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SPECIAL ISSUE ARTICLE

Application of Asparaginase Enzyme in Reducing Acrylamide Formation in Roasted Coffee Beans

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ABSTRACT

Acrylamide is formed through the Maillard reaction between L-asparagine and reducing sugars during thermal processing. The enzyme L-asparaginase can effectively mitigate acrylamide formation by hydrolyzing asparagine into aspartic acid without affecting product quality. This study investigated the effect of asparaginase application methods, enzyme concentrations, and incubation temperatures on acrylamide reduction and pyrazine preservation in roasted Arabica coffee beans. Two enzyme application methods—soaking and spraying—were compared using 4000 U/g asparaginase. Soaking treatment achieved a greater acrylamide reduction (92.8%) compared to spraying (86.8%), and was therefore selected for optimization. Subsequently, enzyme concentrations (2000, 3000, and 5000 U/g) and incubation temperatures (40, 50, and 60 ˚C) were tested. The optimal condition was found at 3000 U/g enzyme concentration and 50 ˚C incubation for 30 minutes, resulting in 96.5% acrylamide reduction and significant conversion of asparagine to aspartic acid, without altering pyrazine concentrations responsible for coffee aroma. The pH of treated beans remained stable (5.38–5.92), indicating no negative impact on sensory attributes. Overall, asparaginase application effectively reduced acrylamide formation in roasted coffee while maintaining desirable flavor compounds, demonstrating its potential as a practical enzymatic strategy for improving the safety and quality of thermally processed coffee products.

Keywords: Acrylamide, Amino acids, Asparaginase, Coffee bean, Enzymes

Introduction

L-asparaginase is an enzyme, an amino hydrolase, that has been extensively studied for decades, primarily for its therapeutic role in depleting the amino acid asparagine and thereby inhibiting the formation of carcinogenic compounds, particularly in leukemia treatment. It has also been investigated for other therapeutic purposes, including the management of acute myeloblastic leukemia, chronic lymphoma, Hodgkin's disease, autoimmune disorders, bacterial infections, collagen-induced arthritis (CIA), necrotizing fasciitis, pharyngitis, and scarlet fever.[1] Its diverse healthcare applications have been comprehensively reviewed by previous researchers.[2] This medical application has driven extensive research on its production, purification, and immobilization. Beyond the medical field, L-asparaginase also plays important roles in the food industry, biosensor development, and several other biotechnological applications.[1]

More recently, L-asparaginase has attracted considerable interest in the food industry due to its ability to reduce acrylamide formation in thermally processed foods. Acrylamide, a potential health hazard, is generated during the Maillard reaction when free asparagine reacts with reducing sugars. Still, the use of L-asparaginase effectively lowers the availability

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of asparagine, thereby mitigating acrylamide levels in fried and baked products, as demonstrated by numerous studies across different food models.[3]

As acrylamide is formed from amino acid L-asparagine and reducing saccharides via the Maillard reaction, elimination of one of these precursors by L-asparaginase enzyme in green beans has led to the suppression of acrylamide content in the final product.[4] The use of the L-asparaginase is also proposed to attain asparagine consumption.[5] L- asparaginase enzyme is claimed to reduce acrylamide levels by up to 90% by converting asparagine into aspartic acid without altering the appearance or taste of the final products.[5] Acrylamide has been distinguished as a poisonous material that induces carcinogenic, reproductive, and genotoxic influences on mammalian cells.[6] Previous studies[7,8] highlighted that L-asparaginase can effectively inhibit acrylamide formation in fried potato products by catalyzing the hydrolysis of L-asparagine to aspartic acid. Additionally, another study[9] discussed various processing strategies, including the use of L-asparaginase, to mitigate acrylamide levels in cereal-based food products. Coffee is one of the most popular beverages worldwide. The high consumption rate of coffee required a high production rate. These findings support the continued use of L-asparaginase as a viable method for reducing acrylamide content in various food products. Reduction of acrylamide content depends on the enzyme dosage, time, and temperature of incubation.[4] The enzyme concentration used in this study was 2000–5000U/g as suggested by Novozymes A/S.

Previous studies have been conducted to reduce acrylamide content in biscuits and French fries by using L-asparaginase enzyme.[5] However, no studies have been carried out on the effectiveness of L- asparaginase from *Aspergillus oryzae* to reduce asparagine and acrylamide concentration in roasted coffee beans without affecting the number of pyrazines. Therefore, the effect of asparaginase in reducing acrylamide formation without sacrificing the number of pyrazines in roasted coffee beans was investigated in this study. The present study was able to establish the relationship between methods of asparaginase application, concentration of asparaginase, and incubation temperature on the development of acrylamide in roasted coffee beans.

Materials and methods

Raw materials and enzyme

Fermented and dried Indonesian arabica coffee beans were obtained from the Indonesian Coffee and Cocoa Research Institute (ICCRI), Jember, Indonesia. This study only used arabica coffee beans since this type of coffee bean was the most consumed coffee bean and contains quite low acrylamide content. Enzyme solutions with enzyme activity of 2000 U/g, 3000 U/g, 4000U/g, and 5000 U/g were prepared using 300 mL deionised water. Prior to roasting and analysis, samples were analysed for moisture content and pH. Treated and untreated samples were analysed for amino acids, acrylamide, and pyrazines. A randomized experimental design was employed in which green coffee bean samples were randomly allocated to different enzyme concentrations and incubation treatments, and the sequence of sample roasting and analysis was randomized to minimize experimental bias.

Determination of amino acids

Treated and untreated samples were analysed for amino acid which was prepared by AccQTag Method using HPLC with Breeze system software (Waters) with chromatographic conditions; (i) column: AccQ Tag Column (3.9 × 150 mm); (ii) Mobile phase: AccQ Tag Eluent A and AccQ Tag Eluent B or 60% acetonitrile; (iii) Derivatization: AccQ Flour Reagents; (iv) Standards: Amino acids standard, Asparagine standard, hydrolysate (Standard H, 'Pierce'; (v) Flow rate: 1mL/min; (vi) Column temperature: 36 °C; (vii) Detection: Fluorescence Detector (Waters 2475) with E_λ = 250 nm, E_m = 395 nm, Gain = 1 and Filter = 1.5 sec, and; (viii) Injection volume: 5 μL. Prior to analysis, Eluent A and B as mobile phase, 6M HCl, 0.1 M HCl, AABA (IS), amino acid standard, asparagine standard, AccQ Flour Reagent, and Borate buffer were prepared. 0.1–0.2g of ground sample was hydrolysed with 5 mL 6M HCl in a test tube, and then placed in the oven at 110 °C for 24 hours. Alpha-aminobutyric acid (AABA) was used as an internal standard (IS), which was added to the amino acid standard and the hydrolysed sample during preparation of the sample before being injected into the HPLC for analysis. Samples were also made using AccQ Flour Reagent to yield highly stable urea that strongly fluoresces at 395 nm during analysis.

Variation in asparaginase application methods

This study compared the variation of asparaginase application methods (soaking and spraying) in reducing acrylamide formation in roasted coffee beans. This study used only the enzyme with an activity level of 4000 U/g. green coffee beans (200g) (single) were soaked immediately in enzyme solution for 5 minutes, while for the spraying method, the solution was sprayed onto green beans thoroughly for

5 minutes. After soaking and spraying, the beans were incubated in a water bath at 40 ˚C for 30 minutes to ensure conversion of asparagine into aspartic acid. The incubated samples were dried in a convection oven at 60 ˚C until they reached the desired moisture content (11–11.12%). After the incubation process, enzyme-treated samples were analysed for amino acids (asparagines and aspartic acid) and roasted using a roaster (PROBAT, Germany) at optimised conditions (167 ˚C temperature, 22 minutes). They were further analysed (triplicate) for pyrazines, acrylamide, amino acids (asparagines and aspartic acid), and pH.

Different asparaginase concentrations

Since the soaking method was selected as an effective means of reducing acrylamide formation in roasted beans, it was further used to investigate different asparaginase concentrations and incubation conditions. In this study, only three enzyme activities (2000 U/g, 3000 U/g, and 5000 U/g) were used since the enzyme activity of 4000U/g was already used for the asparaginase application method. Green coffee beans (200g) were soaked in a series of enzyme activities for 5 minutes. Treated samples were then incubated at three different incubation temperatures (40, 50, and 60 ˚C) for 30 minutes. After incubation, samples were dried in an oven at 60 ˚C until the desired moisture content was attained. Before the roasting phase, treated and untreated samples were analyzed for amino acids. Samples were roasted at 167 ˚C for 22 minutes and further analyzed (triplicate) for pH, pyrazines, acrylamide, and amino acids (asparagine and aspartic acid).

Extraction of pyrazines in roasted coffee beans

Pyrazines were extracted from the coffee sample by SPME and were analysed by using GC-FID. Ground samples (5g) were heated at 60 ˚C, while the SPME fibre was reconditioned for 15 minutes in the GC injection port. Then, reconditioned fibre was introduced to the headspace of the coffee sample at 60 ˚C for 30 minutes. After that, the SPME fibre was desorbed into GC-FID for 15 minutes. The fibre used for the SPME method was Polydimethylsiloxane-Divinylbenzene (PDMS-DVB), which was used for the extraction of volatile compounds (furans, pyrazines, ketones, acids, and pyrroles) in roasted coffee beans and for the characterisation of roasted coffee and coffee beverages in previous researches.[10]

Determination of pyrazines

Only five pyrazine compounds were used as parameters, since 2,5-dimethylpyrazine and 2,6-dimethylpyrazine have quite similar concentrations and aroma characteristics in roasted coffee beans. Flavour compounds (2-methylpyrazine, 2,3-dimethylpyrazine, 2,5-dimethylpyrazine, 2,3,5-trimethylpyrazine, and 2,3,5,6-tetramethylpyrazine) were analysed using GC FID. After extracting the samples using SPME, the fibre extracts were injected into a GC-FID (Agilent Technologies 7890A) equipped with a RTX-5 (dimethylpolysiloxane cross-linked) capillary column coated with a stationary-phase film, and helium was used as the carrier gas with a constant flow of 30 ml/min. The injector temperature was maintained at 260 ˚C, operating in splitless mode. The GC temperature programme fell from 600 ˚C (3 min) to 180 ˚C at 5 ˚C/min for 3 min. Identification of the standard component was performed by comparing the retention time and area of the component with those of the internal standard (4-Picoline). The samples were analysed in triplicate. Concentration of pyrazines was calculated using the relative response factor (F) of the internal standard, using the formula below:

$$\frac{AX}{[X]} = \frac{F\,[AS]}{[S]}$$

where,

AX = Area of analyte in sample

[X] = Concentration of analyte in sample

F = Response factor

AS = Area of internal standard (4-Picoline)

[S] = Concentration of Internal standard (added into the sample).

Data analysis

Statistical analysis was performed in triplicate. Data are presented as mean ± standard deviations. The data were submitted to ANOVA (one-way) followed by Duncan's Multiple Range Test (DMRT) at 5% probability using SPSS 16.0 software. XLStat 2009 was used to compute the multivariate analysis (principal component analysis, hierarchical cluster analysis, and discriminant analysis) and correlation test. The raw data was standardised and the non-detected values were replaced with half the detection limit before being analysed. Linear regression analysis was performed in Microsoft Office Excel 2003. Goodness-of-fit was evaluated by means of the determination coefficients (R^2) and the corresponding

P values, the standard error (SE), and the sum of squares (SS) of residuals. All data were checked for normality and homogeneity of variances prior to conducting ANOVA. The Shapiro–Wilk test was used to assess normality, and Levene's test was used to confirm homogeneity of variance. Only data meeting these assumptions were subjected to one-way ANOVA.

Results and discussion

Variation in asparaginase application methods

The results in Table 1 demonstrate the significant role of L-asparaginase in mitigating acrylamide formation in roasted coffee beans through the hydrolysis of free asparagine. Asparagine is a key precursor for acrylamide generation during the Maillard reaction, and its conversion to aspartic acid by asparaginase effectively reduces acrylamide levels in the final product, as supported by recent literature.[11] In unroasted samples, soaking treatment reduced asparagine concentration by 44.9%, while spraying achieved a 24.6% reduction. This difference is reflected in the corresponding increase in aspartic acid levels, which rose from 0.14 mg/100 g in untreated beans to 0.40 mg/100 g in soaked beans and 0.25 mg/100 g in sprayed beans. The greater efficiency of soaking can be attributed to bean hydration and swelling, which enhanced enzyme penetration and facilitated diffusion of asparaginase into the cellular matrix. Similar findings have been reported in other starchy food systems where soaking promoted effective enzymatic hydrolysis and subsequent acrylamide reduction. For instance, some researchers[12] examined the effects of sequential soaking treatments on Maillard reaction products in vegetable crisps, finding that certain soaking methods effectively decreased acrylamide concentrations. These results were consistent with those reported by previous researchers.[4] They reported that L-asparaginase enzyme was effective in hydrolyzing the amide group of asparagine and converted it into aspartic acid, thus reducing the level of asparagine after treatment.

Upon roasting, acrylamide concentration in untreated beans reached 0.52 mg/100 g, whereas enzyme-treated beans showed significant reductions to 0.07 mg/100 g (sprayed) and 0.04 mg/100 g (soaked). This corresponded to acrylamide reduction rates of 86.8% and 92.8%, respectively. These findings highlight the strong correlation between asparagine depletion and acrylamide mitigation. In particular, the soaking method not only reduced precursor availability more efficiently but also translated into greater acrylamide inhibition during roasting. The U.S. Food and Drug Administration[13] notes that soaking raw potato slices in water for 15–30 minutes before frying or roasting can help reduce acrylamide formation during cooking.

The data in Table 2 show that asparaginase treatment had minimal influence on the overall pyrazine profile of roasted coffee beans. Total pyrazine concentration remained statistically unchanged between untreated beans (2.52 mg/100 g) and soaked beans (2.59 mg/100 g), indicating that the enzyme's action was specific to asparagine hydrolysis and did not disrupt flavor-related compounds. This agrees with previous studies demonstrating that L-asparaginase only reacted on asparagine and did not affect flavour compounds (pyrazines) in food matrices.[4,11] Recent studies have explored the impact of L-asparaginase on acrylamide reduction without significantly affecting flavor compounds like pyrazines. For instance, a study[7] highlighted that L-asparaginase can effectively

Table 1. Amino Acid Asparagine, Aspartic Acid, and Acrylamide Concentration in Samples.

Treatment	Asparagine (unroasted)	Aspartic acid (unroasted)	Acrylamide	Acrylamide Reduction (%)	Asparagine Reduction (%)
Untreated	0.69 ± 0.01^{a}	0.14 ± 0.03^{a}	0.52 ± 0.00^{a}	–	–
Soaking	0.38 ± 0.03^{c}	0.40 ± 0.02^{c}	0.04 ± 0.01^{c}	92.84	44.93 %
Spraying	0.52 ± 0.01^{b}	0.25 ± 0.03^{b}	0.07 ± 0.01^{b}	86.81	24.64 %

Mean values followed by different superscript letters within the same column are significantly different ($p < 0.05$) according to Duncan's Multiple Range Test.

Table 2. Pyrazines concentration in untreated and treated samples.

	2-MP	2,3-DMP	2,5-DMP	2,6DMP	2,3,5-TMP	2,3,5,6-TP	Total pyrazines
Untreated beans	0.49 ± 0.06^{a}	0.06 ± 0.01^{a}	0.19 ± 0.02^{a}	0.39 ± 0.04^{a}	1.24 ± 0.13^{ab}	0.14 ± 0.02^{a}	2.52 ± 0.27^{a}
Soaking	0.44 ± 0.02^{ab}	0.05 ± 0.00^{a}	0.22 ± 0.03^{a}	0.42 ± 0.02^{a}	1.33 ± 0.08^{a}	0.13 ± 0.01^{a}	2.59 ± 0.13^{a}
Spraying	0.40 ± 0.04^{b}	0.05 ± 0.00^{a}	0.18 ± 0.01^{a}	0.16 ± 0.01^{b}	1.13 ± 0.05^{b}	0.12 ± 0.01^{a}	2.05 ± 0.09^{b}

Mean values followed by different superscript letters within the same column are significantly different ($p < 0.05$) according to Duncan's Multiple Range Test.
*MP = methylpyrazine *DMP = dimethylpyrazine *TMP = trimethylpyrazine *TP = tetramethylpyrazine.

inhibit acrylamide formation in fried potato products by catalyzing the hydrolysis of L-asparagine to aspartic acid, thereby preventing acrylamide formation without altering sensory properties. Also, another study[14] investigated the influence of asparaginase on acrylamide content, color, and texture in oat, corn, and rice cookies. The study found that asparaginase treatment significantly reduced acrylamide levels by up to 97% while causing only minor changes in color and texture, suggesting that flavor compounds were largely preserved.

However, in sprayed beans, significant reductions were observed for 2-methylpyrazine (2-MP) and 2,6-dimethylpyrazine (2,6-DMP) compared with both untreated and soaked beans. Consequently, the total pyrazine content in sprayed beans (2.05 mg/100 g) was slightly lower than in untreated (2.52 mg/100 g) and soaked beans (2.59 mg/100 g). This reduction was likely not a direct effect of asparaginase activity, since the enzyme targets asparagine rather than Maillard reaction-derived volatiles. Instead, it may be attributed to secondary processing steps such as drying following enzyme application. The drying operation was one of the most important stages of the coffee process, which could affect the final quality of green coffee. Some researchers[15] examined the effects of different drying techniques on coffee extracts. The findings suggested that drying methods could influence the physicochemical properties of coffee, including flavor compounds like pyrazines.

Table 3 shows that the pH of roasted coffee beans remained acidic across all treatments, with no significant difference between enzyme-treated beans (soaking: 5.38, spraying: 5.34) and untreated beans (5.51). These results indicate that asparaginase activity was highly specific to asparagine hydrolysis and did not affect other chemical components that influence acidity. Coffee acidity is typically derived from chlorogenic acids, citric acid, and other organic acids that remain relatively stable during roasting. Thus, the absence of pH variation suggests that asparaginase treatment does not alter the natural acid balance of coffee, preserving its sensory profile and consumer acceptability. This is consistent with previous studies reporting that L-asparaginase reduces acrylamide precursors without significantly changing physicochemical or sensory attributes of the food matrix.[2]

Table 3. pH analysis of treated and untreated roasted coffee beans.

U/g	Temp (°C)	pH
Soaking	60 °C	5.38 ± 0.11^{a}
Spraying	60 °C	5.34 ± 0.05^{a}
Untreated		5.51 ± 0.04^{a}

Mean values followed by different superscript letters within the same column are significantly different ($p < 0.05$) according to Duncan's Multiple Range Test.

Table 4. Amino Acid Asparagine and Aspartic Acid before and after roasting.

	Unroasted		Roasted	
Treatment	Asparagine	Aspartic acid	Asparagine	Aspartic acid
Untreated	0.69 ± 0.01	0.14 ± 0.03	0.25 ± 0.06	0.11 ± 0.04
Soaking	0.38 ± 0.03	0.40 ± 0.02	0.01 ± 0.00	0.38 ± 0.16
Spraying	0.52 ± 0.01	0.25 ± 0.03	0.01 ± 0.00	0.14 ± 0.05

The amino acid analysis Table 4 highlights the impact of asparaginase treatment on asparagine and aspartic acid levels before and after roasting. In unroasted samples, soaking reduced asparagine from 0.69 mg/100 g (untreated) to 0.38 mg/100 g, while increasing aspartic acid from 0.14 to 0.40 mg/100 g. Spraying produced a smaller reduction (0.52 mg/100 g asparagine) and a moderate increase in aspartic acid (0.25 mg/100 g). These results confirm the enzymatic conversion of asparagine to aspartic acid, with soaking being more effective due to better enzyme penetration facilitated by bean hydration and swelling. It is encouraging to compare this result with that found by some scientist[16] who found that all amino- compounds in their analysis decreased with roasting, although at different rates.

After roasting, asparagine levels declined further across all treatments, consistent with its participation in the Maillard reaction. In untreated beans, asparagine decreased from 0.69 to 0.25 mg/100 g, while in enzyme-treated beans, residual asparagine was nearly eliminated (0.01 mg/100 g for both soaking and spraying). The persistence of aspartic acid in roasted, soaked beans (0.38 mg/100 g) compared with sprayed (0.14 mg/100 g) and untreated beans (0.11 mg/100 g) demonstrates that soaking also favours the substrate diffusion and its contact with the enzyme can lead to greater reduction of acrylamide levels.[5] These results are also in agreement with those reported from a previous study,[4] where asparaginase solution was successfully applied by soaking the potato strips. The technique proved to reduce 60% of acrylamide content in the samples without altering the appearance and taste of the final product.[5] These findings confirm that soaking treatment maximized asparaginase efficiency, leading to greater acrylamide reduction (92.84%) compared with spraying (86.81%), as shown earlier in Table 1.

The overall results on asparagine, aspartic acid, acrylamide, and total pyrazines of untreated, soaked, and sprayed samples can be seen in Fig. 1. This method (soaking) was selected as the asparaginase application method and was used for further study on

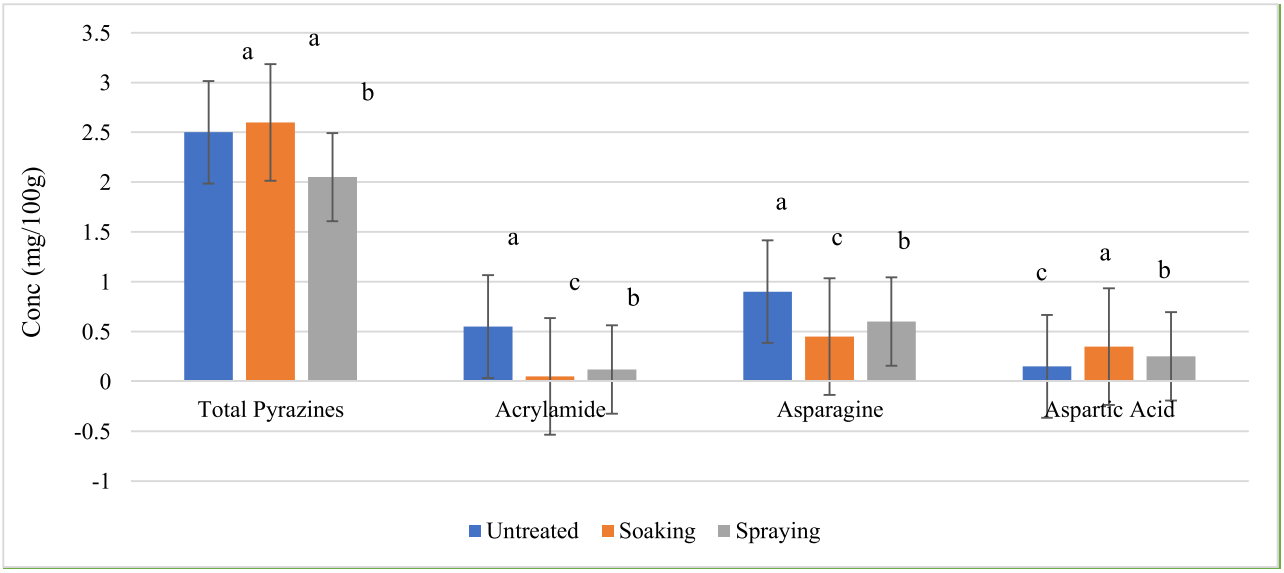

Fig. 1. Concentration of Total Pyrazines, Acrylamide, Asparagine, and Aspartic Acid in Untreated, Soaked, and Sprayed Green Coffee Bean samples (Concentration of Asparaginase 4000 U/g, Incubated at 40 °C for 30 Min).
Note: Mean values with the same alphabet are not significantly different (P > 0.05) according to Duncan's Multiple Range test

different asparaginase concentrations and different incubation temperatures for the reduction of acrylamide in roasted coffee beans.

Different asparaginase concentrations

Since soaking was chosen as the selected method for asparaginase application, this method was used in this stage with different enzyme concentrations. Green coffee bean samples were soaked in a series of enzyme concentrations (2000, 3000, and 5000U/g) and incubated at different temperatures (40, 50, and 60 ˚C) for 30 minutes to study their effect on acrylamide reduction. Analysis of amino acids in green coffee is of the utmost importance since this compound acts as a precursor in the Maillard reaction, in which the colour and aroma are formed. Table 5 showed the amino acid content in green coffee beans before treatment and chromatogram with peaks detected using HPLC using two eluents for almost 45 minutes, highlighting their potential role as precursors in the Maillard reaction and acrylamide formation during roasting. Among the quantified amino acids, glutamic acid was the most abundant (13.321 ± 0.021 mg/100 g), followed by lysine (2.757 ± 0.007 mg/100 g), alanine (1.835 ± 0.065 mg/100 g), and histidine (1.805 ± 0.046 mg/100 g). These findings were consistent with previous results where the three main amino acids in both arabica and robusta green coffees were glutamic acid, aspartic acid, and gamma aminobutyric acid (GABA).[17] Of particular interest is the relatively low concentration of free asparagine (0.687 ± 0.010 mg/100 g), compared to other amino acids such as glutamic acid and lysine. Since asparagine is the primary precursor for acrylamide formation, its presence, though modest, remains critical in influencing acrylamide generation during roasting. The observed level also supports the hypothesis that even small amounts of asparagine can contribute significantly to acrylamide production under high-temperature conditions. Meanwhile, the presence of amino acids such as proline, threonine, valine, methionine, leucine, and phenylalanine underscores their role in flavor development through

Table 5. Amino acid content in arabica green coffee bean.

Amino acids	Mean Concentration (mg/100g)	Standard Deviation (±)
Asparagine	0.687	0.010
Aspartic acid	0.137	0.034
Glutamic acid	13.321	0.021
Glysine	0.772	0.014
Histidine	1.805	0.046
Arginine	1.493	0.012
Threonine	0.974	0.102
Alanine	1.835	0.065
Proline	0.348	0.023
AABA	1.000	0.000
Cysteine	0.475	0.023
Tyrosine	0.338	0.004
Valine	0.324	0.087
Methionine	1.006	0.254
Lysine	2.757	0.007
Ileucine	1.475	0.009
Leucine	0.393	0.135
Phenylalanine	1.209	0.024

Table 6. Amino Acid Asparagine and Aspartic Acid content before and after roasting of Asparaginase treated and untreated coffee beans.

Treatment		Unroasted, Treated		Roasted, Treated	
Concentration of enzyme (U/kg)	Temperature of Incubation (°C)	Asparagine	Aspartic acid	Asparagine	Aspartic acid
Untreated	–	0.68 ± 0.01	0.13 ± 0.03	0.25 ± 0.057	0.10 ± 0.04
5000	60	0.26 ± 0.03	0.51 ± 0.02	0.02 ± 0.003	0.15 ± 0.02
5000	50	0.54 ± 0.02	0.23 ± 0.01	0.01 ± 0.002	0.20 ± 0.05
5000	40	0.27 ± 0.02	0.50 ± 0.05	0.01 ± 0.003	0.16 ± 0.10
3000	60	0.42 ± 0.02	0.26 ± 0.03	0.01 ± 0.006	0.22 ± 0.03
3000	50	0.14 ± 0.04	0.73 ± 0.05	0.00 ± 0.001	0.24 ± 0.02
3000	40	0.25 ± 0.04	0.43 ± 0.01	0.00 ± 0.001	0.18 ± 0.07
2000	60	0.51 ± 0.01	0.20 ± 0.01	0.00 ± 0.001	0.30 ± 0.02
2000	50	0.37 ± 0.04	0.44 ± 0.04	0.00 ± 0.001	0.30 ± 0.02
2000	40	0.24 ± 0.02	0.41 ± 0.04	0.00 ± 0.002	0.21 ± 0.05

Table 7. Amino Acid Asparagine, Aspartic Acid, and Acrylamide Concentration in Unroasted and Asparaginase Treated Samples.

Treatment	Asparagine	Aspartic acid	Acrylamide	% Acrylamide Reduction
Untreated	0.68 ± 0.01	0.13 ± 0.03	0.52 ± 0.002	–
5000,60	0.26 ± 0.03	0.51 ± 0.02	0.02 ± 0.002	94.85
5000,50	0.54 ± 0.02	0.23 ± 0.01	0.07 ± 0.005	86.69
5000,40	0.27 ± 0.02	0.50 ± 0.05	0.04 ± 0.004	86.81
3000,60	0.42 ± 0.02	0.26 ± 0.03	0.03 ± 0.0004	93.98
3000,50	0.14 ± 0.04	0.73 ± 0.05	0.01 ± 0.002	96.53
3000,40	0.25 ± 0.04	0.43 ± 0.01	0.02 ± 0.007	94.41
2000,60	0.51 ± 0.01	0.20 ± 0.01	0.03 ± 0.001	93.00
2000,50	0.37 ± 0.04	0.44 ± 0.04	0.04 ± 0.01	92.28
2000,40	0.24 ± 0.02	0.41 ± 0.04	0.03 ± 0.01	93.92

Maillard and Strecker degradation pathways. These compounds are essential in shaping the sensory attributes of roasted coffee, particularly the generation of aroma-active pyrazines.

Tables 6 and 7 collectively demonstrate the effect of asparaginase treatment on asparagine hydrolysis, aspartic acid accumulation, and acrylamide mitigation in coffee beans. In untreated beans, asparagine was present at 0.68 ± 0.01 mg/100 g, with a corresponding acrylamide content of 0.52 ± 0.002 mg/100 g after roasting Table 7. This confirms the strong correlation between acrylamide formation and the availability of asparagine as a key precursor.

However, treatment with asparaginase at varying concentrations (2000–5000 U/g) and incubation temperatures (40–60 °C) resulted in significant reductions of asparagine (21.3–79.2%) and acrylamide (86.7–96.5%) compared to the untreated control ($p < 0.05$). Simultaneously, aspartic acid concentrations increased across all treatments, confirming enzymatic hydrolysis of asparagine into aspartic acid. Importantly, aspartic acid does not participate in the Maillard reaction, thereby reducing the likelihood of acrylamide formation during roasting. Due to the strong correlation between the acrylamide formation and asparagine concentration, the reduction of asparagine in raw materials subsequently led to the lower level of acrylamide in the final products.[1] The most effective condition was observed at 3000 U/g enzyme concentration and 50 °C incubation for 30 minutes, where asparagine was reduced from 0.68 ± 0.01 mg/100 g to 0.14 ± 0.04 mg/100 g, and acrylamide decreased from 0.52 ± 0.002 mg/100 g to 0.01 ± 0.002 mg/100 g (96.53% reduction). This highlights 3000 U/g at 50 °C as the optimal condition for acrylamide mitigation. Notably, although higher enzyme concentration (5000 U/g) achieved substantial reductions (86.7–94.9%), it did not outperform 3000 U/g at 50 °C. This suggests that enzyme efficiency plateaus at higher concentrations, likely due to substrate saturation or reduced catalytic efficiency at elevated enzyme loads.

Temperature also played a decisive role in enzyme activity. While significant reductions were recorded at all tested conditions, 50 °C provided the best balance between catalytic activity and enzyme stability. At 40 °C, enzyme activity was lower, while at 60 °C, partial denaturation may have occurred, reducing effectiveness. These findings are in agreement with previous reports that asparaginase exhibits optimal

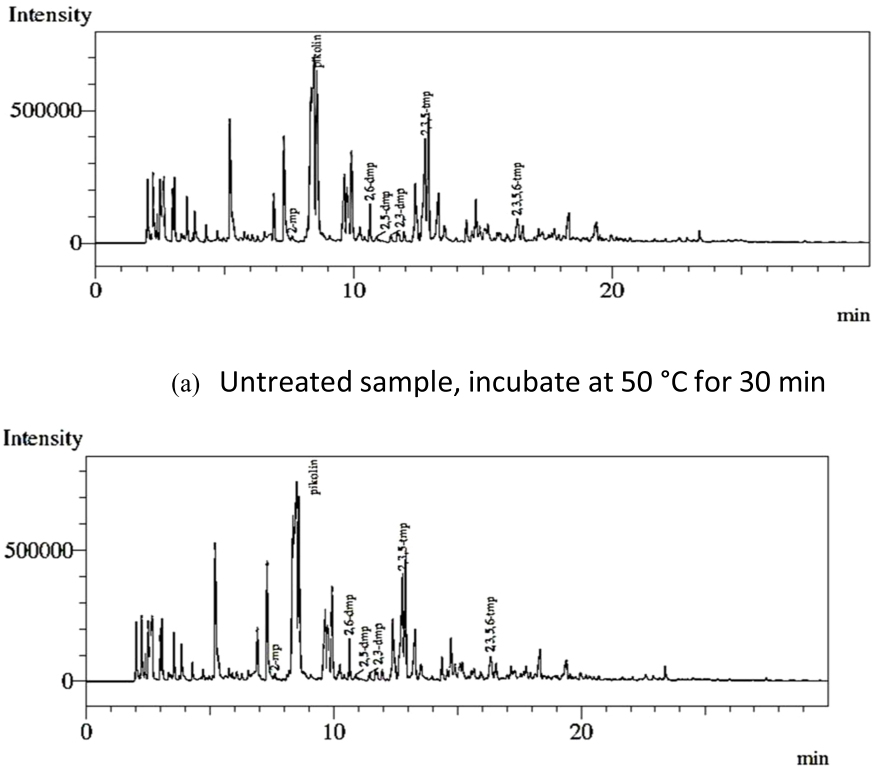

Fig. 2. GC-FID Chromatogram of Pyrazines for Untreated and Treated Samples With 3000 U/g Concentration of Asparaginase Enzyme.

activity within the 20–54 ˚C range for 10–30 minutes of incubation.[5] These findings further supported the idea of a study where the free amino acids that were initially low in the green sample, were massively destroyed during roasting, especially when the temperature reached above 180˚C.[16] While aspartic acid content increased due to the asparaginase reaction, which converted asparagine into aspartic acid. Aspartic acid did not take part in the Maillard reaction5 and thus prevented the formation of acrylamide during roasting.[17] Recent studies have further elucidated the role of L-asparaginase in converting asparagine to aspartic acid, thereby mitigating acrylamide formation during high-temperature processing. For instance, a safety evaluation by the European Food Safety Authority (EFSA) confirmed that asparaginase prevents acrylamide formation by converting asparagine into aspartic acid during food processing.[18]

With regard to the chemical composition, sufficiently high water content facilitated enzyme mobility towards substrate and promoted asparagine hydrolysis and acrylamide mitigation. This is the reason why the enzyme was more effective when added into the aqueous phase of biscuit dough preparation instead of in the powdery phase.[19]

Overall, these results confirm that reducing asparagine in raw coffee beans using asparaginase directly translates into reduced acrylamide levels in roasted beans. The optimal treatment condition (3000 U/g, 50 ˚C, 30 minutes) achieved almost complete mitigation of acrylamide, while preserving amino acid balance and avoiding excessive enzyme use.

Fig. 2 presents the GC-FID chromatograms of pyrazines in untreated roasted beans and beans treated with 3000 U/g of asparaginase, incubated at 50 ˚C for 30 minutes. The chromatographic profiles show that the treated samples exhibited pyrazine peaks that were nearly identical to those of the untreated control. This confirms that asparaginase treatment, under the optimized conditions, did not negatively influence the formation of pyrazines, which are key contributors to the characteristic aroma and flavor of roasted coffee. However, it is noteworthy that treatments involving higher enzyme concentrations and incubation temperatures (e.g., 5000 U/g at 60 ˚C) also achieved significant acrylamide reduction while preserving pyrazines at levels comparable to the untreated samples. However, the use of excessive enzyme concentrations is

Table 8. Concentration of Pyrazines in Asparaginase treated and untreated samples.

Enzyme (U/g)	2-MP (mg/100g)	2,3-DMP (mg/100g)	2,5-DMP (mg/100g)	2,6DMP (mg/100g)	2,3,5-TMP (mg/100g)	2,3,5,6-TP (mg/100g)	Total pyrazines (mg/100g)
Untreated beans	0.490 ± 0.056^{ab}	0.055 ± 0.007^{a}	0.191 ± 0.019^{a}	0.388 ± 0.041^{ab}	1.242 ± 0.129^{ab}	0.143 ± 0.017^{a}	2.517 ± 0.272^{a}
5000,60	0.503 ± 0.035^{a}	0.053 ± 0.015^{a}	0.043 ± 0.006^{c}	0.463 ± 0.055^{a}	1.340 ± 0.095^{a}	0.100 ± 0.01^{de}	2.503 ± 0.204^{a}
5000,50	0.020 ± 0.002^{g}	0.040 ± 0.001^{abc}	0.040 ± 0.003^{c}	0.360 ± 0.017^{ab}	1.017 ± 0.047^{e}	0.120 ± 0.010^{bc}	1.597 ± 0.074^{de}
5000,40	0.393 ± 0.011^{de}	0.040 ± 0.001^{abc}	0.040 ± 0.002^{c}	0.330 ± 0.010^{b}	0.843 ± 0.021^{f}	0.123 ± 0.006^{bc}	1.770 ± 0.036^{cd}
3000,60	0.357 ± 0.011^{ef}	0.040 ± 0.005^{abc}	0.137 ± 0.006^{b}	0.353 ± 0.012^{ab}	1.083 ± 0.055^{de}	0.093 ± 0.012^{e}	2.063 ± 0.055^{b}
3000,50	0.453 ± 0.038^{b}	0.050 ± 0.017^{ab}	0.197 ± 0.015^{a}	0.337 ± 0.012^{ab}	1.267 ± 0.125^{ab}	0.130 ± 0.010^{ab}	2.433 ± 0.098^{a}
3000,40	0.327 ± 0.015^{f}	0.037 ± 0.006^{bc}	0.033 ± 0.006^{c}	0.303 ± 0.021^{b}	0.647 ± 0.025^{g}	0.113 ± 0.006^{cd}	1.460 ± 0.072^{e}
2000,60	0.397 ± 0.032^{de}	0.033 ± 0.006^{c}	0.037 ± 0.006^{c}	0.268 ± 0.204^{b}	1.163 ± 0.065^{cd}	0.107 ± 0.006^{de}	2.003 ± 0.310^{bc}
2000,50	0.353 ± 0.011^{ef}	0.040 ± 0.003^{abc}	0.040 ± 0.005^{c}	0.323 ± 0.006^{b}	0.873 ± 0.015^{f}	0.097 ± 0.006^{de}	1.727 ± 0.035^{de}
2000,40	0.433 ± 0.006^{cd}	0.043 ± 0.006^{abc}	0.047 ± 0.006^{c}	0.363 ± 0.015^{ab}	1.120 ± 0.053^{de}	0.123 ± 0.006^{bc}	2.130 ± 0.087^{b}

Mean values followed by different superscript letters within the same column are significantly different ($p < 0.05$) according to Duncan's Multiple Range Test.
*MP-methylpyrazine *DMP-dimethylpyrazine *TMP-trimethylpyrazine *TP-tetramethylpyrazine.

Table 9. pH of Asparaginase untreated and treated roasted samples.

Concentration of the enzyme (U/g)	Temperature of incubation (˚C)	pH (Mean ± SD)
Untreated	–	5.51 ± 0.04^{a}
2000	60 ˚C	5.50 ± 0.00^{a}
	50 ˚C	5.66 ± 0.02^{a}
	40 ˚C	5.57 ± 0.01^{a}
3000	60 ˚C	5.41 ± 0.03^{a}
	50 ˚C	5.79 ± 0.05^{a}
	40 ˚C	5.92 ± 0.02^{a}
5000	60 ˚C	5.46 ± 0.04^{a}
	50 ˚C	5.61 ± 0.03^{a}
	40 ˚C	5.65 ± 0.03^{a}

Mean values followed by different superscript letters within the same column are significantly different ($p < 0.05$) according to Duncan's Multiple Range Test.

not cost-effective for large-scale applications. Therefore, the condition of 3000 U/g at 50 ˚C for 30 minutes provides the most practical balance between acrylamide mitigation and preservation of sensory quality.

Table 8 presents the concentration of individual and total pyrazines in roasted coffee beans treated with different concentrations of asparaginase and incubation conditions. The results indicate that asparaginase treatment generally reduced acrylamide levels, but in some cases, it also influenced the pyrazine content, which is closely linked to the aroma and flavor quality of coffee. Notably, samples treated with 3000 U/g at 50 ˚C for 30 minutes maintained total pyrazine concentrations (2.433 ± 0.098 mg/100 g) comparable to the untreated beans (2.517 ± 0.272 mg/100 g), showing no significant difference ($p > 0.05$). This suggests that under these conditions, acrylamide reduction can be achieved without compromising the flavor compounds. Conversely, higher enzyme concentrations (5000 U/g at 50 ˚C or 40 ˚C) and lower concentrations at 40 ˚C showed marked reductions in pyrazines, which could negatively impact the sensory properties. These findings align with the GC-FID chromatogram results Fig. 2, confirming that optimal enzyme concentration and incubation conditions are critical for balancing acrylamide reduction with flavor preservation. While all treatments were effective in lowering acrylamide, some led to unintended losses of pyrazines, highlighting the importance of selecting cost-effective yet flavor-preserving conditions, such as 3000 U/g at 50 ˚C for 30 minutes.

Table 9 presents the pH values of roasted coffee beans treated with different concentrations of asparaginase under varying incubation conditions. The results demonstrate that enzyme treatment did not significantly alter the pH of the roasted samples compared to the untreated beans ($p > 0.05$). All samples remained slightly acidic, with pH values ranging from 5.41 to 5.92, which is within the typical range for roasted coffee.

This indicates that the application of asparaginase for acrylamide reduction did not compromise the acidity of the beans, a parameter closely associated with the sensory quality of coffee. The linear correlation observed between acrylamide and pyrazine levels during the Maillard reaction suggests that enzyme treatment effectively reduces acrylamide while maintaining chemical stability, including pH balance.

Fig. 3 summarizes the overall concentration of total pyrazines, acrylamide, asparagine, and aspartic acid in both treated and untreated coffee bean samples. The results demonstrate that soaking green coffee beans in asparaginase at 3000 U/g, incubating at 50 ˚C for 30 minutes, and subsequently drying at 60 ˚C to a final moisture content of 11–11.12% was the most effective strategy for acrylamide reduction. This treatment achieved a dramatic

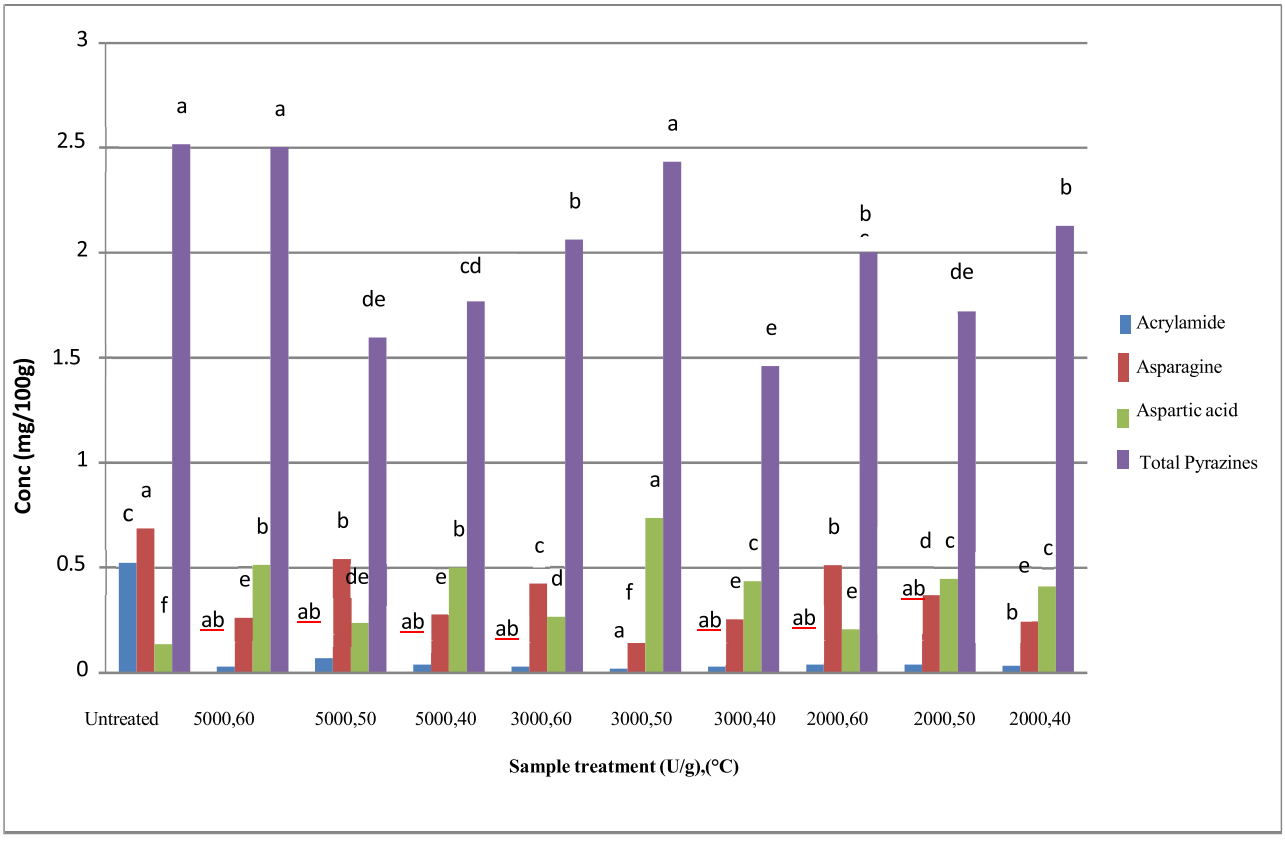

Fig. 3. Overall Concentration for Total Pyrazines, Acrylamide, Asparagine, and Aspartic Acid in Treated and Untreated Samples. Mean values followed by different superscript letters within the same treatment are significantly different ($p < 0.05$) according to Duncan's Multiple Range Test.

decrease in acrylamide levels while simultaneously converting asparagine into aspartic acid, without significantly affecting the concentration of pyrazines responsible for coffee aroma. Statistical analysis further confirmed that no significant differences ($p > 0.05$) were observed in pyrazine concentrations between treated and untreated samples, thereby preserving the characteristic flavor profile of roasted coffee.

Conclusion

Overall, these findings validate the optimized enzyme application as a practical method for reducing acrylamide formation without compromising coffee quality. Asparaginase treatment effectively reduced acrylamide formation in roasted coffee beans while preserving desirable pyrazines responsible for flavor and sensory quality. The enzyme hydrolyzes asparagine into aspartic acid, thereby limiting acrylamide precursors. Optimal reduction was achieved by soaking green coffee beans in a 3000 U/g enzyme solution at 50 °C for 30 minutes.

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Authors' declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images that are not ours have been included with the necessary permission for republication, which is attached to the manuscript.
- No animal studies are present in the manuscript.
- No human studies are present in the manuscript.
- Ethical Clearance: the project was approved by the local ethical committee at Universiti Teknologi MARA, 40450, Shah Alam, Selangor, Malaysia.

Authors' contributions statement

K.M.B performed the Laboratory work. K.M.B and Z.F.A.M wrote the manuscript. M.J provides coffee

samples for this research. Z.F.A.M, Z.A.H, K.M.B and M.J read and approved the final version.

Data availability

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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تطبيق إنزيم الأسباراجيناز في خفض تكوّن الأكريلاميد في حبوب القهوة المحمّصة

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الخلاصة

يتكوّن الأكريلاميد من خلال تفاعل ميلارد بين الحمض الأميني-L أسباراجين والسكريات المختزلة أثناء المعالجات الحرارية. ويمكن لإنزيم الأسباراجيناز الحدّ من تكوّن الأكريلاميد بفاعلية من خلال تحلّل الأسباراجين إلى حمض الأسبارتيك دون التأثير في جودة المنتج. يهدف هذا البحث إلى دراسة تأثير طرق تطبيق الأسباراجيناز، وتركيزات الإنزيم، ودرجات حرارة الحضانة على خفض الأكريلاميد والحفاظ على مركّبات البيرازين في حبوب البن العربي المحمّصة. وقد تمت مقارنة طريقتين لتطبيق الإنزيم—النقع والرش—باستخدام تركيز 4000 وحدة/غرام من الأسباراجيناز. وحققت معالجة النقع خفضًا أكبر في مستويات الأكريلاميد (92.8%) مقارنة بالرش (86.8%)، ولذلك تم اختيارها لغرض التحسين. بعد ذلك، جرى اختبار تركيزات إنزيمية (2000، 3000، و5000 وحدة/غرام) ودرجات حرارة حضانة (40، 50، و60 درجة مئوية). وتبيّن أن الحالة المثلى كانت عند تركيز إنزيم 3000 وحدة/غرام ودرجة حضانة 50 درجة مئوية لمدة 30 دقيقة، مما أدى إلى خفض الأكريلاميد بنسبة 96.5% وتحويل كبير للأسباراجين إلى حمض الأسبارتيك دون تغيير في تركيزات مركّبات البيرازين المسؤولة عن رائحة القهوة. كما بقيت قيمة الأس الهيدروجيني للحبوب المعالجة مستقرة (5.38–5.92)، مما يدل على عدم حدوث تأثير سلبي في الخصائص الحسية. وبوجه عام، أثبت تطبيق الأسباراجيناز فعاليته في الحد من تكوّن الأكريلاميد في القهوة المحمّصة مع الحفاظ على المركّبات النكهية المرغوبة، مما يبرهن على إمكاناته كاستراتيجية إنزيمية عملية لتحسين سلامة وجودة منتجات القهوة المعالجة حرارياً.

الكلمات المفتاحية: الأكريلاميد، الأحماض الأمينية، الأسباراجيناز، حبوب القهوة، الإنزيمات.