due to a compound mixture formed from Maillard reaction, which was dependent on the temperature and roasting time [17]. At high temperature, the longer roasting time can produce a bitter coffee with no characteristic aroma [18]. In our results, the best sensory state was obtained when the green coffee was roasted at 220°C for 7 min.

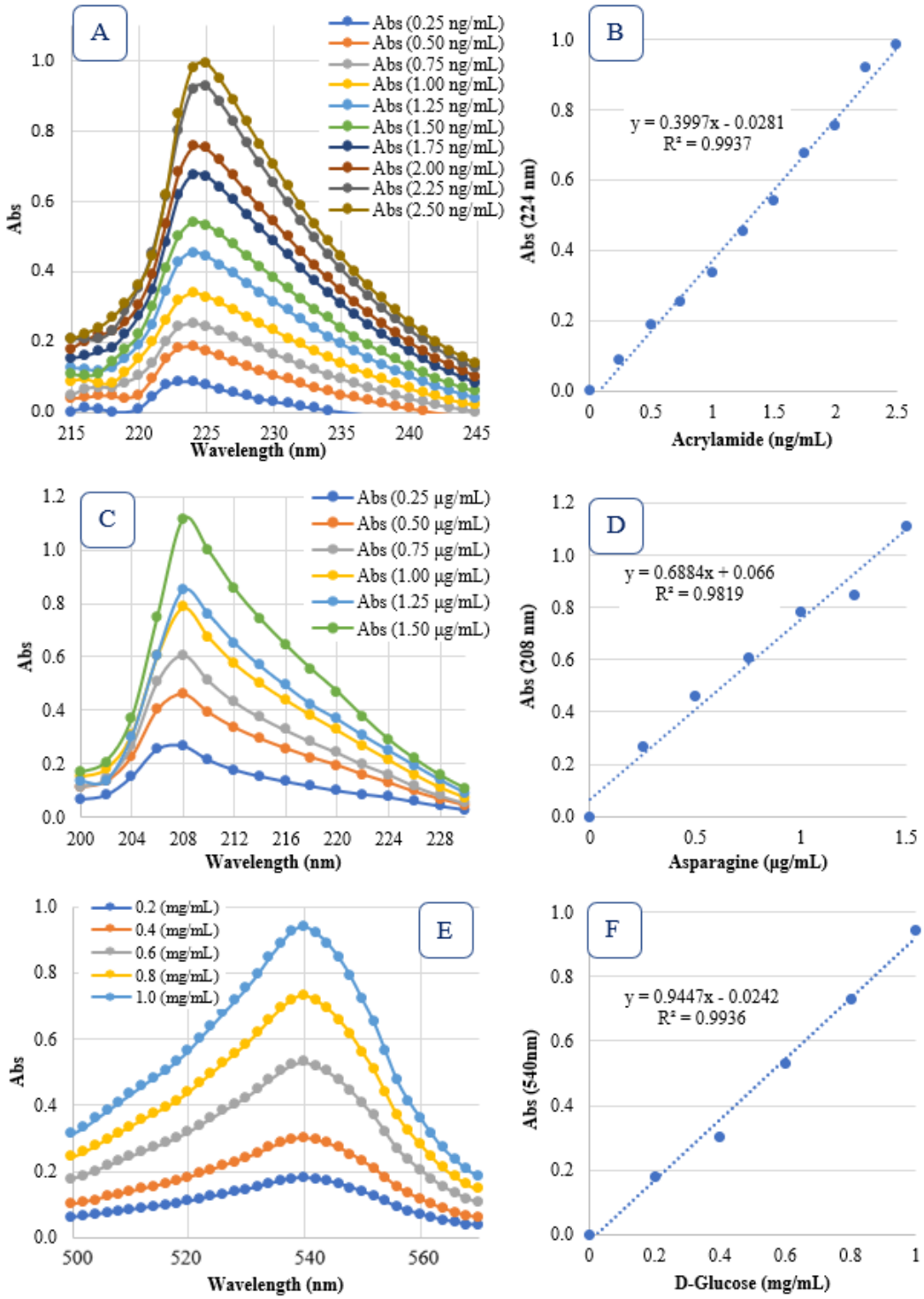


Fig. 2. The absorption spectra and the calibration graphic of acrylamide solutions (A, B), asparagine solutions (C, D) and D-glucose solutions (E, F).

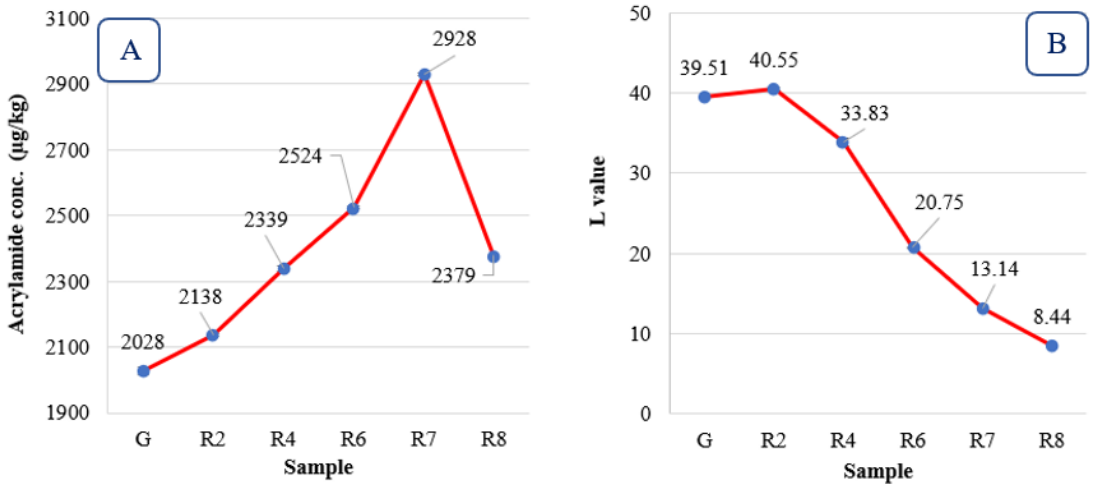


Fig. 3. Changes of acrylamide contents (A) and L values (B) in roasted coffee samples (G-R8) with different roasting time (0-8 min).

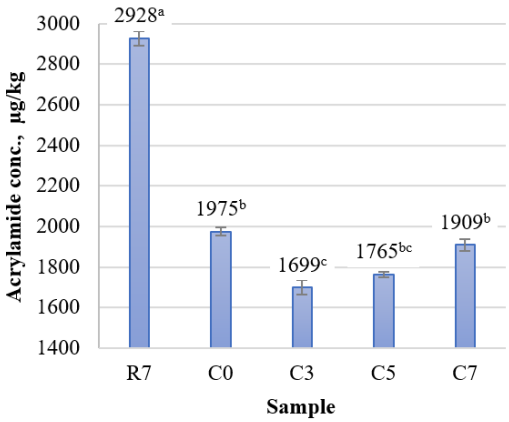
Fig. 4. Appearances of coffee products roasted at 220°C in a range of roasting time from 0 to 8 min (R0-R8).

3.3. Effects of enzyme treatment

3.3.1 Effect of enzyme concentration on acrylamide mitigation

When compared to sample R7 (green coffee roasted at 220 °C for 7 min without enzyme and ultrasound treatment), the enzyme-treated samples under ultrasound (C0-C7) showed a significant reduction of acrylamide level (Fig. 5). The lowest value was achieved at enzyme concentration of 3 IU/mL, resulting in an approximately 42% decrease of acrylamide content. It should be noted that the acrylamide in roasted reference sample C0 (under ultrasound) also decreased by 32.5% compared with the sample control R7. The results can be attributed to the loss of reducing sugar content during immersion time of 40 min in combination with ultrasound. In addition, in the presence of enzyme, the activation of enzyme assisted by ultrasound, e.g., sample C3 speeds up the conversion of asparagine precursor into aspartic acid and ammonia. As a result, the formation of acrylamide due to Maillard reaction in roasted coffee can be reduced [3,19].

The decrease of acrylamide concentration in the final product was not observed at enzyme concentrations higher than 3 IU/mL. Green coffee beans have a very tight structure, pores account for less than 7% of the grain volume, and a diameter of 10 nm. The diameters of enzymes are typically in a range of 5–10 nm and thus, a relatively large amount of the enzyme can be accessible to the coffee bean [20]. However, when the enzyme concentration was higher (C7) and the contact area of the coffee bean remains constant, the penetration of the enzyme into the beans cannot be favorable, leading to a decreased efficiency of enzyme. Here, the enzyme concentration of 3 IU/mL was selected as an appropriate concentration for the treatment of *Robusta* coffee with L-Asparaginase under ultrasound. This concentration value was used for further experiments to come up with a suitable procedure for reducing the acrylamide level of roasted *Robusta* coffee.

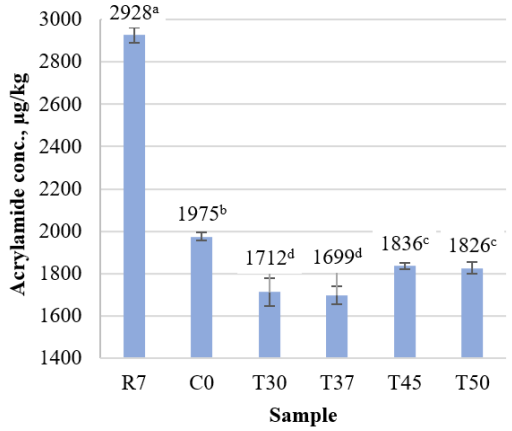


The different letters (a-c) showed significant difference ($p < 0.05$).

Fig. 5. Acrylamide levels formed in roasted coffee samples at various enzyme concentrations.

3.3.2 Effect of temperature on acrylamide mitigation

To evaluate the effects of temperature on enzyme activity, green coffee samples were immersed in enzyme solutions at different temperatures. The temperature-dependence on acrylamide reduction is given in Fig. 6. The acrylamide mitigation was found to be effective when green coffees were immersed in enzyme solution at 30 and 37°C (1712 µg/kg and 1699 µg/kg, respectively). The acrylamide contents decreased approximately by 42 % and 14 % compared to samples R7 and C0. The efficiency of the enzyme decreases with increasing temperature. The acrylamide concentrations were 1836 µg/kg and 1826 µg/kg at 45 °C and 50 °C, respectively. L-Asparaginase derived from *Escherichia coli ATCC 11303* works optimally in a temperature range from 30 to 37 °C and thus, the enzyme becomes inactive at higher temperature [4, 22-24].

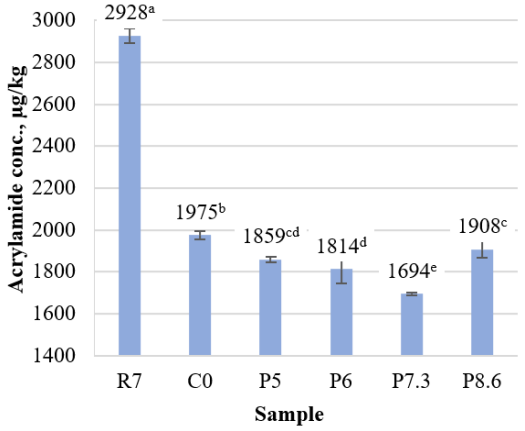


The different letters (a-d) showed significant difference ($p < 0.05$).

Fig. 6. Acrylamide contents of coffee samples treated with enzyme at different temperatures. R7 refers to a sample treated without enzyme and ultrasound. C0 gives a coffee sample treated without L-asparaginase using ultrasound.

3.3.3 Effect of pH on acrylamide mitigation

Fig. 7 refers to the acrylamide levels when green coffees were treated with enzyme at different pH values. The lowest acrylamide level (1694 µg/kg) was obtained in a solution at pH 7.3 (P7.3). This condition led to a 42.1 % and 14.2 % reduction of acrylamide content compared with R7 and C0, respectively. The higher acrylamide concentration (1908 µg/kg) was observed at pH 8.6 (P8.6). In solutions at pH 5 (P5) and 6 (P6), the acrylamide levels reduced by 6-8% in comparison with sample C0. The enzyme activity is dependent on pH values. L-asparaginase is an intracellular enzyme and its optimal activities are in a pH range of 6.3-7.5 [21]. Here, the pH of 7.3 was selected as an optimal pH value for further experiments.

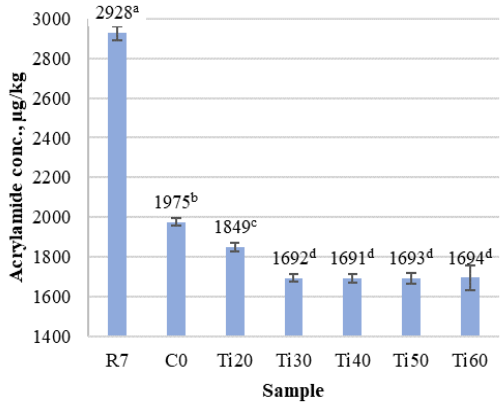


The different letters (a-d) showed significant difference ($p < 0.05$).

Fig. 7. Acrylamide levels in roasted coffee samples at different pH values.

3.3.4 Effects of immersing time on acrylamide mitigation

The acrylamide content in roasted product is dependent on the immersing time of *Robusta* green coffee. Fig. 8 gives the acrylamide content of roasted coffee samples when starting materials were treated with enzyme (3 IU/mL) at various immersion times. The experimental results indicated that the acrylamide mitigation efficiency of enzyme-treated sample in *Robusta* coffee was achieved with an immersion time of 30 min (Ti30). The efficiency levels off when immersion time increases from 30 to 60 (Ti60) min ($p < 0.05$). This observation can be due to the completion of the enzyme penetration into the green coffee, i.e., the enzyme osmosis reaches an equilibrium state after 30 min. The enzyme osmolality capacity was also found in potato chips or biscuits, in which the mitigation of acrylamide in the final products was found to be most effective when enzyme-treated starting material was immersed for 30 min [23, 24].



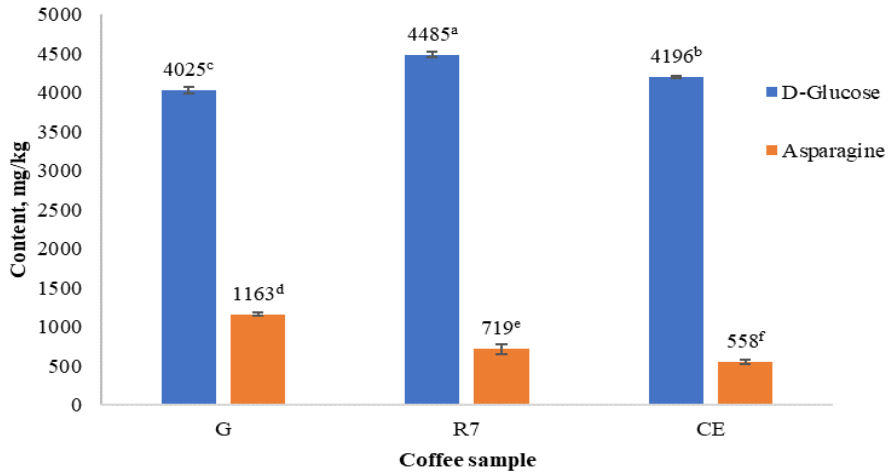
The different letters (a-d) showed significant difference ($p < 0.05$).

Fig. 8. Acrylamide concentrations of roasted coffee samples treated with enzyme at various immersing time.

3.4. Change of reducing sugar and asparagine contents

Fig. 9 depicts the reducing sugar and asparagine levels in green coffee beans (G), non-enzyme roasted coffee samples (R7) and roasted coffee samples (CE) under enzyme-treated conditions (enzyme concentration of 3 IU/ mL, 37 ºC, pH 7.3 in 30 min) using ultrasound. The reducing sugar level of green coffee samples (4025 mg/kg) was lower than that of the roasted coffee samples (R7, CE). The release of reducing sugars due to hydrolysis sucrose (6-9% in coffee) [25] during roasting accounted for the higher reducing sugars in samples R7 and CE. Moreover, the level of reducing sugar in the CE sample (4196 mg/kg) was decreased by 6.4% compared to the non-enzyme-treated coffee (4485 mg/kg).

Asparagine is an acrylamide precursor for acrylamide formation in food. The asparagine content of R7/CE was lower than that of G. The asparagine reduction of 38.2% in R7 (719 mg/kg) compared to green coffee sample (G, 1163 mg/kg). Under roasting conditions at 220 °C for 7 min, asparagine reacts with reducing sugars to form acrylamide, leading to a decrease in asparagine [27]. The reduction of asparagine in CE samples (52%) was found to be higher than that in R7 samples (38.2%). While the acrylamide level in CE samples (1692 µg/kg) was observed to be smaller than that in R7 samples (2928 µg/kg). This result can be attributed to the depletion of asparagine by enzyme using ultrasound.



The different letters (a-f) showed significant difference ($p < 0.05$).

Fig. 9. Reducing sugar and asparagine contents in green coffee (G), non-enzyme roasted coffee (R7) and enzyme-treated roasted coffee (CE) samples.

3.5. Chemical compositions

Chemical compositions of samples G/R7/CE are presented in Table 1. The sample G has the moisture content of 12.5%, ash content of 4.4%, lipid level of 10.6% and protein content of 14.8%, respectively. Those of samples R7/CE have similar values.

Table 1. Chemical compositions of coffee samples.

Chemical composition, %	Green coffee (G)	Regular roasted coffee (R7)	Enzyme-treated coffee (CE)
Moisture	12.5 ± 0.1	1.7 ± 0.2	1.4 ± 0.2
Ash	4.4 ± 0.0	5.1 ± 0.0	4.9 ± 0.0
Lipid	10.6 ± 0.0	10.6 ± 0.0	10.7 ± 0.0
Protein	14.8 ± 0.1	17.5 ± 0.1	17.5 ± 0.1

3.6. Effect of initial moisture content of green coffee beans on acrylamide mitigation

The effects of moisture contents of green coffee on the acrylamide formation in roasted coffee samples with and without enzyme treatment are given in Fig. 10. In the absence of enzyme, there was no change of acrylamide contents in roasted coffee samples with various initial moisture contents ($p > 0.05$). The enzyme-treated samples gave a difference in acrylamide reduction. The lowest value (1525 µg/kg) was obtained when the initial moisture content was 5.5%. The acrylamide reductions were 47.9% and 22.7% when compared to non-enzyme roasted samples R7 (2928 µg/kg) and C0 (1972 µg/kg), respectively. These analytical results were similar to those of Dria G. J *et al*. The acrylamide content decreased when the initial moisture content of *C. arabica* coffee was below 6% [6]. Acrylamide reduction can be explained by the enhanced osmosis capacity of the enzyme when increasing the difference in moisture level between sample and media. As a result, there was an increased catalytic efficiency between the enzyme and the substrate.

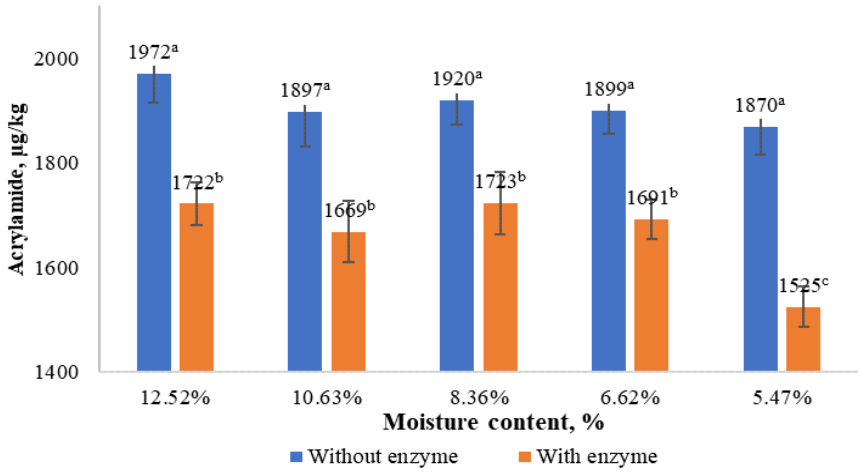


Fig. 10. Effects of initial moisture content of green coffee beans on acrylamide mitigation.

3.7. Sensory evaluation

The sensory evaluation score of coffee products from samples CE/R7 is shown in Fig. 11. Coffee powders were dark brown, fluffy, non-clumping. The final product gave a characteristic taste of

Robusta coffee and no strange smell. The sensory evaluation score for each descriptor of both samples was similar (the average score was 4.4/5, p <0.05). Thus, L-asparaginase not only limits the formation of acrylamide in roasted coffee products, but also ensures safety without affecting the sensory properties of the final product.

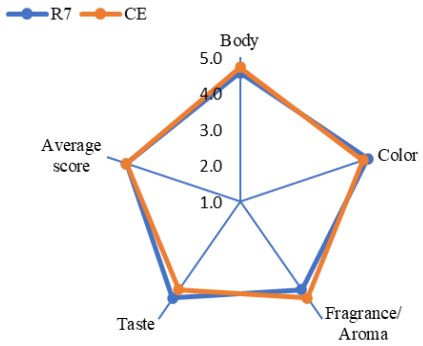


Fig. 11. The sensory evaluation score for enzyme-treated (CE) and regular roasted (R7) coffees.

4. Conclusion

The treatment of L-asparaginase combined with ultrasound on green coffee beans had significant effects on acrylamide mitigation in *Robusta* coffee. The pretreatment conditions: enzyme concentration of 3 IU/mL, immersion time of 30 min, temperature of 37 °C and pH 7.3 and ultrasound with power of 37 kHz showed effectiveness. Furthermore, the initial moisture content of green coffees (5.5%) can give lower acrylamide levels in roasted coffee samples because of different osmosis capacities of the enzyme solution into the samples. There were insignificantly potential adverse effects of asparaginase treatment on sensory properties of enzyme-treated roasted coffee compared to the regular roasted product. However, besides promising results, the effective operation of ultrasound on the acrylamide mitigation has not been indicated. The efficiency of the ultrasound assistance will be systematically compared in our next results.

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List of abbreviations

UV-Vis: Ultraviolet–visible;
Abs: Absorbance

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