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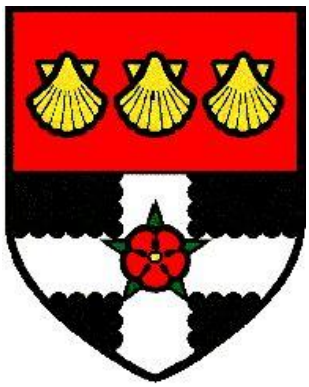

Acrylamide mitigation in coffee by asparaginase application

A thesis submitted in partial fulfilment of the requirement for the degree of Doctor of Philosophy (PhD)

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Declaration

I confirm that this is my own work and the use of all materials from other sources has been properly and fully acknowledged.

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December 2017

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Abstract

Acrylamide is a Group 2B carcinogen compound formed in certain foods when they are heated above 100°C. The use of the enzyme asparaginase is becoming increasingly popular as a means of reducing acrylamide formation in cooked foods. Asparaginase converts the acrylamide precursor asparagine to aspartic acid. Roast coffee is one of the major sources of acrylamide in the human diet. In this thesis, asparaginase solution was initially delivered into green coffee beans by a steam & soak method. Hence, acrylamide in the roasted coffee was significantly reduced (40-91%). However, the steam & soak method showed adverse effects, including precursor loss (amino acids 20–30%, sucrose 38%, glucose 47%) and silver skin loss. To examine the impact of enzyme treatment on the aroma of roasted coffee, volatile determination was carried out. Significant differences were found (10% of identified compounds) in a broad variety of chemical families (aldehydes, ketones, furans, phenols and pyrazines). A series of sensory analyses was carried out to identify the impact of enzyme treatment on coffee organoleptic properties. Sensory analysis using descriptive and discrimination tests found significant differences (12 and 17 out of 39 attributes in light roast and medium roast groups, respectively) between enzyme-treated beans and untreated beans. Preference tests, however, showed that control and treated samples were of similar acceptability, with no unpleasant flavours or aromas associated with the enzyme treated group. Finally, a novel vacuum infusion method was used to reduce the adverse effect of the steam & soak method. The results indicated the vacuum infusion method (21–56% acrylamide reduction) was not as effective as the steam & soak method (40–91%). In conclusion, our research confirmed that the enzymatic approach could successfully reduce acrylamide in coffee. However, as the coffee had tight internal structure, the enzyme treatment process would cause the loss of silver skin on coffee beans. The consequence of this loss was a darker roast in the enzyme treated samples. The shift of roast extent leads to significant differences in volatile compounds profile and organoleptic properties of the coffee beans. The vacuum infusion experiment was carried out to reduce the loss of silver skin issue while maintaining the high acrylamide reduction rate. However, the results indicate that the vacuum failed to allow asparaginase to penetrate the coffee and still resulted in great silver skin loss. Future research should focus on minimising the adverse effect of enzyme treatment while maintaining sufficient contact of enzyme and substrate.

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Table of Contents

List of Figures

List of Tables

Chapter 1 the use of asparaginase to reduce acrylamide levels in cooked food

Abstract

Strategies proposed for reducing the formation of the suspected carcinogen acrylamide in cooked foods often rely on a reduction in the extent of the Maillard reaction, in which acrylamide is formed from the reaction between asparagine and reducing sugars. However, the Maillard reaction also provides desirable sensory attributes of cooked foods. Mitigation procedures that modify the Maillard reaction may negatively affect flavour and colour. The use of asparaginase to convert asparagine to aspartic acid may provide a means to reduce acrylamide formation, while maintaining sensory quality. This review collates research on the use of enzymes, asparaginase in particular, to mitigate acrylamide formation. Asparaginase is a powerful tool for the food industry (80-95% acrylamide reduction rate on various foodstuffs) and it is likely that its use will increase. However, the potential adverse effects of asparaginase treatment on sensory properties of cooked foods and the need to achieve sufficient enzyme–substrate contact remain areas for future research.

Key words

Acrylamide, asparaginase, enzymes, asparagine, reducing sugars, Maillard reaction

1.1 Introduction

It is now over ten years since the Swedish Food Authority and the University of Stockholm confirmed the existence of the suspected carcinogen acrylamide in a variety of heated foods (Tareke, Rydberg, Karlsson, Eriksson, & Törnqvist, 2002).Several months after the announcement, researchers showed that acrylamide is formed from asparagine and reducing sugars during the Maillard reaction(Mottram, Wedzicha, & Dodson, 2002; Stadler, Blank, et al., 2002). As shown in **Figure 1.1**, asparagine and reducing sugars take part in a conjugation reaction resulting in the formation of *N*-glycosylasparagine, which as a result of high temperature treatment will form a decarboxylated Schiff base. The decarboxylated Schiff base may decompose directly to form acrylamide or may hydrolyse to form 3-aminopropionamide (Hedegaard, Frandsen, & Skibsted, 2008). 3-Aminopropionamide is also believed to be an important precursor of acrylamide (Granvogl & Schieberle, 2006).

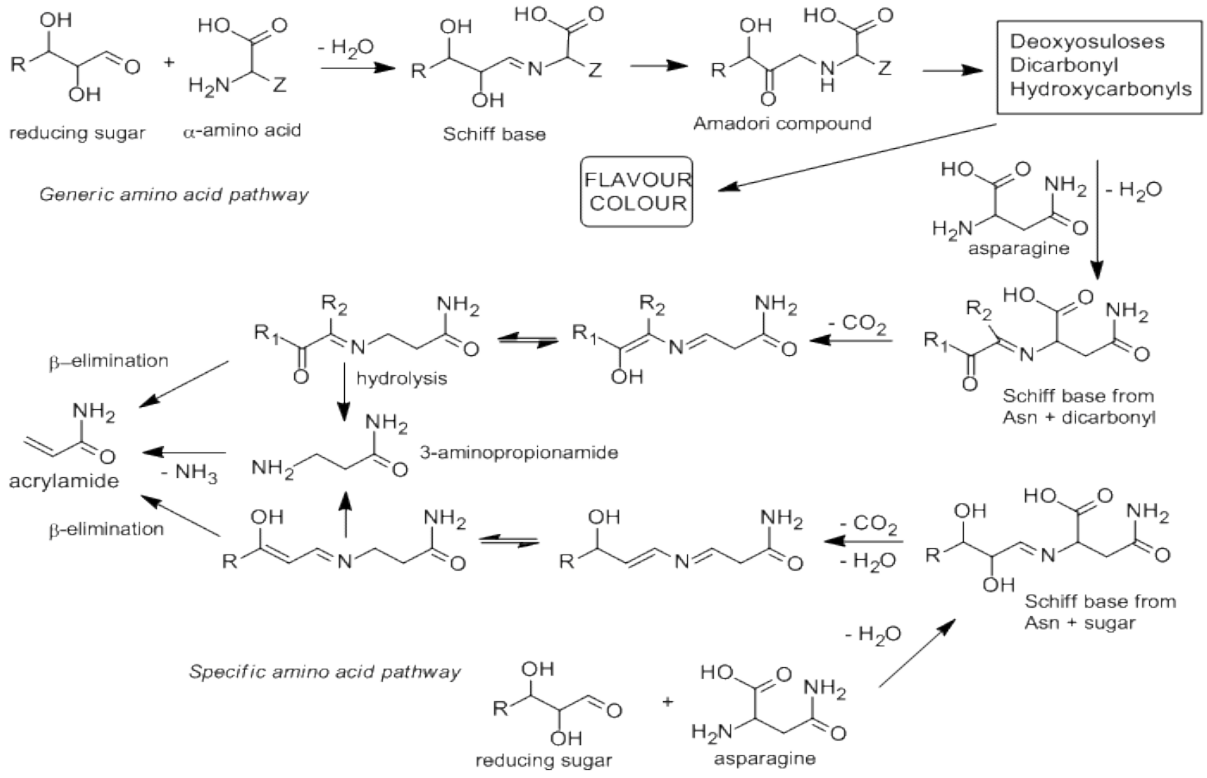

Figure 1.1 *Acrylamide reaction pathway*

Since 2002, the food industry worldwide has collaborated with scientists, in order to reduce the levels of acrylamide in cooked foods. Mitigation techniques can be separated into three different types. Firstly, starting materials low in acrylamide precursors can be used to reduce

the acrylamide in the final product. Secondly, process conditions may be modified, in order to decrease the amount of acrylamide formation. Thirdly, post-process intervention could be used to reduce acrylamide (Pedreschi, Mariotti, & Granby, 2014). While the third approach is not widely considered, an example is the use of supercritical CO_2 extraction to reduce acrylamide levels in coffee. Almost 80% of the acrylamide was removed using this technique(Banchero, Pellegrino, & Manna, 2013), although further sensory tests are needed to validate the effect on food quality.

This review will describe the main mitigation strategies used for acrylamide but will focus on the use of enzymes, in particular asparaginase, to reduce levels of acrylamide precursors. A more general review on acrylamide mitigation has been recently published(Friedman, 2015), while another recent review has covered the latest studies on the sources, purification, and characterisation of L-asparaginase and its application in both the pharmaceutical and food industries (Zuo, Zhang, Jiang, & Mu, 2015a)

1.2 Acrylamide mitigation strategies

1.2.1 Raw materials

Decreasing the amounts of acrylamide precursors will have a huge impact on final acrylamide production (Zyzak et al., 2003). However, the effect will be dependent on the relative levels of precursors. If total reducing sugars are present at higher levels than asparagine in a food, reduction in asparagine will have the greater effect on acrylamide formation, and *vice versa*. Numerous papers have demonstrated that acrylamide formation is proportional to reducing sugar concentrations in potato (Elmore et al., 2015; Ohara-Takada et al., 2005; Vinci, Mestdagh, Van Poucke, Van Peteghem, & De Meulenaer, 2012), while in cereals, such as rye and wheat, acrylamide formation is proportional to asparagine content (Curtis et al., 2010; Halford, Curtis, et al., 2012). Hence potato varieties low in reducing sugars and cereal varieties low in asparagine are sought. Storage may increase levels of reducing sugars in stored potatoes, particularly under low-temperature conditions (Rak, Navarro, & Palta, 2013)while levels of fertilisation, for example nitrogen and sulfur, may have effects on reducing sugars and asparagine levels in both potatoes and cereals (Elmore et al., 2007; Muttucumaru et al., 2006;

Muttucumaru, Powers, Elmore, Mottram, & Halford, 2013). It is clear, however, that little or no acrylamide will form in the absence of asparagine, while other components of the food matrix, such as lipid-derived aldehydes (Zamora & Hidalgo, 2008), amino acids such as serine and threonine (Shu, 1999), and other carbonyl-containing molecules (Hamzalioglu & Gokmen, 2012; Zamora, Delgado, & Hidalgo, 2011), can react with asparagine to form acrylamide.

1.2.2 Process-based mitigation

Initial mitigation methods involved the control of processing conditions; for instance, lowering pH, reducing cooking temperature and shortening the processing time (Palazoğlu & Gökmen, 2008). Although these methods achieved an effective reduction of acrylamide, sensory properties of the food were compromised. As the Maillard reaction begins when food is heated, the first option in this type of mitigation method is to lower the temperature and time of heating. However, as the Maillard reaction is also responsible for generating desirable taste and smell in cooked food, sensory properties become unacceptable when cooking temperature is substantially reduced (Masi, Dinnella, Barnaba, Navarini, & Monteleone, 2013).

Besides temperature, another important parameter, pH, has also been studied. In a model system, the acrylamide content will reach a maximum amount when the pH is around 8, which is near to the pK_a value of asparagine and leads to an enhancement in the initial steps of acrylamide formation (Rydberg et al., 2003). Several authors have reduced acrylamide formation by reducing the pH, using compounds such as citric acid (Gama-Baumgartner, Grob, & Biedermann, 2004), although product quality has suffered generally using this approach (Vinci, Mestdagh, & De Meulenaer, 2012)

1.2.3 Use of additives

Adding other chemicals prior to or after heating could also decrease the final acrylamide amount in a cooked product. For example, adding glycine before heating will compete with asparagine to lower the final acrylamide amount (Bråthen, Kita, Knutsen, & Wicklund, 2005). It could also be added after the Maillard reaction, to react with acrylamide directly, thus lowering its amount in the final product (Liu, Man, Zhu, Hu, & Chen, 2013). However, this method also has negative effects on the sensory properties of the product, as added glycine will react with reducing sugars to increase levels of odour-active alkylpyrazines (Low, Parker, &

Mottram, 2007).

The addition of divalent cations has also been shown to be an effective means of reducing acrylamide. Nixtamalisation, the traditional cooking of corn grains in calcium hydroxide solution prior to milling, is traditionally used in the preparation of tortillas (Salazar et al., 2014). A calcium chloride dip for potatoes reduced acrylamide in French fries by 95%, with no adverse effects on product quality reported. The effect is due to the divalent cations inhibiting formation of the Schiff base (Gökmen & Senyuva, 2007). Monovalent cations at low concentrations may also exhibit a mitigation effect. Addition of 1–2% sodium chloride to a bread mix led to a substantial reduction in acrylamide in baked rolls. Higher salt concentrations inhibited yeast growth, resulting in increased acrylamide formation (Claus, Mongili, Weisz, Schieber, & Carle, 2008). A 2% sodium chloride dip pre-treatment (60 min, room temperature) resulted in a 78% reduction in acrylamide in fried potato discs (Sansano, Juan-Borras, Escriche, Andres, & Heredia, 2015).

Antioxidants could also be added to inhibit the formation of acrylamide. However, effects were variable in the several studies carried out during the past decade, due to the various types of antioxidants used (Jin, Wu, & Zhang, 2013). For instance, rosemary added to corn or olive oil could effectively lower the amount of acrylamide in fried potato slices (Becalski, Lau, Lewis, & Seaman, 2002), while some commonly used antioxidants, such as BHT, sesamol and Vitamin E, had an enhanced effect on acrylamide formation in cooked meat (Rydberg et al., 2003). The reducing or promoting effects could be attributed to differences in reaction conditions, antioxidant dosage and different reaction pathways.

1.2.4 Enzymatic approaches for acrylamide reduction

Fermentation methods use specific microorganisms to consume the asparagine or reducing sugar before the food processing step (Sadd, Hamlet, & Liang, 2008). For instance, a starter medium containing lactic acid bacteria was used in the preparation of wholemeal rye bread and substantially reduced acrylamide levels in the final product (Bartkiene et al., 2013). As well as lowering the pH, the lactic acid bacteria reduced the levels of reducing sugars in the dough.
However, there are several points that need to be considered in a fermentation approach. To begin with, the temperature and pH need to be controlled in order to maximise the activity of the microorganism. Even if the reducing sugar consumed was added back after processing, the

sensory quality of the final product may still be affected by the fermentation step (Bartkiene et al., 2013). Secondly, fermentation predominantly works in bakery products, with limited application in potato-based products and coffee (Kamkar et al., 2015).

The use of an enzymatic approach to modify reaction pathways was first proposed by Amrein et al. (Amrein, Schönbächler, Escher, & Amadò, 2004), who used asparaginase to hydrolyse asparagine to aspartic acid and ammonia (Ciesarova, Kiss, & Boegl, 2006). This approach is considered to be effective because asparagine is not considered a major contributor to the overall flavour and colour of cooked foods (Parker et al., 2012), so desirable sensory properties are maintained.

1.2.5 Acrylamidase

Rather than hydrolysing the precursors of acrylamide, acrylamidase focuses on acrylamide itself. Acrylamidase can hydrolyse acrylamide to acrylic acid and ammonia. Cha and Chambliss carried out the first research using acrylamidase from *Ralstonia eutrophia* to reduce acrylamide in food (Cha & Chambliss, 2011). This enzyme had an optimum pH of 6.3, and optimum temperature of 55 °C. A batch culture of AUM-01 completely converted 28.0 mM acrylamide to acrylic acid in 8 h. *Moraxella osloensis* MSU11 converted a 40 mM acrylamide solution to acrylic acid within 2448 h (Jebasingh, Lakshmikandan, Rajesh, & Raja, 2013), while the bacterium *Stenotrophomonas acidaminiphila* MSU12 degraded a 30 mM acrylamide solution within 48–54 h (Lakshmikandan et al., 2014). Acrylamidase from *Arthrobacter* sp. DBV1 degraded a 30 mM acrylamide solution within 42 hours (Bedade & Singhal, 2017).

Though some of the research mentioned the potential usage of acrylamidase in foodstuffs, none of the previous research carried out acrylamide reduction in real foods. Several issues raised concern for the real food application. To begin with, the enzyme delivery method needed to be developed. On one hand, most food has tighter internal structure, which makes sufficient enzyme and substrate contact more difficult. On the other hand, food grade application requires high specificity of the enzyme. Especially for acrylamidase, which was applied in the processed food, which made residual acrylamidase exist after the acrylamide hydrolysis reaction. Secondly, even though acrylamidase achieved enough contact with acrylamide in heated food, the acrylic acid that generated in the acrylamide hydrolysis reaction may pose another problem for safety concern. The European Union risk assessment report provided an oral NOAEL value

(No-observed-adverse-effect-level) of 40 mg/kg bw/d in male rats (EC-JRC, 2002).

Furthermore, acrylamidase might be inhibited by the presence of amino acids. For instance, acrylamidase activity in *Ralstonia eutrophia* was partially inhibited by 20 amino acids, with several amino acids reducing 50% of its relative activity (alanine 48%, methionine 45%, phenylalanine 44%, proline 48%,threonine 39%, tryptophan 47% and valine 35%) (Cha & Chambliss, 2011). However, this situation was greatly improved with much more higher relative activity with most of the amino acids for a new acrylamidase purified from *Geobacillus thermoglucosidasius* in these researchers' following study, which increased the likelihood for its application in food (Cha & Chambliss, 2013).As researchers overcome its limitations, acrylamidase may have potential application for acrylamide mitigation in food.

1.3 Asparaginase

Asparaginase (L-asparagine amidohydrolases EC 3.5.1.1) is an enzyme widely distributed in animals, plants and living organisms (Wriston, 1985). It has been shown that asparaginase catalyses the hydrolysis of asparagine into aspartic acid and ammonia by hydrolysing the amide group in the side chain of asparagine (Hendriksen, Kornbrust, Ostergaard, & Stringer, 2009). Aspartic acid will then enter the citric acid cycle, playing a vital role in amino acid metabolism. Asparagine is responsible for nitrogen storage in most plants; therefore, asparaginase plays an important role in energy utilisation (Sieciechowicz, Joy, & Ireland, 1988)

L-Asparaginase has been used as a therapeutic treatment for certain kinds of cancer, such as leukaemia (Bushman, Palmieri, Whinna, & Church, 2000). In leukaemia sufferers malignant cells depend more on exogenous asparagine and glutamine to survive than normal cells. Asparaginase injected into the bloodstream hydrolyses free asparagine into aspartic acid and ammonia, and glutamine to glutamic acid and ammonia (Friedman, 2003). In this way, the growth of malignant cells is inhibited.

L-Asparaginase is an intracellular enzyme which is obtained from a variety of microorganisms: *Escherichia coli, Erwinia carotovora, Bacillus* sp., *Enterobacter aerogenes, Corynebacterium glutamicum, Pseudomonas stutzeri* and *Candida utilis*(Qin & Zhao, 2003). For pharmaceutical

uses, L-asparaginase is typically obtained from *Escherichia coli* (Pritsa & Kyriakidis, 2001). However, the production of the enzyme is complex with low yield. Until now, there is no medium that has been specifically established for the optimum production of L-asparaginase from different microorganisms. To maximise enzyme production, each organism has its own optimum conditions. Research on *E. coli* using response surface methodology achieved a 10-fold enhancement in asparaginase production (Kenari, Alemzadeh, & Maghsodi, 2011)

Most asparaginases are quite specific for asparagine. Optimal activity is usually achieved at pH 5–7 and 37 °C. However, as glutamine has similar structure to asparagine, some enzymes also have a low activity towards glutamine (Krasotkina, Borisova, Gervaziev, & Sokolov, 2004). A small group of enzymes, called glutaminase-asparaginases, have activities for both asparagine and glutamine but prefer glutamine as a substrate (Roberts, Dolowy, & Holcenbe.Js, 1972). Crystallographic study has shown that both types of asparaginase, common asparaginase and glutaminase-asparaginase, have the same basic structure and catalytic mechanism but differ in working conditions (pH and temperature) (Yao, Yasutake, Morita, & Tanaka, 2005). Researchers believe that glutaminase activity caused by glutaminase-asparaginase will exert serious adverse effects on human health, such as liver dysfunction, pancreatitis and leucopoenia (Mahajan, Saran, Kameswaran, Kumar, & Saxena, 2012). Therefore, this specific type of asparaginase should be strictly avoided in the food industry.

Commercially, there are two asparaginase products currently available for acrylamide mitigation in the food industry. These are PreventASe® from DSM (Heerlen, The Netherlands) and Acrylaway® from Novozymes A/S (Bagsvaerd, Denmark). PreventASe® was the first, launched in 2007. It was obtained after analysing the gene sequence of *Aspergillus niger* and produced recombinant in the *Aspergillus niger* host. It has an acidic profile (optimum pH 4–5, temperature 50 °C). Acrylaway® on the other hand, is obtained from *Aspergillus oryzae* and has an almost neutral profile (optimum pH 7, temperature 37 °C).

Regarding safety, these enzymes are produced by specific fungal strains of *A. oryzae* and *A. niger*, fungi that have been widely used in commercial products for several decades and have been proved to be safe by JECFA (JECFA, 2007). Acrylaway® and PreventASe® have shown high specificity and therefore minimum activity towards glutamine and other amino acids. Ultimately, these enzymes will be deactivated during the heating process, ensuring their safe

application in foodstuffs (Hendriksen, Kornbrust, Ostergaard, & Stringer, 2009).

Asparaginase has received "generally recognized as safe" status from the US government. It has also been given a favourable evaluation as a food additive by the Joint FAO/WHO Expert committee (JECFA, 2007) and it is currently used in several countries, including United States, Australia, New Zealand, China, Russia, Mexico and several European countries. As different dosages of asparaginase will be used in different types of food, there is no unified standard for the maximum dosage.

1.3.1 Use of asparaginase in acrylamide mitigation

As shown in **Table 1.1**, over the last decade there have been numerous studies monitoring the reduction of acrylamide formation by means of asparaginase treatment. The first study was carried out by Zyzak et al. in 2003, immediately after the formation mechanism was revealed. However, Zyzak's research was focused on the formation mechanism rather than the mitigation efficiency. He used commercial asparaginase from Aldrich (A2925 from *Erwinia chrysanthemi*), 50 U added to 60 g of mashed potato slurry (15 g potato, 45 g water), to hydrolyse the asparagine, in order to verify that asparagine is indeed the precursor of acrylamide. The asparaginase achieved an 88% asparagine reduction that led to 99% acrylamide reduction in a microwaved mashed potato snack, heated at full power until brown (Zyzak et al., 2003).

Table 1.1 Results published on enzymatic mitigation of acrylamide using asparaginase

foodstuff	enzyme source	enzyme dosage	processing conditions	acrylamide reduction	reference
potato	*Escherichia coli*	not stated	not stated	99%	Zyzak et al. (2003)
gingerbread	*E. coli*	4 U/kg	various time/temperature combinations	55%	Amrein et al.(2004)
potato	*E. coli*	0.2–1 U/g	180 °C, 20 min	50–90%	Ciesarová et al. (2006)
French fries	*A. oryzae*	10000 ASNU/L*	175 °C, 3 min	67%	Pedreschi et al. (2008)
semi-sweet biscuits, ginger biscuits, crispbread, French fries, potato crisps	*A. oryzae*	various dosages	various time/temperature combinations	semi-sweet biscuits: 65–84% ginger biscuits: 34–90% crispbread: 84–92% French fries: 59% potato crisps: 60%	Hendriksen et al. (2009)
fried dough model system	*A. oryzae*	100, 500, 1000 U/kg	180 or 200 °C; 4, 6 or 8 min	90%	Kukurová et al. (2009)
bread	enzymes from *Pisum sativum* L.	not stated	220 °C, 22–25 min	wheat bran bread: 57% whole-grain bread: 68%	Tuncel et al. (2010)
potato chips	*A. oryzae*	10000 ASNU/L	170 °C, 5 min	90%	Pedreschi et al. (2011)
biscuits	*A. oryzae*	100–900 U/kg	200 °C, final moisture content 2%	7–88%	Anese et al. (2011a)
biscuits	*A. oryzae*	900 U/kg	200 °C, final moisture content 2%	69%	Anese et al. (2011b)
potato	*Bacillus licheniformis*	30 IU/mL	175 °C, 15 min	80%	Mahajan et al. (2012)
lebkuchen, tortilla chips, potato snack, French fries, coffee	*A. oryzae*	various dosages	lebkuchen: 200 °C, 14 min tortilla chips: 190 °C, 60 s French fries: 175 °C, 3 min others not specified	lebkuchen: 95% tortilla chips: 90% potato snack: 40% French fries: 57%	Hendriksen et al. (2013).

				coffee: 55–74%	
cookies	*A. oryzae*	500 U/kg	205 °C, 11 or 15 min	23–75%	Kukurová et al. (2013)
wheat-oat bread	*Aspergillus niger*	500 U	220, 230 and 250 °C; 10, 30 and 40 min	90%	Ciesarová et al. (2014)
sweet bread	*Cladosporium sp.*	50–300 U	220 °C; 25 min	sweet bread crust: 97% sweet bread crumb: 73%	Kumar et al. (2014)
biscuits, bread	*Rhizomucor miehei*	0.5–10 U	200 °C; 15 min	biscuits: 81.6% bread: 94.2%	Huang et al. (2014)
potato crisps	*Bacillus subtilis*	0–40 U	170 °C; 90 s	80%	Onishi et al. (2015)
French fries	*Thermococcus zilligii*	0–20 U	175 °C; 5 min	80%	Zuo et al. (2015b)
potato slices	*Bacillus subtilis*	0-47 U	170 °C; 6 min	95%	(Gaurav Sanghvi1 & Sheth1, 2016)
potato chips	*Pseudomonas oryzihabitans*	0.6-2.3U/ml	175°C; 10 min	90%	(Bhagat, Kaur, & Chadha, 2016)
French fries	*Aquabacterium sp. A7-Y*	0-458.9U/mg	170 °C; 5 min	88.2%	(Sun et al., 2016)
potato chips & mooncakes	*Paenibacillus barengoltzii*	0-80 U/ml	170 °C; 5 min	potato chips：86% mooncake： 52%	(Shi, Liu, Mu, Jiang, & Yang, 2017)
French fries	*Aspergillus oryzae CCT 3940*	50 U/ml	170 °C; 8 min	72%	(Dias, Bogusz, Hantao, Augusto, & Sato, 2017)

* ASNU is defined as the amount of asparaginase that produces 1 μmol of ammonia per min under the conditions of the assay (pH = 7 ± 0.005; 37 ± 0.5 °C) using Acrylaway®

The following year, the first paper on the use of asparaginase as an acrylamide mitigation method was published. Asparaginase (from *E. coli,* 4 U/kg) added to gingerbread hydrolysed approximately 75% of the free asparagine, leading to a 55% acrylamide reduction in the final product. The acrylamide-reduced product was identical to a control product in both colour and taste(Amrein et al., 2004). Though this enzyme application formed only a small part of the research, it stressed the advantage of the enzymatic method on mitigating acrylamide while maintaining the organoleptic properties of the product.

Ciesarová et al. set up a model system to examine the importance of all the related factors, such as temperature, dosage and application time (Ciesarova et al., 2006). However, there were insufficient time and temperature points studied to determine optimum activity. Applying asparaginase to dried potato powder led to a 90% acrylamide reduction in cooked product. However, instead of considering the effect of the cut and shape of the potato products, this research focused more on potato varieties. Although this research showed great success in acrylamide reduction, the agronomic factors were discussed more than the enzyme itself.

Pedreschi, Kaack, and Granby were the first to publish results using a commercial asparaginase (Acrylaway®) (Pedreschi, Kaack, & Granby, 2008). They established that the optimum temperature and pH for this enzyme were 60 °C and 7.0, respectively. A reduction of 67% in acrylamide was achieved in French fries under these conditions. In this study, the importance of blanching and temperature control of asparaginase treatment was highlighted. It is known that blanching will change the microstructure of the potato strips and increase the contact probability of asparaginase and asparagine (Lisińska, Tajner-Czopek, & Kalum, 2007), so blanching is highly recommended for increasing the performance of the enzyme.

Another study by the same group focused on the combination of asparaginase (Acrylaway®) and conventional blanching, alongside their individual usage. Blanching using hot water at 85 °C to treat the potato tuber samples for 3.5 min was compared with enzymatic mitigation using an asparaginase solution (10000 ASNU/L) at 50 °C for 20 min. One ASNU is defined as the amount of asparaginase that produces one micromole of ammonia per minute under standard conditions (pH 7; 37 °C). Experimental results showed that blanching and enzyme treatments have a similar effect on acrylamide reduction (17%). By combining the two methods, almost 90% of acrylamide was mitigated. The authors assumed that the microstructure of the potato tissues was changed in the blanching process, causing the asparagine in the cell to have

a more effective interaction with the enzyme outside the cell (Pedreschi, Mariotti, Granby, & Risum, 2011). Although acrylamide in this research was significantly reduced, no sensory analysis of the product was performed.

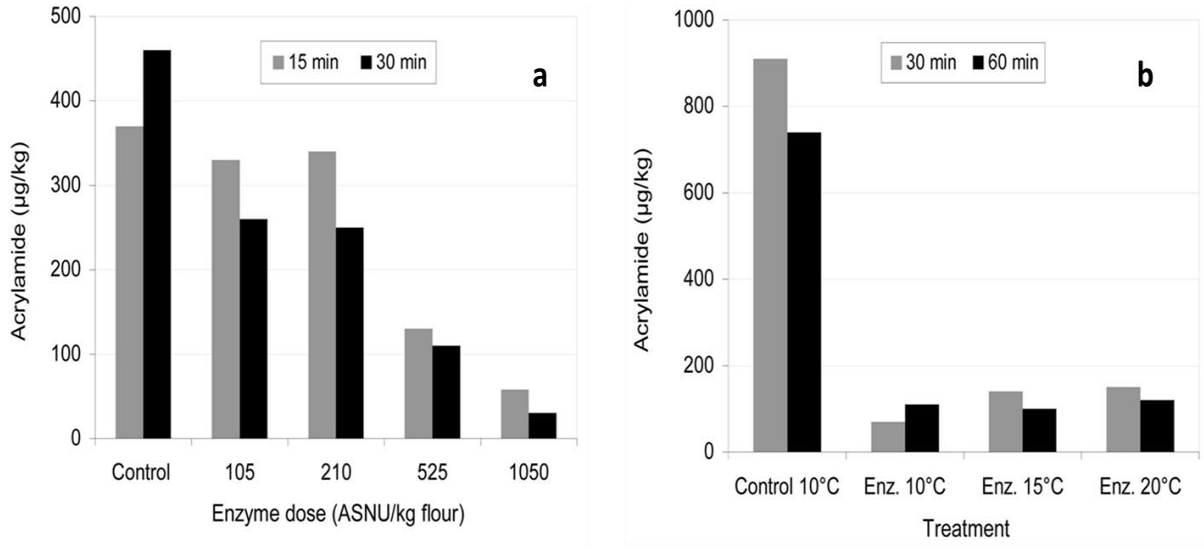

Figure 1.2 Acrylamide formation in (a) semi-sweet biscuits and (b) crisp bread, influenced by enzyme dosage and incubation temperature, respectively (adapted from Hendriksen, Kornbrust, Ostergaard, and Stringer, 2009)

In 2009, another study involving Acrylaway® was carried out on a much wider range of foods, including gingerbread, crispbread, semi-sweet biscuits, French fries and crisps (Hendriksen et al., 2009). Again the optimum conditions of temperature and pH were 60 °C and 7.0. In this study, other factors were also taken into consideration, depending on the food matrix. In semi-sweet biscuits, the dosage was the variable and the temperature was set at 40 °C. Asparaginase treatment took place at the dough resting time before the biscuits were baked at 260 °C for 5.5 min (**Figure 1.2a**). For the crispbread trial, the temperature was held at 10, 15, or 20 °C for 30 or 60 min and the dosage was set at 2100 ASNU/kg of flour. Then the crispbread was baked at 250 °C for 11 min (**Figure 1.2b**). By changing the dosage of enzyme and time, the influences of each factor are revealed. Besides dosage and temperature, water content is another important factor in a cereal-based product like gingerbread, as higher water activity will provide sufficient contact for the enzyme with the substrate. Therefore, in order not to compromise final product sensory quality, a higher water content is recommended, although a further drying step may be needed subsequently. For potato-based products, a reduction was achieved in both French fries and crisps. Potatoes made into French fries were treated with 10500 ASNU/L and then fried

for 3 min at 175 °C. Sliced potatoes used to make potato crisps were treated with various concentrations of enzyme for 15 min at 40 °C. Then frying was conducted for 2.5 min at 180 °C. In the French fries experiment, the authors prepared a sample set with a one-minute dip and 20 minutes soaking in the enzyme bath. Although the acrylamide reduction in the one-minute dip (59% maximum) was less than for the samples in the 20-min soak (85% maximum), the results were still meaningful for the practical continuous process. Results indicated a broad range of enzyme applications. The authors suggested that by combining modified processing conditions with an enzymatic approach, acrylamide could be mitigated at a fairly low cost. The key point in this research is that the authors tried to assess all related variables and generate specific solutions for each type of product from an enormous data-set.

Also in 2009, Kukurová, Morales, Bednáriková, and Ciesarová (2009) used two levels of Acrylaway® (100 U/kg and 500 U/kg flour) in the preparation of fried bread rolls. The asparaginase treatment (15 min, 37 °C) removed at least 96% of the asparagine from the dough (Kukurova, Morales, Bednarikova, & Ciesarova, 2009); acrylamide could not be quantified in the fried rolls with Acrylaway® added, while levels of 215 µg per kg were present in control rolls fried at 200 °C for 8 min. The same group also studied acrylamide formation in cookies treated with Acrylaway® (500 U/kg flour) and different raising agents (Kukurova, Ciesarova, Mogol, Acar, & Gokmen, 2013). The raising agents increased the pH of the dough, reducing the effectiveness of the asparaginase in reducing asparagine. When applied for less than 30 minutes, the asparagine had no effect on the sensory properties of the cookies.

Hendriksen, Budolfsen, and Baumann (2013) used Acrylaway® to study potato and cereal products and also carried out the first experiments on the effect of asparaginase on acrylamide in coffee. For cereal products, the mitigation efficiency reached 95% in lebkuchen and 90% in tortilla chips, whereas in coffee a 70–80% reduction was achieved (Hendriksen, Budolfsen, & Baumann, 2013). In potato products, experiments on potato tuber snack pellets and French fries were carried out. In potato tuber snack pellets, the enzyme was added directly to a dough based on 29% potato starch, 27.6% potato granules, 15% potato flakes, 1.4% salt and 27% water, while in the French fries test, Bintje or Maris Piper potatoes were manually peeled and cut into 8 mm × 8 mm strips. Two innovative points of this study stand out. First, asparaginase is added at the disodium acid pyrophosphate (SAPP) dipping stage rather than as a separate step. SAPP is commonly used in potato processing to prevent after-cooking darkening. Integrating the two treatments could reduce time and cost. Secondly, an industrial scale trial was carried out to test

the mitigation efficiency of asparaginase in continuous processing. Such trials will push the industrial application of asparaginase forward. Reductions in potato product were comparatively low, due to the insufficient contact of asparagine and enzyme. However, the industrial scale experiment (8 tonne/h) still achieved satisfactory results; a 43% reduction in 10 mm × 10 mm and 53% reduction in 7 mm × 7 mm potato pieces was achieved. A dye-based experiment indicated that asparaginase could only penetrate 1 mm into the potato, again highlighting the importance of incorporating a blanching step when treating potatoes (Hendriksen et al., 2013)

For coffee, increased acrylamide mitigation could be achieved by incubating the wetted green beans. Typically, green coffee beans are steamed to decrease the caffeine content. The decaffeination process is usually carried out by a water or solvent partition system. Firstly, green coffee beans are steamed to make the caffeine available. Then, a solvent is used to extract the caffeine. Finally, the green beans are steamed again to remove any residual solvent (Spiller, 1997). Hence, asparaginase could be infused during these steps with minor changes to the processing conditions. A laboratory-scale experiment indicated that a low dosage (2000–6000 ASNU) of asparaginase could achieve 55–74% acrylamide reduction in coffee beans (Hendriksen et al., 2013), while work in our laboratory showed that both the steaming step and the asparaginase treatment caused a reduction in free asparagine when the coffee was roasted, which was reflected in acrylamide losses from 69% to 86% using dosages of 2600 to 20000 ASNU, respectively (Xu, Khalid, Oruna-Concha, & Elmore, 2015).

The effect of asparaginase on acrylamide mitigation in biscuits has also been examined by Anese, Quarta, and Frias (2011). The authors used asparaginase levels from 100 to 900 ASNU (Acrylaway®) with 20–54 °C incubation temperature and 10–30 min incubation time, 15 treatments in all. By analysing the results from each treatment, the influence of each factor was considered and optimum conditions could be obtained. This study not only demonstrated a method that could assess the effect of the enzyme but compared the effect obtained with the cost for each treatment. This paper also contained valuable advice on the practical application of asparaginase. For instance, acrylamide development was at a minimum at intermediate asparaginase concentrations and increased asparaginase addition did not significantly affect the colour of the final product.

We are only aware of one publication where PreventASe® was used as the asparaginase source.

A solution of 500 ASNU in 10 mL of water was spread onto the surface of a wheat/oat bread loaf prior to baking. The enzyme was effective during a proofing step of 15 min at 32 °C. This treatment led to a 46% reduction in the acrylamide content of the baked bread crust (Ciesarová et al., 2014)

1.3.2 New sources of asparaginase

Recent papers have identified new sources of asparaginase for acrylamide reduction. Tuncel, Yılmaz, and Şener (Tuncel, Yilmaz, & Sener, 2010) used asparaginase from a vegetable source. They germinated pea flour to enhance asparaginase activity and remove beany flavour. The pea flour was finely ground and then added to wheat flour in white wheat bread, wheat bran bread and wholegrain white bread at three different levels (1%, 3% and 5%). The bread was baked at 220 °C for 22–25 min. In white wheat bread acrylamide reduction was less than 10% in all cases, while addition of 5% pea flour to bran bread and grain bread reduced acrylamide levels in crust by 57% and 68%, respectively. Although the sensory panel showed detectable differences in the final products, there was no significant negative impact on sensory properties. The extraction of asparaginase from fungus is relatively costly; therefore this approach provides an effective alternative means to produce asparaginase.

Asparaginase with low glutaminase activity was successfully extracted from *Bacillus licheniformis* and was used to reduce acrylamide in fried potato strips by up to 80% (Mahajan et al., 2012). Asparaginase produced from *Cladosporium* sp. was used in the crumb and crust of sweet bread (Kumar, Shimray, Indrani, & Manonmani, 2014). The dosage used varied from 50–300 U. However, the units were not defined. Reductions of 97% and 73% were achieved at 300 U in bread crust and crumb, respectively. The authors also measured the formation of 5-(hydroxymethyl) furfural, which is another potential toxicant formed in the Maillard reaction, and it was also decreased. This research also showed a new possible source for asparaginase, although yield data were not disclosed.

Asparaginase has also been extracted from *Rhizomucor miehei* (Huang, Liu, Sun, Yan, & Jiang, 2014) The extracted asparaginase, designated as RmAsnase, was optimally active at pH 7.0 and 45 °C and was stable at this temperature for 30 min. RmAsnase was cloned and expressed in *Escherichia coli* and proved highly specific towards asparagine. The researchers demonstrated that a low concentration of asparaginase (0.5 U/g flour) had much better

mitigation efficiency in bread (40%) than biscuits (15%). However, an 80% reduction was achieved in both products at 10 U/g flour. Overall, this new enzyme showed remarkable potential both as an acrylamide mitigator and also in leukaemia therapy.

Asparaginase from food-grade *Bacillus subtilis* was applied to potato chips (Onishi et al., 2015). Compared to a control sample, 40 U of asparaginase led to 80% reduction in acrylamide. One unit of the enzyme was defined as the amount that catalysed the formation of 1 µmol ammonia per min; additional details were not provided by the authors. They suggested that BAsnase, as the enzyme was christened, could be used to spray potatoes prior to cooking at home. Similar reductions in acrylamide in French fries were also obtained using an asparaginase from *Thermococcus zilligii*. The purified enzyme displayed a maximum activity at pH 8.5 and 90 °C (Zuo, Zhang, Jiang, & Mu, 2015b) and retained 70% of its original activity after 2 hours incubation at 85 °C. Another asparaginase with high temperature stability was recently isolated from *Pyrococcus furiosus* (Kundu, Bansal, & Mishra, 2013). In addition, at the end of 2013 Novozymes launched Acrylaway®® HighT, which is specifically designed for higher temperature processing (Novozymes, 2013). Enzymes stable at such temperatures can be incorporated into the blanching step of a commercial process, which would increase their applicability.

Immobilised asparaginase may not convert as much asparagine as free asparaginase but its stability is improved, meaning that blanching water containing immobilised enzyme can be re-used several times without loss of asparaginase activity. The asparaginase is immobilised by crosslinking with glutaraldehyde on an inert silica-based carrier (Hendriksen, Puder, & Olsen, 2014)

L-Asparaginase was extracted from *Bacillus subtilis sp.* strain KDPS1(Sanghvi et al., 2016). In this case, the highest asparaginase activity (47 IU/mL) was obtained using orange peel as substrate. The extracted asparaginase (47U) was tested on potato slices, which were then fried at 170 °C for six minutes. A maximum 95% acrylamide reduction was achieved. An L-asparaginase purified from *Pseudomonas oryzihabitans* (Bhagat et al., 2016) showed an activity range of 0.6–2.3 U/mL. The purified enzyme displayed a maximum activity at pH 8.0 and 37–40 °C and when tested on potato chips 90% acrylamide reduction was achieved. Compared to Acrylaway®, the optimum temperature of this asparaginase was lower (40°C vs 60 °C) and the optimum pH was higher (pH 8.0 *vs* pH 7). These conditions will limit the range

of foodstuffs that this enzyme could be applied to.

Asparaginase extracted from *Aquabacterium sp.* A7-Y (Sun et al., 2016) displayed optimum enzyme activity at pH 9.0 and 60 °C. and when tested on potato chips 88.2% acrylamide reduction was achieved. An asparaginase extracted from *Paenibacillus barengoltzii* CAU904 (Shi et al., 2017) possessed an optimum activity at pH 8.5 and 45 °C. with a maximum acrylamide reduction of 86% in potato chips and 52% in Chinese mooncakes. Trace activity towards glutamine and relatively low optimum temperature restrict the possible applications for this enzyme.

An asparaginase was extracted from *Aspergillus oryzae* CCT 3940 (Dias et al., 2017). This microorganism is also the source of Acrylaway®, however from a different strain. This asparaginase had an optimum pH at 6–8 and temperature at 40–50 °C. The researchers claimed that the new asparaginase had better specificity than commercial asparaginase (potentially Acrylaway®). In this research, this newly purified asparaginase was compared with an unspecified commercial asparaginase on the reduction rate of acrylamide in French Fries soaked at 50 °C for 30 minutes. It was observed that for the same dosage (50 U mL^{-1}) 72% reduction for the new asparaginase and 92% for the commercial asparaginase was achieved. For commerical asapraginase like Acrylaway®, the glutaminase activity had been tested by the manufacturer and government bodies. The specificity issue discussed by the author needed justification and validation from a third party researcher.

1.3.3 Asparaginase activity under different conditions

To quantify the effect of asparaginase in food applications, its activity needs to be determined. For the two commercially available enzymes (Acrylaway® by Novozymes and PreventASe® by DSM), two different methods to determine the activity of the enzyme have been used. Both methods are based on measuring the ammonia that is generated from the asparagine hydrolysis. However, in the method used to measure the activity of Acrylaway®, ammonia subsequently reacts with α-ketoglutarate to form L-glutamic acid. The reaction is catalysed by glutamate dehydrogenase in the presence of NADH, which is oxidised to NAD+ with the concomitant loss of absorbance measured at 340 nm. The asparaginase activity is measured as the rate of NADH consumption under standard conditions (pH = 7; 37 °C). The activity of asparaginase is expressed in ASNU activity units. One ASNU is defined as the amount of asparaginase that

produces one micromole of ammonia per minute under standard conditions (Hendriksen et al., 2009). The activity of PreventASe® is measured by a different method. The liberated ammonia subsequently reacts with phenol nitroprusside and alkaline hypochlorite resulting in a blue colour. This is known as the Berthelot reaction (Rhine, Sims, Mulvaney, & Pratt, 1998). The activity of asparaginase is determined by measuring the absorbance of the reaction mixture at 600 nm. Asparaginase activity is expressed in ASPU activity units. One ASPU is defined as the amount of asparaginase that liberates one micromole of ammonia from L-asparagine per minute under standard conditions (pH = 5.0; 37 °C).

By setting up a standard activity determination method, the activity of the enzyme under different conditions of pH and temperature can be measured. From the work done by Hendriksen's group in Novozymes, it was shown that Acrylaway® has almost two times the activity at 60 °C compared to its activity at 37 °C. Also, the activity of the enzyme at pH 7 is almost two times its activity at pH 5. These differences in activity should be considered prior to the application of the enzyme to a substrate.

1.3.4 Practicalities of asparaginase application

The practicalities of asparaginase application can be assessed from three different aspects: raw material composition, processing and commerce. The amount of asparagine in a foodstuff should be considered when deciding the dosage of asparaginase. For example, concentrations of free asparagine in potato may vary by a factor of 10 or more, and can be affected by variety and growing conditions, (Halford, Muttucumaru, et al., 2012)

When using asparaginase in food manufacture, factors like temperature, time and substrate ratio are of importance. For example, the optimal temperature for Acrylaway® is around 60 °C and its activity will decrease significantly above this temperature (Hendriksen et al., 2009). Hence asparaginase will be denatured and inactivated during food processing. The dwell time for enzyme and foodstuff before heat treatment should be optimised before application. Enzyme–substrate ratio is also an important factor and dosage of the enzyme should be determined, so that maximum mitigation is achieved with minimum enzyme concentration. Extra water may be used to ensure the delivery of the enzyme (Beate.Kornbrust, 2009).

Even though asparaginase has advantages over other mitigation methods, its use by manufacturers may not be commercially viable at present. There are also issues with the industrial application of asparaginase in a continuous process, which can achieve good results in a relatively short time. Pilot-scale experiments have been carried out using a continuous process for French fries production (Hendriksen et al., 2009). High levels of acrylamide reduction were achieved (60–85% in French Fries and 60% in potato chips). However, more research is needed to better incorporate asparaginase usage into industrial-scale food production. To maximise the overall effect of the enzymatic method, pre- and post-treatment procedures may need to be adapted; for instance, reduction of starting material dimensions and blanching before enzyme treatment and modification of the process conditions after the enzyme treatment by, for example, changing cooking temperature and pH.

1.4 Conclusion

Asparaginase has become a powerful tool for acrylamide mitigation in the food industry. With the success of commercial products, it is likely that asparaginase will be used more and more. The first commercially available “acrylamide-free” product, biscuits treated with PreventASe®, was announced to launch shortly in Germany for Christmas, 2008. This information was released by DSM Food Specialities, although the manufacturer’s name was not disclosed(Foodingredients1st, 2007). However, there was no more news subsequently.

In November 2013 Novozymes launched Acrylaway® HighT and claimed it could maintain its activity at higher temperature than Acrylaway®(Novozymes, 2013).An optimum temperature of 90 °C has been claimed for Acrylaway® HighT (C-lEcta, 2007).

The potential adverse effects of asparaginase treatment on sensory properties of cooked foods and the need to achieve sufficient enzyme–substrate contact are areas for future research. However, if the application of asparaginase becomes commercially attractive, its use alongside raw materials low in asparagine may provide the solution to the acrylamide problem.

Chapter 2 Acrylamide reduction in enzyme-treated coffee samples

Abstract

This chapter included several experiments concerned with the steam & soak enzyme processed coffee, such as enzyme activity determination, precursor determination, acrylamide determination and colour determination. After the activity of the enzyme was determined, enzyme treatment was carried out with calculated dosages. To ensure sufficient contact of the enzyme and the substrate, coffee beans were steamed first to open the pores. Then, coffee beans were soaked in an enzyme bath. Precursor determination include amino acids determination and sugar determination. The results showed that asparagine decreased (40-91%), aspartic acid increased (167-267%) and sugar content was partially lost (Sucrose-38%, Glucose-47%). Acrylamide determination for a different batch of the experiment showed that different dosages (2000-20000 units) of commercial asparaginase Acrylaway® & PreventASe® managed to reduce acrylamide by 40% to 91% in a variety of Arabica and Robusta coffees. Besides the chemistry impact of precursor loss, as a negative impact of steam & soak enzyme treatment, the silver skin of coffee beans was lost in the enzyme process. Without the protection of silver skin, enzyme treated coffee was more exposed in the roast process. Colour determination confirmed that the enzyme treated coffee was significantly darker (ΔE=1.92-10.42) than the control coffee.

Keywords

Steam & Soak, Precursor, Silver Skin, Colour

2.1 Introduction

Summarizing the knowledge gap indicated by the previous chapter, asparaginase could successfully reduce acrylamide in a variety of foodstuffs. However, few experiments had been carried out in coffee. In green coffee beans, cell walls are thick with minimum intracellular space (Luigi Poisson, 2017). In addition, as shown in **Figure 2.1**, green coffee beans are covered by the silver skin. Silver skin refers to the thin seed skin that covered the coffee cherry(Narita & Inouye, 2014).

In the processing of green coffee beans, parchment is totally removed either by a wet or dry process. Then, green coffee beans with silver skin on are transported to the roasting company. In the roast process, the only by-product of coffee is burnt silver skin (Saenger, Hartge, Werther, Ogada, & Siagi, 2001). Coffee silver skin, the thin tegument of the outer layer of green coffee beans, contains 80% of the total fibre (16% of soluble fibre and 64% of insoluble fibre). The major components of the fibre tissues are cellulose and hemicellulose (16.7%). Research carried out on the thermal degradation of silver skin showed great thermal stability of silver skin as the cellulose started to decompose from 280 °C (Alghooneh, Amini, Behrouzian, & Razavi, 2017).

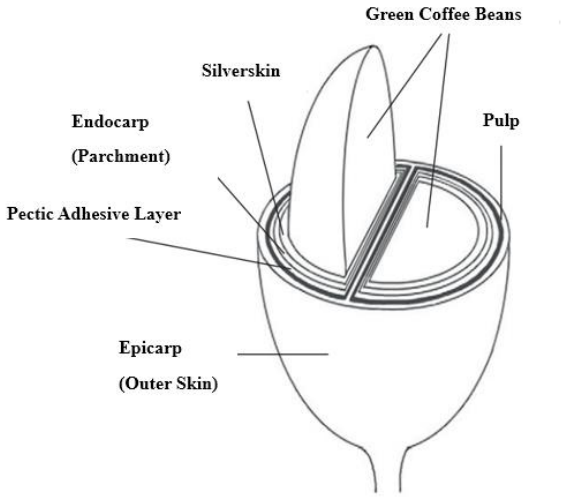

Figure 2.1 Typical structure of coffee cherry (Narita & Inouye, 2014).

The chemical composition of green coffee beans is shown in **Table 2.2**. The differences between Arabica and Robusta coffee were demonstrated by the proportions of specific compounds. Green coffee beans of both varieties were principally composed of carbohydrates, nitrogen (N)-containing compounds (mainly proteins, trigonelline, and caffeine), lipids, organic acids, and water. Major flavour precursors included sugars, proteins, free amino acids, trigonelline, and chlorogenic acids (CGA)(Luigi Poisson, 2017).

Table 2.2 Chemical composition of green Arabica and Robusta coffee (Luigi Poisson, 2017).

	Content (% Based on Dry Weight)	
Constituents	Arabica	Robusta
Soluble Carbohydrates	9-12.5	6-11.5
Monosaccharides	0.2-0.5	0.2-0.5
Oligosaccharides	6-9	3-7
Polysaccharides	3-4	3-4
Insoluble Carbohydrates	46-53	34-44
Hemicellulose	5-10	3-4
Cellulose	41-43	32-40
Acids and Phenols		
Organic Acids	2-2.9	1.3-2.2
Chlorogenic Acids	6.7-9.2	7.1-12.1
Lignin	1-3	1-3
Lipids	15-18	8-12
Coffee oil	15-17.7	8-11.7
Wax	0.2-0.3	0.2-0.3
N-compounds	11-15	11-15
Free amino acids	0.2-0.8	0.2-0.8
Proteins	8.5-12	8.5-12
Caffeine	0.8-1.4	1.7-4.0
Trigonelline	0.6-1.2	0.3-0.9
Minerals	3-5.4	3-5.4

Currently, besides the asparaginase approach, coffee acrylamide mitigation strategies include blending of Arabica and Robusta coffee, and optimisation of roast conditions. For coffee manufacturers, Robusta coffee, which is generally more acidic and astringent than Arabica coffee and cheaper than Arabica coffee, was mixed with Arabica coffee to achieve a perfect balance of cost and flavour for the final product. For asparagine content, as showed in **Figure 2.2**, Robusta coffee generally had higher asparagine content than Arabica coffee. So, to lower acrylamide in roasted coffee products, the relative proportions of Arabica/Robusta were increased. Regarding the dietary exposure data of 2007-2009 collected by European Food Safety Agency, coffee showed a great contribution to the dietary exposure to acrylamide. As shown in **Table 2.1**, potatoes and bread contributed the 1st and 2nd sources of acrylamide intake in the UK diet. However, in France, coffee was the second largest source of acrylamide.

Table 2.1 Acrylamide content in the diet in UK & France (EFSA, 2009)

Food Product	Acrylamide content as percentage of total dietary acrylamide	
	UK	France
Potato	67.1	18.3
Bread	17	31
Cereal	8.6	2.3
Coffee	0.7	31
Biscuit	6.3	7.6
mean acrylamide consumption (µg/kg bw)	0.63	0.41

Though coffee products were ranked high for dietary exposure in European countries, few asparaginase applications have been carried out in coffee. A possible reason behind this circumstance is the tight internal structure of green coffee beans.

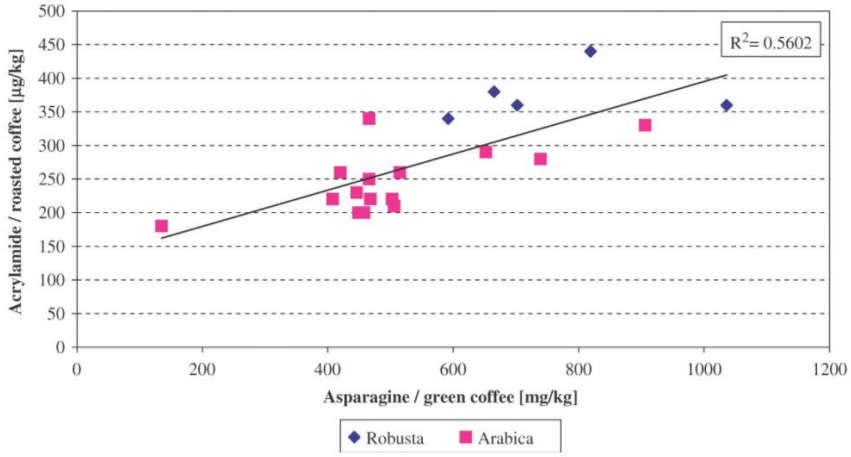

Figure 2.2 Asparagine content in the green coffee beans of a variety of Arabica and Robusta coffees (from Guenther, Anklam, Wenzl, & Stadler, 2007)

The other method which coffee manufacturers like to use is to modify the roast profile (time and temperature). As showed in **Figure 2.3**, acrylamide in coffee increases at the start of the roast process, then decreases after reaching a peak point. Theoretically, higher roast degree would lead to the lower acrylamide. However, as the flavour profile of coffee is closely related to the roast conditions, roast conditions were strictly limited to a narrow range to avoid a potential significant difference in sensory properties.

Coffee has a very tight internal structure (Pittia et al., 2011); therefore steam & soak pre-treatments have been recommended to ensure sufficient contact between enzyme and substrate (Hendriksen et al., 2013). In Hendriksen`s study, coffee bean samples were firstly steamed for 45 mins at 100 °C to open the pores of the coffee bean and then placed in an enzyme bath where the beans were mixed with enzyme by stirring. At the end of the experiment, water used in the enzyme bath had been totally absorbed by the samples. In Hendriksen`s research, 4000, 6000, 8000 ASNU/kg coffee beans were used in a trial experiment on coffee. After the enzyme treatment, coffee beans were roasted to Light Roast-100 light reflectance units (LRU) and as shown in **Figure 2.4**, 70-80% asparagine reduction and 55-74% acrylamide reduction were observed with the three different enzyme dosages. The roasting degree could be measured by light reflectance and expressed in LRU. In theory, coffee roast LRU values can range from very

dark roast (41) to very light roast (116). In practice, values for roasted coffees in Europe range from 55 (dark roast) to 105 (light roast) (Guenther et al., 2007).

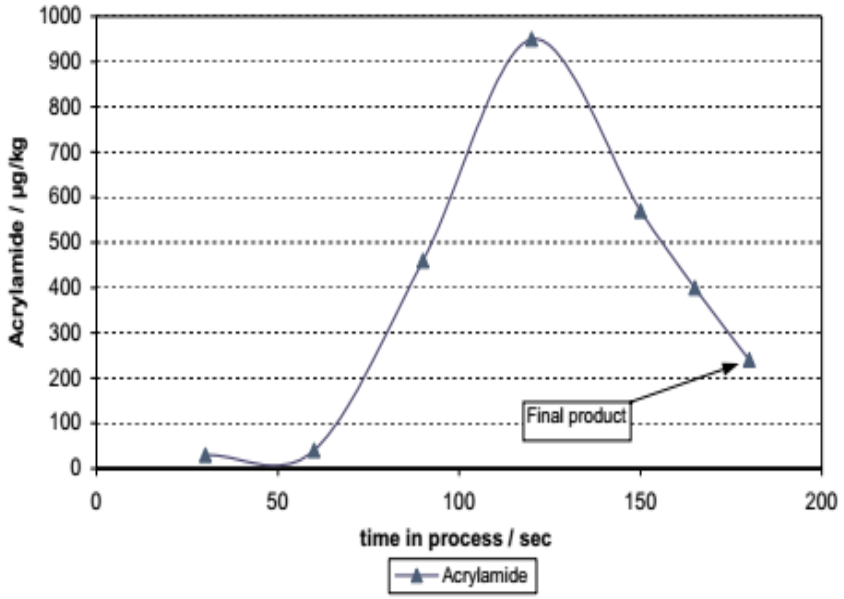

Figure 2.3 Acrylamide levels of partially roasted Colombian coffees, by prematurely stopping the process of roasting(Lantz et al., 2006).

At the beginning of this project, there were two commercial food grade asparaginases available. Acrylaway® produced by Novozymes (Bagsværd, Denmark) from *Aspergillus oryzae* with an activity of 3500 ASNU/g at pH 7 and 37 °C; and PreventASe® produced by DSM (Heerlen, Netherlands) from *Aspergillus niger*, with an activity of 2500 ASPU/g at pH 5 and 37 °C. In the standard enzyme activity assay protocol, body temperature (37 °C) serves as the assay temperature (Bisswanger, 2014). **Figure 2.5** and **Figure 2.6** show the enzyme activities under various conditions for Acrylaway® and PreventASe®, respectively. As shown in these figures, 60 °C is reported to be the optimal temperature for both enzymes, although they show their maximum activities at different pH values. As the maximum activity of both enzymes is at 60 °C, this temperature was used for enzyme incubation in this study. Because green coffee has a pH of around 6 (Flament & Bessière-Thomas, 2002), it was assumed that the activities of both enzymes would be close to their optimum.

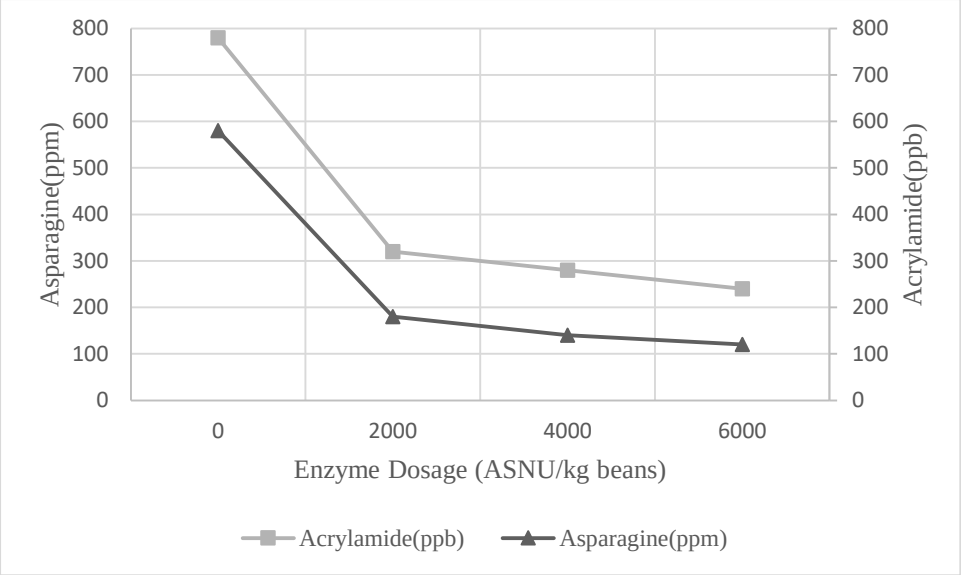

Figure 2.4. 'Effect of enzyme dosage (Acrylaway®) on asparagine levels in green coffee beans and acrylamide levels in roasted coffee beans (Hendriksen et al., 2013)

As the research of Hendriksen (2013) showed that the steam & soak method could successfully reduce acrylamide, this method was used in experiments 1, 2, 3 in this chapter. Three separate experiments were performed to investigate the effectiveness of asparaginase as an acrylamide mitigation strategy:

1. Acrylaway® application in Arabica coffee experiment

The first experiment used Acrylaway® at 2600, 12600, 20000 ASNU/kg coffee beans, to examine the effects of both steam processing and dosage of the enzyme on the asparagine content of green coffee beans and on the acrylamide content of roasted coffee beans.

2. Acrylaway® application in Arabica & Robusta coffee experiment

The second experiment used 2000, 4000, 6000 ASNU/kg coffee beans Acrylaway® in various types of coffees, including two Arabica coffees and two Robusta coffees, to investigate acrylamide reduction rate with enzyme dosages suggested by Novozymes.

3. Acrylaway® & PreventASe® application in Arabica coffee experiment

The third experiment used Acrylaway® at 2000, 4000, 6000 ASNU/kg coffee beans and PreventASe® at 2000, 4000, 6000 ASPU/kg coffee beans on Brazilian Santos coffee, with the purpose of comparing the acrylamide reduction rate between the two commercial enzymes. Also, a process control sample (the sample treated with water using the exact same procedure as enzyme-treated sample) was included in this experiment.

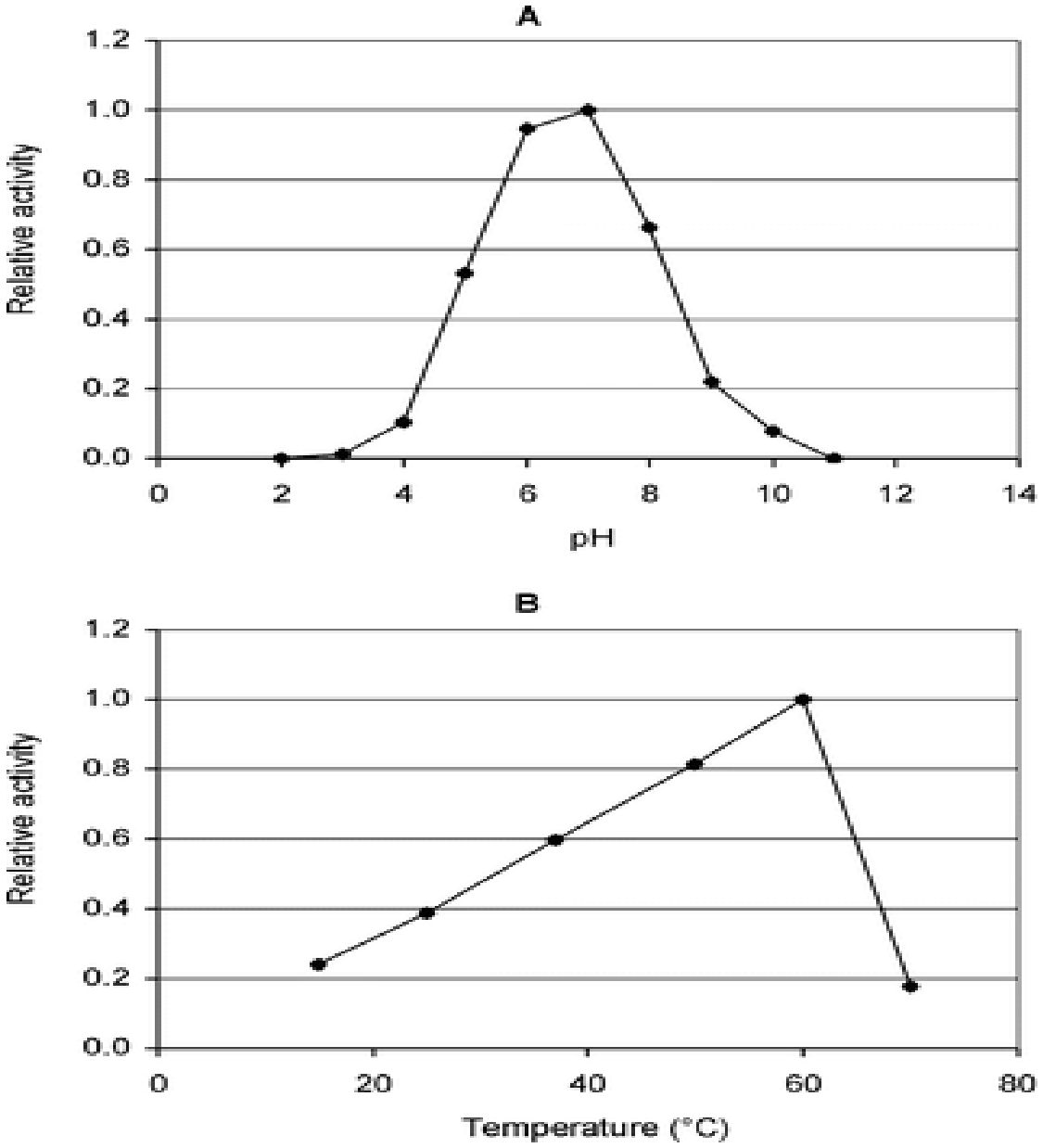

Figure 2.5. Enzyme activity of Acrylaway® showing optimum temperature and pH values; (**A**) pH activity profile at 37 °C; (**B**) Temperature activity profile at pH 7. Activities are represented relative to optimum values (Hendriksen et al., 2009).

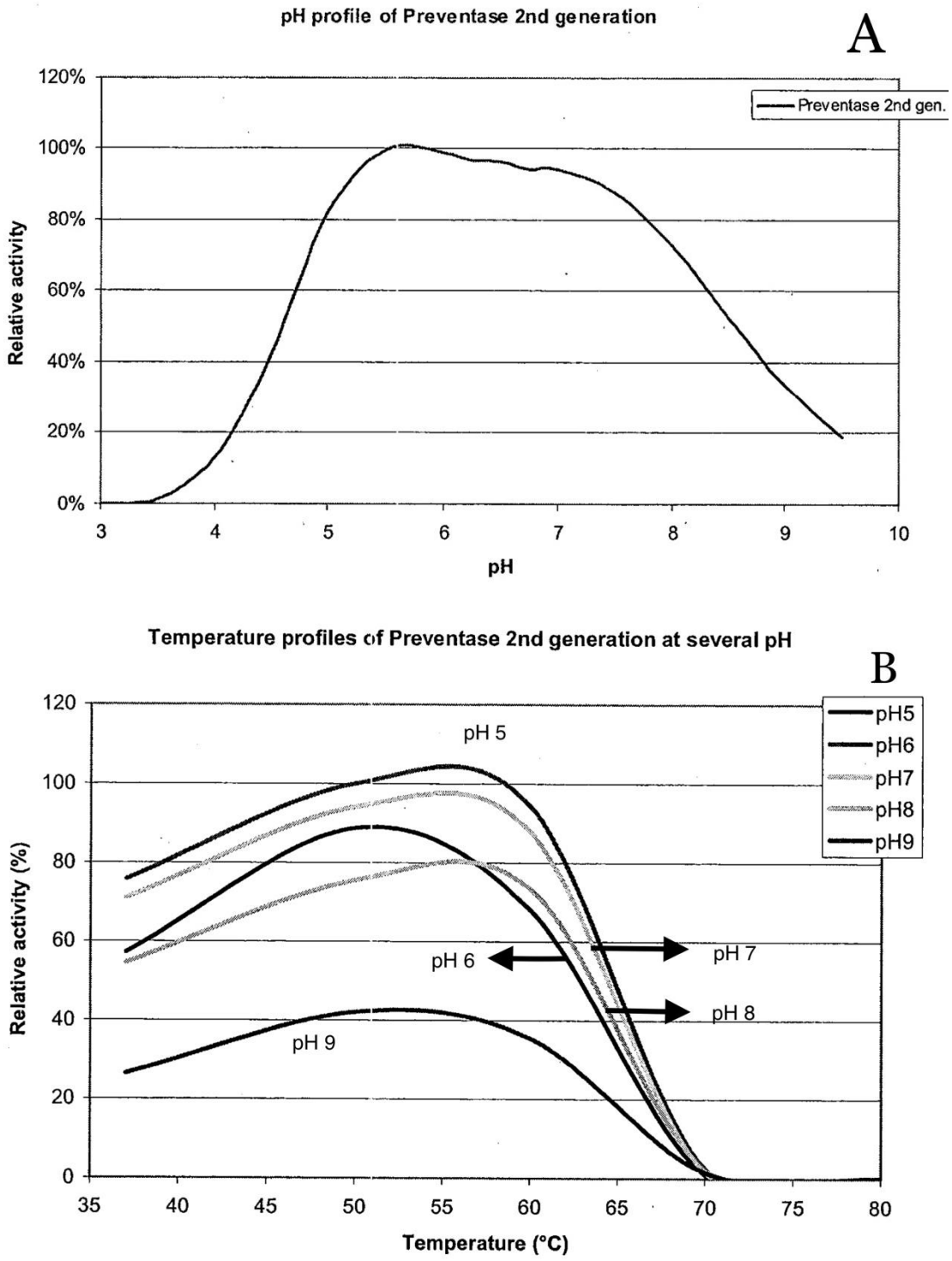

Figure 2.6 (**A**) PreventASe® activity between pH 3 and 9.5 at 37 °C (**B**) Temperature profile of PreventASe® activity at different pH values, relative to the measurement at 50 °C, pH 6, which was set at 100% (Yingling, 2012).

Before and after enzyme treatment, the precursors in the green coffee were determined. Free asparagine and its breakdown product aspartic acid were determined by EZ-Faast (Phenomenex, Torrance, CA) derivatisation followed by gas chromatography-mass spectrometry (GC-MS). Sugar composition was determined by ion chromatography with pulsed amperometric detection. Acrylamide and colour were measured in samples roasted at one level first then three roast levels in the later experiment. Acrylamide was measured by high-performance liquid chromatography with triple quadrupole mass spectrometry (LC-MS/MS), while colour was measured using a HunterLAB® LAB colorimeter.

Before the analysis by LC-MS/MS, an extraction step was needed to remove the acrylamide from the coffee brew. Two methods were utilised in this series of the experiment. The first method was the EU official solid-phase extraction (SPE) method using Multimode and ENV+ cartridges. The second method was a newly developed supported liquid extraction (SLE) method using SLE+ cartridges. Both methods used cartridges produced by Biotage (Uppsala, Sweden). In Acrylaway® application in Arabica coffee experiment, both methods were tested and both achieved great acrylamide extraction. As the SPE method was more expensive and time-consuming, the SLE method was used in experiments 2 and 3.

2.2 Materials & Methods

2.2.1 Materials

2.2.1.1 Enzymes

Both enzymes (Acrylaway® and PreventASe®) were gifts from the manufacturers. Acrylaway® from Novozymes (3500ASNU/g) and PreventASe® (2500ASPU/g) from DSM were used. Both ASNU and ASPU are defined as the amount of asparaginase that produces one μmol of ammonia per minute under the conditions of the assay (pH = 7±0.005; T = 37±0.5 °C) using Acrylaway® or PreventASe®, respectively.

2.2.1.2 Coffee

For the acrylamide extraction method comparison experiment commercially available "extra special coffee" samples ASDA brand were analysed. These included Fairtrade Colombian (FC), Indian Elephant Farm (IEF) and Java Volcanic Island (JVI).

Three different experiments were carried out as listed in the introduction. Experiment 1 (Acrylaway® application in Arabica coffee experiment) and Experiment 3 (Acrylaway® & PreventASe® application in Arabica coffee experiment) used Brazilian Santos coffee which was sourced from Rave Coffee UK (Cirencester, Gloucestershire). Experimanet 2 (Acrylaway® application in Arabica & Robusta coffee experiment) used four Brazilian coffees. Two Robusta coffees, Robusta Tardio (2015) and Robusta Comum (2015), were sourced from Rodonia state. An unspecified Arabica (2014) was from Mina Geras state and Arabica Safra (2014) was from Rio de Janeiro state.

2.2.1.3 Reagents

Enzyme activity determination: Tris buffer, L-asparagine solution, ammonium sulphate standard solution, trichloroacetic acid, and ammonia colour reagent were obtained from Sigma-Aldrich (Gillingham, UK). All reagents were HPLC grade.

Acrylamide determination: LC-MS grade ^{13}C-acrylamide in water (1000 ng/mL) was sourced from LGC Standards (London, UK). HPLC-grade cyclohexane, ethyl acetate, ammonium hydroxide, tetrahydrofuran and ethylene glycol were sourced from Sigma-Aldrich. Methanol and HPLC-grade water were from Fisher (Loughborough, UK). Supported Liquid Extraction cartridges (SLE+) and Solid Phase Extraction cartridges (Multimode & ENV+ cartridges) were sourced from Biotage (Uppsala, Sweden).

2.2.2 Methods

2.2.2.1 Determination of Free Amino Acids

Freeze-dried green coffee bean and roasted coffee beans samples (0.20 ± 0.001 g) were weighed into 7-mL vials. Hydrochloric acid (0.01 M) was added (5 mL) to the vial, and the sample was stirred for 15 minutes at room temperature. After stirring, the sample was left to

settle for 45 minutes. An aliquot of the supernatant (2 mL) was then centrifuged at 7200 rpm for 30 minutes.

One hundred microlitres of the centrifuged supernatant were then derivatised using the EZ-Faast amino acid derivatisation technique (Phenomenex, Torrance, CA) and the derivatised products were analysed by GC-MS. Each sample was extracted and analysed in triplicate. The preparation of a sample for GC-MS began with the addition of 20 nmol of norvaline internal standard (100 µL in 0.1 M HCl), followed by a solid-phase extraction and then a two-step derivatisation at room temperature. The derivatised amino acids were extracted into isooctane/chloroform (100 µL) and analysed in electron impact mode at 70 eV using an Agilent 5975 GC-MS system. An aliquot of the derivatised amino acid solution (1 µL) was injected at 250 °C in split mode (5:1) onto a 10 m × 0.25 mm Zebron ZB-AAA capillary column. The oven temperature was 110 °C for 1 minute, then increased at 30 °C/minutes to 320 °C, and held at 320 °C for 2 minutes. The transfer line was held at 320 °C, and the carrier gas flow rate was kept constant throughout the run at 1.1 mL/min. The ion source was maintained at 220 °C. Samples and standards were analysed in triplicate.

Standards of 19 non-basic amino acids included in the kit (Ala, β-Ala, AABA, GABA, Asp, Glu, Gly, His, Ile, Leu, Lys, Met, Orn, Phe, Pro, Ser, Thr, Val) in 0.1 M hydrochloric acid and 3 basic amino acids (Asn, Gln, and Try) in water were used. A calibration curve (0.005 mM, 0.01 mM, 0.02 mM, 0.05 mM, 0.1 mM, 0.2 mM) was plotted for asparagine and aspartic acid, and the gradient of this curve was used to calculate the amount of asparagine and aspartic acid in the coffee samples. Specific mass spectral fragment ions were chosen for quantification of asparagine (m/z = 69) and aspartic acid (m/z = 216). The peak areas of these ions in asparagine and aspartic acid were measured relative to the area of the m/z 158 ion of norvaline (internal standard).

2.2.2.2 Analysis of Sugars by Ion Chromatography

Each coffee powder sample (0.200 ± 0.005 g) was weighed into a 14-mL screw-top vial. Aqueous methanol (50%, 10 mL) containing 100 mg/L trehalose as an internal standard was

added to the vial, and the sample was stirred for 15 minutes at room temperature. After a further 15 minutes, 1.5 mL of supernatant were removed from the bottle and centrifuged at 7200 *g* for 15 minutes. Aliquots (500 µL) of the centrifuged supernatant were diluted 10-fold in water; 2 mL of the diluted extract were then filtered through a 0.2 µm syringe filter. The extracts were analysed using a Dionex ion chromatography system with a 250 × 4 mm Carbopac PA1 column (Dionex Corp., Sunnyvale, CA), operated using Chromeleon software. The ion chromatography system consisted of an AS50 autosampler, an LC25 column oven, GS50 pumps, and an ED50 pulsed amperometric detector, running in internal amperometric mode. Injection volume was 25 µL. A gradient program was set up using 200 mM NaOH (solvent **A**) and water at a flow rate of 1 mL/minute: 50% solvent **A**, held for 10 minutes and then increased to 100% at 40 minutes. The column was then washed for 8 minutes with 500 mM sodium acetate in 125 mM NaOH and re-equilibrated with 50% solvent **A** for 7 minutes. The waveform of the pulsed amperometric detector was as follows: 400 ms at 0.1 V, 20 ms at –2.0 V, 10 ms at 0.6 V, and 60 ms at –0.15 V. Standards of glucose, fructose and sucrose (100 mg/l, 50 mg/l, 25 mg/l, 10 mg/l, 5 mg/l, 1 mg/l) were used for quantification with $R^2 > 0.999$ for each standard. Each sample was extracted and analysed in triplicate

2.2.2.3 Enzyme activity determination

Comparing the optimum pH (7) and official activity (3500ASNU/g) of Acrylaway®, PreventASe® has a lower optimum pH (5) and lower official activity (2500 ASPU/g). As enzymes lose activity each day, even when stored in at 4 °C, enzyme activity determination was carried out before each experiment, to determine the correct dosage to use. After the enzyme determination, optimum dosage for the enzyme treatment was calculated in this study.

The claimed activity of Acrylaway® is 3500 ASNU/g. The activity of asparaginase is expressed in ASNU activity units. One ASNU is defined as the amount of asparaginase that produces one micromole of ammonia per minute under standard conditions (pH = 7, 37 °C). The claimed activity of PreventASe® produced by DSM is 2500 ASPU. The ASPU is defined as the amount of asparaginase that produces one micromole of ammonia per minute under standard conditions (pH = 5, 37 °C). For coffee samples analysed in this experiment, ASNU and ASPU refer to the amount of enzyme used per kilogram of coffee beans (ASNU/kg coffee beans and ASPU/kg coffee beans).

Two methods to determine the enzymatic activity of asparaginase were used in this study. The two methods were based on the quantification of ammonium released in the hydrolysis reaction of asparagine. For the first method (Olempska-Beer, 2006), in the presence of glutamate dehydrogenase (GDH) and reduced nicotinamide-adenine dinucleotide (NADH), ammonia reacts with 2-oxoglutarate to L-glutamate, whereby NADH is oxidised. The amount of NADH oxidised is stoichiometric to the amount of ammonia. NADH is determined by means of its light absorbance at 334, 340 or 365 nm. For the second method (Shirfrin et al., 1974), Nessler`s Reagent was used to determine the ammonia generated; the activity of enzyme was calculated from the ammonia content detected in the reaction. Method 1 was used to determine Acrylaway® activity in Acrylaway® application in Arabica & Robusta coffee experiment (Experiment 1 & 2). Method 2 was used to determine Acrylaway® and PreventASe® activity in Acrylaway® & PreventASe® application in Arabica coffee experiment (Experiment 3).

Method 1:

Preparation of the reagents: Solution 1 60ml (triethanolamine buffer and 150mg 2-oxoglutarate) and solution 2 1.2ml (1000 Unit glutamate dehydrogenase) were prepared and used without dilution. 0.4mg NADH tablet was dissolved with 1 mL of solution from solution 1. This resulted in reaction mixture 1 which was used later. L-Asparagine (189 mM, 10 mL) solution and enzyme solution (2 units/mL) were prepared as sample solution. One millilitre of reaction mixture 1 was mixed with 2 mL water to provide a blank. Then for samples 1.0 mL of mixture 1 was mixed with 0.1 mL of sample solution, 1.9 mL water were added to make the same volume as the blank. The absorbances of these two groups were measured after 5 minutes. Then 0.2 mL solution 2 were added to the blank and sample group. After 20 minutes, the second absorbances were read. From the absorbance data, ammonia in the sample solution was calculated. Then the activity of the enzyme was calculated from the amount of ammonia generated.

Method 2:

L-Asparagine (189 mM, 10 mL) solution and 1 mL ammonium sulphate standard (6 mM) were added to a 20-mL Falcon tube. The Falcon tubes were then equilibrated to 37 °C and enzyme solution (2 units/mL) was added to the tubes. The tubes were immediately shaken and

incubated at 37 °C for 30 minutes. Trichloroacetic acid (1.5 M) was then added to the test tubes. The solutions were mixed and centrifuged (Eppendorf Mini Spin; Eppendorf UK Ltd, Stevenage, UK) for 2 minutes at 3600 rpm. The supernatant obtained was mixed with 0.5 mL Ammonia Colour Reagent (1.01 g/mL) immediately. After one minute the absorbance at 436 nm was recorded (SigmaAldrich, 1997). From the absorbance data, ammonia in the sample solution was calculated. Then the activity of the enzyme was calculated from the amount of ammonia generated.

2.2.2.4 Steam & Soak enzyme treatment experiment

Table 2.3 Experimental procedure for the three experiments.

	Experiment 1	Experiment 2	Experiment 3
Experiment Design	Group 1. Control Group 2. Steam Control* Group 3. Enzyme treated without prior steam Group 4. Enzyme treated with prior steam	1. Control 2. Process Control** 3. Enzyme Treated	
Enzyme	Acrylaway®	Acrylaway®	Acrylaway®, PreventASe®
Enzyme Dosage (Units)	2600, 12600, 20000	2000, 4000, 6000	2000, 4000, 6000
Roasting (Gene Coffee CBR-101, South Korea)	17 minutes at 220 °C	light: 12 minutes at 230 °C, medium: 14 minutes at 240 °C and high: 17 minutes at 250 °C	
Storage	vacuum-sealed in dedicated 250mm× 250mm vacuum plastic pouch (The vacuum pouch company LTD, Walshaw) and stored at 4 °C		

*Beans were only steamed than dried to the original weight

**Beans were processed like the treated group with enzyme solution replaced by water

Experiment

1. Acrylaway® application in Arabica coffee
2. Acrylaway® application in Arabica & Robusta coffee
3. Acrylaway® & PreventASe® application in Arabica coffee

Unwashed green coffee beans (150 g) were steamed for 45 minutes at 100 °C. The beans were mixed with 103.5 mL of heated water (60 °C) containing various dosages of enzyme. For the process control 103.5 mL water were used instead of the enzyme solution. Samples were incubated at 60 °C for 60 minutes with stirring. After incubation, all liquid had been absorbed by the beans. Then, the treated beans were transferred to an aluminium tray and spread on the tray. The trays were placed in a drier (Gallenkamp, Loughborough, UK) for 4-6 hours until the coffee beans were reduced to their original weight of 150 g.

2.2.2.5 Acrylamide Analysis

2.2.2.5.1 Acrylamide Extraction - Solid Phase Extraction (SPE)

Ground (0.1 mm particle size), roasted coffee sample (2.00 ± 0.01 g) was weighed into a 50-mL Falcon tube, where 5 mL of *n*-hexane followed by 40 mL of water were added. Samples were spiked with 400 µL of internal standard solution (1000 ng/mL ^{13}C-acrylamide in water); tubes were shaken for 15 seconds by hand then 15 seconds by vortex shaker (IKA MS1 Minishaker, UK) and then 60 minutes on a mechanical shaker (Heidolph, MultiReax, UK). Samples were centrifuged (sigma 3K10, UK) at 10 °C, at 3600 rpm for 20 minutes. After centrifugation, the top layer (*n*-hexane) was discarded and 10 mL of the aqueous layer were transferred to a clean vial.

Multimode cartridges were fitted to the vacuum manifold, and then cartridges were conditioned with 3 mL of methanol and twice with 3 mL of water. Then, 2 mL of supernatant were passed through the cartridge followed by 3 mL of water; in total 5 mL of combined eluate was collected.

Then, the ENV+ cartridges were fitted to the vacuum manifold and the cartridges were conditioned by 5 mL of methanol followed by 5 mL of water. Then approximately 5 mL of the

extract from the previous step were loaded onto each cartridge and the eluate solution was discarded. Then, 2 mL of 60% *v/v* methanol in water passed through the ENV+ cartridge and the eluate collected in a 4-mL vial. the solvent was then evaporated to 500 µL under a gentle stream of nitrogen at 40 °C, then this 500 µL solution was transferred to a 2 mL vial for LC-MS/MS analysis(Wenzl, Szilagyi, Rosen, & Karasek, 2009).

2.2.2.5.2 Acrylamide Extraction-Supported Liquid Extraction (SLE)

Fine ground (0.1 mm particle size), roasted coffee sample (2.00 ± 0.01 g) was weighed into a 50-mL Falcon tube; 40 mL of water were added to the tube. Samples were spiked with 400 µL of the internal standard solution (1000 ng/mL ^{13}C-acrylamide in water); tubes were shaken for 15 seconds by hand then 15 seconds by vortex shaker (FB15013 TopMix by Fisher Scientific) and then 60 minutes on a mechanical shaker (Multi Reax by Heidolph). Samples were centrifuged (Sigma 3K10, UK) at 10 °C and 3600 rpm for 20 minutes. After centrifugation, 0.625 mL of supernatant were transferred to a 7-mL glass vial containing 12.8 µL of a saturated solution of ammonium hydroxide in water. The vials were briefly shaken, and then 0.5 mL of the mixture were transferred to an SLE cartridge placed on a vacuum manifold. Cartridges did not require a conditioning step. An initial vacuum was applied to allow the sample to be absorbed for 5 minutes. Then a mixture of ethyl acetate and tetrahydrofuran (1:1, 2 × 2.5 mL) was applied and allowed to flow under gravity into a tube containing 2 µL ethylene glycol, to extract the acrylamide that had been retained. Finally, the tubes containing the eluates were dried in a stream of nitrogen and reconstituted in water (500 µL) prior to the analysis (Biotage, 2014).

2.2.2.5.3 Analysis of acrylamide by LC-MS/MS

Samples were analysed by liquid chromatography-triple quadrupole mass spectrometry (LC-MS/MS) using an Agilent 1200 HPLC system with a 6410 triple-quadrupole mass spectrometer with electrospray ion source in positive ion mode. An isocratic separation was carried out at room temperature using a 100 × 3.0 mm Hypercarb column with a 10 × 3.0 mm Hypercarb precolumn (both 5 µm particle size; Thermo Fisher, Waltham, MA). The mobile phase was 0.1% aqueous formic acid at a flow rate of 0.3 mL/minute. The injection volume was 25 µL. The eluent from the column was run to waste from 0 to 4.5 minutes, and data were collected from 4.5 to 8 minutes. Acrylamide was eluted at around 6 minutes. A run time of 20 minutes allowed

the clean-up of the column for the following sample. The transitions m/z 72 → 55 and 72 → 27 were used for acrylamide analysis, and the transition m/z 75 →58 was used to measure the internal standard ^{13}C-acrylamide (1 mg/L). Peaks were symmetrical with no interference from impurities. A calibration curve (5000 µg/L, 1000 µg/L, 200 µg/L, 40 µg/L, 10 µg/L, 2 µg/L acrylamide in water) with $r^2 > 0.999$ was prepared. ^{13}C-acrylamide solution (50 µg/L) was also present in the standard.

2.2.2.6 Colour determination by LAB method

A HunterLAB® CT1100 ColourQuest colorimeter (Hunter Associate Laboratories, Inc., Reston, VA) was used for the colour measurements. The colorimeter set-up was as follows: total transmittance mode, Illuminant D65, and 10 degree observer angle. All measurements were conducted in triplicate. Fine ground coffee powders (particle size 0.1 mm) were packed in a transparent plastic snap bag with a width of 1 cm. After calibration of the colorimeter using the equipment`s reference white plate, colour profiles of the coffee samples were obtained by measuring the coffee powder directly in the bags.

The HunterLAB® machine measured the *L* (lightness), *a* (redness) and *b* (yellowness) values of the samples to locate a specific colour in the three-dimensional colour scheme. In this scheme, Δ*E* (Total Colour Difference) was introduced to illustrate the distance between two specific colours. Δ*L,* Δ*a,* Δ*b* refers to the difference between control and processed samples on *L*, *a, b* respectively. Δ*E* equals the square root of the sum of square Δ*L*, square Δ*a* and square Δ*b*.

$$\Delta E = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2}$$

2.2.2.7 Statistical Analysis

All analyses were done in triplicate and expressed as means with standard deviations. XLStat 2015 (Addinsoft, Paris, France) was used to perform analysis of variance (one-way and two-way ANOVA). For those compounds that exhibited a significant difference in the ANOVA, multiple pairwise comparison tests (Tukey`s HSD) were applied to determine which sample means differed significantly ($p < 0.05$).

2.3 Results

2.3.1 Enzyme activity determination results

In Experiment 1, the enzyme was immediately used after it was produced and delivered from Novozymes. No enzyme activity determination was carried out. In Acrylaway® application in Arabica coffee experiment, Acrylaway® was stored in the fridge. Then, the enzyme was used at calculated dosages from the official claimed activity of 3500 ASNU/g.

In Acrylaway® application in Arabica & Robusta coffee experiment (Experiment 2), a new batch of Acrylaway® was used. Enzyme activity determination experiment showed that this batch of Acrylaway® had an activity of 3000 ASNU/g All experiment was carried out by using the new batch of Acrylaway® with calculated dosage.

In the Acrylaway® & PreventASe® application in Arabica coffee experiment (Experiment 3) the PreventASe® activity was 2200 ASPU/g and its activity were measured one month after its production date. A new batch of Acrylaway® had an activity of 3200 ASNU/g when it arrived. Both enzymes were used with calculated dosages based on the measured enzyme activity.

Compared to the official activity values, both Acrylaway® and PreventASe® maintained good activity under the proper storage conditions. However, because the two enzymes lose activity each day after production, proper storage conditions and enzyme activity measurements were needed for the correct dosages to be used in each experiment.

2.3.2 Precursors Determination

2.3.2.1 Asparagine and Aspartic Acid Analysis

In Guenther`s research, asparagine content in Arabica coffee ranged from 170 to 350 μg/g. Results are shown in **Figure 2.2**. (Guenther et al., 2007). In Murkovic`s research, asparagine

content ranged from 280 to 960 µg/g in both Arabica and Robusta coffee (Murkovic & Derler, 2006). The value for the asparagine level in the control green coffee beans (300 ±15 µg/g) in Acrylaway® application in Arabica coffee experiment (Experiment 1) was in the range discovered in previous research. In this experiment, the effect of Acrylaway® on asparagine and aspartic acid levels in green coffee beans was measured (**Figure 2.7**). Asparagine showed a 43% loss at 2600 ASNU/kg coffee beans, increasing to 91% at 20000 ASNU/kg coffee beans. Compared to the data obtained from previous research (**Figure 2.4**), a similar trend was observed. Asparagine decreased to 57% of the control sample in Hendriksen`s research (770µg/g in control group; 330µg/g in 2000 ASNU/kg coffee beans sample).

A steaming process prior to enzyme treatment is necessary for sufficient enzyme and substrate contact. When Acrylaway was used without steaming, the asparagine reduction rate was very low. In fact, steaming without asparaginase was more effective than the enzyme treated without prior steaming. Also, compared the steamed groups to groups without prior steam treatments (Group 3 vs Group 4), the significant difference between these two groups indicated that enzyme did not have sufficient contact with substrate without steam treatment. Compared the control and steam control group (Group 1 vs Group 2), the steam treatment caused 26% of asparagine reduction alone.

The steaming process before enzyme treatment was vital to ensure the sufficient contact of enzyme and substrate and the steaming process alone could reduce asparagine content. However, comparing the steamed group with the steamed, enzyme treated groups, the asparagine reduction by steaming alone was relatively low. Aroma precursors other than asparagine might also be lost in the steaming process. Hence sugar content determination was necessary to examine the impact of the enzymatic process.

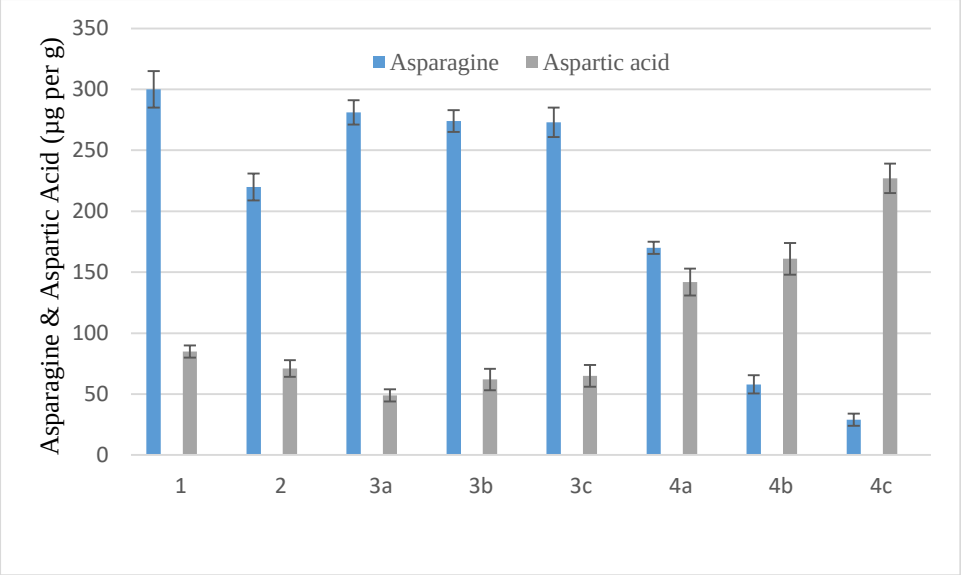

Figure 2.7: Effect of asparaginase on asparagine and aspartic acid concentrations (µg per g) in Brazilian Santos (Arabica) green coffee (1: control, 2: steam-processed control, 3: enzyme-treated without prior steam treatment, 4: enzyme-treated with prior steam treatment. For groups 3 and 4: a = 2600 ASNU/kg coffee beans, b = 12600 ASNU/kg coffee beans, c = 20000 ASNU/kg coffee beans)

2.3.2.2 Sugar analysis

Sucrose is the major free sugar found in coffee, both Arabica and Robusta green beans. The amount of sucrose present in Arabica coffee (6.25–8.45%) outweighs that in Robusta coffee (0.9–4.85%) (Arya & Rao, 2007b). Steam treatment can cause the hydrolysis of sucrose into glucose and fructose (Luger, Steinhart, & Assoc Sci Int, 1995). In the coffee roasting process, sucrose is either hydrolysed or rapidly degraded. As a product of hydrolysis reaction, glucose and fructose would increase (Luigi Poisson, 2017). However, glucose and fructose are degraded more rapidly than sucrose. Therefore, both sucrose and hydrolysed products are very low in roasted coffee beans (Illy & Viani, 2005).

Sugar content determination was carried out only for the green coffee samples used in Acrylaway® & PreventASe® application in Arabica coffee experiment (Experiment 3). Results shown in **Table 2.4** are presented as percentage on a dry weight basis to be able to compare them with previously published data. The control coffee contained 6.45% sucrose and 0.21% glucose and 0.22% fructose. In Arabica and Robusta coffees, 6.3% to 9.0% of sucrose

and 0.22% of glucose were found by Murkovic and Derler (Murkovic & Derler, 2006), while 7.3% sucrose and 0.7% reducing sugar were found by Arya and Rao (Arya & Rao, 2007a).

In process control and enzyme (Acrylaway® & PreventASe®) processed samples, the average value of sucrose was 4% while glucose and fructose were 0.01%. Statistical analysis showed sugar content was not related with the different enzyme dosages ($p>0.05$) and different enzyme brands ($p>0.05$). No sugar content determination in enzyme processed green coffee beans had been carried out before. However, considering the 26% of asparagine content loss in the previous steam control samples, the sucrose content also fell from 6.45% in control sample to 4% in process control sample. For samples treated by Acrylaway® and PreventASe®, no significant difference was found in the sugar content. As well as sucrose, reducing sugars (glucose and fructose) were also affected by the steam & soak treatment (0.2% to 0.1%). Therefore, steam & soak process leads to less sugar participating in the Maillard reaction, with possible effects on flavour and colour in roasted coffee.

Table 2.4 Sugar content in green coffee beans (%, dry weight basis; $n = 3$)

Green Coffee Beans	Sucrose	SD	Glucose	SD	Fructose	SD
Control	6.45^{a}	±0.08	0.21^{a}	±0.02	0.22^{a}	±0.02
Process Control	4.01^{b}	±0.06	0.11^{b}	±0.01	0.10^{b}	±0.01
Enzyme Treated (Average*)	3.98^{b}	±0.04	0.10^{b}	±0.01	0.10^{b}	±0.01

a, b values in the same column with different letters are statistically different ($p < 0.05$).

*As Acrylaway® and PreventASe® treated samples had similar results, average value of the two groups were showed in the table.

2.3.3 Acrylamide extraction method comparison

The SPE method used in this work to measure acrylamide has been validated by 11 European laboratories with high reproducibility and repeatability (Wenzl et al., 2009). However, it involves the use of two SPE cartridges: The first Multimode cartridge (octadecyl (non-endcapped), sulfonic acid (SO^{3-}) and quaternary amine ($-NR^{3+}$)) is used to remove the interference components (acidic, basic and non-polar) and let the analyte (small, polar and neutral) elute to the ENV+ cartridge (hydroxylated polystyrene-divinyl benzene copolymer)

where only polar analytes are collected. The SLE method, on the contrary, only uses one cartridge and two steps are involved in the extraction of acrylamide: sample load and elution of the compound of interest. In this case, cartridge conditioning step is not needed. The biggest difference in the design is the material (modified diatomaceous earth) which will hold the interference components while the analyte is eluted.

Before Experiments 1, 2 and 3, acrylamide extraction method comparison was carried out. Both the SPE method and SLE method used cartridges produced by Biotage (Uppsala, Sweden). The SPE method is relatively expensive and time-consuming to carry out, so the novel SLE method was recommended by the manufacturer. The extraction methods comparison experiment was carried out using coffee powders purchased from the supermarket. As results indicate in **Table 2.5**, in three samples tested, 2 samples showed no statistical significantly difference. To summarise when comparing supported liquid extraction with the standard SPE method, SLE was quicker, easier and cheaper. Hence, SLE was used in the following experiments

Table 2.5 Amount of acrylamide (μg/kg) in commercial coffee samples (n = 3). SD = standard deviation (Fairtrade Colombian (FC) and Java Volcanic Island (JVI)).

Coffee	SPE	SD	SLE	SD	P value
FC	182.3	± 6.8	181.2	± 5.5	0.138
JVI	178.5	± 4.9	178.8	± 5.8	0.157

2.3.4 Acrylamide determination results

2.3.4.1 Acrylaway® application in Arabica coffee experiment (Experiment 1)

In Acrylaway® application in Arabica coffee experiment, 2600, 12600, 20000 ASNU of Acrylaway® were used for enzymatic asparagine reduction. Medium-high roast (220 °C, 17 min) was chosen for this experiment. There were two reasons why this roast time and temperature combination was picked. Firstly, as Hendriksen`s work had showed that asparaginase could successfully reduce acrylamide in a light roast coffee, this experiment investigated the enzymatic approach on acrylamide reduction rate under darker roast conditions.

Secondly, as shown in Lantz`s research, acrylamide in coffee would increase first as roast began, then decrease with increased roast after reaching a maximum (Lantz et al., 2006).

The aim of this experiment was to examine acrylamide reduction with extremely high enzyme dosage and the steam process effect for enzyme delivery. When comparing the control and steam process control, it was clear that the steaming step itself had achieved 29% acrylamide reduction. Steaming prior to enzyme treatment will open the pores of the coffee beans (Beate.Kornbrust, 2009). When comparing the enzyme-treated samples and the control samples, a 22% reduction of acrylamide was observed in the steamed control samples ($p < 0.05$), indicating that the steaming step was effective on delivering the enzyme into the beans.

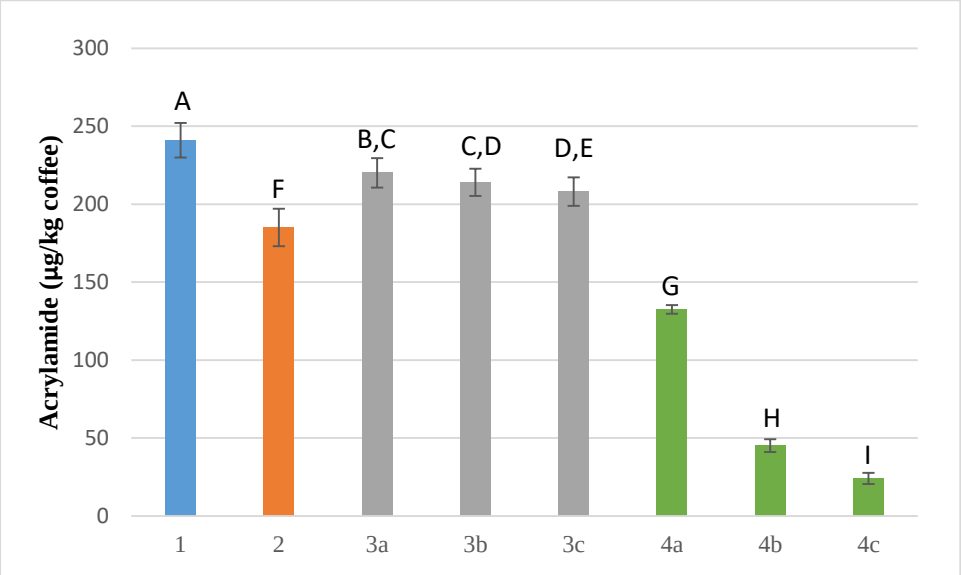

Figure 2.8 Acrylamide content (µg/kg coffee) in Brazilian Santos coffee in Experiment 1. (1: control, 2: steam processed control sample, 3: enzyme-treated without prior steam treatment, 4: enzyme treated with prior steam treatment. For groups 3 and 4: a = 2600ASNU/kg, b = 12600ASNU/kg, c = 20000ASNU/kg. Coffee was processed by Acrylaway® then roasted for 17 minutes at 220 °C) ($n = 3$)

As shown in **Figure 2.8**, because it was roasted to a higher level the control coffee showed a lower acrylamide content compared to that used in Hendriksen`s research (241 µg/kg in compared to 800 ppb). Comparing group 2 to group 1 (control vs steamed control,

respectively), the steaming process showed 29% acrylamide reduction. Group 3 and group 4 were both treated with threes dosages of the enzyme. However, without the steam treatment, group 3 showed a maximum 11% acrylamide reduction with the highest dosage (20000ASNU/kg) of enzyme. While with the pre-steam treatment, sufficient contact of enzyme and substrate led to significant acrylamide reduction in group 4, which was more noticeable as the acrylamide dose was increased, 45%, 81% and 91% for 2600 ASNU/kg, 12600 ASNU/kg and 20000 ASNU/kg, respectively.

Comparing the maximum acrylamide reduction rate in Experiment 1 to previous research, even though coffee varieties and roast levels were different, a dosage between 2000ASNU/kg and 6000 ASNU/kg was considered to be most dosage-effective (81% acrylamide reduction rate achieved by 12600ASNU/kg in medium roast and 74% achieved by 6000ASNU/kg in Hendrikesn`s research in light roast).

To summarize Experiment 1, an extremely high enzyme dosage did not achieve 100% acrylamide reduction. Hence, a lower enzyme range was suggested for subsequent study. Moreover, the steaming process prior to asparaginase application showed the important effect of opening the coffee pores and ensuring sufficient contact between asparaginase and asparagine. This steaming process was recommended for subsequent studies.

2.3.4.2 Acrylaway® application in Arabica & Robusta coffee experiment (Experiment 2)

The Acrylaway® application in Arabica & Robusta coffee experiment (Experiment 2) focused on testing the recommended dosages (2000ASNU/kg, 4000ASNU/kg and 6000ASNU/kg) in the most common coffee species (Arabica and Robusta). The steam & soak enzyme treatment process was used on two Arabica coffees (an unspecified Arabica and Arabica Safra) and two Robusta (Robusta Comum & Robusta Tardio) coffees.

As shown in **Figure 2.9**, in light roast Arabica, asparaginase had achieved identical acrylamide reduction rate in two species. Averagely, 2000 ASNU/kg achieved 54%, 4000 ASNU/kg achieved 68% and 6000 ASNU/kg achieved 80% acrylamide reduction. In Hendriksen`s work, where coffee was also light roasted, 2000 ASNU/kg achieved 58%, 4000 ASNU/kg achieved

66% and 6000 ASNU/kg achieved 72% acrylamide reduction; the reduction rate was almost identical (Hendriksen et al., 2013).

In medium and high roast Arabica coffee, the acrylamide concentration in enzyme-treated coffee was lower than the light roast coffee. For medium and high roast groups, similar enzyme dosage generally showed similar acrylamide reduction to the light roast. However, as the control for both medium and high roast groups had lower acrylamide levels, the acrylamide that was detected in enzyme-treated group was less than in the light roast group. Therefore, confirming with the literature, roast level factor showed dominant effect on acrylamide generation in coffee (Lantz et al., 2006).

As shown in **Figure 2.10**, Robusta coffee had more acrylamide in the control group. Based on literature results, higher asparagine content was found in Robusta coffee; higher asparagine content in green coffee beans tends to generate more acrylamide in roasted coffee beans (Guenther et al., 2007). Acrylamide in the control group of both Robusta coffee cultivars was higher than in the Arabica coffee. For enzyme-processed light roast Robusta Comum coffee, 2000 ASNU/kg achieved 61% acrylamide reduction, while 4000 ASNU/kg achieved 71% and 6000 ASNU/kg achieved 78%. Compared to light roast Arabica coffee (58% at 2000 ASNU/kg, 66% at 4000 ASNU/kg and 72% at 6000 ASNU/kg), at the same dosage, enzyme treatment showed slightly higher acrylamide reduction. However, in medium and high roast Robusta Comum coffee, enzyme treatment achieved similar acrylamide reduction to Arabica coffee. For all three dosages and three roast levels, statistical analysis found no significant difference for acrylamide reduction comparing the Robusta Comum to average acrylamide reduction rate in Arabica coffee.

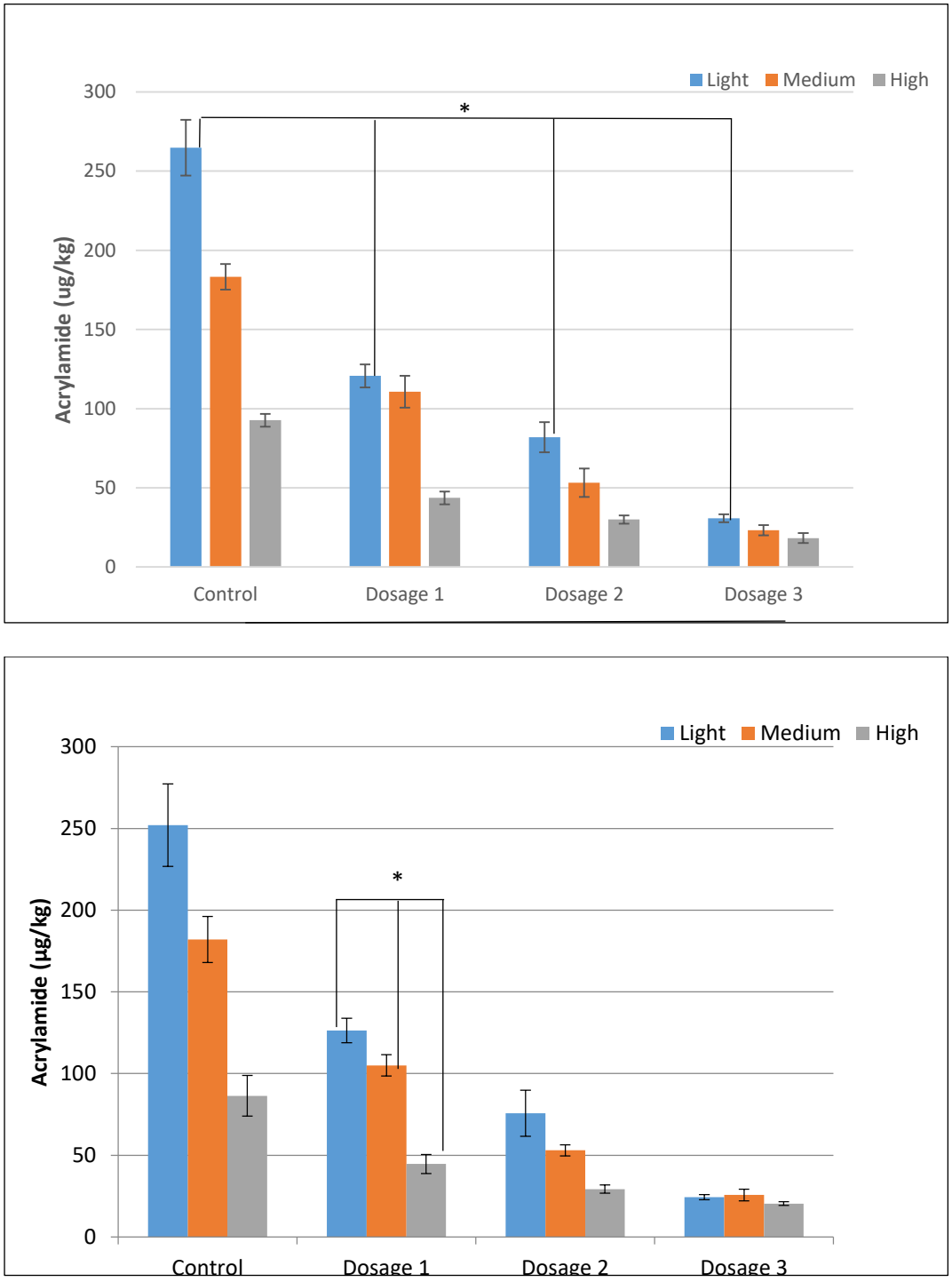

Figure 2.9 Acrylamide content (µg/kg coffee) in two Brazilian Arabica (top – Arabica Safra, bottom – unspecified Arabica) coffees after Acrylaway® application (Dosage 1 – 2000 ASNU/kg, Dosage 2 – 4000 ASNU/kg, Dosage 3 – 6000 ASNU/kg. Light: 12 minutes at 230°C, Medium: 14 minutes at 240°C, High: 17 minutes at 250°C. * = significant difference with $p < 0.05$).

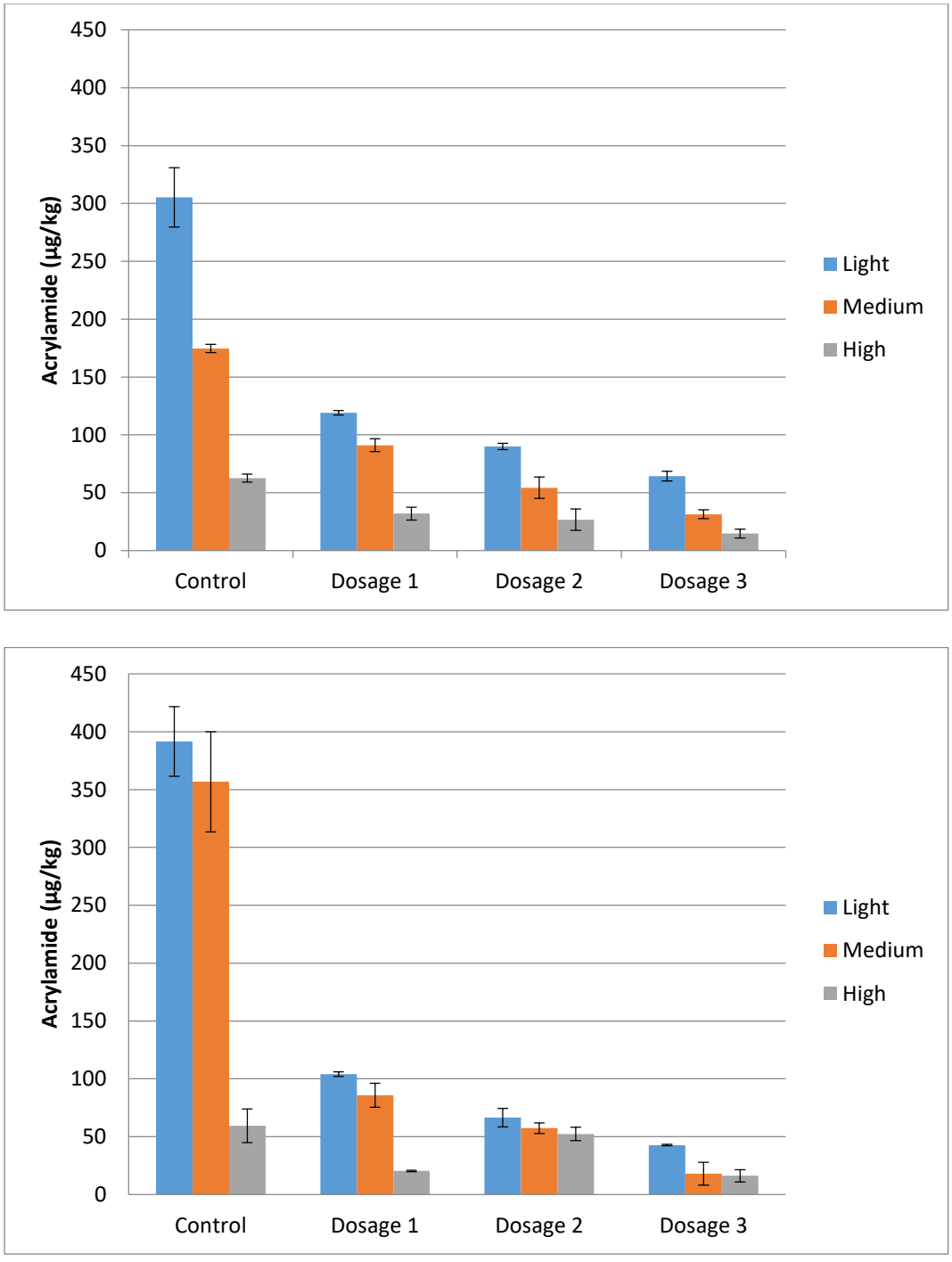

Figure 2.10 Acrylamide content (µg/kg coffee) in two Brazilian Robusta (top – Robusta Comum, bottom – Robusta Tardio) coffees after Acrylaway® application (Dosage 1 – 2000 ASNU/kg, Dosage 2 – 4000 ASNU/kg, Dosage 3 – 6000 ASNU/kg. Light: 12 minutes at 230°C, Medium: 14 minutes at 240°C, High: 17 minutes at 250°C.

For Robusta Tardio, a huge increase in acrylamide reduction was found in medium roast coffee. For instance, at 2000 ASNU/kg dosage, acrylamide reduction was 76%. Compared to other types of coffee, this acrylamide reduction was much higher (unspecified Arabica 42%, Arabica Safra 40%, Robusta Tardio 47%). The reason for the high value of acrylamide reduction rate was because the high amount of acrylamide found in the medium roast control coffee. However, if this unusual value may be an outlier: there was no significant difference in acrylamide reduction in medium roast Robusta Tardio coffee compared to the other varieties of coffee. However, asparaginase (Acrylaway®) treatment showed successful acrylamide mitigation in all coffee varieties.

Using ANOVA for each coffee variety (unspecified Arabica, Arabica Safra, Robusta Tardio, Robusta Comum), enzyme dosages (2000, 4000 and 6000U) and roasting conditions (light, medium and high) had a highly significant impact on acrylamide levels with all *p*-values less than 0.001 (**Table 2.6**). In conclusion, the different dosages of enzyme treatment successfully reduced acrylamide in different roast conditions regardless of the coffee varieties.

Table 2.6 Significance levels for enzyme dosages and roast levels on acrylamide content in 2 Arabica and 2 Robusta coffee samples after Acrylaway® application

Coffee Variety	Enzyme dosages	Roast Levels
Unspecified Arabica	<0.001	<0.001
Arabica Safra	<0.001	<0.001
Robusta Comum	<0.001	<0.001
Robusta Tardio	<0.001	<0.001

2.3.4.3 Acrylaway® & PreventASe® application in Arabica coffee experiment (Experiment 3)

Figure 2.11 shows the effect of two commercially available asparaginases Acrylaway® and PreventASe® on acrylamide reduction in Brazilian Santos coffee. As no PreventASe® acrylamide reduction experiment in coffee was found in the literature, this experiment was the

first one in the world that utilised PreventASe® in coffee and compared it with another commercial asparaginase Acrylaway®

For this experiment, a new process control was introduced. In Arabica coffee experiment (Experiment 1), steam control group samples were steamed only without soaking. It was set to examine the importance of steam process. Admitting the importance of steam process, experiment 2 had been carried out without control groups. In this experiment (experiment 3), the process control coffee was steamed and soaked without the addition of enzyme, then dried and roasted. This process control was introduced to more clearly demonstrate the impact of the enzyme delivery method on the coffee.

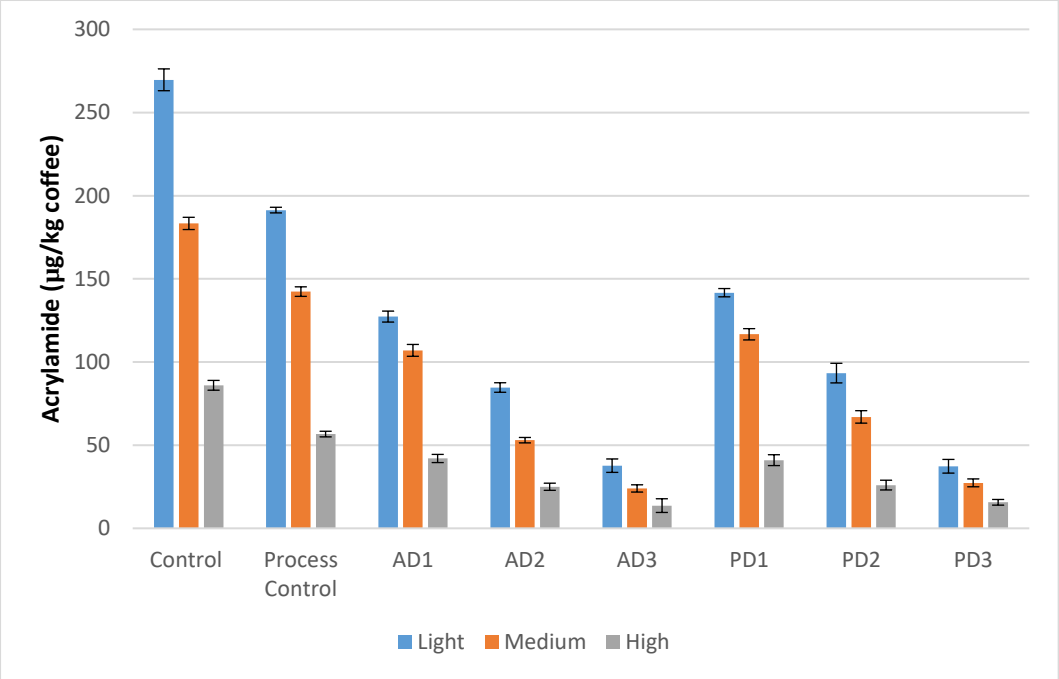

Figure 2.11 Acrylamide content in Acrylaway® and PreventASe® processed Brazilian Santos coffee (µg/kg coffee bean) (AD1, 2, 3: Acrylaway Dosage 1, 2, 3. Dosage 1 – 2000 ASNU/kg, Dosage 2 – 4000 ASNU/kg, Dosage 3 – 6000 ASNU/kg. PD1, 2, 3: PreventASe® Dosage 1, 2, 3. Dosage 1 – 2000 ASNU/kg, Dosage 2 – 4000 ASNU/kg, Dosage 3 – 6000 ASNU/kg. Light: 12 minutes at 230°C, Medium: 14 minutes at 240°C, High: 17 minutes at 250°C. $n = 3$.)

As shown in **Figure 2.11**, both Acrylaway® and PreventASe® showed a good inhibitory effect on the levels of acrylamide formed on coffee at different roasting levels. In light roast coffee, dosage 1 (2000 ASPU) PreventASe® achieved 52%, dosage 2 (4000 ASPU) achieved 68% and dosage 3 (6000 ASPU) achieved 86% acrylamide reduction. Compared to the acrylamide reduction of Acrylaway® (57% at 2000 ASNU/kg, 71% at 4000 ASNU/kg, 86% at 6000 ASNU/kg), the reduction of PreventASe® was relatively lower at the 2000 (52% to 57%) and 4000 (68% to 71%) unit dosages.

Similar reduction rate results of the two enzymes were observed in the medium and high roast coffee. The acrylamide reduction rate of Acrylaway® was slightly higher than that of PreventASe®. From the activity data presented in **Figure 2.5** and **Figure 2.6**, the reason for this may potentially be the pH of the enzyme soaking step; the Brazilian Santos coffee low acidic profile (pH = 6.2) favoured the relative activity of the Acrylaway® (optimum pH = 7.0) compared to PreventASe® (optimum pH = 5.0). As shown in **Figure 2.5** and **Figure 2.6**, PreventASe had much less activity compared to Acrylaway under these pH and temperature conditions.

For the statistical analysis of Acrylaway® & PreventASe® application in Arabica coffee (**Table 2.7**), Tukey test was performed. Four factors concerned with the enzyme process had been analysed: Roast level (light, medium, high), Enzyme dosage (2000, 4000, 6000U), Enzyme brand (Acrylaway®, PreventASe®), enzyme treatment (control vs treated). All 4 factors had significant effects on the acrylamide content. Roast level was the dominant factor for acrylamide generation. Regarding the enzyme treatment and enzyme dosage, significant impacts were found for both Acrylaway® and PreventASe®. Although Acrylaway® and PreventASe® performance was similar considering the dosages and roast conditions, a significant difference was still found.

Table 2.7 Significance levels for enzyme dosages and roast levels in Brazilian Santos coffee samples after Acrylaway® and PreventASe® application.

Factors	*p*-value
Enzyme Brands	<0.05
Roast Level	<0.001
Enzyme treatment	<0.05
Enzyme Dosages	<0.05

2.3.5 Colour determination results

Roasted colour is not only an important appearance attribute that is closely related to consumer acceptability, but also an important parameter for roast process control (Merken, Merken, & Beecher, 2001). Increased brown pigments are a symbol of the complex reactions that take place in coffee beans while roasting (Saklar, Katnas, & Ungan, 2001). Therefore, monitoring and determining the colour value are vital in the coffee roast process.

Colour determination results were carried out in the Acrylaway® & PreventASe® application in Arabica coffee experiment (Experiment 3). The average values for light, medium and high roast colour are shown in **Table 2.8**. Each value (*L*, *a* and *b*) progressively decreased as the roast extent increased. *L* value showed a greater reduction than the other two values. Statistical analysis showed that enzyme treatment had a significant effect on each value in each roast group.

In order to examine if the enzyme process had a significant impact on the integrated colour, the ΔE value was calculated by pairwise comparison of *L*, *a* and *b* of the control with *L*, *a* and *b* of each of the other samples. The higher the ΔE value is, the larger is the difference in colour between two samples. From the table below (**Table 2.9**), it is easy to identify that in the light roast, a huge leap of ΔE value was found in the enzyme-treated samples compared to the process control samples. However, in the medium and high roast, the colour difference was much smaller than in the light roast samples. This is because the control sample in medium and high roast was already dark in colour. Therefore the impact of enzyme treatment on roast extent and colour change was less pronounced than for the control sample.

Table 2.8 *Lab* values of Acrylaway® and PreventASe® treated Brazilian Santos coffee at three roast levels (Dosage 1 – 2000 U, Dosage 2 – 4000 U, Dosage 3 – 6000 U).

Light Roast	Colour Parameters		
Samples	L^*	a^*	b^*
Control	42.15	6.38	8.97
Process Control	36.27	4.46	8.86
Acrylaway® Dosage 1	34.93	2.71	6.56
Acrylaway® Dosage 2	36.03	3.02	7.46
Acrylaway® Dosage 3	35.90	2.95	7.57
PreventASe® Dosage 1	34.65	3.16	6.05
PreventASe® Dosage 2	35.74	3.83	7.22
PreventASe® Dosage 3	34.45	2.78	5.35
Medium Roast			
Control	35.53	3.73	6.87
Process Control	31.83	0.89	3.45
Acrylaway® Dosage 1	32.25	-0.37	3.83
Acrylaway® Dosage 2	32.26	-0.28	3.83
Acrylaway® Dosage 3	32.25	-0.15	3.73
PreventASe® Dosage 1	32.76	0.90	4.17
PreventASe® Dosage 2	31.87	0.18	3.49
PreventASe® Dosage 3	32.61	0.42	3.53
High Roast			
Control	32.21	0.87	3.27
Process Control	30.27	-0.74	2.38
Acrylaway® Dosage 1	30.95	-1.32	2.40
Acrylaway® Dosage 2	30.07	-1.27	2.96
Acrylaway® Dosage 3	31.42	-0.86	3.02
PreventASe® Dosage 1	30.71	-0.87	3.10
PreventASe® Dosage 2	30.41	-1.06	2.68
PreventASe® Dosage 3	30.22	-0.63	2.48

Table 2.9 ΔE of different treatment groups compared to control group in Acrylaway® & PreventASe® application in Arabica coffee experiment (Dosage 1 – 2000 U, Dosage 2 – 4000 U, Dosage 3 – 6000 U)

ΔE	Light Roast	Medium Roast	High Roast
Process control	9.19	5.79	2.68
Acrylaway® Dosage 1	9.98	6.12	2.71
Acrylaway® Dosage 2	10.15	4.79	2.31
Acrylaway® Dosage 3	10.04	5.53	2.62
PreventASe® Dosage 1	10.42	6.07	2.67
PreventASe® Dosage 2	10.22	6.01	3.04
PreventASe® Dosage 3	10.39	5.98	1.92

As shown in **Table 2.10**, roast conditions (low, medium and high), enzyme dosages (2000, 4000 and 6000 U) and enzyme brand (Acrylaway® and PreventASe®) were selected as three factors. Enzyme brand showed no significant effect on ΔE value with a *p*-value of 0.206, while the roasting condition and enzyme dosages had significant impacts with *p*-values of 0.001 and 0.025 respectively. The only interaction that showed significant impact was the roast condition and enzyme dosage. This significant difference was caused by the value of the process control in the light roast group. The impact of enzyme treatment on the coffee beans was more significant under the light roast conditions.

Table 2.10 Statistical analysis of the ΔE value in Acrylaway® & PreventASe® application in Arabica coffee experiment

Factors	*p*-value
Enzyme Brands	0.206
Roast Conditions	0.001
Enzyme Dosages	0.025

2.4 Discussion

2.4.1 Enzyme activity determination and precursors determination

It is crucial to determine the activity of the enzyme before applying it to the foodstuff. As a bioactive compound, enzyme loses its activity over time even when stored at 4 °C. The activity of the enzyme needs to be determined, so that the correct dosage of enzyme is applied. Furthermore, enzyme activity varies with temperature and pH (**Figure 2.3** & **Figure 2.4**) (Hendriksen et al., 2009). The optimum temperature of both Acrylaway and Preventase is 60 °C. Therefore, the enzyme treatment on green coffee needs to be carried out at 60 °C to achieve the highest activity of the enzyme. Another important parameter is pH. Acrylaway® optimum pH is 7 while PreventASe® optimum is 5 (**Figure 2.3** & **Figure 2.4**). The official activity of the two enzymes was determined at 37 °C and pH 7. The official claimed activity of Acrylaway® is 3500 ASNU/g and PreventASe® is 2500 ASPU/g. The enzyme activity determination experiment was carried out by two spectrophotometric methods, which were based on the determination of ammonia generated in a reaction.

Asparaginase should be inactivated in the later heat treatment (Hendriksen et al., 2009). Although trace amounts of aspartic acid and ammonia are generated from the action of the asparaginase, this enzymatic reaction should have a minimal effect on the composition of the coffee beans. However, similar to the blanching process in potatoes, the steam & soak enzyme treatment process could potentially cause a considerable amount of aroma and flavour precursors loss (Pedreschi et al., 2011). Hence, it was essential to determine the amount of these precursors in untreated and treated green coffee, not only to check whether the enzyme had successfully reacted with the asparagine substrate, but also to check whether there had been a decrease in other free amino acids and sugars after the steam & soak process.

Regarding the precursor determination, sugars, asparagine and aspartic acid were determined in both green and process coffee beans to demonstrate the impact of enzyme and enzyme treatment on precursor reduction. As the results indicated above, under the effect of asparaginase, asparagine was decreased and aspartic acid increased. However, in the process

control group, asparagine was also reduced (27%) and sugar content was lower (40%) compared to the control. This significant change in precursors was an effect of the steam & soak process. As sugar content would take part in the Maillard reaction during the roasting process, this adverse effect may lead to differences in the aroma profile in roasted coffee. In this experiment, the levels of acrylamide precursors in roasted coffee were also measured. As results showed above, most of asparagine and sugar content was lost as a result of the roasting process.

Regarding the other amino acids, compared to the control sample, the processed sample showed around 20–30% reduction. These amino acids, such as glutamine, glycine, leucine, phenylalanine, proline and valine, play important roles in the Maillard reaction and Strecker degradation and hence flavour formation in roast coffee. Also, a change in ratio of asparagine relative to the other amino acids may facilitate or mitigate acrylamide generation, as shown in model system studies (Koutsidis et al., 2009). Amino acids loss could have potential adverse effects on volatile compounds generation and acrylamide mitigation.

2.4.2 Acrylamide Reduction

The steam & soak process is necessary for the enzyme treatment process. In the literature review, little enzymatic acrylamide reduction research has been carried out in coffee compared to other foodstuffs. A possible reason for this phenomenon is that the tight internal structure of the coffee beans makes enzyme delivery very difficult to achieve. Therefore, the steam & soak process was deployed to increase the surface area of coffee beans by opening the internal pores. In potato products, free asparagine was found to leak out of the cells after the blanching process. In Acrylaway® application in Arabica coffee experiment (Experiment 1), the steaming process caused around 30% acrylamide reduction compared to the control. In coffee beans that were treated with enzyme without the prior steam treatment, a maximum 11% acrylamide reduction was achieved. In the Patent owned by Novozymes (LYNGLEV, 2017), an unsteamed sample only achieved 3% acrylamide reduction. Consequently, to ensure sufficient contact of enzyme and substrate, a prior steaming process is necessary.

Different enzyme dosages were used at the different stage of the experiment. In the beginning, an extremely high enzyme dosage was used to achieve maximum acrylamide reduction: 91% using Acrylaway® at 20000 ASNU/kg. Considering its potential impact on the sensory properties of the product, this dosage (20000ASNU) was not recommended by the manufacturers. However, this dosage demonstrated that there was a limit for acrylamide reduction. The goal of achieving 100% of acrylamide reduction was not achieved even at 20000 ASNU/kg. Consequently, in the following Experiments 2 and 3, more practical and cost-effective enzyme dosages were used (2000–6000 ASNU/kg or ASPU/kg).

For different varieties of coffee and different brands of enzyme that was used in the experiment, 35% to 80% acrylamide reduction was achieved. Comparing the acrylamide reduction rates, enzyme dosages lower than 6000 units (ASNU/kg or ASPU/kg) per kilogram of coffee beans were recommended for the application. The relationship of acrylamide reduction rate and enzyme dosage is shown in **Figure 2.12**. Despite the seasonal variation between the different batches of Brazilian Santos coffee, the combination of Acrylaway® application in Arabica coffee experiment and Acrylaway® & PreventASe® application in Arabica coffee experiment results demonstrated the highest effective dosage for the laboratory-scale experiments was between 4000ASNU ASPU/kg and 6000 ASNU ASPU/kg.

Regarding the price of asparaginase, the recommended retail price of PreventASe® is €80 per kilogram. A dosage of 6000ASPU (around 1.2 g) asparaginase achieved great acrylamide reduction rate for 150 g coffee. Hence, around 10 g enzyme would need to be used for 1 kg green coffee, which would cost around €0.80. Hence, the price of asparaginase and the energy bill for the enzyme treatment process would incur a considerable cost on the food manufacturer.

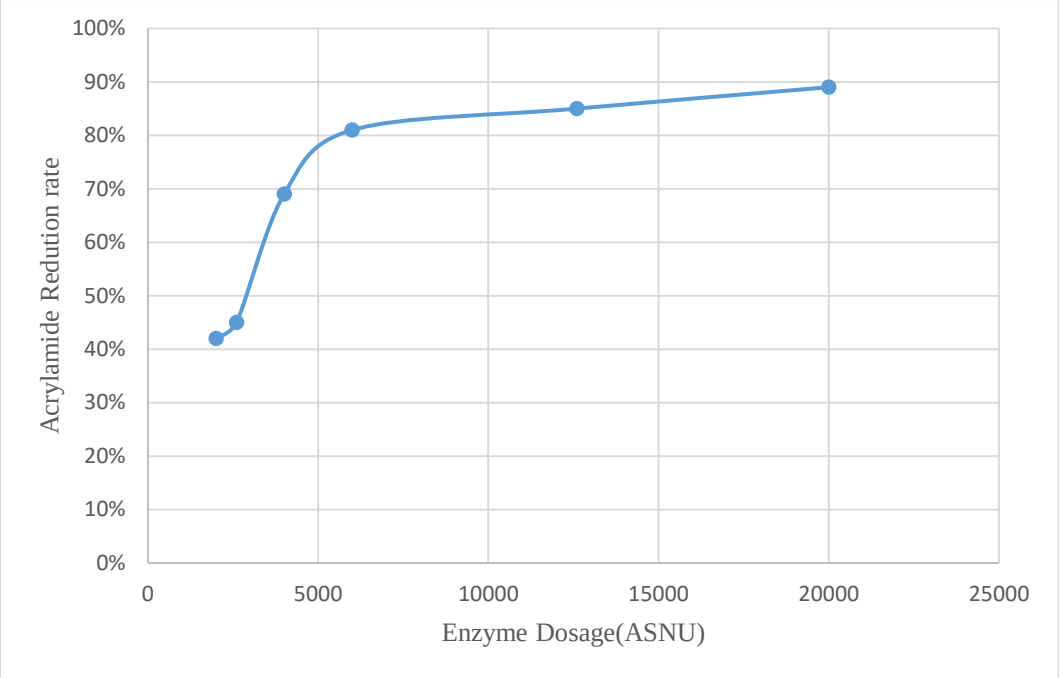

Figure 2.12 Acrylamide reduction rate under different Acrylaway® dosages.

Acrylaway® application in Arabica & Robusta coffee experiment focused on the acrylamide reduction rate in a variety of coffees. Asparagine content in Robusta coffee is higher than in Arabica (Guenther et al., 2007), suggesting that acrylamide content in Robusta roast coffee will be higher than the equivalent roast Arabica coffee, as long as asparagine was the limiting reagent for acrylamide formation in coffee. Comparing the control group data in **Figure 2.10** and **Figure 2.9**, this assumption was confirmed with the results found in Brazilian Robusta and Arabica coffee. It was of interest to see whether the enzyme would perform similarly in these two types of coffee. the incubation temperature was set to 60°C, while the pH was not altered. Generally, the pH of Arabica coffee is significantly lower than that of Robusta coffee (Bicho, Leitao, Ramalho, de Alvarenga, & Lidon, 2013). However, in Acrylaway® application in Arabica & Robusta coffee experiment (Experiment 2), the pH of the selected Robusta coffee was not significantly different to that of the Arabica coffee. Consequently, as is discussed in the results section, no significant differences in acrylamide reduction percentages were found between Arabica and Robusta coffees.

In Acrylaway® & PreventASe® application in Arabica coffee experiment (Experiment 3), comparison of the performances of the two enzymes was carried out. An important difference

between the two enzymes was the optimum pH (Acrylaway® pH7 and PreventASe® pH5). As the enzyme was delivered with water, the closer to neutral environment may have favoured the performance of Acrylaway® compared to that of PreventASe®. In fact, the acrylamide results indicated that the influence of this pH difference was not significant. Comparing the acrylamide reduction rate of the two enzymes, Acrylaway® achieved slightly higher reduction rate than the PreventASe® but this advantage of Acrylaway® became weaker as the roast level increased and acrylamide in control samples decreased. At the same dosage, statistical analysis found a significant difference between the two enzymes in light roast coffee ($p < 0.001$). However, no significant difference was found at the high roast level ($p = 0.746$).

Asparaginase treatment after the steam & soak processing of coffee beans achieved around 80% acrylamide reduction. The steaming and soaking step was designed to open the tight internal structure of the green coffee bean and let the asparaginase have a better contact with the substrate. However, the silver skin was partially lost during the steam & soak process. This silver skin loss caused differences in the later roast process. When the same heat treatment has been applied to the control and treated samples, the treated sample will have the greater extent of roast level because of a lack of silver skin. This difference in the roast level caused the difference in the following colour determination experiments. The silver skin became loose because of the steam treatment, then the soak step and the physical stir force that applied in the soak step made silver skin peeled off from the green bean at the end of the steam & soak process.

2.5 Conclusions

Acrylaway® in a range from 2000 to 6000 ASNU ASPU/kg showed good acrylamide reduction in Arabica coffee, compared to higher dosages. A steaming process was necessary to ensure sufficient contact of enzyme and substrate. Although Robusta coffee had more acrylamide than Arabica coffee under the same roast condition, Acrylaway® showed great acrylamide reduction rate in both Arabica and Robusta coffee. When comparing Acrylaway® with PreventASe® in Arabica coffee both showed a great acrylamide reduction at relatively low dosage.

An adverse effect of enzyme treatment, as shown by the total colour difference score, was the silver skin loss from the coffee beans, which made the enzyme treated coffee beans darker when roasted. In addition, the asparagine loss in the process control also suggested the potential loss of the aroma precursors. However, whether this impact (silver skin loss & aroma precursor loss) was great enough to cause a significant difference to the volatile compounds profile and sensory properties of the final product, the experiment in the following chapter will explain in detail.

Chapter 3 Influence of asparaginase treatment of coffee beans on the aroma of coffee with different degree of roasting

Abstract

The original aim of the project was to reduce acrylamide in coffee while maintaining the organoleptic properties. For coffee, aroma profile was a vital parameter on the coffee quality determination. Hence, volatile compounds determination was carried out in this chapter. Two experiments were done, the first one used only Acrylaway® and roasted to one roast level, while the second one used Acrylaway® and PreventASe® and roasted to three roast levels. Over 100 compounds were identified in these two experiments. Statistical analysis found less than 10% of identified compounds were significantly affected by enzyme treatment (light roast: 5 out of 51 compounds, medium roast: 2 out of 28 compounds, high roast: 3 out of 27 compounds). A literature review showed that only pyrazines were associated with the enzyme (reduction in asparagine and increased aspartic acid content). So, the adverse effect of enzyme treatment (precursor loss & roast level shift) caused a significant difference in aroma profiles. However, as different compounds had different flavour impact when perceived by human consumers, sensory tests need to be carried out to determine whether the differences in volatile composition reflected significant difference in organoleptic properties.

Keywords

Chemical families, Pyrazine, Precursor loss, Roast level shift, Flavour impact

3.1 Introduction

In the previous chapter, the influence of the enzyme asparaginase on acrylamide formation in coffee beans was discussed. Levels of acrylamide precursors, namely asparagine and reducing sugars, as well as the reaction product aspartic acid were also monitored. Results demonstrated the significant reduction of asparagine content and significant increase of aspartic acid content in enzyme-treated coffee beans. Also, processed green coffee beans showed 40% less sucrose compared to the control group. Acrylamide determination showed that the asparaginase treatment significantly mitigated the acrylamide content in the treated samples compared to the control group with a reduction rate, which ranged from 45% to 88% depending on the enzyme dosage that was used. Furthermore, significant colour differences were found between the control and treated samples, with the enzyme treated coffee being darker than the control, due to silver skin loss during the enzymatic treatment. This roast level difference may cause significant differences in coffee aroma profile. Therefore, in this chapter, the analysis of aroma volatile compounds of enzymatically treated and non-treated coffee beans will be evaluated.

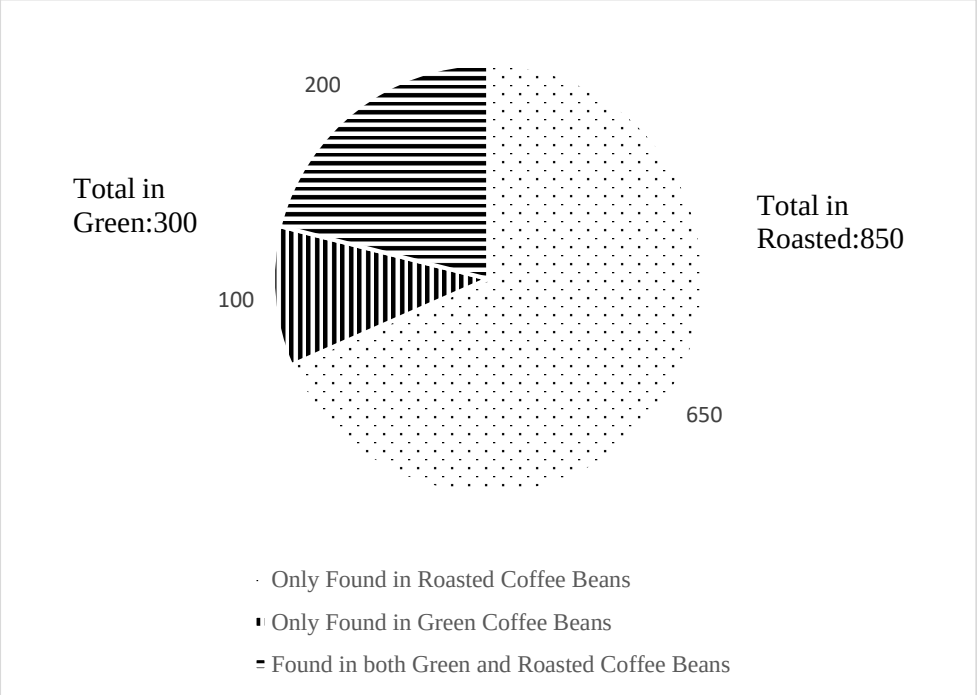

Figure 3.1 Distribution of aroma constituents in green and roasted coffee (Flament & Bessière-Thomas, 2002)

As shown in **Figure 3.1**, around 300 compounds have been associated with green coffee beans, whereas more than 850 have been associated with roasted coffee beans (Flament & Bessière-Thomas, 2002); 200 compounds can be found in both, green and roasted coffee beans. Flament & Bessiere-Thomas`s research summarised the proportions of each coffee aroma constituents: aldehydes (50.7%), ketones (21.5%), esters (10.3%), heterocyclic compounds (7.0%), sulfur compounds (4.3%), alcohols (2.4%) and nitriles.

During roasting, the reactions that take place are very complex, which makes the identification and quantification of coffee volatiles very challenging (Toledo, Pezza, Pezza, & Toci, 2016). **Figure 3.2** summarises the reactions that occur at different stages of the roast process, including pyrolysis, Strecker reaction, Maillard reaction, oxidation and thermal-degradation (Toci, 2010; Toledo et al., 2016). Ten reaction pathways were identified in previous research (Buffo & Cardelli-Freire, 2004). However, four major reactions were described in all of them (Illy & Viani, 2005). Firstly, caramelization and degradation of carbohydrates, forming mainly aldehydes and volatile acids (Yeretzian, Jordan, Badoud, & Lindinger, 2002). Secondly, Maillard reaction, which encompasses denaturation of proteins and reaction of free amino acids with carbohydrates and their degradation products. Thirdly, production of phenols and taste-active compounds from chlorogenic acids. Fourthly, degradation of trigonelline (Stadler, Varga, Hau, Vera, & Welti, 2002).

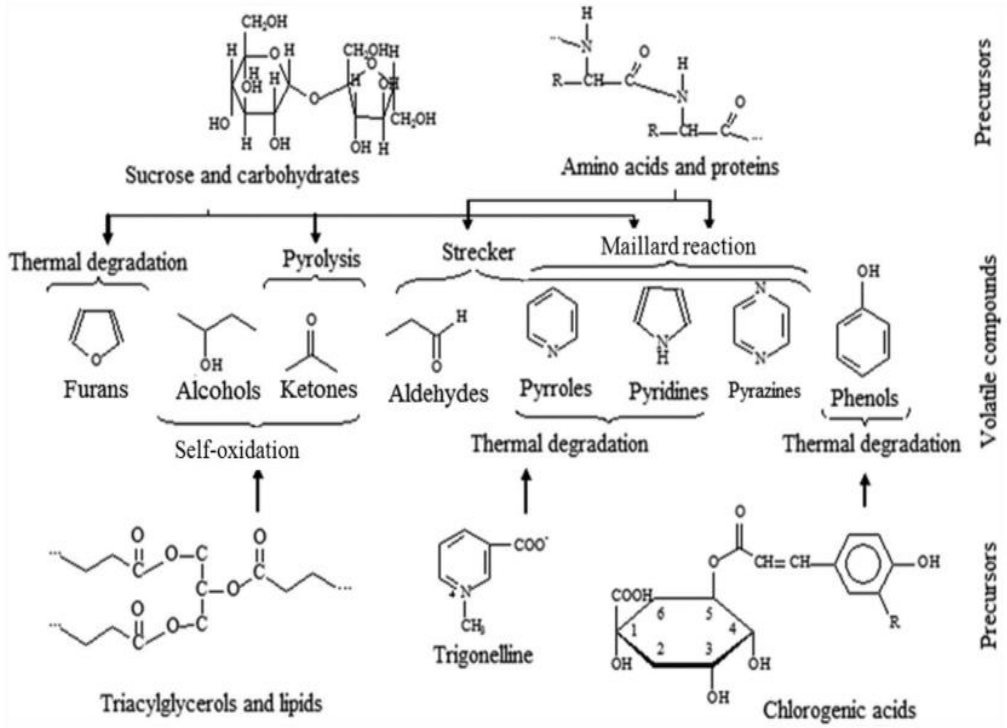

Figure 3.2: Schematic representation of the relationship between precursor compounds and volatile compounds, Adopted from Toledo et al., 2016.

Reactions described above happen in both Arabica and Robusta coffees. However, as the composition of the two varieties of coffee is different, the volatile compounds are also slightly different. Robusta coffee, which has more phenolic compounds (guaiacol and 4-vinylguaiacol), has more earthy notes, originating from the astringent note from the chlorogenic acids (Vitzthum, Weisemann, Becker, & Kohler, 1990). Research on glycosides has shown that these aroma precursors could enzymatically liberate the bound aroma compounds during post-harvest treatment and roasting (Weckerle, Gati, Toth, & Schreier, 2002), while different degrees of roasting produce differences in the aromatic profiles. Lighter roasts are richer in compounds that are more volatile, for instance, furan compounds which are responsible for fresh and floral notes. Then as the roast process continues, compounds like pyrazines and pyridines increase that are responsible for characteristic roasted and earthy notes.

At the beginning of flavour research, it was assumed that every volatile compound contributes equally to the aroma of food. However, later research found out that only a fraction (<5%) of volatile odorants were potent (Grosch, 2001; Yeretzian, Jordan, & Lindinger, 2003). To

determine the potency of odorant, gas chromatography with olfactometry detection has been carried out. Based on the conventional detector, trained panellists would sniff the odorant and give a sensory impression of individual compounds. Dilution techniques were used in GC-O(Mayer & Grosch, 2001). Flavour Dilution (FD) value was defined as the lowest concentration at which a compound was still sensorial detectable. Higher FD value indicated that the compound has higher flavour impact on the total flavour profile. Compounds with high FD value could be sensed by the consumer even at a very low concentration. As shown in **Table 3.1**, potent compounds have been analysed using GC-O, and different FD values have been obtained. As the values demonstrate, the volatile compounds had very different flavour impact (Schenker et al., 2002). For instance, in Holscher`s research about roasted Colombian coffee, after isolation by means of vacuum distillation or simultaneous distillation/extraction the aroma extracts were submitted to an aroma dilution analysis to characterize the key components. Compounds with the highest FD value (500) were 2-methyl-3-furanthiol, 2-furfurylthiol, methional, 3-mercapto-3-methyl-butyl formate, 2-isobutyl-3-methoxy-pyrazine, 2/3-methylbutanoic acid and (*E*)-β-damascenone (Holscher, Vitzthum, & Steinhart, 1990). As the results of Mayer`s work showed, the flavour impact of potent odorants in coffee can be quantified (Mayer & Grosch, 2001), to build up a model aroma that can be assessed by sensory analysis. Their results demonstrated that coffee aroma could be successfully simulated by a model made up from the key potent odorants.

Table 3.1 Aroma Impact compounds in roasted coffee powder. Peak sorted by retention time on DB-WAX column. Compounds with FD⩾32 are reported(Illy & Viani, 2005).

Odorant	Odour Impression	Holscher	Grosch	Schenker	Mayer
Methanethiol	Putrid, Cabbage-like	25			4500
2-Methylpropanal	Pungent, malty	100			24000
2-Methylbutanal	Pungent, fermented	100		16-128	28600
2,3-Butanedione	Buttery	200		256-1024	55700
2,3-Pentanedione	Buttery	100		4-128	28300
3-Methyl-2-buten-1-thiol	Animal-like, skunky	200		64-256	13
2-Methyl-3-furanthiol	Roasted meat-like	500	128	32	60
Mercaptopentanone	Sweaty, Catty	100			
2,3,5-Trimethylpyrazine	Roasty, Musty	200	64	16-32	6000
2-Furfurylthiol	Roasty, coffee-like	500	256	1024	1350
2-Isopropyl-3-methoxypyrazine	Peasy	100	128		
Acetic Acid	Vinegar-like	100			
Methional	Cooked Potato	500	128	1024	148
2-Ethyl-3,5-dimethylpyrazine	Roasty, musty	200	2048	1024	55
(*E*)-2-Nonenal	Fatty	5	64		100
2-Vinyl-5-methylpyrazine	Roasty, musty	200			53
3-Mercapto-3-methylbutyl formate	Catty, roasted coffee-like	500	2048	1024	130
2-Isobutyl-3-methoxypyrazine	Paprika-like	500	512	4-64	84
2-Phenylacetaldehyde	Honey-like	25	64		2500
3-Mercapto-3-methyl-1-butanol	Soup-like	100	32		
2/3-Methylbutanoic acid	Sweaty	500	64	1024	25000
(*E*)-β-Damascenone	Honey-like, fruity	500	1024	16-128	258
Guaiacol	Phenolic, burnt	200		512-1024	3420
4-Ethylguaiacol	Clover-like	25	256		1780
4-Vinylguaiacol	Clover-like	200	512	256	45100
Vanilline	Vanilla-like		32		4050

To our knowledge, there is scarce information of the impact on the aroma of coffee of enzymatic treatment to reduce the formation of acrylamide in coffee. All the research has been focused on whether the enzymatic treatment could reduce the formation of acrylamide without considering the sensorial effects on coffee (Beate.Kornbrust, 2009; Hendriksen et al., 2013). Therefore, in this chapter, the aroma volatile profile of enzymatically treated and non-treated coffee will be discussed. Extraction of coffee volatile compounds will be done by SPME with identification and quantification by GC-MS.

Two volatile compounds experiments are described in this chapter. First an Acrylaway® experiment that used three dosages of Acrylaway® and roasted to one roast level. Four groups of samples were set in the Acrylaway® experiment design to reveal the impact of enzyme treatment and steam process. A steaming process was introduced, as it would help open the green coffee pores and ensure sufficient contact between the enzyme and substrate. This experiment had two main objectives: (1) to assess the impact of the enzyme treatment (by comparing groups 1 and 3, (control vs enzyme treated, not steamed) and groups 2 and 4 (process control vs enzyme treated, both steamed) and (2) to assess the impact of the steaming process by comparing groups 1 and 2 (control vs process control, control not steamed) and groups 3 and 4 (enzyme treated without steam vs enzyme treated with steam). Furthermore, by comparing groups 1 and 4 (control vs steamed enzyme treated), the impact of prior steaming before enzymatic treatment will be assessed.

The second experiment was a comparison between Acrylaway® and PreventASe®. Coffee beans were treated with three dosages of the two enzymes then roasted to three roast levels. The aim of this experiment was to examine different dosages of the different enzymes in coffees roasted to different roast levels, so that the impact of enzyme treatment would be revealed.

3.2 Materials & Methods

3.2.1 Materials

3.2.1.1 Coffee

The coffee used in this chapter was the same batch of Arabica coffee (Brazilian Santos) that was used in acrylamide determination and sensory analysis respectively. Both batches of Brazilian Santos coffee were sourced from sourced from Rave Coffee UK (Cirencester, Gloucestershire).

3.2.1.2 Enzymes

Both enzymes (Acrylaway® and PreventASe®) were gifts from the manufacturers. Acrylaway® from Novozymes (3500ASNU/g) and PreventASe® (2500ASPU/g) from DSM were used. Both ASNU and ASPU are defined as the amount of asparaginase that produces one μmol of ammonia per minute under the conditions of the assay (pH = 7±0.005; T = 37±0.5 °C) using Acrylaway® or PreventASe®, respectively.

3.2.2 Methods

3.2.2.1 Enzymatic treatment of coffee beans

Unwashed green coffee beans (150 g) were steamed for 45 minutes at 100 °C. The beans were mixed with 103.5 mL of heated water (60 °C) containing various dosages of enzyme. For the process control 103.5 mL water were used instead of the enzyme solution. Samples were incubated at 60 °C for 60 minutes with stirring. After incubation, all liquid had been absorbed by the beans. Then, the treated beans were transferred to an aluminium tray and spread on the tray. The trays were placed in a drier (Gallenkamp, Loughborough, UK) for 4-6 hours until the coffee beans were reduced to their original weight of 150 g.

3.2.2.2 Acrylaway® Experiment coffee processing

In the first year, a preliminary enzyme (only Acrylaway®) treatment was carried out on Brazilian Santos coffee. Four treatment groups were prepared in this experiment as indicated below:

1: Control group – no steaming or enzyme treatment;

2: Beans are steamed without enzymatic treatment and then dried to 150 g;

3: Beans are treated with enzyme without steaming then dried to 150 g; 3a = 2600 ASNU/kg, 3b = 12600 ASNU/kg, 3c = 20000 ASNU/kg;

4: Beans are steamed, enzymatically treated and then dried to 150 g; 4a = 2600 ASNU/kg, 4b = 12600 ASNU/kg, 4c = 20000 ASNU/kg.

In all treatments, coffee beans were roasted at 220 °C for 17 min. Analysis was done in triplicate.

3.2.2.3 Acrylaway® & PreventASe® experiment coffee processing

Three treatment groups were prepared in this experiment as indicated below:

1: Control Group: no steaming or enzyme treatment.

2: Process Control Group: Beans were steamed first and then treated with distilled water (instead of enzyme solution) and then dried to 150 g.

3: Enzyme Treated Group: Beans were steamed first, then treated with either Acrylaway® or PreventASe® (Dosage1-2000U, Dosage 2-4000U, Dosage 3-6000U) for 30 minutes then dried to 150 g.

All coffee samples were then roasted to three different roast levels: light (230 °C for 12 minutes), medium (240 °C for 14 minutes) and high (250 °C for 17 minutes)

3.2.2.4 Aroma analysis of roasted coffee using SPME-GC-MS

A 1-cm 50/30 DVB/Carboxen on PDMS fibre from Supelco (Bellefonte, PA) was used for automated solid-phase microextraction (SPME), followed by gas chromatography-mass spectrometry (GC-MS) using a 7890A GC coupled with 5975C mass spectrometer (Agilent Technologies), to obtain the aroma composition of the coffee. The DB-wax column (Agilent Technologies) was fitted in the GC-MS system. Samples (1 ± 0.001g of ground roasted coffee) were incubated for 5 minutes at 30 °C and then extracted at 30 °C for 60 mins. Splitless injection was used for sample introduction. Desorption time was set to 10 minutes. GC oven temperature was set to 40 °C for 2 minutes, then increased to 250 °C at a rate of 4 °C/min. Helium was used as carrier gas with a flow rate of 1.2 mL/min. Mass spectrometer scan range

was set from *m/z* 29 to 250 with the temperature set at 230 °C for the source and 150 °C for the quadrupole.

3.2.2.5 Statistical Analysis

Analyses were done in triplicate and compound amounts were expressed as mean peak areas with standard deviations. XLStat 2015 (Addinsoft, Paris, France) was used to perform analysis of variance (one-way and two-way ANOVA). For those compounds that exhibited a significant difference in the ANOVA, multiple pairwise comparison tests (Tukey's HSD) were applied to determine which sample means differed significantly ($p < 0.05$).

3.3 Results

3.3.1 Effect of Acrylaway® on the volatile profile of roasted coffee

Then volatile compounds determination was carried out to determine the impact of enzyme treatment on the aroma profile. Four groups of samples comprised the experimental design, to reveal the impact of enzyme treatment and steaming process on roast coffee aroma compounds.

As shown in **Table 3.2**, the samples tested have shown around 90 volatile compounds in total. After comparing the results between different groups, 18 major compounds were selected (Table 3). These 18 compounds were either considered important odorants in each chemical family (2 aldehydes, 6 furans & furanones, 3 ketones, 6 pyrazines and 1 pyridine) or showed high flavour impact in other odorant potency researches.

As shown in **Table 3.3**, the response of each group was shown. The peak areas of 18 compounds in 4 groups (in triplicate) were present at high levels in the SPME extracts of roasted ground coffee and were compared using analysis of variance (ANOVA). The mean showed the average value among 24 variants in all groups.

One-way ANOVA on the 8 treatments was carried out using Fisher and Tukey test. The *p*-values are shown in Table 3 below. In the 18 compounds that were identified in the three roast levels, only 2-furanmethanol was significantly difference between the control and the enzyme-treated group.

Table 3.2: Major compounds identified in Acrylaway® experiment and Acrylaway® & PreventASe® comparison experiment.

KI	Compound	KI	Compound	KI	Compound
	ALDEHYDES & KETONES		FURANS		PYRIDINES
704	acetaldehyde	776	furan	1202	pyridine
774	propanal	853	2-methylfuran	1250	methyl pyridine
822	2-methylpropanal	886	3-methylfuran	2450	3-hydroxypyridine
888	butanal	956	2,5-dimethylfuran		PYRROLES
925	2-methylbutanal	1079	2-vinylfuran	1524	pyrrole
1060	3-hexanone	1221	2-furfuryl methyl sulphide	1542	1-methylpyrrole
914	2-butanone	1552	furfuryl acetate	1281	1,2-dimethylpyrrole
1065	2,3-pentanedione	1596	5-methylfurfural	1683	2-acetyl-1-methylpyrrole
1137	3-penten-2-one	1628	furfuryl alcohol	2022	2-acetylpyrrole
1194	cyclopentanone		FURANONES		PHENOLS
971	2,3-butanedione	1282	dihydro-2-methyl-(2*H*)-furanone	1886	2-methoxyphenol
1320	1-hydroxy-2-propanone	1707	5-methyl-2(5*H*)-furanone	2030	phenol
	ALCOHOLS	1847	dihydro-4-methyl-2(3*H*)-furanone		MISCELLANEOUS COMPOUNDS
928	ethanol	2062	2,5-dimethyl-4-hydroxy-3(2*H*)-furanone	1261	acetoin
1120	2-pentanol		PYRAZINES	1048	toluene
1315	3-methyl-2-buten-1-ol	1231	pyrazine	1181	trimethyloxazole
1485	2-ethyl-1-hexanol	1286	2-methylpyrazine	1650	2-acetylthiazole
	ACIDS	1346	2,5-dimethylpyrazine	1085	2-methylthiophene
1486	acetic acid	1352	2,6-dimethylpyrazine	1032	thiophene
1529	propanoic acid	1357	2-ethylpyrazine	1465	acetoxyacetone
	CYCLOPENTENONES	1414	2-ethyl-3-pyrazine	1616	acetic acid, methyl ester
1511	4,4-dimethyl-2-cyclopenten-1-one	1415	2-ethyl-5-pyrazine	1658	propanoic acid, methyl ester
1573	2,3-dimethyl-2-cyclopenten-1-one	1371	2,3-dimethylpyrazine	1624	propanoic acid, 2-propenyl ester
1857	2-hydroxy-3-methyl-2-cyclopenten-1-one	1408	2-ethyl-6-methylpyrazine	1323	butanediol
1924	3-ethyl-2-hydroxy-2-cyclopenten-1-one	1429	2,3,5-trimethylpyrazine		

Table 3.3 Effect of asparaginase treatment on major volatile compounds in roasted coffee samples

	KI	1	2	3a	3b	3c	4a	4b	4c	*p*-value
Pyrazine										
pyrazine	1160	2.14E+07	2.00E+07	1.86E+07	1.68E+07	1.55E+07	1.96E+07	2.36E+07	2.26E+07	0.938
methylpyrazine	1278	1.25E+08	1.15E+08	1.20E+08	9.89E+07	1.05E+08	1.39E+08	1.55E+08	1.55E+08	0.912
2,6-dimethylpyrazine	1437	1.07E+08	1.01E+08	1.05E+08	9.47E+07	1.08E+08	1.09E+08	1.32E+08	1.33E+08	0.830
ethylpyrazine	1348	3.01E+07	3.08E+07	2.97E+07	2.70E+07	2.85E+07	3.07E+07	3.72E+07	3.73E+07	0.914
2-ethyl-6-methylpyrazine	1002	1.62E+07	1.54E+07	1.59E+07	1.53E+07	1.78E+07	1.51E+07	1.93E+07	2.03E+07	0.674
2-ethyl-5-methylpyrazine	1002	1.85E+07	1.89E+07	1.82E+07	1.77E+07	1.96E+07	1.58E+07	2.07E+07	2.07E+07	0.902
Pyridine										
pyridine	1161	1.70E+08	1.88E+08	1.64E+08	1.53E+08	1.35E+08	1.81E+08	2.17E+08	2.00E+08	0.916
Furan & Furanone										
dihydro-2-methyl-3(2*H*)-furanone	1488	7.19E+07	7.31E+07	7.54E+07	6.54E+07	5.76E+07	7.88E+07	8.25E+07	8.16E+07	0.834
furfural	1481	6.77E+07	5.57E+07	6.48E+07	5.21E+07	7.21E+07	8.16E+07	9.29E+07	9.40E+07	0.287
2-furanmethanol	1666	2.01E+08	1.80E+08	1.48E+08	1.61E+08	1.90E+08	1.53E+08	2.13E+08	1.98E+08	0.028*
furfuryl formate	1495	1.84E+07	1.88E+07	1.75E+07	1.79E+07	1.87E+07	2.03E+07	2.65E+07	2.57E+07	0.496
5-methyl-2-furancarboxaldehyde	1582	3.01E+07	2.55E+07	2.83E+07	2.53E+07	3.49E+07	3.33E+07	4.05E+07	4.22E+07	0.152
2-furanmethanol acetate	1539	4.94E+07	5.49E+07	4.54E+07	4.71E+07	4.51E+07	4.57E+07	5.63E+07	5.40E+07	0.927
Aldehyde										
3-methyl butanal	928	1.78E+07	1.99E+07	4.07E+07	3.65E+07	3.23E+07	3.04E+07	3.26E+07	3.12E+07	0.471
2-methyl-butanal	925	4.16E+07	4.42E+07	6.36E+07	4.84E+07	4.89E+07	6.05E+07	6.32E+07	6.07E+07	0.87
Ketone										
1-hydroxy-2-butanone	1381	2.03E+07	2.05E+07	2.12E+07	1.92E+07	1.75E+07	2.32E+07	2.62E+07	2.62E+07	0.548
1-(acetyloxy)-2-propnone	1535	6.97E+07	7.32E+07	6.79E+07	7.27E+07	7.18E+07	7.33E+07	9.60E+07	9.08E+07	1.425
2,3-pentanedione	1065	4.85E+07	4.84E+07	6.30E+07	4.67E+07	4.71E+07	7.72E+07	7.48E+07	7.30E+07	0.586

1: Control group – no steaming or enzyme treatment; 2: Beans are steamed without enzymatic treatment and then dried to 150 g; 3: Beans are treated with enzyme without steaming then dried to 150 g; 3a = 2600 ASNU/kg, 3b = 12600 ASNU/kg, 3c = 20000 ASNU/kg; 4: Beans are steamed, enzymatically treated and then dried to 150 g; 4a = 2600 ASNU/kg, 4b = 12600 ASNU/kg, 4c = 20000 ASNU/kg; *=p value<0.05

3.3.2 PreventASe® and Acrylaway® Comparison Experiment

A new batch of the Brazilian Santos coffee was used for the comparison experiment after the PreventASe® was sourced from DSM. In this experiment, two enzymes with 3 dosages (2000 U, 4000 U and 6000 U) were used and then roasted to three roast levels. Compared to the previous experiment, enzyme dosages were set to a more cost-effective level based on the acrylamide reduction results from chapter 2, and the coffee samples were roasted to three levels. Because the volatile compounds varied with the different roast level, the impact of enzyme treatment on volatile compounds in different roast levels was investigated.

A new process control (where the sample was steamed first and then soaked in distilled water (instead of the enzyme solution) replaced the previous process control where no soaking had occurred) in the experimental design. The process control was added to investigate the effect of enzyme treatment. In this experiment, silver skin loss in the enzyme process (steaming & soaking) was observed, so the weight of the waste chamber in the roast machine was weighed after each roast. The enzyme treated coffee had 50% less burnt silver skin in the waste chamber, which implied that the silver skin was lost during the enzyme treatment prior to roasting (steaming & soaking).

The Acrylaway® & PreventASe® comparison experiment showed around 100 volatile compounds in total (**Table 3.2**). In this experiment, the factors that could have potential impacts on volatile compounds amount include roast levels, enzyme, enzyme treatment, enzyme dosages and enzyme brands. These five factors were added to the statistical analysis to investigate the potential effect that they may pose on the volatile profiles. The statistical analysis had been carried out to examine the significant impact of each factor and the implications these factors may indicate. The confidence level was set at 95% while the corresponding significance level was 0.05.

As different roasting conditions were studied, the first step would be to compare the results under the same roasting conditions. As mentioned above, most of the volatile compounds were generated during the heating process. Therefore the roast extent was closely related to the

compounds that were present. For instance, 51 compounds were detected in light roast coffee, both control and enzyme treated; 65 compounds were detected in the medium roast coffee, while 80 compounds were detected in the high roast. However, not all compounds were found at every roast level.

3.3.2.1 Light roast

So, summarize the compounds that were found in both control and enzyme treated coffee. The number of compounds that could be found in every sample was same or less than the number of compounds that were detected. It indicated that the compounds list variation increased with the roast degree. As it showed in **Tables 3.4, 3.5 & 3.6**, light roast had 51 compounds, medium roast had 28 compounds while high roast had 27 compounds. As it mentioned above in the introduction, most of the compounds detected in the roasted coffee beans were generated in the roast process. So, only 18 compounds could be found in three roast level.

The light roast coffee volatile profile represents the initial stage of the roast process. On the one hand, volatile compounds like furan compounds that represent the fruity and floral note of the coffee were at its peaks. These compounds which existed in both green coffee and roasted coffee beans began to pyrolysis as the roast extent increased. On the other hand, typical compounds that generated from the Maillard reaction began showing up. For instance, heterocyclic compounds like pyrazines, pyridines and pyrroles. These Maillard reaction products which had pungent and roast flavour impression increased as the roast process progressed.

When process control samples were compared with enzyme treated samples by Fisher and Tukey tests, no significant differences were observed in any of the 51 compounds. When the unprocessed control was compared with the enzyme-treated group 5 out of 51 volatile compounds, namely carbon disulfide, 1-hydroxy-2-propanone, methylpyrazine, 3-methylbutanoic acid and 2,5(or 6)-dimethyl-3-ethylpyrazine were found to be significantly different between the control and treated groups.

Three compounds (methylene chloride, 2-methylpropanal and 2-methylbutanal) out of 51 showed significant difference due to the brand of enzyme, while 2 compounds (1-hydroxy-2-

propanone and 2,3-hexanedione) out of 51 had significant differences among the beans treated with different dosages of enzyme

3.3.2.2 Medium roast

The medium roast was a transitional phase of all three roast levels. Reactions that took place in the light roast were still occurring. Consequently, compounds responsible for roasted and cooked flavour increased. As the roast process continued, more compounds were generated. One of the symbolic chemical families is the phenol family. Phenol and 2-methoxyphenol (guaiacol) began to be detected in the medium roast coffee. Especially guaiacol, as an iconic compound in coffee, loads of researches had been carried out about it (Czerny, Mayer, & Grosch, 1999; Mayer, Czerny, & Grosch, 2000; Toci & Farah, 2014). In medium roast coffee samples, 28 compounds which were detected in both control and enzyme treated group, were picked for the statistical analysis. As described above, the first analysis considered the influence of enzyme (process control vs treated) and enzyme treatment (control vs treated).

Five compounds showed a significant difference (acetic acid, acetol, 2,3-pentanedione, 2,3-butanediol, 5-methylfurfural) between unprocessed control and enzyme-treated samples, while 2 compounds showed a significant difference (acetol & 5-methylfurfural) between processed control and enzyme-treated samples. Two compounds (2-methylbutanal & 2,5 (or 6)-dimethyl-3-ethylpyrazine) were significantly affected by the enzyme brand while only one compound (2-methylbutanal) showed a significant difference due to the enzyme dosage.

Table 3.4: *p*-Value for each compound in the light roast samples (Fisher & Tukey test results, Confidence level set at 95% while the corresponding significance level was 0.05. *=p value<0.05). Enzyme brand factor (Acrylaway® vs PreventASe®), Enzyme dosage factor (Dosage 1 vs Dosage 2 vs Dosage 3), Enzyme factor (process control vs treated), Enzyme treatment factor (control vs treated**)**

Compounds	KI	Enzyme Treatment	Enzyme	Enzyme Brand	Enzyme Dosages
Acetaldehyde	704	0.075	0.783	0.083	0.359
carbon disulphide	732	0.015*	0.961	0.456	0.115
methyl formate	764	0.053	0.893	0.167	0.382
Furan	776	0.174	0.813	0.359	0.349
Acetone	817	0.566	0.835	0.354	0.438
2-methylpropanal (isobutyraldehyde)	822	0.447	0.254	0.006*	0.194
methyl acetate	832	0.821	0.882	0.076	0.981
3-methylfuran	911	0.188	0.999	0.518	0.198
methylene chloride	914	0.252	0.614	0.044*	0.602
2-methylbutanal	925	0.561	0.252	0.015*	0.181
Ethanol	928	0.482	0.625	0.797	0.136
3-methylbutanal	928	0.084	0.691	0.309	0.077
3,3-dimethyl-2-butanone,	936	0.366	0.695	0.751	0.144
2,3-butanedione	971	0.845	0.624	0.172	0.613
2,3-pentanedione	1065	0.191	0.181	0.281	0.722
2,3-pentanedione	1065	0.921	0.735	0.247	0.481
2-vinylfuran	1079	0.411	0.575	0.412	0.544
2,3-hexanedione	1111	0.604	0.611	0.748	0.018*
Pyridine	1202	0.319	0.594	0.738	0.168
Pyrazine	1231	0.078	0.571	0.306	0.754
4-methylthiazole	1256	0.774	0.864	0.219	0.406
Acetoin	1261	0.163	0.648	0.263	0.683

dihydro-2-methyl-3[2*H*]-furanone	1261	0.078	0.291	0.385	0.861
2-methyl-3(2*H*)-furanone	1261	0.082	0.905	0.162	0.365
methyl pyrazine	1278	0.050*	0.736	0.247	0.479
3-heptanol	1293	0.078	0.736	0.507	0.479
1-hydroxy-2-propanone (acetol)	1320	0.025*	0.339	0.261	0.001*
Ethylpyrazine	1323	0.727	0.256	0.254	0.759
2,5-dimethylpyrazine	1327	0.283	0.218	0.316	0.775
2,3-dimethylpyrazine	1340	0.531	0.229	0.134	0.56
1-methyl-2-pyrrolealdehyde	1374	0.655	0.938	0.613	0.123
1-hydroxy-2-butanone	1381	0.121	0.749	0.663	0.157
2-ethyl-3-methylpyrazine	1414	0.995	0.972	0.331	0.707
2-ethyl-5-methylpyrazine	1456	0.655	0.328	0.872	0.322
Acetoxyacetone	1465	0.698	0.401	0.058	0.125
3-furancarboxaldehyde	1473	0.067	0.337	0.491	0.719
2-hexanol	1485	0.208	0.736	0.247	0.479
acetic acid	1486	0.998	0.436	0.967	0.089
furfuryl formate	1497	0.626	0.741	0.245	0.476
furfuryl acetate	1506	0.871	0.729	0.187	0.752
Pyrrole	1524	0.789	0.817	0.596	0.171
1-acetyloxy-2-butanone	1535	0.874	0.642	0.307	0.559
1-methylpyrrole	1542	0.733	0.587	0.487	0.438
5-methyl-2-furancarboxaldehyde (5-methylfurfural)	1574	0.628	0.919	0.653	0.441
2,3-butanediol	1580	0.428	0.342	0.236	0.077
2,5(or 6)-dimethyl-3-ethylpyrazine	1586	0.038*	0.228	0.029*	0.239
2-furanmethanol (furfuryl alcohol)	1628	0.078	0.975	0.207	0.252
3-methylbutanoic acid	1687	0.044*	0.721	0.256	0.491

Table 3.5 *p*-Values for each compound in medium roast coffees (Fisher & Tukey test results, significance level set at 0.05).

Compounds	KI	Enzyme Treatment	Enzyme	Enzyme Brand	Enzyme Dosage
Acetaldehyde	704	0.322	0.669	0.191	0.582
Furan	776	0.869	0.665	0.202	0.977
Acetone	817	0.768	0.657	0.208	0.994
2-methylpropanal (isobutyraldehyde)	822	0.215	0.733	0.138	0.663
2-butanone	914	0.601	0.728	0.162	0.752
2-methylbutanal	925	0.175	0.993	0.003*	0.031*
3-methylbutanal	928	0.211	0.709	0.151	0.696
2,3-pentanedione	1065	0.023*	0.214	0.341	0.977
1-(2-methylpropyl)pyrrole	1115	0.174	0.797	0.168	0.131
Pyridine	1202	0.751	0.359	0.411	0.964
Pyrazine	1231	0.521	0.476	0.319	0.997
Acetoin	1261	0.101	0.201	0.484	0.748
dihydro-2-methyl-3[2*H*]-furanone	1261	0.282	0.202	0.513	0.962
Methylpyrazine	1278	0.244	0.819	0.095	0.536
1-hydroxy-2-propanone (acetol)	1320	0.026*	0.018	0.679	0.991
Ethylpyrazine	1323	0.299	0.744	0.141	0.631
2,3-dimethylpyrazine	1340	0.864	0.855	0.114	0.167
1-methyl-2-pyrrolealdehyde	1374	0.841	0.321	0.485	0.763
2-ethyl-3-methylpyrazine	1414	0.486	0.494	0.417	0.493
Acetoxyacetone	1465	0.465	0.526	0.294	0.864
2-furancarboxaldehyde (furfural)	1481	0.619	0.814	0.137	0.257
acetic acid	1486	0.045*	0.052	0.621	0.939
furfuryl acetate	1506	0.842	0.628	0.233	0.732
Pyrrole	1524	0.872	0.412	0.983	0.499

1-acetyloxy-2-butanone	1535	0.365	0.766	0.146	0.259
5-methyl-2-furancarboxaldehyde (5-methylfurfural)	1574	0.006*	0.041*	0.484	0.988
2,3-butanediol	1580	0.001*	0.541	0.101	0.367
2,5(or 6)-dimethyl-3-ethylpyrazine	1586	0.193	0.935	0.034*	0.103

Table 3.6: *p*-Values for each compound in high roast coffee (Fisher & Tukey test results, significance level set at 0.05).

Compounds	KI	Enzyme Treatment	Enzyme	Enzyme Brand	Enzyme Dosage
Trimethylamine	570	0.447	0.442	0.587	0.699
Acetaldehyde	704	0.572	0.947	0.031*	0.229
Furan	776	0.965	0.675	0.196	0.937
N,*N*-dimethylethanamine	803	0.963	0.875	0.856	0.744
Acetone	817	0.774	0.997	0.002*	0.007*
2-methylpropanal (isobutyraldehyde)	822	0.098	0.257	0.405	0.883
methyl acetate	826	0.732	0.618	0.236	0.899
2-butanone	914	0.327	0.713	0.161	0.697
2-methylbutanal	925	0.082	0.364	0.317	0.823
2,3-butanedione	971	0.138	0.113	0.682	0.878
2,3-pentanedione	1065	0.021*	0.028*	0.636	0.945
1-(2-methylpropyl)pyrrole	1115	0.672	0.179	0.873	0.736
1-methyl-1,2,3,6-tetrahydropyridine	1150	0.482	0.633	0.367	0.316
Pyridine	1202	0.708	0.642	0.223	0.84
Pyrazine	1231	0.146	0.353	0.409	0.675

dihydro-2-methyl-3[2*H*]-furanone	1261	0.038*	0.034*	0.682	0.948
Methylpyrazine	1278	0.041*	0.112	0.531	0.865
1-hydroxy-2-propanone (acetol)	1320	0.146	0.252	0.454	0.886
Ethylpyrazine	1323	0.205	0.109	0.803	0.657
2,3-dimethylpyrazine	1340	0.124	0.368	0.342	0.768
2-ethyl-3-methylpyrazine	1414	0.883	0.822	0.133	0.219
Acetoxyacetone	1465	0.058	0.042*	0.689	0.917
Pyrrole	1524	0.411	0.326	0.466	0.864
1-acetyloxy-2-butanone	1535	0.363	0.181	0.537	0.754
2,5(or 6)-dimethyl-3-ethylpyrazine	1586	0.13	0.125	0.594	0.862
guaiacol (2-methoxyphenol)	1886	0.315	0.919	0.094	0.051
Phenol	2030	0.759	0.227	0.554	0.905

3.3.2.3 Dark roast

In high roast coffee, some volatile compounds that had floral and grassy notes are consumed in the reaction, while some compounds responsible for the roasted flavour reached their peak. Researcher Joon-Kwan roasted Ethiopian coffee to different degrees. Compounds like acetylpyrrole reached a peak at medium roast then decreased (Moon & Shibamoto, 2009).

In high roast coffee samples, 27 compounds which were detected in both the unprocessed control and enzyme treated group, were picked for statistical analysis. three compounds showed a significant difference (2,3-pentanedione, dihydro-2-methyl-3[2*H*]-furanone and methylpyrazine) between unprocessed control and enzyme-treated samples, and 3 compounds showed a significant difference (2,3-pentanedione, dihydro-2-methyl-3[2*H*]-furanone and acetoxyacetone) between processed control and enzyme-treated samples. Two compounds (acetaldehyde & acetone) were significantly affected by the enzyme brand while only one compound (acetone) showed a significant difference due to the enzyme dosage. Summarising the medium and high roast results, significant differences found in the medium and high roast coffee were fewer than in light roast coffee.

Even though the whole reaction mechanism of all the volatile compounds in coffee was not fully elucidated, the 17 compounds that were present at every roast level illustrate the reaction trend by their amounts at the different roast levels. Some volatile compounds generated in the roast may increase as the roast level increases, while other compounds that participate in reactions may decrease as the roast level increase. As shown in **Table 3.7**, 3 compounds increased with the roast level, and 7 compounds decreased with roast level, while 8 compounds increased first in light roast level, reached a peak at the medium roast level and decreased at the high roast level. Four out of these 8 compounds had a significant difference between different roast levels. The potential theory behind this phenomenon could be the roast level increase; the pyrolysis speed outweighs the generation speed. Hence the compound amount would increase then decrease along with the coffee beans roasted for longer time

Table 3.7 Compounds variance trend and significance level for 18 compounds that were present in every roast level. (Confidence level set at 95% while the corresponding significance level was 0.05. *= p <0.05)

compounds increasing with roast level	*p*-value
Furan	0.335
Pyridine	<0.001*
Pyrrole	<0.001*
compounds decreasing with roast level	*p*-value
isobutyraldehyde	0.021*
Acetol	<0.001*
2-methylbutanal	0.034*
dihydro-2-methyl-3[2*H*]-furanone	0.243
1-acetyloxy-2-butanone	0.601
2-ethyl-3-methylpyrazine	0.009*
compounds increasing first then decreasing	*p*-value
acetaldehyde	0.669
Acetone	0.010*
Pyrazine	0.722
methylpyrazine	0.362
acetoxyacetone	0.013*
ethylpyrazine	< 0.001*
2,3-dimethylpyrazine	0.249
2,5(or 6)-dimethyl-3-ethylpyrazine	0.034*

3.4 Discussion

From the statistical analysis of the volatile compounds data, several conclusions could be summarized. To begin with, degree of roast affected the compounds identified (light roast: 51, medium roast: 65, high roast: 80) and compounds that existed in both control and processed groups (light roast: 51, medium roast: 28, high roast:27). Secondly, significantly different

compounds were found at each roast level. For the light roast, between un processed control and enzyme-treated groups, 5 significantly different compounds were found in the 51 compounds that were identified, while 2 compounds were found among 28 identified in the medium roast and 3 compounds were found among the 27 in the high roast group. Regarding the chemistry families of the significantly different compounds, aldehydes, ketones, furans, phenols and pyrazines compounds were involved.

Among the reaction pathways discussed in the introduction, only pyrazines have a chemical relationship with asparagine/aspartic acid. In model systems which focused on the reaction pathway of acrylamide, highly significant correlations were obtained for the relationship between pyrazine and acrylamide formation (Koutsidis et al., 2008). Regarding the asparagine and aspartic acid contributions to the whole aroma profile, several research was carried out before the millennium(Chun & Ho, 1997; Ho, Hwang, Yu, Zhang, & Assoc Sci Int, 1993). Deamidation of glutamine and asparagine would react with reducing sugars generating pyrazines. At that time, researchers believed that Maillard reaction products contributed to the majority of the volatile compounds in coffee (Holscher & Steinhart, 1993). However, other formation pathways were revealed in later research that proved the Maillard reaction products were not the most important aroma contributors in coffee.(Flament & Bessière-Thomas, 2002). The product compounds that generated in other reaction pathways (**Figure 3.2**) were equally important. Summarizing the literature about asparagine and aspartic acid, only pyrazine family were chemically associated. So, clearly, for the compounds in other chemical families that were significantly different in the enzyme treated group compared to the control, the absence of asparagine and increased aspartic acid content were not the reason that caused these differences.

In theory, the absence of asparagine was not likely to cause significant differences in aroma volatiles. Hence, enzymatic acrylamide reduction appeared to be attractive. There must be other reasons that triggered the differences. In Kornbrust`s research, the author stressed that the steam & soak process would rearrange the solutes and concentrate sugars and amino acids at the surface (Beate.Kornbrust, 2009). As a result, a change in roast kinetics was suggested by the researcher after the enzyme treatment. To discuss the impact of enzyme treatment on roast coffee volatile profiles, three aspects needed to be considered.

The first aspect would be the chemical differences that were caused by the enzyme. This potential difference related to the change that was caused by the absence of asparagine and presence of aspartic acid. The added enzyme was mostly consumed and residual enzyme would be inactivated in the later heat treatment. The increased amount of aspartic acid would take part in the Maillard reaction. The chemical impact of the enzyme is not the key factor for the variations in aroma profile.

The second aspect relates to the adverse effect of the steam & soak process. In the previous chapter, acrylamide determination data in the only steamed control group showed that steam process alone would cause acrylamide reduction. As the results in Chapter 2 showed, while steam process opened the pores of the coffee beans, asparagine and sugar content came to the surface of coffee beans and washed away in the following soak process. Steam treatment of green coffee bean was patented for a method to improve the flavour quality of Robusta coffee (Becker, 1989; Darboven A., 1995). In a recent publication, this method was described as using saturated steam to provoke changes in the precursor composition. The main aim of the method was to diminish the formation of undesirable substances, such as catechol, pyrogallol and hydroquinone, pyrazines and phenols (Luigi Poisson, 2017). Precursor loss caused by the steam & soak process was a vital factor for the variation in volatile compounds.

The third aspect would be the silver skin loss because of the enzyme treatment. As mentioned in Chapter 2, the silver skin loss leads to a darker roast of the enzyme-treated beans. The roast level has a dominant influence on the coffee beans flavour profile (Baggenstoss, Poisson, Kaegi, Perren, & Escher, 2008; Moon & Shibamoto, 2009; Somporn, Kamtuo, Theerakulpisut, & Siriamornpun, 2011).

The enzyme factor (absence of asparagine and increased amount of aspartic acid) had a minimal effect while the roast level shift and precursor loss had the greatest effect. So, combining the internal chemical redistribution or loss and external silver skin loss together, the effect of enzyme treatment was magnified or diminished depending on the specific compound. For instance, precursor loss caused less sugar and other precursors to participate in the reactions in

the roast process, while the roast level shift meant more reactants were consumed as the roast level was higher. There was an interaction between these two factors that may have magnified the enzyme treatment impacts. The adverse effect caused by precursor loss and roast level shift explained why the compounds that showed significant differences had no chemical relationship with the absence of asparagine.

3.5 Conclusion

At all three roast levels, enzyme-treated coffee had volatile compounds that were significantly different from the unprocessed control. Considering the whole aroma profile, the enzyme-treatment affected compounds were less than 10% of all compounds identified. It is likely that the significant differences are mainly caused by the adverse effect of the enzyme treatment, such as precursor loss and roast level shift. Because of the precursor loss and roast level shift, the significantly different compounds involved a variety of chemical families, such as aldehydes, ketones, furans, phenols and pyrazines.

However, as different volatile compounds have very different potency levels, the differences caused by enzyme treatment on the high flavour impact compounds may result in a significant impact on the aroma that could be perceived by the consumers. Therefore, a new enzyme treatment experiment and sensory tests are presented in the following chapter. The sensory test aimed to find out whether the significant differences in volatile compounds would lead to significant differences that could be perceived by trained panellists and consumers. Regarding the volatile compounds determination, further work should include key compounds reaction pathway research, and GC-O analysis of the enzyme treated coffee should be carried out to fully understand the influence of the enzyme treatment on coffee aroma.

Chapter 4 Sensory Analysis of Enzyme-Treated Coffee

Abstract

Discriminative, descriptive tests were carried out to examine the whether there was significant difference between control and processed samples. In the Discriminative test, the coffee profile had been separated to two parts (Taste & Smell) and used specific methods for each part (Same-Different & Tetrad) to increase the statistical power and reduce consumer fatigue. Consumers form the University and outside university had participated the test. The descriptive test used professional panellists and evaluated enzyme treated coffee samples from four aspects: taste, aroma, basic taste and after taste. Preference Tests were carried out to test whether there were preference difference because of the enzyme treatment. Preference test used 9-point scale test (1-extremely dislike to 9-extremely like). Significant differences were found in the discriminative test in both taste and smell parts. In the descriptive test, for 39 attributes tested in each roast level, 12 attributes found significant difference in light roast and 17 attributes found significant difference in a medium roast. Preference test showed no significant difference between control and enzyme treated coffee and no unpleasant flavour were reported from participants` feedback. In conclusion, discriminative and descriptive test found significant differences between control and treated coffee. However, as no significant preference difference and unpleasant flavour or taste were found, enzyme treated coffee appeared to be acceptable to the consumers.

Keywords

Tetrad, Same-different, Preference, Consumer fatigue, Significant difference and consumer acceptance.

4.1 Introduction

In the previous chapter, coffee treated with Acrylaway® and PreventASe® (steam & soak) had shown a significant reduction (40%-91%) of acrylamide when roasted. In addition, acrylamide was reduced by around 30% in the process control sample (steam & soak in water). Also, colour measurement using the HunterLAB® spectrophotometer had revealed that the conventional enzyme treatment process, which included steam and soak steps, had made an impact on the colour of roasted coffee. Whether the enzyme treatment would also have a noticeable impact on the sensory properties of roasted coffee would be assessed in this chapter.

In theory, asparaginase will be inactivated and decompose during heat treatment. Additional aspartic acid and ammonia generated by the asparaginase treatment should not cause a noticeable difference in the sensory properties of the treated food (Hendriksen et al., 2009). Green coffee has a very tight internal structure so, a conventional process like steam and soak had to be carried out to enhance the enzyme contact with the substrate (Beate et al., 2009). However, this enzyme treatment may lead to diffusion of flavour precursors out of the bean. Therefore, as well as examining the effect of asparaginase, it is also necessary to examine if the steam and soak process also changes the sensory properties of the coffee.

Although previous research have used sensory analysis to examine the flavour properties of coffee, only the work carried out by ….. has tried to relate acrylamide mitigation with sensory quality. Conventionally, sufficient roast, which were enough for desirable flavour to be generated, was defined from 170 to 240°C for 10 to 15 minutes as normal roast and 140-170°C for 12 to 20 minutes as under roast. Under roast concept was initially brought up by the researchers to maximize the preservation of endogenous antioxidant compounds (Perrone et al., 2010). In this paper, the authors prepared several samples of under-roasted coffee, which were then compared in sensory quality to a regular roast (Masi et al., 2013). The under-roasted coffees were roasted at 140, 145, 150, 155, 160, and 165 °C for 20 minutes, while the regular roast was prepared at 220 °C, also for 20 minutes. The authors assumed that the levels of potential acrylamide generated would be lower in the under-roasted coffee than the regular roasted coffee. However, no acrylamide was measured in any of the coffee samples. As it has

been mentioned in previous research, the acrylamide content in coffee will rise first and then decrease as the extent of roasting continues (Lantz et al., 2006). So, there was a possibility that under roast treatment may increase the acrylamide content rather than decrease it. Though there was no acrylamide determination data in this research, the consumers still identified the differences on major coffee characteristics between control and under-roast coffee samples.

Before carrying out any sensory experiments, an appropriate discriminating method needs to be selected. There are several major discriminating methods, including *n*-AFC, duo-trio, triangle, tetrad and same-different. As coffee has an intense aroma and flavour, it is difficult for the consumer to discriminate many samples without suffering fatigue (de Oliveira, Cabral, Eberlin, & Cordello, 2009; Masi et al., 2013; Nebesny & Budryn, 2006).

N-AFC stands for the *n*-alternative forced-choice method, where *n* is the number of samples presented to the assessor in each test. In this method, participants are given a criterion by which they are required to select one sample. Typical methods used are 2-AFC and 3-AFC. In the duo-trio method, participants are presented with one known sample and two unknown samples. The participant identifies which unknown sample is the same as the known sample. In the triangle method participants are presented with three unknown samples, two of which are the same and one is different. The participant is instructed to identify the odd one out (Civille & Carr, 2015). Because the criterion was given to the participant, the discriminating power of n-AFC is far superior than that of duo-trio and triangle test. However, as the n-AFC test is based on a given criterion, the range of applications was smaller than duo-trio and triangle test (Ennis, 1993).

As it showed in **Figure 4.1** below, tetrad method is a relatively new method with high discriminating power (Ennis & Jesionka, 2011). In the Tetrad method, participants are presented with four samples (two samples of one type and the other two of another type). The participants are instructed to separate the samples into two pairs (O'Mahony & Rousseau, 2003).

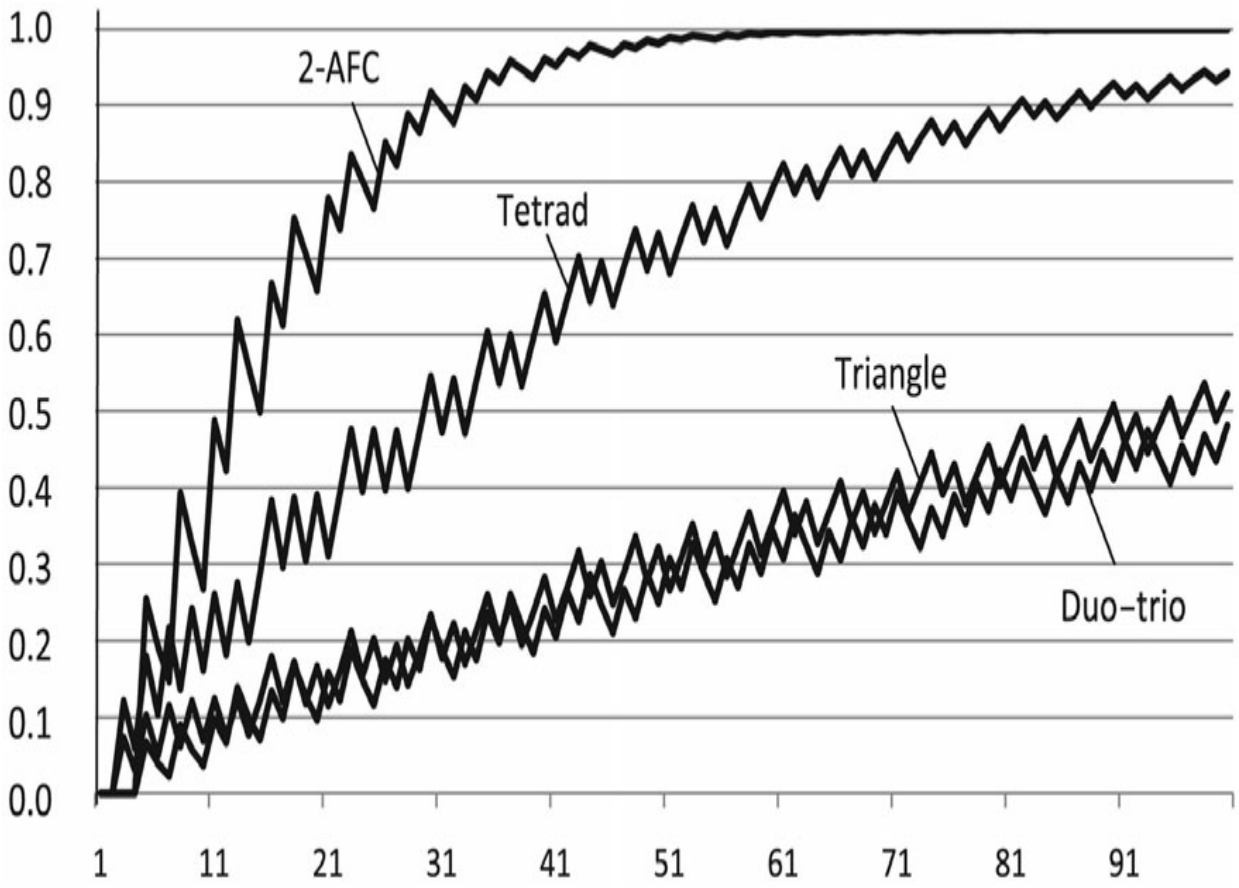

Figure 4.1 Power as a function of sample size for the 2-AFC, DUO-TRIO, TRIANGLE AND TETRAD; δ=1.0. The Tetrad substantially outperforms the TRIANGLE and DUO-TRIO (X-Number of Consumers, Y-Power).

Same-different (or simple difference test) is another discriminating method where participants are presented with two samples and need to identify whether the samples are the same or different. The same-different method has a discriminating power between n-AFC and triangle test. The same-different method is a useful discriminating method to determine the sensorial difference between two products, especially when the other methods (triangle and duo-trio) are unsuitable; for instance, samples that have strong or lingering flavour, samples that are applied to the skin in half-face tests and samples that have very complex stimuli and are mentally confusing to the panellists. Coffee has intense flavour and possesses complex stimuli. Therefore, the same-different method could be an appropriate method to use for coffee discrimination.

In order to examine the effect of asparaginase treatment on coffee sensory quality, the tetrad method was selected for studying coffee aroma whereas the same-different method was selected for studying coffee taste. As shown in **Figure 4.1**, the advantage of the tetrad approach is that it has more discriminating power for the same number of assessors, compared to other discriminating methods, such as triangle and duo-trio (Ennis & Jesionka, 2011). A forced-choice method is not suitable for this test, because the sensory differences between the treated and control samples were unknown. The disadvantage of the tetrad test is that, as there are four samples in each test, the higher number of samples will easily trigger fatigue and may lead to poor reproducibility, compared to other discriminating methods. To take advantage of the tetrad approach and avoid the disadvantages, the tetrad method was only used to test coffee aroma. For the taste test, as the panellists were drinking the coffee samples, the same-different method was used. This technique required fewest comparisons of samples, thus minimising the possibility of fatigue.

The key objective of the sensory analysis was to determine whether there was a difference between enzyme treated and control coffee samples. Three experiments were carried out with the sensory method development and test completion. Detailed information was provided in **Table 4.1** and **Table 4.2** below. The first experiment was the discrimination test carried out in Embrapa (Brazil) for which three coffees were used (one Arabica, two Robusta). The second experiment was the discrimination test carried out at the University of Reading where the Arabica coffee samples treated in Brazil were used. A second discrimination test was carried out in University of Reading separating between smell test and taste test. Each test will only focus on the odour and flavour respectively. The third experiment included a discrimination test, descriptive test and preference test using a new batch of coffee which was processed in University of Reading. Descriptive test used professional panellists was carried out in University of Reading. The first and second experiment used medium roast coffee while the third experiment used light roast coffee. The results obtained from the different tests will allow determining the potential differences between the treated coffee and control samples.

4.2 Materials & Methods

4.2.1 Materials

4.2.1.1 Coffee

As it showed in **Table 4.1**, two Robusta (Canephora & Tardio) and one Arabica (Mundo Novo) coffees were used at Embrapa. Brazilian Santos coffee sourced from Rave Coffee UK (Cirencester, Gloucestershire) was used for the preliminary consumer test and major profiling and consumer test carried out at Reading University.

Thirty kilogrammes of coffee were treated, roasted and vacuum-packed in Embrapa and were transported to the University of Reading for the following test (2 Arabica: Unspecified Arabica and Arabica Safra; 2 Robusta: Robusta Tardio and Robusta Comum). Samples were stored at 4 °C.

4.2.1.2 Enzymes

Both enzymes (Acrylaway® and PreventASe®) were gifts from the manufacturers. Acrylaway® from Novozymes (3500ASNU/g) and PreventASe® (2500ASPU/g) from DSM were used. Both ASNU and ASPU are defined as the amount of asparaginase that produces one μmol of ammonia per minute under the conditions of the assay (pH = 7±0.005; T = 37±0.5 °C) using Acrylaway® or PreventASe®, respectively.

4.2.1.3 Palate Cleansers

Carr`s table water biscuits and Yeo Valley natural yoghurts (Bristol, UK) were used as palate cleansers for participants to clean their mouth between samples.

Table 4.1 Coffee Samples Used in Sensory Tests

Used at	Variety	Species	Harvest Year	Experiment year	Origin	Enzyme applied	Test carried out	Supplier
Embrapa	Robusta	Canephora	2015	2015	Espirito Santos State	Acrylaway®	Consumers Discrimination Test (Brazil)	Embrapa (Rio de Janeiro, Rio de Janeiro State, Brazil)
	Robusta	Tardio	2015	2015	Rodonia State	Acrylaway®	Consumers Discrimination Test (Brazil)	Embrapa (Rio de Janeiro, Rio de Janeiro State, Brazil)
	Arabica	Mundo Novo	2014	2015	Rio de Janerio	Acrylaway®	Consumers Discrimination Test (Brazil) Consumers Discrimination Test 1(University of Reading)	Embrapa (Rio de Janeiro, Rio de Janeiro State, Brazil)
University of Reading	Arabica	Santos	2016	2016	Mina Gerais State	Acrylaway® PreventASe®	Consumers Discrimination Test 2(University of Reading) Panellists Descriptive Test (University of Reading)	Rave Coffee (Gloucester, Gloucestershire, UK)

Table 4.2 Consumer Tests Set up and experiment design

	Participants	Coffee Samples	Enzymes	Experiment Design
Preliminary Consumer Test	58 (30 males and 28 females)	Light roast Arabica coffee processed in Embrapa	Acrylaway® by Novozyme	One visit Smell session 1 Control vs Dosage1. Control vs Dosage 2, Control vs Dosage 3 Demographic Information Taste Session Control vs Dosage 1, Control vs Dosage 3 Smell Session 2 Dosage 1vs Dosage 2, Dosage 1 vs Dosage 3, Dosage 2 vs Dosage 3
Major Consumer Test	77 (44 males and 33 females)	Light and Medium roast Arabica (Santos) coffee processed at Reading University.	Acrylaway® by Novozyme & PreventASe® by DSM	Two visits First Visit Smell session 1-Meidum Roast Coffee Control- Acrylaway® Dosage 1, Control- Acrylaway® Dosage 2, Acrylaway® Dosage 1-Acrylaway® Dosage 2 Demographic Information Smell session 2- Medium Roast Coffee Control- PreventASe® Dosage 1, Control- PreventASe® Dosage 2 PreventASe® Dosage 1- PreventASe® Dosage 2 Preference test Liking test for light roast coffee Second Visit Taste Test-Medium Roast Coffee Control- Acrylaway® Dosage 1, Control-Acrylaway® Dosage 2 Process Control-Acrylaway® Dosage1, Process Control-Acrylaway® Dosage 2 Control- PreventASe® Dosage 1, Control- PreventASe® Dosage 2 Process Control- PreventASe® Dosage1, Process Control- PreventASe® Dosage 2 Preference test Liking test for medium roast coffee

Table 4.3 Coffee samples used in the profiling test (n=3).

	Control	Process Control	Acrylaway® Dosage 1	Acrylaway® Dosage 2	PreventASe® Dosage 1	PreventASe® Dosage 2
Light	3 (CL)	3 (PCL)	3 (AD1L)	3 (AD2L)	3 (PD1L)	3 (PD2L)
Medium	3 (CM)	3 (PCM)	3 (AD1M)	3 (AD2M)	3 (PD1M)	3 (PD2M)

Annotation: C-Control, PC-Process control, L-Light Roast, M-Medium Roast, A-Acrylaway®, P-PreventASe®, D1 & D2- Dosage 1 & Dosage 2. light roast 230℃-12mins & Medium Roast 240℃-14mins. Dosage 1-2000 Unit, Dosage 2-4000 Unit.

4.2.2 Methods

4.2.2.1. Treatment of Coffee Samples

All samples were prepared as follows:150 g green coffee beans were firstly steamed for 45 min (Tefal 8 in 1 cooker). Then the steamed the coffee was transferred to a Stuart® heated stirrer to carry out the enzyme treatment. The coffee was treated either by Acrylaway® or PreventASe® (Dosage 1-2000 U, Dosage 2-4000U, Dosage 3-6000U) for 45 minutes under 60 °C. Then the coffee was dried in the Gallenkamp® drier at 60 °C for 4-6 hours to remove the moisture content (from around 200g to 150g). After drying, coffee samples were roasted (Gene Café® coffee roaster) to three different roasting conditions (Low- 12 min at 230 °C, Medium- 14 min at 240 °C, High- 17 min at 250 °C). The roasting temperature and time combination were recommended by the roasting machine supplier. Also, these roasting conditions were selected considering it went beyond the second crack and all laid at the very end of the acrylamide generation slope to avoid the potential acrylamide increase risk of under-roast coffee (Lantz et al., 2006). Then coffee beans were stored in 300-g plastic vacuum sealed bags in a cold room (4°C) one week before the test. Control samples were green coffee beans roasted to the above set levels. Processed control samples had undergone the same procedure as the treated samples without the addition of enzyme in the soaking step.

4.2.2.2 Preparation of coffee brews

Treated coffee beans (50 g) were ground to 0.1 mm particle size in an Andrew James coffee grinder half an hour before sensory tests. Brewed coffees were prepared in a SCAE (Special Coffee Association of Europe) recommended ratio of 31.2 g coffee/500 ml water at 100 °C using a plunger cafetière (SCAE, 2013). After brewing for five minutes, the filtered coffee was poured into a 1-L Thermo flask. The testing sessions lasted three hours during which the temperature of the coffee brew was checked every 15 minutes. A drop of less than 5 °C was observed during that time. Before each serving, the container was vigorously shaken for 10 s before the pouring step.

4.2.2.3 Consumer Test Method

Two consumer tests were carried out to find out whether there was a significant difference between control and treated coffee beans. The first consumer test was carried out in Embrapa and used both Robusta coffee and Arabica coffee, both of which had been enzyme-treated in Embrapa. Results indicated that the Arabica coffee was sweeter and less astringent than Robusta. Consequently, differences caused by the enzyme treatment were more easily noticed. Hence, the following tests in University of Reading only used Arabica coffee. The second consumer test carried out in University of Reading used Brazilian Santos coffee sourced from Rave Coffee UK.

4.2.2.3.1 Embrapa-Brazil

In Embrapa, the panellists were invited to give a summary of the flavour and taste characteristics of the treated and control coffee samples. The coffee was prepared and served following the protocol described above. The 15 panellists were trained at Embrapa and had more than six months experience in coffee tasting. Also, all the panellists used a particular cupping method in the coffee test where coffee was used to rinse the entire palate and then spat out. Because the coffee samples were not swallowed, it was considered that the after taste would pose less effect on discriminating coffee between different samples. The panellists were presented with Acrylaway®-treated Arabica and Robusta coffee of three roasting conditions (low, medium and high) and three dosages (2000, 4000 and 6000ASNU/kg).

Following the feedback from the panellists, a Difference-From-Control test with similarity index (7-point scale, 1-identical, 7-extremely different) was used in the consumer test (Civille & Carr, 2015). One Arabica coffee and two Robusta light roast Acrylaway® treated coffee were presented to the 50 consumers in the dedicated sensory booths in Embrapa with controlled temperature (25°C) and relative humidity (50%).

4.2.2.3.2 University of Reading-UK

The second series of consumer tests was carried out at the University of Reading and it was divided into two parts. The first test (preliminary test) used light roast samples that had been prepared in Embrapa. The second test (major test) used light and medium roast samples that had been prepared at the University of Reading using a preference test. The preliminary test not only had the purpose of find out whether there was significant difference between control and treated coffee, but also to validate the experiment design. Although the preliminary test was performed to discover any noticeable difference between the control and treated samples,

the preference test was crucial in demonstrating whether there was any unpleasant flavour in the enzyme-treated samples.

As shown in **Table 4.2**, 58 untrained consumers (30 males and 28 females) recruited from in and around of University of Reading participated in the preliminary test. The test was carried out in the dedicated sensory booth in the Sensory Science Centre at University of Reading with controlled temperature (25°C) and relative humidity (50%). Like the test carried out in Embrapa, light roast Acrylaway®-treated Arabica samples, which were processed in Brazil, were used in the test, where a same-different method was used for the taste test and tetrad method was used for the odour test.

As shown in **Table 4.2**, for the major consumer test, 77 participants (44 men and 33 women) During the first visit, two smell tests were carried out using tetrad method and preference test for light roast coffee. During the second visit, same test was carried out on medium roast Brazilian Santos coffee. All consumers signed a consent form before the test. All consumers that participated in the test were aged 18-65, non-smokers and not lactose intolerant (yoghurt was used as a palate cleanser). However, unlike in Embrapa, consumers in the University of Reading were asked to swallow the coffee to experience the whole flavour profile of the sample.

4.2.2.4 Descriptive Analysis

Sets of sensory descriptors for coffee were established using an expert panel of eleven sensory assessors (see **Table 4.4** for definitions of terms used). Panellists were selected and trained by ISO standards for sensory analysis (ISO 8586:2012) and are subject to performance monitoring (ISO 11132:2012). All panellists had a minimum of 6 months experience in sensory evaluation.

The assessment included sensory vocabulary development, subject training and subjects' reproducibility. For the vocabulary development, 12 different samples blind coded (**Table 4.3**) were presented randomly to the panellists for a one-hour session on 4 consecutive days. Brazilian Santos coffee processed with two dosages of enzyme, two enzyme brands and two levels of roasting was used. From the feedback from the panellists, the high roast level burnt

the coffee beans, which led to a roasted coffee with much less flavour and aroma characteristics to assess. Therefore, the high roast coffee was excluded from the descriptive tests.

The medium enzyme dosage had already caused a great reduction (75%-90%) in acrylamide formation and there was no significant increase in the medium to high dosages (85%-90%), therefore, it was decided that samples treated with the high dosage of enzyme should be excluded from the descriptive test. The samples were presented in a random, blind coded fashion. In the vocabulary decision stage, all samples (20 ml each panellist) were presented each day in a 50-ml plastic disposable cup. During the test session, 12 samples were randomly separated into 3 sets (4 samples each set) and presented twice. It was six test days, two replicates, each one lasted three days with a set randomly tested each day. At the beginning of the vocabulary session, panellists were encouraged to discuss which attributes were associated with various aspects of coffee, including taste, aroma and aftertaste. As shown in **Figure 4.2**, the coffee flavour wheel (SCAA, 2016) was also used as an aid to develop the list of attributes that were sensed by the panellists.

Figure 4.2 Coffee Flavour Wheel (SCAA, 2016).

A number of reference foodstuffs were provided to the panellists, in order to make sure that the panellists were using the vocabulary in a constant manner. For instance, in the first vocabulary sessions, one flavour/aroma was described by half of the panel as molasses and by the other half as treacle. Then, treacle and molasses were provided in the next vocabulary session; after comparing the two products, panels agreed that the flavour/aroma was closer to molasses than treacle. Hence, molasses was the term used for the following sessions.

Reference samples were also served at the beginning of each session for every vocabulary and test session carried out. As roast level had the most dominant effect on coffee flavour profile, the sample that had been roasted to the medium level for all samples was recommended as the reference sample. Colour results, as previously discussed in Chapter 2, had showed that the medium roast process control sample had a roast colour value laid around the medium of all samples presented. This means that the medium roast process control samples would have a flavour profile where every attribute will sit around the middle of the line scale (from none to extreme). At the end of the vocabulary session, reference samples were tested to generate a standard score. Then, in each test session, before the test was carried out, the reference sample was served to each panellist together with the standard score that they had previously recorded. Therefore, by taking a sip of the reference sample and review the standard score, they could compare the sample to the reference and score each attribute accordingly. This reference sample served as a benchmark and helped panellists to recall their memory and the standard for scores.

After six sessions, the panellists reached a consensus for the final vocabulary. The final vocabulary was decided as indicated in **Table 4.4** with attributes in basic tastes, odour, flavour and aftertaste. The experimental design started with a training session that lasted for four days to reach consensus on the vocabulary, followed by a test session which lasted six days to test all samples twice. It included two replicates and each test session lasted half an hour including four samples (3 sessions tested all 12 samples; all samples were random three-digit coded and randomly presented). Before each day's test, the reference samples and the average scores were provided to the panellists to recall the memory and to justify the scoring standard for samples. After the reference standard sample, the brewed samples were presented, and the panellists scored the intensity of each attribute using Compusense software (version 5.2; Guelph, ON, Canada). The intensity on the software was scored on anchored unstructured line scales (15 cm,

scaled 0-100 points from zero to the extreme). After two replicates by all 11 panellists (n = 22), all descriptive analysis data were collected and stored. Evaluation sessions were carried out under simulated daylight conditions in an air-conditioned room (~22 °C), in isolated sensory booths within the Sensory Science Centre at the Department of Food & Nutritional Sciences, University of Reading, UK. Panellists were provided with water (room temperature) and natural yoghurt (Yeo Valley Farms (Production) Ltd., Bristol, UK) for palate cleansing between samples. Each day test was limited to 4 coffee samples to avoid fatigue of the panellists` palate/trigeminal nerves. A one-minute time delay was also set in the Compusense programme between the finishing of one sample and the presenting of the next.

4.2.2.5 Ethics Approval

In Embrapa (Brazil), ethics for the tests were approved by the head of sensory division and ethics committee. At the University of Reading, two ethics proposals were submitted to the Department Ethics Committee and approved (Appendix III-Study Number 41/05 for first discrimination test and Appendix IV-Study Number 20/16 for tests including second discrimination test, descriptive test and preference test).

4.2.2.6 Statistical Analysis

To analyse the sensory profiling data, two-way analysis of variance (ANOVA; with accessions and assessors as treatment effects, and these main effects tested against their interaction) was carried out using Senpaq (Qi Statistics Ltd., Reading, UK). ANOVA was conducted using a 95% confidence interval and a tolerance of 0.01%. A post-hoc Tukey's HSD test was used for multiple pairwise comparisons. This was chosen for the higher level of stringency than other pairwise comparison tests, such as Fisher's LSD Test.

Table 4.4 Agreed definition for the final attributes

Attribute	Agreed definition
Basic Tastes	
Astringency	Degree to which samples induced drying and/or the sensation of shrinkage of the tongue and soft palate
Sweet	A fundamental taste factor of which sucrose is typical
Bitter	A fundamental taste factor associated with a caffeine solution
Salty	A basic taste factor of which sodium chloride is typical
Odour	
Floral	A sweet, light, slightly fragrant aromatic associated with fresh flowers
Black Tea	A somewhat brown, musty, dried plant and dried bark aromatic associated with the oxidisation of tea leaves
Fruity/Blackcurrant	A sweet, floral, aromatic blend of a variety of ripe fruits
Elderflower/off note	An off aromatic associated with elderflower
Sour	An aromatic, acidic sensation associated with vinegar
Green Vegetable	An aromatic characteristic of fresh, plant-based material
Woody/Dusty	The sweet, brown, musty, dark aromatic associated with a bark of a tree
Musty/Wet Paper/Earthy	The somewhat sweet, heavy aromatic associated with decaying vegetation and damp, black soil
Medicinal	A clean, sterile aromatic characteristic of antiseptic-like products such as Band-Aids, alcohol and iodine
Petroleum	A specific chemical aromatic associated with crude oil and its refined products, which have heavy oil characteristics
Brown Roast	A rich, full, round, aromatic impression always characterised as some degree of darkness, generally associated with attributes such as toasted, nutty, roasted and sweet
Smoky/Ashy	An acute, pungent aromatic that is a product of the combustion of wood, leaves or a non-natural product
Nutty	A slightly sweet, brown, woody, oily, musty, astringent and bitter aromatic commonly associated with nuts, seeds, beans and grains

Cocoa	A brown, sweet, dusty, musty, often bitter aromatic associated with cocoa bean, powdered cocoa and chocolate bars
Brown Sugar-Molasses	A rich, full, round, sweet aromatic impression characterised by some degree of darkness
Flavour	
Floral	A sweet, light, slightly fragrant taste associated with fresh flowers
Black Tea	A somewhat brown, musty, dried plant and dried bark taste associated with the oxidisation of tea leaves
Fruity/Blackcurrant	A sweet, floral, taste blend of a variety of ripe fruits
Elderflower/off note	An off-taste associated with Elderflower
Sour	An acidic taste sensation associated with vinegar
Green Vegetable	An aromatic characteristic of fresh, plant-based material
Woody/Dusty	The sweet, brown, musty, dark taste associated with a bark of a tree
Musty/Wet Paper/Earthy	The somewhat sweet, heavy taste associated with decaying vegetation and damp, black soil
Medicinal	A clean, sterile taste characteristic of antiseptic-like products such as Band-Aids, alcohol and iodine
Petroleum	A specific chemical taste associated with crude oil and its refined products, which have heavy oil characteristics
Brown Roast	A rich, full, round, taste impression always characterised as some degree of darkness, generally associated with attributes such as toasted, nutty, roasted and sweet
Smoky/Ashy	An acute, pungent taste that is a product of the combustion of wood, leaves or a non-natural product
Nutty	A slightly sweet, brown, woody, oily, musty, astringent and bitter taste commonly associated with nuts, seeds, beans and grains
Cocoa	A brown, sweet, dusty, musty, often bitter taste associated with cocoa bean, powdered cocoa and chocolate bars
Brown Sugar-Molasses	A rich, full, round, sweet taste impression characterised by some degree of darkness

After effects	
Bitter	A persistence of bitter taste after swallowing coffee samples
Sweet	A persistence of pleasant, sugary taste
Drying	A persistence feeling lack of moisture
Smoky	An acute, pungent aromatic that is a product of the combustion of wood, leaves or a non-natural product
Cocoa	A persistence of sweet and bitter taste associated with cocoa bean

4.3 Results

4.3.1 Sensory tests in Brazil

In Embrapa, enzyme treated Arabica coffee were presented to the panellists to seek for their opinions. After tasting and smelling the samples, the panellists discussed the samples from five different aspects: aroma, appearance, flavour and odour, basic taste, mouth sensation. Fifteen panellists reached a consensus that high and medium roast Arabica coffee was burnt and lacked sufficient characteristics to evaluate. For the light roast coffee, after visual inspection, panellists agreed that despite the colour difference in coffee powders, the colour and taste difference in brewed coffee was difficult to find.

When Robusta coffee was presented to the panel to evaluate the differences between the control and enzyme-treated coffee, panellists' feedback showed that differences in taste and colour still existed. However, panellists reached a consensus that this difference was less pronounced than for the Arabica coffee. As a result, and to quantify the difference and determine whether it was significant or not, consumer discrimination tests were carried out for Arabica and Robusta light roast coffees.

As shown in **Table 4.5** from the data collected from the 50 consumers, significant differences were found in Arabica coffee (p=0.029) between control and 2000ASNU/kg Acrylaway®

treated sample. However, no significant differences were detected for the two Robusta coffees ($p^1 = 0.8779$, $p^2 = 0.9889$). Combining the feedback from panellists and data from consumers, the difference between treated and control samples in Arabica coffee was easier to be detected than for the Robusta coffee. In the experiment, consumers reported that they were more familiar with the Arabica coffee taste than the Robusta coffee taste. Therefore, consumers` judgement may have been influenced by the fact that Arabica coffee is the main variety consumed in Brazil. As a result, consumers are most likely to notice the difference in Arabica coffee rather than the unfamiliar Robusta coffee (Crocitti & Vallance, 2012).

Arabica coffee accounts for three-quarters of worldwide cultivation and consumption. Robusta coffee contains 40-50% more caffeine than Arabica coffee, and is considered to be much bitter and with less characteristic flavour and taste (Flament & Bessière-Thomas, 2002). Hence, Arabica variety was better than the Robusta coffee for discrimination test for the familiarity reason.

Table 4.5 Significance for the Embrapa consumer Difference-From-Control test

Arabica Mundo Novo					
Level	Name	Size	Mean	Std. Dev.	P value (Variety)
1	D1	54	5.04	1.91	
2	D2	54	4.48	1.86	0.0292
3	D3	54	4.43	1.97	
Robusta Canephora					
Level	Name	Size	Mean	Std. Dev.	P value (Variety)
1	D1	50	3.64	1.95	
2	D2	50	3.7	1.95	0.9889
3	D3	50	3.64	2.13	
Robusta Tardio					
Level	Name	Size	Mean	Std. Dev.	P value (Variety)
1	D1	53	4.19	1.88	
2	D2	53	4	2.04	0.8779
3	D3	53	4.23	1.83	

D1, 2, 3- Dosage 1-2000ASNU/kg, Dosage 2-4000ASNU/kg, Dosage 3-6000ASNU/kg. light roast 230℃-12mins. Confidence level set at 95% while the corresponding significance level was 0.05

4.3.2 Sensory tests at the University of Reading

4.3.2.1 Preliminary Consumer Test

The consumer test in Brazil showed significant differences between the control and enzyme-treated samples for the light-roast Arabica coffee. It was decided to carry out the same evaluation with consumers in the UK because the UK consumers may provide different results. As stated by the coffee supplier staff in Embrapa, the Arabica coffee that was tested in Embrapa and the Arabica Safra tested at the University of Reading are the same type of coffee but planted in different Brazilian states with relatively small differences regarding bitter flavour and taste. The two coffees were of the same species but harvested at different time.

As shown in **Table 4.2**, the tetrad method was used in the two sessions where the odours of the coffees were compared. Session one compared the control sample to samples with different Acrylaway® dosages. Session two compared within the different dosages samples. As it can be seen in **Table 4.6,** for the smell tests, significant differences were only found in the higher dosages group compared to the control samples ($P^{\text{control vs dosage2}} = 0.02$, $P^{\text{control vs dosage 3}} = 0.01$). whereas significant differences were found between all the treated samples ($P^{\text{dosage 1 vs dosage 2}} = 0.01$, $P^{\text{dosage 1 vs dosage 3}} = 0.01$, $P^{\text{dosage 2 vs dosage 3}} = 0.01$). In the taste tests, where control samples were compared with enzyme treated samples (dosage 1 & 3) using the same-different method, significant differences were also found between the treated groups and control groups ($P^{\text{control vs dosage 1}} = 0.01$ and $P^{\text{control vs dosage 3}} = 0.01$) samples.

Table 4.6 Statistical analysis results for the preliminary consumer test (smell and taste) at the University of Reading on light roast Brazilian Arabica Safra samples in which 50 consumers participated.

Smell Test	
Groups	P value
Control-Dosage 1	0.06
Control-Dosage 2	0.02
Control-Dosage 3	0.01
Dosage 1-Dosage 2	0.01
Dosage 1-Dosage 3	0.01
Dosage 2-Dosage 3	0.01
Taste Test	
Groups	P value
Control-Dosage 1	0.01
Control-Dosage 3	0.01

P value for each group were calculated by V-power programme (Virginia, USA). D1, 2, 3- Dosage 1-2000ASNU/kg, Dosage 2-4000ASNU/kg, Dosage 3-6000ASNU/kg. light roast 230℃ -12mins. Confidence level set at 95% while the corresponding significance level was 0.05

4.3.2.2 Major Profiling Test

For this test, as it can be seen in **Table 4.3**, twelve samples subject to two enzymes, Acrylaway® and PreventASe®, at two enzyme dosages (Dosage 1 and Dosage 2) and two roasting levels (Light and Medium) were used. Process control sample refers to the sample that have been process like the enzyme treated samples but with no enzyme in the soaking step. This control is added to illustrate the impact of the enzyme treatment on the sensory properties of coffee. As aforementioned in the methods and material section, 39 attributes were picked by the panellists (4 in basic tastes, 15 in odour, 15 in flavour and 5 in aftertaste). The results will be presented by the different roast conditions and different enzyme conditions to show the influences by the various factors.

For all 39 attributes (**Table 4.4 & Table 4.7**), 12 attributes in light roast coffee were significant different while in medium roast coffee 17 attributes were significant different (**Figure 4.3-4.6**). For all these significant different attributes, 11 attributes had significant differences in both light and medium roast coffee. Brown sugar-Molasses (Odour) was significantly different only in light roast samples while Sour (Odour), Cocoa (Odour), Woody-dusty (Flavour), Petroleum (Flavour), Brown Sugar-Molasses (Flavour), Cocoa (After Effects) were significantly different only in medium roast samples. For the 11 attributes that were significant different in both roast conditions, 3 were in basic tastes (Bitter, Metallic and Astringent), 2 in odours (Brown Roast, Smoky/Ashy), 3 in flavours (Sour, Brown Roast, Smoky/Ashy) and 3 in after effects (Bitter, Drying, Smoking).

4.3.2.2.1 Basic Tastes

The light roast coffee was less bitter, metallic and astringent compared to medium roast coffee. For each roasting condition, high dosage enzyme treated coffee scored greater than the small dosage enzyme treated coffee. Comparing the two enzymes, in the light roast condition, Acrylaway® treated samples had higher marks on these attributes than the PreventASe® treated samples at the same dosage. However, the PreventASe® treated samples scored greater than the Acrylaway® treated samples in the medium roast level samples.

For the panellist's performance, as the medium roast coffee had stronger bitter, metallic and astringent taste, Generally, panellists` performance for the medium roast were worse than the light roast samples. Especially the p-value of the medium roast bitter attribute was below 0.05; indicating that the assessor had a different trend on scoring this attribute. This result could be therefore associated to the genetic differences amongst assessors regarding bitter taste sensitivity (Bachmanov et al., 2014).

4.3.2.2.2 Odour and Flavour

Two attributes in odour (Brown Roast, Smoky/Ashy) and three attributes in flavour (Sour, Brown Roast, Smoky/Ashy) were significantly different at both light and medium roast conditions. Both attributes (Brown Roast, Smoky/Ashy) had p-value less than 0.001 for both samples and assessors in light and medium roast conditions. The values that panellists scored fluctuated. Though the medium roast group have higher values on these two attributes, no clear trends could be summarised regarding roast conditions. Regarding the enzyme, Acrylaway®

treated samples scored slightly higher in the light control group. However, in the medium roast level, Acrylaway® treated group scored close to the value for the PreventASe® treated group. For these four attributes (Brown roast-odour, Brown roast-flavour, Smoky/Ashy-odour, Smoky/Ashy-flavour), the potential error may occur from the vocabulary stage, these two words (Brown Roast, Smoky/Ashy) were not acknowledged and defined by every panellist. Besides these four attributes, there was another sour flavour attribute that had a significant difference. In theory, as roast level increases, the acidic compounds in coffee will be destroyed, such as quinic acid, citric acid, chlorogenic acid and phosphoric acid (Flament & Bessière-Thomas, 2002; Illy & Viani, 2005; Luigi Poisson, 2017). Most of the acids had melting points around 150°C, so medium to dark roast would diminished the acids in coffee. Consequently, dark roast coffee had a lower acidic note compared to the light roast coffee. Though the sour score of the light and medium roast coffees are fairly close, it is reasonable considering to its low acidic note. As Brazilian Santos coffee is famous for its low acidic profile (pH=6.2), so low and medium roast pH variance might be too small for the panellists to notice. This difference, less acid and less marked aroma of Brazilian coffee compared to other Central and South America coffee has also been studied by other researchers (Illy & Viani, 2005; Mayer et al., 2000).

4.3.2.2.3 After effect

Three after effects attributes (bitter, drying and smoky) had significant differences in both light and medium roast sample. For the bitter after-effects, panellists did not reach a uniform standard, and therefore no trend could be summarised from the data regarding the roast level and enzyme dosage. The drying and smoky attributes showed an opposite trend in the light and medium roast coffee in terms of the enzyme processing. As it can be seen in **Table 4.7**, in light roast coffee, control sample had the lowest score compared to the treated samples. For treated samples, Acrylaway® treated sample gave higher values than the PreventASe® treated samples. However, the opposite could be observed in the medium roast coffee samples. The control sample had the highest value, and PreventASe® treated samples scored higher than the Acrylaway® samples.

Besides the attributes that exhibit significant differences in both light and medium roast samples, one attribute (Brown sugar-Molasses in odour) showed significant difference only in

light roast coffee and six attributes (Sour and Cocoa in odour/ Woody-dusty, petroleum and Brown sugar-Molasses in flavour/ Cocoa in aftertaste) showed significant differences only in medium roast coffee. Light roast samples, had much higher scores for Brown Sugar-Molasses in odour for the enzyme treated samples compared to the control samples. For the medium roast coffee samples, odour attributes, flavour attributes and after effects attributes exhibited the same pattern, with the control samples scoring higher than the treated sample, and PreventASe® treated samples having a higher rating than the Acrylaway® sample.

Several conclusions could be summarised from the data analysis. Firstly, between the process control and control groups, significant differences were observed for the light roast level. However, for the medium roast samples, the difference between these two groups was much smaller. Secondly, Acrylaway® treated samples scored slightly higher in the light roast group. However, in the medium roast level, Acrylaway® treated group scored close to the value of the PreventASe® treated group. Thirdly, certain attributes reflect the difficulty in the reproducibility of coffee data. Even with a variety of measurements in place to maintain the consistency, the strong flavour and odour still made it difficult to assess. However, despite the fatigue issue, data analysis still concluded that the significant difference between enzyme-treated coffee samples indeed exists. The different enzyme and dosage would result in different sensory profiles even with the same roast condition and enzyme treatment process.

4.3.2.3Major Consumer Test

Following the panellists profiling test, a consumer test using medium roast coffee was conducted. Admittedly, there were aroma and flavour differences caused by enzyme treatment. However, whether the significant differences made coffee aroma and flavour unacceptable to consumers were still unknown. So, the preference test for both light and medium roast coffee was carried out.

Table 4.7 Average values of sensory traits of light roast enzyme treated coffee samples accessions rated by trained panel assessors

Sensory Trait	Accession						Significance (P values)	
	CL	PCL	AD1L	AD2L	PD1L	PD2L	Sample	Sample*Assessor
Basic tastes								
Sweet	18.6	17.1	14.9	14.7	16.3	16.4	0.7559	0.5472
Bitter	15.2^{c}	21.7^{ab}	18.2^{bc}	23.9^{a}	16.8^{c}	18.4^{bc}	0.0013*	0.7963
Metallic	2.5^{c}	5.4^{ab}	3.8^{bc}	6.7^{a}	4.0^{abc}	2.7^{bc}	0.0303*	0.7963
Astringent	8.9^{b}	12.4^{b}	10.6^{b}	17.3^{a}	10.0^{b}	9.2^{b}	0.0008*	0.3013
Odour								
Floral	1.7^{abc}	3.4^{ab}	1.8^{abc}	0.4^{c}	3.7^{a}	1.2^{bc}	0.0625	0.3019
Black Tea	3.1^{b}	4.5^{b}	5.5^{ab}	7.6^{a}	4.2^{b}	4.4^{b}	0.0515	0.0559
Fruity/Blackcurrant	1.7	3.3	1.9	3.0	1.1	1.1	0.2220	0.0101*
Elderflower/off note	1.2	0.6	2.7	1.5	2.0	0.7	0.3661	0.7958
Sour	3.7	4.2	4.9	4.9	5.1	4.9	0.6463	0.9482
Green Vegetable	4.1	0.8	1.8	0.8	1.8	1.2	0.3848	0.0783
Woody/Dusty	8.7	11.3	13.2	12.5	11.9	12.2	0.5682	0.0017*
Musty/Wet Paper/Earthy	6.1^{ab}	6.9^{ab}	8.4^{a}	4.2^{b}	3.6^{b}	3.8^{b}	0.0513	0.8509
Medicinal	0.0	0.0	0.4	0.1	0.0	0.6	0.2902	0.7333
Petroleum	0.0	1.2	2.0	1.8	1.1	1.0	0.5055	0.5983
Brown Roast	23.2^{bc}	27.2^{ab}	24.5^{bc}	29.5^{a}	22.9^{bc}	21.8^{c}	0.0028*	0.9747
Smoky/Ashy	5.2^{c}	10.4^{ab}	10.2^{ab}	13.4^{a}	8.6^{bc}	8.3^{bc}	0.0079*	0.8022
Nutty	10.8^{a}	8.3^{ab}	5.7^{b}	4.7^{b}	6.1^{b}	5.4^{b}	0.1050	0.9233
Cocoa	10.1	10.2	7.1	10.4	9.9	7.2	0.1506	0.9944
Brown Sugar-Molasses	6.5^{c}	12.6^{a}	6.9^{bc}	10.3^{ab}	9.7^{abc}	8.4^{bc}	0.0065*	0.5737

Table 4.7 Average values of sensory traits of light roast enzyme treated coffee samples accessions rated by trained panel assessors

Sensory Trait	Accession						Significance (P values)	
	CL	PCL	AD1L	AD2L	PD1L	PD2L	Sample	Sample*Assessor
Flavour								
Floral	2.1	2.7	1.0	1.3	3.2	0.9	0.3558	0.0012*
Black Tea	2.8^{b}	3.3^{b}	4.4^{ab}	6.6^{a}	5.4^{ab}	3.2	0.0515	0.3925
Fruity/Blackcurrant	1.5	2.8	2.0	2.9	1.4	1.8	0.4854	0.1279
Elderflower/off note	1.2^{ab}	0.4^{b}	0.5^{b}	0.7^{b}	1.9^{a}	0.6	0.0531	0.9145
Sour	4.3^{d}	11.0^{a}	7.8^{bc}	9.1^{ab}	8.1^{bc}	5.7	0.0003*	0.6020
Green Vegetable	3.1	0.4	2.1	0.9	2.4	1.5	0.5995	0.0485*
Woody/Dusty	9.5^{b}	13.8^{a}	14.4^{a}	12.4^{ab}	10.9^{ab}	13.1^{ab}	0.1151	0.6320
Musty/Wet Paper/Earthy	6.4	7.7	7.8	4.5	4.5	4.9	0.2834	0.8776
Medicinal	0.4	0.0	0.0	1.0	1.3	0.9	0.4267	0.7397
Petroleum	0.0^{a}	2.1^{ab}	2.4^{a}	1.7^{abc}	0.5^{bc}	1.1^{abc}	0.0678	0.8859
Brown Roast	21.2^{b}	29.2^{a}	23.3^{b}	29.6^{a}	20.7^{b}	23.8^{b}	0.0002*	0.8909
Smoky/Ashy	5.6^{c}	12.6^{a}	11.4^{ab}	13.5^{a}	8.9^{bc}	8.5^{bc}	0.0003*	0.9507
Nutty	10.6^{a}	8.8^{ab}	5.6^{b}	5.3^{b}	6.8^{ab}	5.8^{b}	0.1179	0.5729
Cocoa	9.1	7.9	8.5	9.4	9.5	7.2	0.8018	0.0464*
Brown Sugar-Molasses	6.5^{b}	11.7^{a}	8.2^{ab}	11.2^{a}	8.6^{ab}	9.5	0.1517	0.6887
Aftereffects								
Sweet	14.5	14.1	12.8	15.7	13.7	14.9	0.6469	0.9474
Bitter	13.6^{bc}	18.1^{ab}	16.2^{abc}	19.1^{a}	12.2^{c}	14.1^{bc}	0.0158*	0.0734
Drying	11.1^{b}	17.8^{a}	12.2^{b}	14.5^{ab}	12.6^{b}	13.0^{b}	0.0084*	0.7108
Smoky	3.7^{c}	10.6^{a}	9.7^{ab}	11.4^{a}	8.0^{ab}	6.0^{bc}	0.0010*	0.5750
Cocoa	8.2^{a}	8.3^{a}	7.9^{a}	9.5^{a}	6.4^{a}	6.2^{a}	0.3535	0.2251

Table 4.7-cont Average values of sensory traits of medium roast enzyme treated coffee samples accessions rated by trained panel assessors

Sensory Trait	Accession						Significance (P values)	
	CM	PCM	AD1M	AD2M	PD1M	PD2M	Sample	Sample*Assessor
Basic tastes								
Sweet	16.1	16.2	18.6	15.1	17.3	15.9	0.5798	0.5311
Bitter	30.0^{a}	24.0^{bc}	13.8^{d}	24.4^{abc}	19.0^{cd}	25.9^{ab}	<.0001*	0.0483*
Metallic	7.8^{a}	8.2^{a}	3.3^{c}	5.3^{abc}	4.5^{bc}	6.8^{ab}	0.0164*	0.5588
Astringent	18.2^{a}	16.0^{ab}	7.3^{c}	17.5^{a}	11.9^{b}	17.3^{a}	<.0001*	0.1359
Odour								
Floral	0.8	0.6	0.5	1.2	2.1	1.0	0.4721	0.8731
Black Tea	8.6^{ab}	9.0^{a}	4.7^{b}	7.0^{ab}	5.8^{ab}	7.4^{ab}	0.3069	0.2275
Fruity/Blackcurrant	2.1^{ab}	2.5^{ab}	1.1^{b}	4.3^{a}	1.9^{ab}	2.5^{ab}	0.3951	0.7880
Elderflower/off note	0.6	1.3	1.2	1.0	0.2	1.6	0.3985	0.8764
Sour	7.1^{a}	5.4^{ab}	3.7^{b}	7.2^{a}	5.0^{ab}	6.8^{a}	0.0119*	0.9453
Green Vegetable	0.3^{b}	0.3^{b}	1.4^{ab}	0.0^{b}	2.4^{a}	0.6^{ab}	0.1056	0.5688
Woody/Dusty	14.3^{a}	13.2^{ab}	10.4^{b}	13.4^{ab}	12.4^{ab}	15.5^{a}	0.1719	0.9147
Musty/Wet Paper/Earthy	8.4^{a}	6.9^{ab}	7.0^{ab}	4.5^{ab}	3.3^{b}	8.6^{a}	0.0538	0.1582
Medicinal	0.0	0.7	0.6	0.6	0.0	0.0	0.5512	0.0975
Petroleum	2.6	2.2	1.2	1.2	0.9	3.1	0.4319	0.7065
Brown Roast	42.1^{a}	34.6^{b}	18.3^{d}	36.9^{ab}	28.4^{c}	37.3^{ab}	<.0001*	0.3936
Smoky/Ashy	17.1^{a}	11.0^{b}	6.9^{b}	17.4^{a}	10.5^{b}	17.2^{a}	0.0001*	0.3570
Nutty	4.3^{b}	2.7^{b}	9.7^{a}	5.3^{ab}	5.8^{ab}	3.3^{b}	0.0517	0.0159*
Cocoa	12.9^{a}	10.1^{a}	6.4^{b}	11.8^{a}	10.0^{ab}	12.2^{a}	0.0078*	0.9189
Brown Sugar-Molasses	13.7^{a}	9.8^{ab}	6.8^{b}	11.9^{a}	10.3^{ab}	10.2^{ab}	0.0571	0.0113*

Table 4.7-cont Average values of sensory traits of medium roast enzyme treated coffee samples accessions rated by trained panel assessors

Sensory Trait	Accession						Significance (P values)	
	CM	PCM	AD1M	AD2M	PD1M	PD2M	Sample	Sample*Assessor
Flavour								
Floral	0.3	0.6	1.7	0.8	2.1	0.5	0.3688	0.5527
Black Tea	8.0	8.2	5.8	4.5	4.5	6.2	0.4479	0.5135
Fruity/Blackcurrant	1.9^{b}	1.7^{b}	2.4^{b}	4.6^{a}	2.1^{b}	2.6^{ab}	0.0915	0.7271
Elderflower/off note	2.2	1.3	0.8	1.3	0.4	1.3	0.4669	0.5217
Sour	12.7^{a}	11.7^{ab}	4.8^{c}	11.5^{ab}	8.7^{b}	9.3^{ab}	0.0008*	0.1689
Green Vegetable	0.4	0.0	2.9	0.7	2.3	0.5	0.2530	0.0003*
Woody/Dusty	16.5^{a}	13.8^{ab}	9.3^{c}	14.3^{ab}	12.2^{bc}	15.5^{ab}	0.0044*	0.8408
Musty/Wet Paper/Earthy	7.0	6.9	5.6	5.4	5.6	8.3	0.5925	0.0568
Medicinal	0.0^{b}	1.6^{a}	1.1^{ab}	0.0^{b}	0.7^{ab}	0.0^{b}	0.0862	0.0193*
Petroleum	5.4^{a}	3.9^{ab}	0.7^{c}	1.4^{bc}	1.4^{bc}	3.4^{abc}	0.0099*	0.4155
Brown Roast	41.7^{a}	37.0^{ab}	17.1^{d}	35.3^{b}	27.5^{c}	35.6^{ab}	<.0001*	0.0437*
Smoky/Ashy	18.3^{a}	17.0^{a}	5.5^{b}	16.8^{a}	11.0^{b}	17.0^{a}	0.0001*	0.2525
Nutty	4.6^{ab}	3.2^{b}	8.7^{a}	5.8^{ab}	5.1^{ab}	4.8^{ab}	0.1742	0.0170*
Cocoa	13.6^{a}	12.7^{a}	7.3^{b}	12.4^{ab}	10.8^{ab}	11.5^{ab}	0.2577	0.1506
Brown Sugar-Molasses	16.2^{a}	10.0^{bc}	6.2^{c}	14.5^{ab}	10.8^{bc}	9.6^{bc}	0.0052*	0.0189*
Aftereffects								
Sweet	15.9	14.6	15.8	13.6	15.7	12.7	0.3734	0.1712
Bitter	24.2^{a}	21.4^{ab}	9.9^{d}	18.4^{bc}	16.7^{c}	20.0^{bc}	<.0001*	0.7863
Drying	19.2^{a}	17.0^{ab}	10.4^{c}	16.4^{ab}	15.5^{b}	14.6^{b}	<.0001*	0.9087
Smoky	13.3^{a}	13.2^{a}	5.1^{b}	12.0^{a}	7.1^{b}	12.9^{a}	0.0002*	0.4895
Cocoa	13.0^{a}	10.8^{a}	5.7^{b}	11.2^{a}	9.8^{a}	10.1^{a}	0.0191*	0.9173

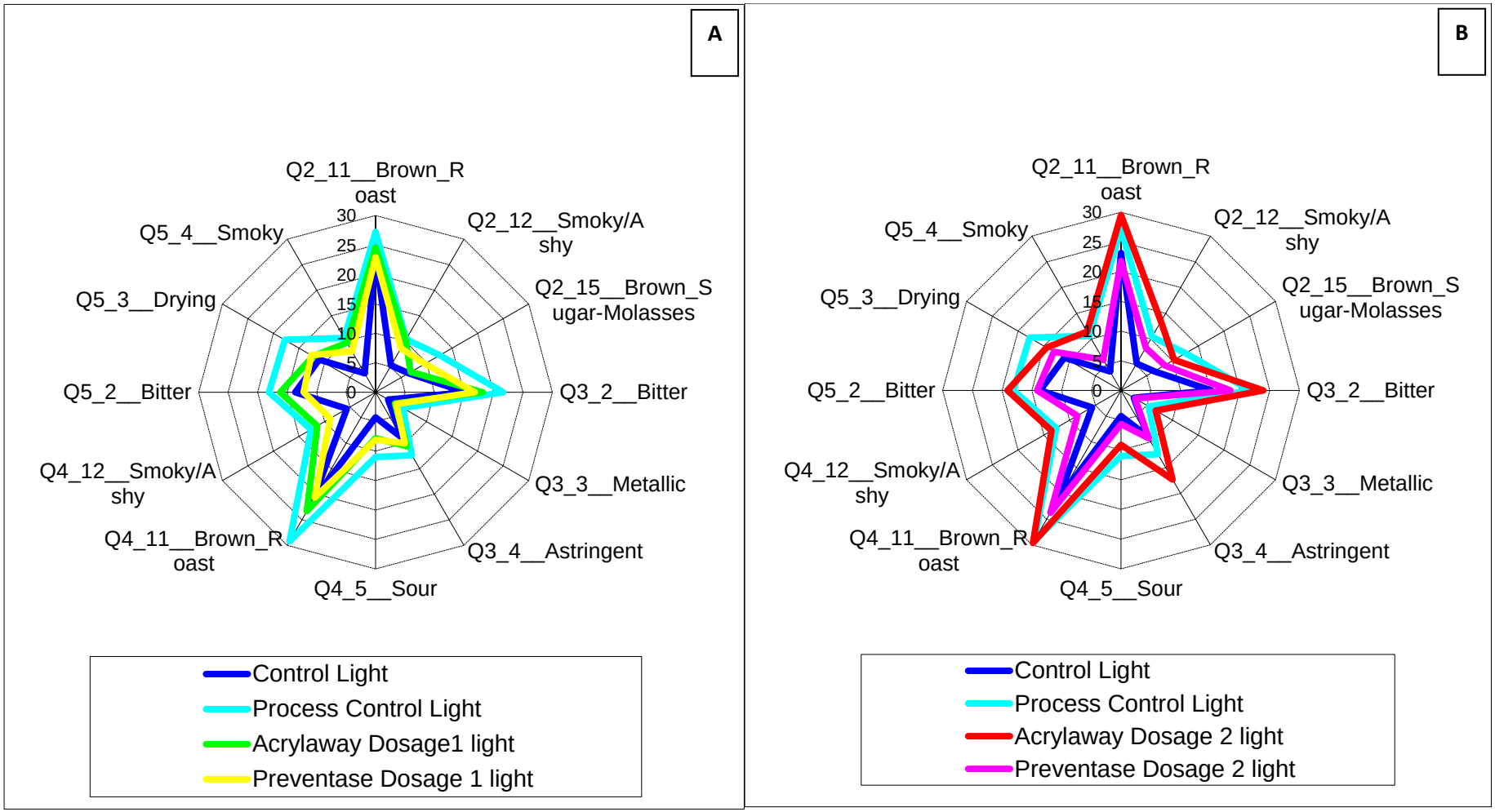

Figure 4.3 Spider web representing 12 significant attributes found in light roast (Dosage 1-A, Dosage 2-B) coffee samples (Light-12 min at 230°C) at two different dosages (Dosage 1=2000 ASNU or ASPU/kg and Dosage 2 = 4000 ASNU or ASPU/kg) in descriptive test.

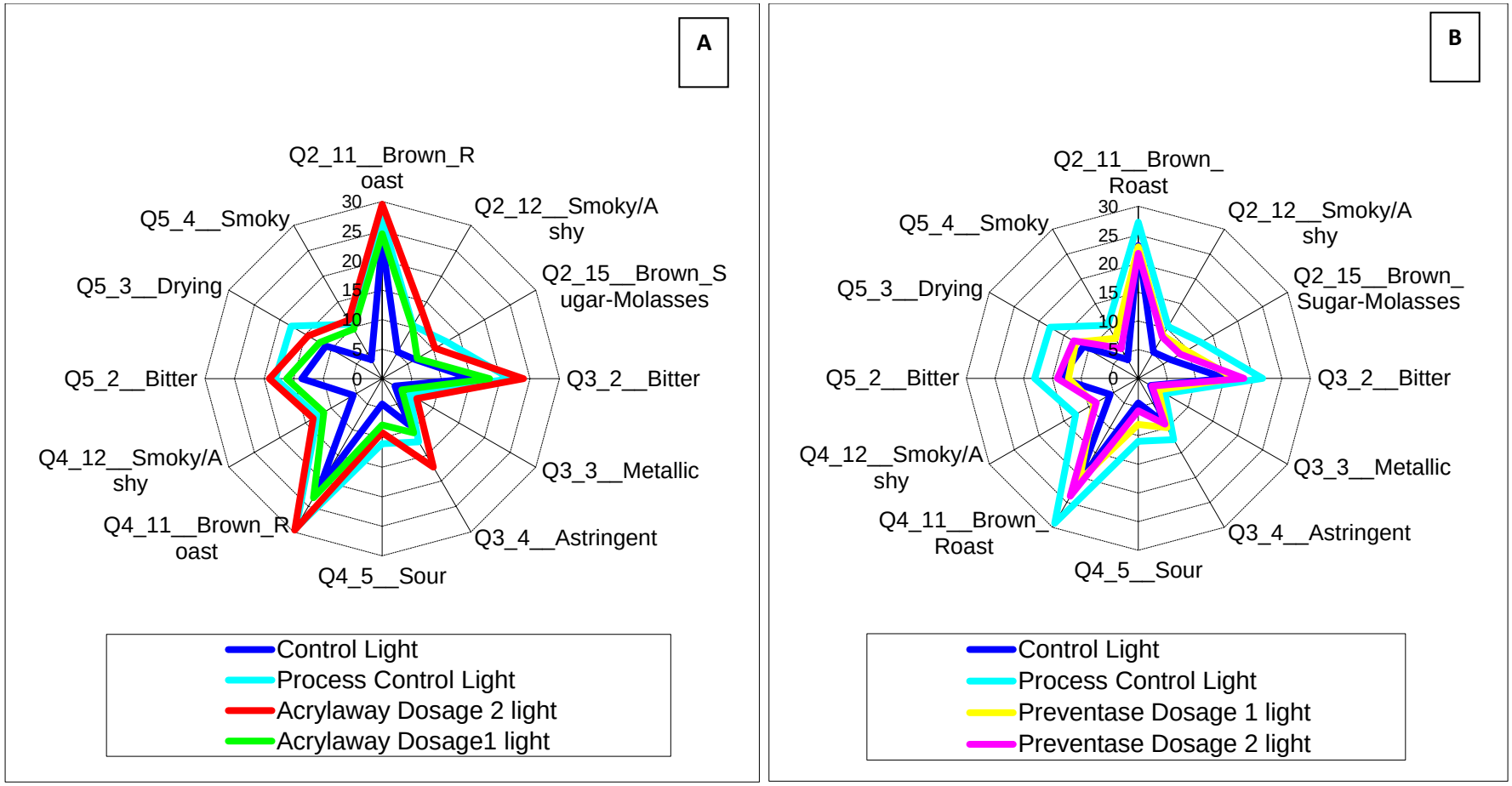

Figure 4.4 Spider web representing 12 significant attributes found in light roast (Acrylaway®-A, PreventASe®-B) coffee samples (Light-12 min at 230°C) at two different dosages (Dosage 1=2000 ASNU or ASPU/kg and Dosage 2 = 4000 ASNU or ASPU/kg) in descriptive test.

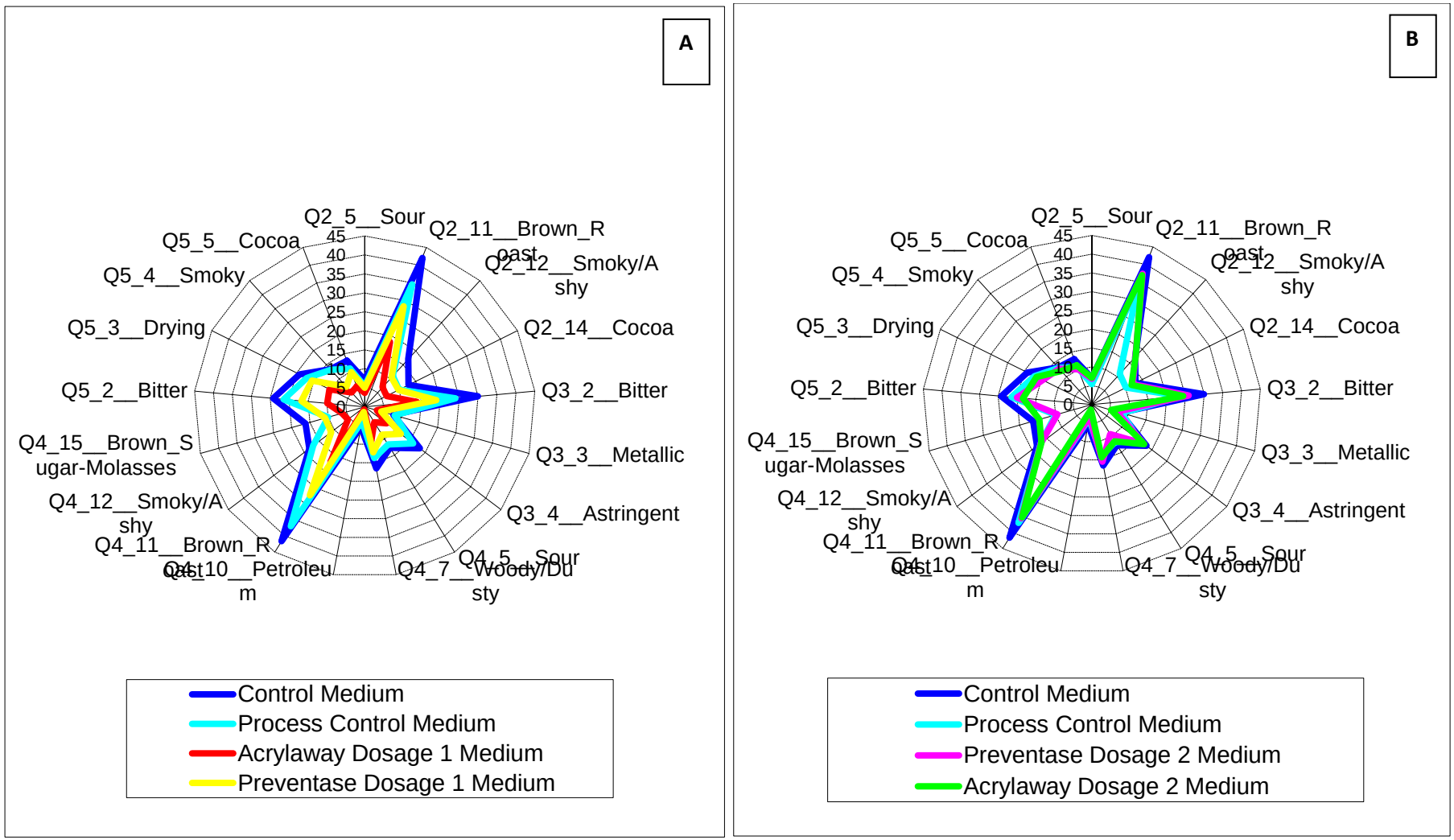

Figure 4.5 Spider web representing 17 significant attributes found in Medium roast (Dosage 1-A, Dosage 2-B) coffee samples (Medium-14 min at 240 °C) at two different dosages (Dosage 1=2000 ASNU or ASPU/kg and Dosage 2 = 4000 ASNU or ASPU/kg) in descriptive test.

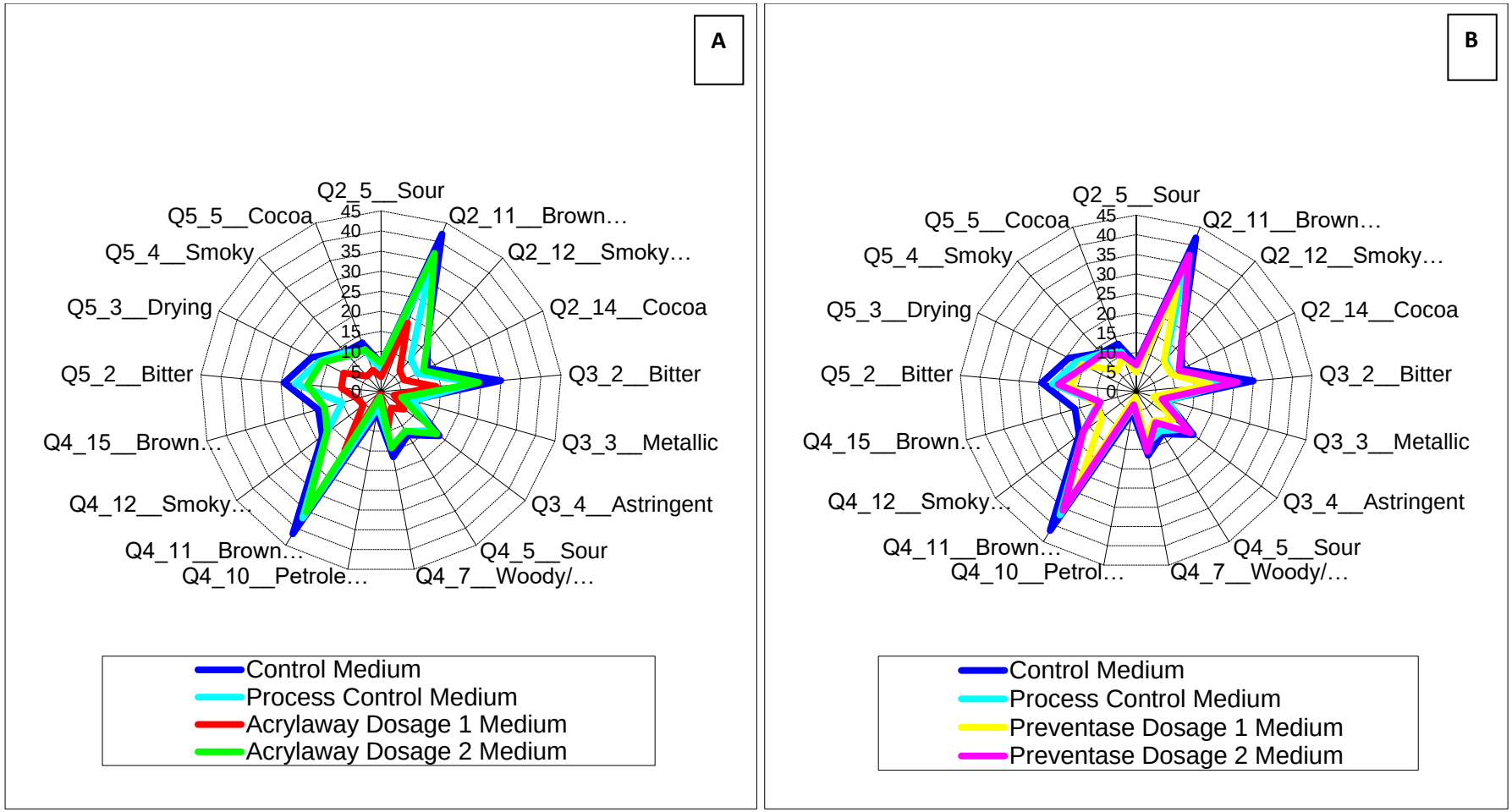

Figure 4.6 Spider web representing 17 significant attributes found in Medium roast (Acrylaway®-A, PreventASe®-B) coffee samples (Medium-14 min at 240 °C) at two different dosages (Dosage 1=2000 ASNU or ASPU/kg and Dosage 2 = 4000 ASNU or ASPU/kg) in descriptive test.

For the smell test, a significant difference was found in Acrylaway® treated samples ($P^{\text{Control-Acrylaway® Dosage 1}}$=0.01). However, though a significant difference was found in the PreventASe® treated samples, the p value was larger than the Acrylaway® group. ($P^{\text{Control-PreventASe® Dosage 1}}$=0.04). Comparing this result to the profiling test result, consumers and panellists reached a consensus that the PreventASe® treated samples were closer to the control samples on the odour perspective. In relation to taste, a significant difference was found between the control and enzyme treated samples (for both Acrylaway® and PreventASe®) whereas, no significant difference was found between the process control, and enzyme treated samples (for both Acrylaway® and PreventASe®). These results indicated that the enzyme treatment process rather than the enzyme treatment had more influence on the noticeable differences in the final coffee samples.

Table 4.8 Statistical analysis results for major consumer test for medium roast Brazilian Santos sample in which 77 consumers participated.

Smell Test	
Groups	P value
Control-Acrylaway® Dosage 1	0.01
Control-Acrylaway® Dosage 2	0.01
Acrylaway® Dosage 1-Acrylaway® Dosage 2	0.01
Control-PreventASe® Dosage 1	0.04
Control-PreventASe® Dosage 2	0.03
PreventASe® Dosage 1-PreventASe® Dosage 2	0.56
Taste Test	
Groups	P value
Control-Acrylaway® Dosage 1	0.01
Control-PreventASe® Dosage 1	0.01
Process Control-Acrylaway® Dosage 1	0.08
Process Control-PreventASe® Dosage 1	0.11

p value for each group was calculated by V-power programme (Virginia, USA). Dosage 1- 2000ASNU/kg or ASPU/kg, Dosage 2-4000 ASNU/kg or ASPU/kg. Medium Roast Level-14 min at 240 °C. Confidence level set at 95% while the corresponding significance level was 0.05

4.3.2.4 Preference Test

9-point liking scale (1-extremely dislike to 9 extremely like) was utilized in the preference test. For the preference assessment (**Table 4.9**), the light roast coffee average score was 4.5, whereas medium roast coffee average score was 5. The higher preference score of medium coffee indicated that for the same coffee, panellists and consumer in the UK liked medium roast more than the light roast.This phenomenon was the opposite to the Brazilian panellist's feedback.

Regarding the preference differences between the control and treated coffee, from the feedback and comments gathered from the consumers, no unpleasant flavour or taste was observed on any of the treated coffee samples. Statistical data also showed no significant differences between the control and treated samples regardless of the enzyme brand (P^{light}=0.19, P^{medium}=0.26). Therefore, though the enzymatic treatment had a significant impact on the sensory properties of the treated coffee samples, the reduced acrylamide coffee samples were still acceptable to the consumers.

Table 4.9 Preference score for light and medium roast enzyme treated Brazilian Santos samples.

Preference Score	Light	Medium
Control	4.54	5.12
Process Control	4.42	5.07
Acrylaway Dosage 1	4.59	5.03
Acrylaway Dosage 2	4.47	4.96
Preventase Dosage 1	4.52	4.93
Preventase Dosage 2	4.44	5.09
Average	**4.5**	**5**

Dosage 1- 2000ASNU/kg or ASPU/kg, Dosage 2-4000 ASNU/kg or ASPU/kg. Light- 12 min at 230°C, Medium Roast Level-14 min at 240 °C

4.4 Discussion

The consumer tests confirmed that the enzyme-treated Brazilian Arabica coffee, both at the light and medium roast level, was significantly different from the control group. The novel experiment design used tailored discriminative method for taste and smell part respectively. This set up achieved great discriminating power while without causing significant fatigue to the consumers. In the separated smell and taste section, significant differences were observed. So, the enzyme treatment had caused significant impacts on both taste and smell of the processed coffee beans. As it can be seen in Figure 4.7, as the roast continues, the coffee brew become bitterer and less acidic. The bitter, acidic and roasty attributes that showed significant differences in sensory chapter reflected the volatile compounds analysis results in previous chapter. For instance, pyrazines which generated in the Maillard reaction had roasty note, while phenols which generated in the chlorogenic acids degradation had smoky/ashy note. So, the adverse effect (silver skin loss & precursor loss) had caused significant impact to the roasted coffee composition both chemically and physically.

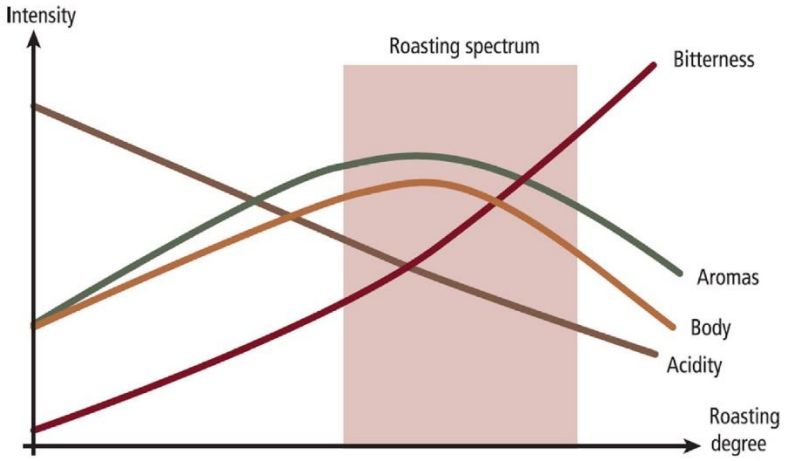

Figure 4.7 Schematic representation of the kinetics of flavour evolution in coffee during roasting (Luigi Poisson etal., 2017)

The direct consequence was that with less protection from the silver skin, the same extent of heat treatment on enzyme treated beans would lead to a greater extent of the roast level compared to the control coffee beans. So, the process control or enzyme treated coffee samples would have had a higher roast degree than the control coffee samples. This difference may be the key reason that leads to the difference in the final coffee products. Besides, this difference could also be reflected by the

weight loss of roasted coffee. Weight loss is an important variant that could relate to the roast degree and compositional change (Perrone et al., 2010). The higher weight losses of the green coffee beans in the enzyme-treated group also indicated the loss of silver skin and greater extent of roast level. This loss was also reflected by the data from the LAB colour test results. The higher roast level led to a darker roast colour.

Therefore, the steaming and soaking step peeled the silver skin off the coffee beans, leading to the difference in the extent of the roast. As the descriptive results indicated, because of the roast level shift, the processed sample which was roasted to higher extent showed similar attributes value to the control coffee at the next roast level. For instance, the enzyme treated light roast coffee was like the medium roast control coffee and this phenomenon could explained the feedback obtained from the panellists. The smells and or tastes enzyme treated light coffee more like the medium roast control coffee, so enzyme treated light coffee had more established coffee flavour and aroma than the light control.

However, for the medium roast group, as the medium roast enzyme treated coffee is more like high roast control coffee, the burnt flavour masked the other attributes. So, panellists reported few attributes could be sensed from the medium roast enzyme processed coffee. In the high roast level, as the heat treatment increased to a stage that bitter and burnt taste has overwhelmed other tastes, so the enzyme treated medium had fewer values scored on the significant attributes compared to the medium roast.

Regarding the preference test, the statistical analysis showed that the consumers accepted the significant differences between the control and processed samples. However, the results of the preference test were country or region specific. As it discussed above, the panellists in UK reached a consensus that the light roast coffee was insipid and medium roast coffee were full of characters. However, the panellists in Brazil showed contrary preference on roast level. The enzyme treatment which caused the processed coffee to be bitterer and less acidic matched the preference of UK panellists. Hence, potentially, no significant differences were found in Preference test. However, the same test may have achieved different results if it were carried out in countries that have opposite roast level preference.

4.5 Conclusion

To conclude this chapter, there were several points that could be summarised from these sensory tests. Significant differences between the control and enzyme treated coffee samples indeed exist. The potential reason is enzyme treatment (steam and soak) process made enzyme treated and process control samples roasted to a higher extent, the roast level differences were sensed and reported by panellists. Besides, different commercially available enzymes could also lead to differences in the sensory attributes for a particular product as seen in the present study.

Despite the significant aroma and taste difference that enzyme treatment had done to the treated samples, no unpleasant flavour was reported in the preference test. With high acrylamide reduction rate, enzyme treatment was still a powerful and effective tool for acrylamide mitigation in coffee.

Chapter 5 Enzyme vacuum infusion method on acrylamide mitigation

Abstract

In this chapter, an alternative enzyme delivery method, namely vacuum infusion, was investigated to reduce the adverse effects of steam & soak method while maintaining the high acrylamide reduction rate. Without the prior steam treatment, this experiment aimed to use vacuum force to push the enzyme inside the coffee beans. Acrylamide determination and colour determination was carried out for processed samples. Acrylamide data indicated that enzyme vacuum processed coffee had significant lower acrylamide content compared to the control. However, the reduction efficiency (12%-56%) was not as high as the steam & soak method (40%-91%). The colour determination showed that the colour difference between processed samples and control groups was similar to the steam & soak method. Besides, long-time incubation experiment was also carried out to examine the importance of incubation time. However, the results of acrylamide and colour determination implied that the incubation time was not the vital factor. In conclusion, vacuum infusion method provided another approach of enzyme delivery. With the optimised parameters, more sufficient contact may be achievable by the vacuum infusion method.

Keywords

Vacuum infusion, diffusion hindrances, silver skin

5.1 Introduction

In chapter two, a pretreatment method involving steaming and soaking of coffee beans with asparaginase to reduced acrylamide in coffee was discussed. It involved steaming the beans to open the pore and increase the surface areas followed by a soak step where coffee beans are submerged in an enzyme bath to let the enzyme react with the substrate. Acrylamide determination results have shown that the steam & soak enzyme delivery method had great acrylamide reduction efficiency for two food grade commercially available enzymes tested Acrylaway® and PreventASe®. With this process, asparagine was reduced (43%-91%) in enzyme (2000-20000 Units) treated green coffee beans and acrylamide was reduced (40%-91%) in the roasted coffee beans. However, it had a negative impact on the colour characteristics of the enzyme treated coffee beans as they became darker than the control coffee beans for every roast level that was studied. This effect was mainly due to the loss of the silver skin of the coffee beans which was lost during the enzymatic treatment. Besides, sugar determination in the processed green coffee beans showed significant reduction in the enzyme processed coffee beans.

Furthermore, this process had a significant impact on 10% of the volatile compounds of the coffee beans, particularly for key aroma compounds (pyrazines, aldehydes, ketones, furans and phenols). Sensory tests also confirmed that significant differences on the odour, flavour and after taste aspects existed between the control and treated coffee. Therefore, although the steam & soak method had great acrylamide reduction rate, the significant differences in the overall coffee flavour profile indicated that an alternative enzyme delivery method should be investigated to reduce the adverse effects (silver skin loss and precursor loss) while maintaining the acrylamide reduction efficiency.

As a result, an alternative enzyme delivery method with minimum impact on the green coffee beans was investigated. Whether the new method would work efficiently on mitigating acrylamide, it mostly depended on whether the enzyme had sufficient contact with the substrate (asparagine). Green coffee beans have a very tight internal structure (Schenker, Handschin, Frey, Perren, & Escher, 2000) with less than 7% of the pore volume having a diameter of 10 nm or more ((Pittia et al., 2011), **Figure 5.1** and **Figure 5.2.)** The bean porosity characteristics changes dramatically during the roasting process

with the green coffee beans having fewer pores with smaller pore volume compared to the roasted beans, indicating that the internal structure of the green coffee beans is more compact.

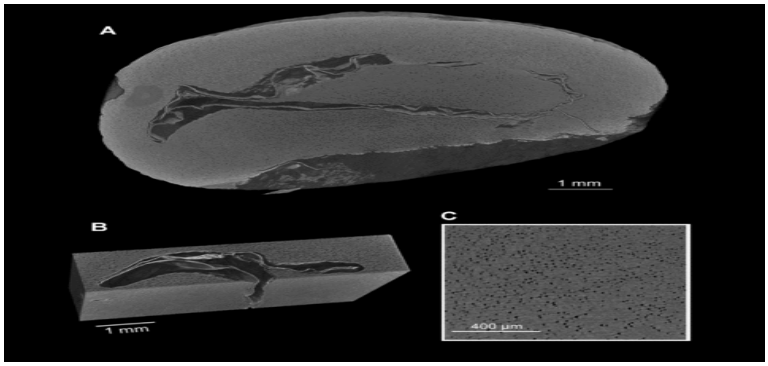

Figure 5.1 Green bean 3D rendering images: (A) volume rendering of a portion of the green bean to show global features, (B) volume rendering of the tegumentum zone, (C) zoom of a slice to highlight the bean porosity characteristics (Pittia et al., 2011).

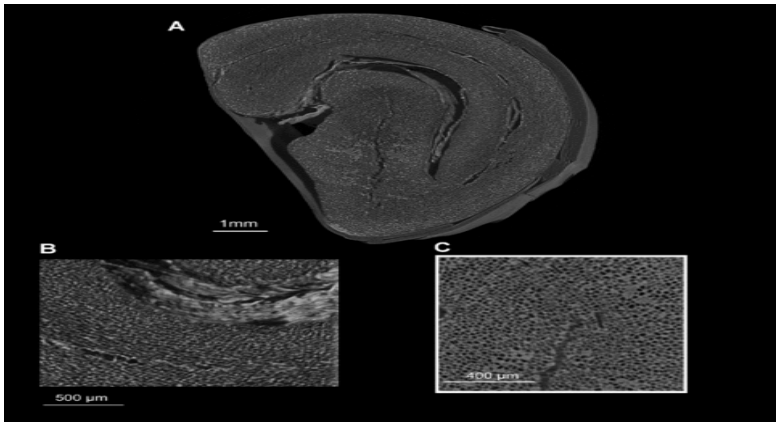

Figure 5.2 Roasted bean 3D rendering images (A) Volume rendering of a portion of the dark bean to show global features, (B) volume rendering of detail to show the porosity and the tegumentum, (C) detail is taken from an axial slice to show the porosity and cracks features (Pittia et al., 2011).

Therefore, the tight internal structure of coffee beans could potentially make it difficult for the enzyme to react with the substrate efficiently. On the other hand, the size of the enzyme needs to be considered too, as asparaginase is a relatively large enzyme. The research by Hendriksen showed that the size of Acrylaway® was around 5-10 nm (Beate et al., 2009), which made it difficult to access the cell wall. The steam and soak method was aiming at helping the asparagine to diffuse to the surface or out of the cell wall and let it contact with the enzyme in the enzyme solution (Beate et al., 2009). Acrylamide determination results indicated good sufficient contact between substrate and enzyme. However, during this process, adverse effects (precursor loss and silver skin loss) were caused by the enzyme treatment. Therefore, to reduce the adverse effects of the process and enhance the contact of enzyme

and asparagine, vacuum infusion method aimed to force the enzyme to penetrate into the beans to increase contact with the asparagine.

Scarce data is available regarding vacuum infusion for acrylamide reduction in coffee, and it mainly relates to the work carried out by Hendriksen`s group (Beate et al., 2009). In this case, the coffee beans were vacuum sealed and then incubated with Acrylaway® (enzyme dosage: 4000 ASNU/kg beans) at 50 °C for 18 hours. Then the samples were roasted for 3, 4 and 4.5 minutes which represented medium or darker roasts. The enzyme treatment achieved up to 70% acrylamide reduction. A process control group was included where the coffee beans were vacuum-infused with water (**Figure 5.3).** However, the results were questionable as per the roasting time that was chosen by the researcher. As shown in **Figure 5.4**, the research carried out by Lantz (Lantz et al., 2006) confirmed that the acrylamide amount would decrease as the roast time is increased. Though enzyme treated groups were lower than the control group, roast level factor showed a far more dominant effect than the enzyme treatment. The author did not explain the further details of the experiment set up. Also, as no replication was stated, and acrylamide reduction rate was high comparing to other researches, increased the doubt of the feasibility or reproducibility of the enzyme infusion method.

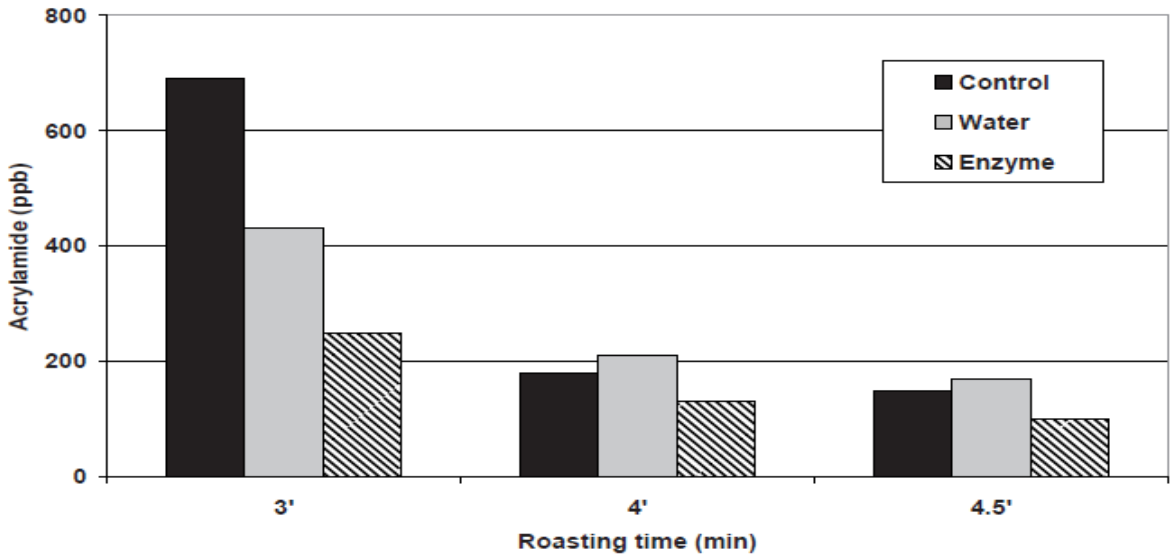

Figure 5.3 Acrylamide reduction in vacuum-infused coffee (Beate et al., 2009).

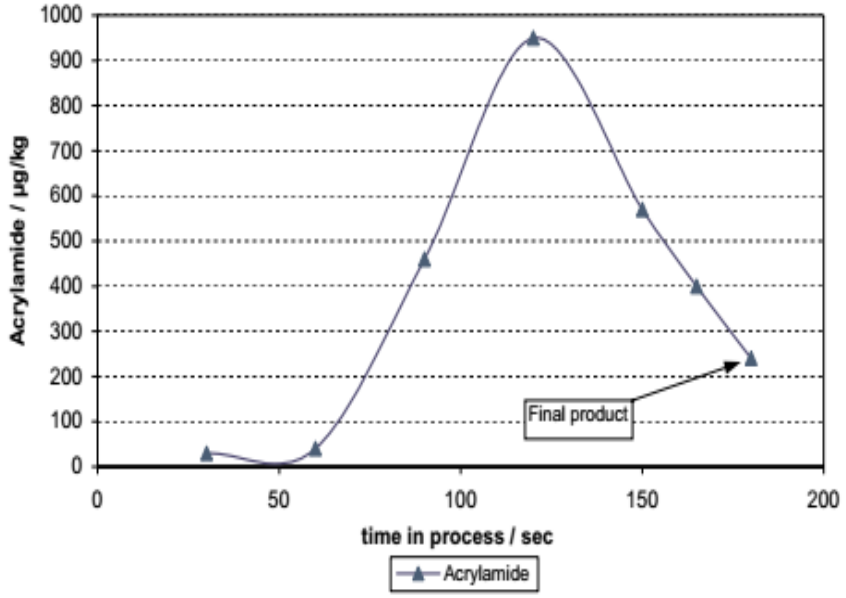

Figure 5.4 Acrylamide levels of partially roasted Colombian coffees, by prematurely stopping the process of roasting(Lantz et al., 2006).

Overall, the main aim of this chapter was to examine whether the vacuum infusion method could successfully reduce acrylamide in coffee, while reducing the loss of silver skin. Colour tests were carried out to examine the colour difference of vacuum infused coffee compared to the control. Also, long incubation time was stressed by other researchers as a vital parameter on overcoming the diffusion hindrance, long incubation time experiment would also be carried out to examine the effect of incubation time.

Therefore, two experiments that used vacuum infusion method with different incubation time were carried out:

First experiment used three dosages (2000, 4000, 6000 ASNU/kg coffee beans) of two enzymes (Acrylaway® & PreventASe®) with 45 minutes incubation then roast to three different levels. This incubation time was set to match the incubation time that used in the previous experiment. Then compare the acrylamide reduction results at the same incubation time basis.

Long-time incubation experiment focused on the effect of incubation time on acrylamide mitigation. It carried out vacuum infusion process with long incubation time including 3 hours, 6 hours and 18 hours. As this experiment was designed to examine the effect of different incubation time, the long incubation time group only used Acrylaway® at 2000 ASNU/kg coffee beans then roasted to three roast levels.

5.2 Materials & Methods

5.2.1 Materials

5.2.1.1 Enzymes

Both enzymes (Acrylaway® and PreventASe®) were gifts from the manufacturers. Acrylaway® from Novozymes (3500ASNU/g) and PreventASe® (2500ASPU/g) from DSM were used. Both ASNU and ASPU are defined as the amount of asparaginase that produces one μmol of ammonia per minute under the conditions of the assay (pH = 7±0.005; T = 37±0.5 °C) using Acrylaway® or PreventASe®, respectively.

5.2.1.2 Coffee

Brazilian Santos Arabica coffee supplied by Rave Coffee UK (Cirencester, Gloucestershire) was used for this study.

5.2.1.3 Reagents

Both enzymes (Acrylaway® and PreventASe®) were gifts from the manufacturers. Acrylaway® from Novozymes (3500ASNU/g) and PreventASe® (2500ASPU/g) from DSM were used. Both ASNU and ASPU are defined as the amount of asparaginase that produces one μmol of ammonia per minute under the conditions of the assay (pH = 7±0.005; T = 37±0.5 °C) using Acrylaway® or PreventASe®, respectively.

5.2.2 Methods

5.2.2.1 Volume determination of enzyme solution

Asparaginase (Acrylaway®) was mixed with 25, 50, 75 ml of water and sprayed evenly on 150g of coffee beans. The concentration of Acrylaway® used was 2000 ASNU/kg. The beans were rolled over to increase the contact area with asparaginase during spraying. Then, beans were sealed in a dedicated 250mm× 250mm vacuum plastic pouch (The vacuum pouch company LTD, Walshaw). The vacuum bags were then laid to one layer in a Henkelman Falcon 52 vacuum machine. Then, the coffee bean bags were vacuumed to -1 bar. Next, the coffee beans were placed into a 60°C (optimum temperature for enzyme activity) water bath for 45 minutes. After the treatment, samples were removed from the vacuum bag and placed into a plastic tray. Afterwards, coffee samples were dried in the Gallenkamp® drier to the original weight (150g) at 60°C for 4-6 hours. Asparaginase treated coffee beans were then roasted at 230°Cfor 12 min (light roast) and then stored in 300g plastic vacuum sealed bags in a cold room (4ºC)

5.2.2.2 Preparation of coffee sample

Asparaginase (Acrylaway®, PreventASe®) was mixed with 50 ml of water (optimum amount of water) and sprayed evenly on 150g of coffee beans. Three different concentrations of enzyme were tested for both Acrylaway® and PreventASe® (2000, 4000, 6000 ASNU/kg and ASPU/kg, respectively). The beans were rolled over to increase the contact area with asparaginase during spraying. Then, beans were sealed in a dedicated 250mm× 250mm vacuum plastic pouch (The vacuum pouch company LTD, Walshaw) with 50ml of asparaginase solution sprayed on them. Then the coffee bean bags were vacuumed to -1 bar. Next, the coffee beans were placed into a 60°C (optimum temperature for enzyme activity) water bath for 45 minutes. After the treatment, samples were removed from the vacuum bag and placed into a plastic tray. Afterwards, coffee samples were dried in the Gallenkamp® drier to the original weight (150g) at 60°C for 4-6 hours. Then coffee beans were roasted at 230°C for 12 min (light), at 240°C for 14 min (medium); at 250°C for 17 min (high), respectively and stored in 300g plastic vacuum sealed bags in cold room (4ºC)

5.2.2.3 Enzyme Activity determination

Compared to the optimum pH (7) and official activity (3500ASNU) of Acrylaway®, PreventASe® had a relatively lower optimum pH (5) and lower official activity (2500ASPU). As enzymes lose activity each day, even when stored in at 4 °C, enzyme activity determination was carried out before

each experiment, in order to determine the correct dosage to use. After the enzyme determination, optimum dosage for the enzyme treatment was calculated in this study.

10ml L-Asparagine solution (189 mM) and 1ml ammonium sulphate standard (6mM) was added to 20 ml falcon tube. The falcon tubes were then equilibrated to 37° C and enzyme solution (2 units/mL) was added to the tubes. The tubes were immediately shaken for 15 seconds by vortex shaker (FB15013 TopMix by Fisher Scientific) and incubated at 37 °C for 30 minutes. 10ml Trichloroacetic acid (1.5 M) were added to the test tubes. The solutions were mixed and centrifuged (Eppendorf mini spin, UK) for 2 minutes at 3600 rpm. The supernatant obtained was mixed with 0.5 mL Ammonia Colour Reagent (1.01 g/mL) immediately. After one minute the absorbance at 436 nm was recorded (SigmaAldrich, 1997).

5.2.2.4 Supported Liquid Extraction (SLE)

Fine ground (0.1 mm particle size), roasted coffee sample (2.00 ± 0.01 g) was weighed into a 50-mL Falcon tube; 40 mL of water were added to the tube. Samples were spiked with 400 µL of the internal standard solution (1000 ng/mL ^{13}C-acrylamide in water); tubes were shaken for 15 seconds by hand then 15 seconds by vortex shaker (FB15013 TopMix by Fisher Scientific) and then 60 minutes on a mechanical shaker (Multi Reax by Heidolph). Samples were centrifuged (Sigma 3K10, UK) at 10 °C and 3600 rpm for 20 minutes. After centrifugation, 0.625 mL of supernatant were transferred to a 7-mL glass vial containing 12.8 µL of a saturated solution of ammonium hydroxide in water. The vials were briefly shaken, and then 0.5 mL of the mixture were transferred to an SLE cartridge placed on a vacuum manifold. Cartridges did not require a conditioning step. An initial vacuum was applied to allow the sample to be absorbed for 5 minutes. Then a mixture of ethyl acetate and tetrahydrofuran (1:1, 2 × 2.5 mL) was applied and allowed to flow under gravity into a tube containing 2 µL ethylene glycol, to extract the acrylamide that had been retained. Finally, the tubes containing the eluates were dried in a stream of nitrogen and reconstituted in water (500 µL) prior to the analysis (Biotage, 2014).

5.2.2.5 Acrylamide determination by LC-MS/MS

Samples were analysed by liquid chromatography-triple quadrupole mass spectrometry (LC-MS/MS) using an Agilent 1200 HPLC system with a 6410 triple-quadrupole mass spectrometer with electrospray ion source in positive ion mode. An isocratic separation was carried out at room

temperature using a 100 × 3.0 mm Hypercarb column with a 10 × 3.0 mm Hypercarb precolumn (both 5 µm particle size; Thermo Fisher, Waltham, MA). The mobile phase was 0.1% aqueous formic acid at a flow rate of 0.3 mL/minute. The injection volume was 25 µL. The eluent from the column was run to waste from 0 to 4.5 minutes, and data were collected from 4.5 to 8 minutes. Acrylamide was eluted at around 6 minutes. A run time of 20 minutes allowed the clean-up of the column for the following sample. The transitions m/z 72 → 55 and 72 → 27 were used for acrylamide analysis, and the transition m/z 75 →58 was used to measure the internal standard ^{13}C-acrylamide (1 mg/L). Peaks were symmetrical with no interference from impurities. A calibration curve (5000 µg/L, 1000 µg/L, 200 µg/L, 40 µg/L, 10 µg/L, 2 µg/L acrylamide in water) with $r^2 > 0.999$ was prepared. ^{13}C-acrylamide solution (50 µg/L) was also present in the standard.

5.2.2.6 Colour determination by LAB method

A HunterLAB® CT1100 ColourQuest colorimeter (Hunter Associate Laboratories, Inc., Reston, VA) was used for the colour measurements. The colorimeter set-up was as follows: total transmittance mode, Illuminant D65, and 10 degree observer angle. All measurements were conducted in triplicate. Fine ground coffee powders (particle size 0.1 mm) were packed in a transparent plastic snap bag with a width of 1 cm. After calibration of the colorimeter using the equipment`s reference white plate, colour profiles of the coffee samples were obtained by measuring the coffee powder directly in the bags.

The HunterLAB® machine measured the *L* (lightness), *a* (redness) and *b* (yellowness) values of the samples to locate a specific colour in the three-dimensional colour scheme. In this scheme, Δ*E* (Total Colour Difference) was introduced to illustrate the distance between two specific colours. Δ*L,* Δ*a,* Δ*b* refers to the difference between control and processed samples on *L*, *a, b* respectively. Δ*E* equals the square root of the sum of square Δ*L*, square Δ*a* and square Δ*b*.

$$\Delta E = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2}$$

5.2.2.7 Long incubation time experiment

Asparaginase (Acrylaway®), 2000 ASNU/kg, was mixed with 50 ml of water and sprayed evenly on 150g of coffee beans. The beans were rolled over to increase the contact area with asparaginase during spraying. Then, beans were sealed in a dedicated 250mm× 250mm vacuum plastic pouch (The vacuum pouch company LTD, Walshaw) with 50ml of asparaginase solution sprayed. Then the coffee bean bags were vacuumed to -1 bar. Next, the coffee beans were placed into 60°C (optimum

temperature for enzyme activity) water bath for 45 minutes, 3 hours, 6 hours and 18 hours. After the treatment, samples were released from vacuum bag and placed into a plastic tray. Afterwards, coffee samples were dried in the Gallenkamp® drier to the original weight (150g) at 60°C for 4-6 hours. Then coffee beans were roasted at 230°C for 12 min (light), at 240°C for 14 min (medium); at 250°C for 17 min (high), respectively and stored in 300g plastic vacuum sealed bags in cold room (4ºC)

5.2.2.8 Statistical Analysis

All analyses were done in triplicate and expressed as means with standard deviations. XLStat 2015 (Addinsoft, Paris, France) was used to perform analysis of variance (one-way and two-way ANOVA). For those compounds that exhibited a significant difference in the ANOVA, multiple pairwise comparison tests (Tukey`s HSD) were applied to determine which sample means differed significantly ($p < 0.05$).

5.3 Results

5.3.1 Volume determination of the enzyme solution

Prior to carrying out the vacuum infusion experiment, the amount of water needed to treat the coffee beans was calculated. Previously, as discussed in Chapter 2, the coffee beans had been steamed first followed by a soaking step however this induced the silver skin loss as a result. The goal for this new way of delivering the enzyme therefore was to reduce the amount of water (originally 50ml) and therefore 25, 50 and 75 ml of water were initially tested to determine the optimum amount to be used in the subsequent experiment. After 45 minutes of incubation, it was observed that all water had been absorbed when using 25 and 50 ml solutions whereas for the 75ml solution there was still water left in the vacuum bag to be absorbed. When assessing acrylamide reduction 25 ml had achieved around 10% acrylamide reduction whereas 50 ml and 75 ml treatments performed similarly, achieving a reduction percentage of 27% and 29%, respectively (**Table 5.1**). Though the 75ml group had slightly higher acrylamide reduction rate, 75ml enzyme solution could not be absorbed by un-steamed coffee beans. As a result, 50ml was selected as the water amount to be used for the vacuum infusion experiment.

Table 5.1 Acrylamide content in coffee infused with different volumes of enzyme containing solution.

	Control*	25ml	50ml	75ml
Acrylamide (μg/kg)	225±8.29	204±3.46	168±4.58	160±5.09

Coffee beans treated with 2000ASNU/kg of Acrylaway® and roasted for 12 min at 230°C (n=3).* Control group refer to coffee processed without treatment.

5.3.2 Enzyme activity determination

Before carrying out the experiment, the activity of enzyme was determined so correct dosages of asparaginase could be used in the vacuum infusion experiment. At the time, PreventASe® activity was 1800 ASPU/g (seven months after the production date) whereas a new batch of Acrylaway® was obtained which had an activity of 2400 ASNU/g (six months after the production date). As a result, the dosages used for this experiment were calculated based on their activity.

5.3.3 Vacuum Infusion

For the vacuum infusion experiment, the incubation time was set to 45 minutes to compare the results with previous steam & soak experiment. As per with steam & soak experiments, three dosages of the two commercially available asparaginase enzymes, Acrylaway® and PreventASe®, were used for this experiment followed by roasting to light, medium and high conditions. Same batch of Brazilian Santos coffee was used in vacuum infusion experiment and long incubation time vacuum infusion experiment. The reduction results are shown in **Figure 5.5** and **Table 5.2**.

Table 5.2 Acrylamide reduction rate in steam & soak experiment and vacuum infusion experiment.

	Steam & Soak						
	Process Control	Acrylaway® Dosage 1	Acrylaway® Dosage 2	Acrylaway® Dosage 3	PreventASe® Dosage 1	PreventASe® Dosage 2	PreventASe® Dosage 3
Light	40%	53%	69%	86%	47%	65%	86%
Medium	22%	42%	71%	87%	36%	63%	85%

High	34%	51%	71%	84%	52%	70%	82%

Vacuum Infusion

	Process Control	Acrylaway® Dosage 1	Acrylaway® Dosage 2	Acrylaway® Dosage 3	PreventASe® Dosage 1	PreventASe® Dosage 2	PreventASe® Dosage 3
Light	14%	21%	24%	25%	17%	22%	24%
Medium	12%	20%	27%	35%	17%	17%	31%
High	15%	28%	48%	56%	28%	32%	36%

For each roast level, statistical analysis was carried out taking into account several factors including enzyme brand (Acrylaway® vs PreventASe®), enzyme dosages (dosage 1, 2, 3), enzyme treatment (control vs treated) and enzyme (process control vs treated). Enzyme factor focuses on the impact caused by enzyme alone while the enzyme treatment factor focuses on the impact of enzyme treatment process alone. Enzyme brand and enzyme dosages focus on the differences triggered by the various conditions of different enzymes. As it is shown in **Table 5.3**, all factors had a significant effect, but most noticeably enzyme and enzyme treatment ($p<0.0001$ and $p<0.001$, respectively)

Table 5.3 Significance of enzyme, enzyme treatment, enzyme dosage and enzyme brand for the vacuum infusion experiment.

	Enzyme	Enzyme Treatment	Enzyme Dosages	Enzyme Brands
Light	<0.0001	<0.001	0.01	0.028
Medium	<0.0001	<0.001	0.001	0.004
High	<0.0001	<0.001	0.004	0.006

Dosage: 1- 2000ASNU/kg or ASPU/kg, 2-4000 ASNU/kg or ASPU/kg, 3-6000 ASNU/kg or ASPU/kg. Light- 12 min at 230°C, Medium- 14 min at 240 °C, High- 17 min at 250°C. Confidence level was set at 95% while the corresponding significance level was 0.05

As it can be seen in Figure **5.5** & **Figure 5.6**, because of the seasonal variation and batch differences, though the same type of Brazilian Santos coffee were used in the two experiment, the control groups of the steam & soak processed samples were relatively higher than the vacuum infusion. In steaming & soaking process experiment, in light roasting conditions, the reduction rate increased from 49% (Acrylaway®) and 45% (PreventASe®) to 86% (Acrylaway®) and 84% (PreventASe®) as the dosage of enzyme rose from 2000U to 6000U (**Table 5.3**). It had been observed that the steaming process was necessary for conventional enzyme treatment as it ensured sufficient contact between

enzyme and substrate. Hence, under the same roast condition, increasing the enzyme dosage will be reflected as a decrease of acrylamide content in the final roasted coffee. Acrylamide in vacuum infusion method processed coffee beans was shown in **Figure 5.5**. The treated group for both enzymes showed a reduction of around 30% in light roast samples to 20% in high roast samples. Unlike the steam & soak treated samples, the acrylamide reduction rate was independent from the various enzyme dosages used in the vacuum infusion experiment.

The relationship between the acrylamide reduction rate and enzyme dosages indicated that for the part of the coffee beans that enzyme solution had entered, lowest dosage of enzyme solution was already abundant for the free asparagine present. As it was discussed in the introduction, the vacuum infusion aims to push enzyme solutions into the coffee beans. However, because of the tight internal structure, it was still in doubt whether the vacuum force could make the enzyme solution penetrate to the core of the green coffee beans. Therefore, after the incubation step, processed coffee beans were transversely cut into halves to examine the extent of the enzyme solution had entered, Visual examination, indicated that the enzyme solution had partially reached the core of the green beans and (Evidence transverse cut photo attached in Appendix V). because no steam was used in the process, the inner part of the coffee bean was still dry at the end of the incubation step.

To differentiate the impact of enzyme and enzyme treatment, process control samples were included in the experiment design. In the conventional steaming and soaking process, process control samples were coffee beans that were firstly steamed for the same amount of time as enzyme treated samples and then soaked in a water bath at the same temperature for the same amount of time. In the conventionally processed coffee beans (**Figure 5. 7)**, the steam and soak process reduced acrylamide by an average of 30%. As stated in Chapter 2, the free asparagine was leaked during the steam process and then washed away in the enzyme soaking process. In the vacuum infusion process, the process control samples were coffee beans that were infused with the same amount of water with no enzyme in it. As results showed in **Figure 5.5**, acrylamide reduction in the process control was around 15%. Considering that the water solution could not fully penetrate into the coffee beans, only 15% reduction of acrylamide was observed. Comparing the process control in steam & soak experiment and vacuum infusion experiment, the acrylamide reduction rate showed that the vacuum infusion method was likely to have lower precursor loss than the steam & soak method.

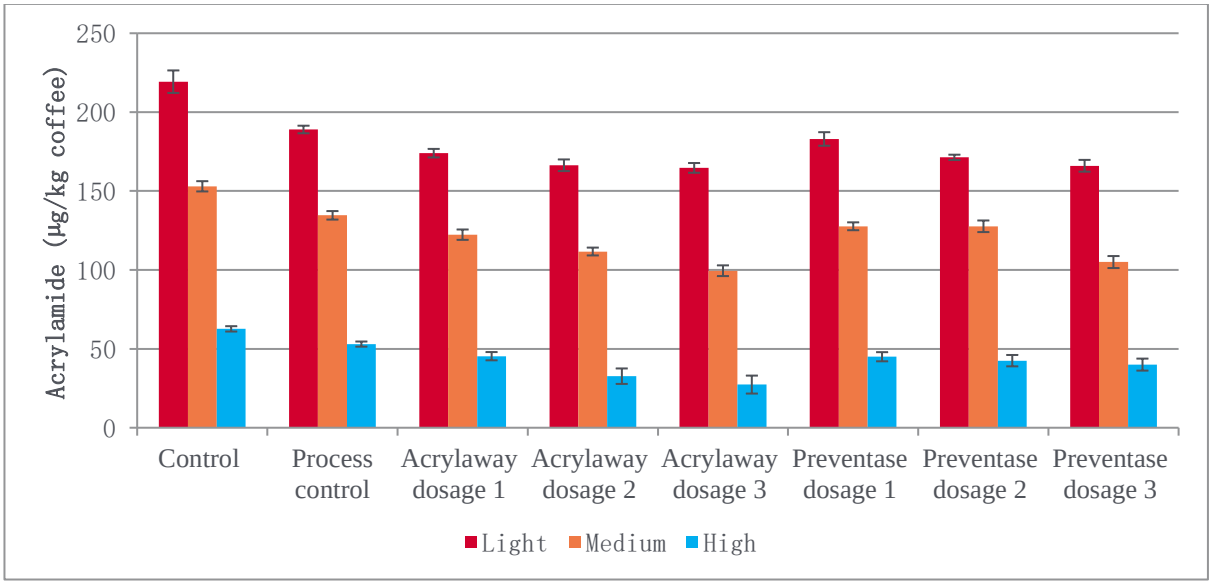

Figure 5.5 Acrylamide content by vacuum infusion enzyme process. Acrylamide was calculated as µg/kg coffee (Dosage 1-2000 U, Dosage 2-4000U, Dosage 3-6000U. Roast conditions: Light- 12 min at 230°C, Medium- 14 min at 240 °C, High- 17 min at 250°C). (n=3)

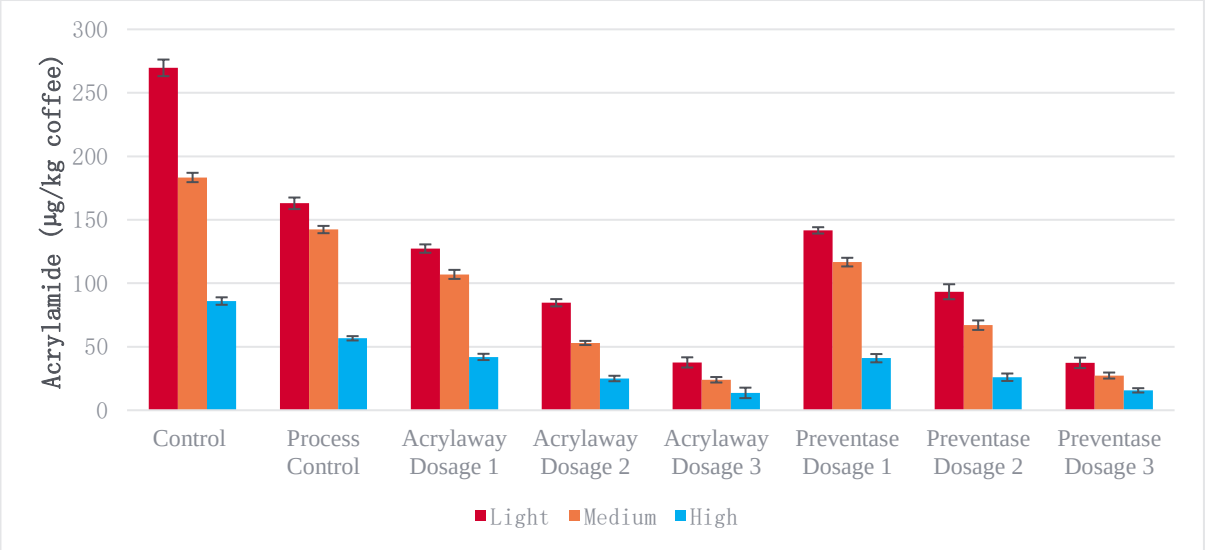

Figure 5.6 Acrylamide content by steaming and soaking enzyme process (Dosage 1-2000 U, Dosage 2-4000U, Dosage 3-6000U). Acrylamide was calculated as µg/kg coffee (Light- 12 min at 230°C, Medium- 14 min at 240 °C, High- 17 min at 250°C). This figure refers to the **Figure 2.11** in Chapter 2

5.3.4 Long Incubation Time Vacuum Infusion Experiment

In the research that was carried out by Hendriksen`s group (Beate et al., 2009), long time incubation was recommended as the author stated that 18 hours were used as it was long enough to overcome infusion hindrances (Beate et al., 2009). Therefore, an experiment was set up to study whether different incubation times would have an impact on acrylamide reduction. Therefore, incubation times were therefore set up to 3 hours, 6 hours and 18 hours. As it showed in **Figure 5.7**, in all three roasted groups, acrylamide content for the enzyme treated group was lower than the control. Also, as the incubation time increased, the acrylamide reduction rate increased slightly.

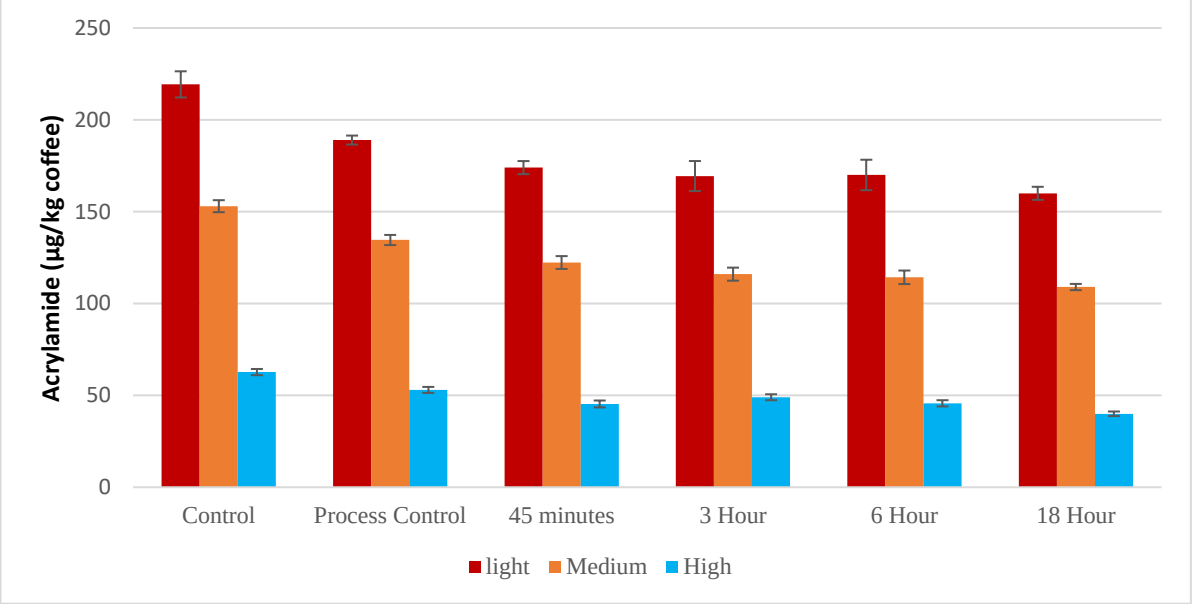

Figure 5.7 Acrylamide content by vacuum infusion enzyme process in process control and long incubation time groups. Acrylamide was calculated as µg/kg coffee (Acrylaway® Dosage-2000 U. Roast conditions: Light- 12 min at 230°C, Medium- 14 min at 240 °C, High- 17 min at 250°C) (n=3)

Statistical analysis was carried out where three factors were taken into consideration: enzyme, enzyme treatment and incubation time. As previously stated, enzyme factor focuses on the impact caused by an enzyme (process control vs treated) whereas enzyme treatment factor focused on the impact caused by the enzyme treatment (control vs treated). As it showed in **Table 5.4**, incubation time, enzyme and enzyme treatment factors all have shown significant impact for all three roast levels. Though the acrylamide reduction rate in different incubation time group did not have great difference, statistical

test showed long incubation time factor in vacuum enzyme treatment indeed had significant impact on acrylamide reduction rate

Table 5.4 Statistical analysis results for the long incubation experiment of coffee.

	Enzyme	Enzyme Treatment	Incubation time
Light	0.001	0.001	0.0243
Medium	0.0001	0.001	0.017
High	0.001	0.001	0.007

2000ASNU/kg of Acrylaway® were used. Light- 12 min at 230°C, Medium- 14 min at 240 °C, High- 17 min at 250°C. Confidence level set at 95% while the corresponding significance level was 0.05

In vacuum infusion method, after the vacuum step, the coffee beans were incubated in the water bath. Comparing to the steam & soak method, physical stir was applied during the incubation time. As there was no physical stir step applied in this experiment, once the vacuum was applied, the prolonged incubation time won`t help the enzyme solution to penetrate deeper into the coffee bean. Increased incubation time would only allow more sufficient contact between the enzyme and substrate where the enzyme solution had already penetrated. The slightly increased acrylamide reduction rate (20.66%-45 minutes to 27.28%-18 hours) indicated that the long incubation time leads to slightly more contact of enzyme with the substrate where the enzyme had penetrated within the coffee bean.

5.3.3 Vacuum infusion colour determination results

In order to reveal the impact of vacuum infusion method and long incubation time on the colour of roasted coffee beans, ΔE values between control and enzyme treated samples were calculated. In Chapter 2, high ΔE values showed that the steam & soak enzyme treatment caused the enzyme treated coffee beans to be roasted to a higher extent than the control group due to the silver skin loss. Therefore, if the vacuum infusion method caused less impact, the ΔE expected values should be lower than those observed for the conventional method.

Table 5.5 ΔE values for the steam & soak and vacuum infused coffee samples compared to their corresponding control groups.

ΔE	Steaming & Soaking			Vacuum Infusion		
	Light	Medium	High	Light	Medium	High
Process control	6.19	5.78	2.67	11.02	3.25	2.95
PreventASe® Dosage 1	10.16	4.86	2.31	12.62	4.05	3.47
PreventASe® Dosage 2	9.98	6.12	2.71	12.84	3.99	3.28
PreventASe® Dosage 3	10.04	5.54	2.61	13.15	3.97	3.55
Acrylaway® Dosage 1	10.42	6.07	2.67	11.12	2.89	1.34
Acrylaway® Dosage 2	10.22	6.01	3.04	12.03	3.14	2.26
Acrylaway® Dosage 3	10.39	5.98	1.92	12.52	3.48	2.46

Dosage: 1- 2000ASNU/kg or ASPU/kg, 2-4000 ASNU/kg or ASPU/kg, 3-6000 ASNU/kg or ASPU/kg. Light- 12 min at 230°C, Medium- 14 min at 240 °C, High- 17 min at 250°C. Confidence level set at 95% while the corresponding significance level was 0.05 (n=3)

As it showed in **Table 5.5,** the colour difference was reduced as the roast level increased. However, comparing the value for the two enzyme treatment methods, vacuum infusion processed samples had a slightly higher difference in light and medium roast at the same enzyme dosage, whereas in the high roast, the two methods processed samples had similar value at the same enzyme dosage. Statistical analysis showed that for all roast levels, enzyme brand and enzyme dosage had no significant impact and only the process method showed significant differences for all roast levels **(Table 5.5)**. Comparing the values of the two methods, the vacuum infusion processed samples had similar ΔE values to the steam & soaking method processed samples. Contrary to the hypothesis, vacuum infusion method failed to reduce the adverse impact on roasted coffee colour.

Table 5.6 Statistical analysis of the ΔE values in vacuum infused coffee samples

	Enzyme Brands	Enzyme Dosages	Process Method
light	0.645	0.898	0.001
medium	0.588	0.776	0.001
high	0.924	0.912	0.001

Dosage: 1- 2000ASNU/kg or ASPU/kg, 2-4000 ASNU/kg or ASPU/kg, 3-6000 ASNU/kg or ASPU/kg. Light- 12 min at 230°C, Medium- 14 min at 240 °C, High- 17 min at 250°C. Confidence level set at 95% while the corresponding significance level was 0.05

5.3.4 Long incubation experiment colour determination

Long incubation group and process control for the vacuum infusion samples were also tested. As it showed in **Table 5.7**, the ΔE values of the long incubation time group were higher than the vacuum infusion group in three roast levels. Also, the colour difference increased as the incubation time became longer. The 3 hours group had identical colour data to the 45 minutes enzyme treated samples. While the 6 hours and 18 hours had a significantly higher value (**Table 5.7**), indicating the longer incubation time increased the intensity of roasting. The incubation time factor had significant influences on the $\Delta \boldsymbol{E}$ values with a P value of 0.001.

The long incubation time group have shown that the impact on colour differences increase as the incubation time becomes longer. Though the acrylamide reduction rate increased with incubation time, the longer processing time also brought a greater colour difference. In the previous chapter, colour difference was caused by the silver skin loss. While in the long incubation experiment, though no physical forces were applied after vacuum process, the increased incubation time also caused the processed coffee had a higher colour difference under the same roast level.

Table 5.7 ΔE values of the vacuum infusion processed coffee samples compared to the control.

ΔE	**Vacuum Infusion**		
	Light	**Medium**	**High**
Process Control	11.02	3.25	2.95
45 minutes	11.12	2.89	1.34

3 hours	12.57*	4.51	4.41
6 hours	13.24*	4.64	4.56
18 hours	14.06*	4.89*	4.59

2000ASNU/kg of Acrylaway® were used. Light- 12 min at 230°C, Medium- 14 min at 240 °C, High- 17 min at 250°C. Confidence level set at 95% while the corresponding significance level was 0.05 *=p value<0.05 (n=3)

5.4 Discussion

As previously discussed in chapter 2, the adverse effect of steam & soak method was the loss of the silver skin and aroma precursors. So, rather than use steam to open the internal structure of coffee, a physical force could be applied to push the enzyme into the coffee beans. Both, vacuum and pressure methods were considered before carrying out this experiment (Beate et al., 2009). Therefore, vacuum infusion method experiment was carried out to reduce acrylamide while without causing the adverse effect of steam & soak enzyme treatment.

Acrylamide determination and colour determination experiment were carried out on vacuum infused samples to test the vacuum infusion method impact on silver skin loss,. In this experiment, 45 minutes incubation time was used to match the incubation time used in the previous steam & soak experiment. As the same dosage was applied while lower acrylamide reduction rate was achieved, the results indicated that the enzyme didn`t have sufficient contact with the substrate. Comparing to the Hendriksen`s experiment (Beate.Kornbrust, 2009), the major difference in the present study was the incubation time. The author stated that 18 hours were used to overcome the diffusion hindrance. However, other factors that may had affected the experiment results were not mentioned including coffee variety, vacuum pressure and enzyme solution volume used. Regarding incubation time, the importance of this parameter was in dispute. As steam & soak experiment results indicated, once the enzyme was pushed into the coffee beans, 45 minutes were enough for the enzyme reaction to happen. So, diffusion hindrance was considered to be related to the factors stated above rather than the incubation time.

For the parameters that were not mentioned in Hendriksen`s experiment, several factors raised doubts for the low acrylamide reduction rate in this experiment including coffee variety, vacuum pressure, enzyme solution volume and enzyme dosage. Firstly, coffee variety factor is related to the differences in coffee internal structure and therefore, differences in coffee bean cell wall have been shown to determine the difficulty for the enzyme to penetrate the cell wall (Flament & Bessière-Thomas, 2002). Secondly, vacuum pressure factor was related to the maximum physical power of vacuum machine. This difference decided the extent of contact between enzyme and substrate. Then, enzyme solution volume refers to the water solution that was used as enzyme courier. In the steam & soak experiment, 100ml was used for the soak process and although 100ml were absorbed by the coffee beans at the end of the process, the steam & soak still led to adverse effects like silver skin loss and aroma precursor loss. In the vacuum infusion experiment, however, as coffee was not steamed, 50 ml of water were used instead. In Hendriksen`s vacuum infusion experiment, the author stated the enzyme solution volume as proper amount but did not specify the volume. Finally, regarding the enzyme dosage, the author stated 2500-10000 ASNU/kg of Acrylaway® was used, but no specific value was stated for the acrylamide reduction achieved in the work.

As these factors were not discussed or stated by Hendriksen, it was difficult to reproduce his results. As incubation time was stated by the author as the key element to overcome the infusion hindrance and ensure the success acrylamide reduction, different incubation times were tested to examine its effect on acrylamide reduction rate. As the results showed, the acrylamide reduction rate increased from 21% in 45 minutes group to 28% in the 18 hours group. However, compared to the 49% reduction in the steam & soak method, even after the 18 hours this achievement in acrylamide reduction remained much lower. The results implied that though acrylamide reduction rate increased from 21% to 28% as incubation time increased, the contact of enzyme and substrate was still not as sufficient as it was in the steam & soak experiment.

Furthermore, there were significant differences in the colour values observed in the two experiments, with the vacuum infusion method having greater colour difference between control and treated samples. This result was contrary to the hypothesis that vacuum infusion could successfully reduce acrylamide while reduce the adverse effect of steam & soak process. As no steam process was used, the vacuum infusion process was predicted to have less impact on silver skin loss of the green coffee beans. Potential reasons could be the vacuum force which damaged the silver skin and made it easier

to separate from the coffee beans in the coffee bean roast process. In the long incubation time experiment, the ΔE value increased as the incubation time increased. As the increased incubation time was the only parameter that was changed compared to the previous experiment, the increased time for coffee beans soaked in the enzyme solution led to the higher loss of silver skin.

5.5 Conclusion

The objective of the experiments in this chapter was to test the vacuum infusion method acrylamide reduction efficiency and impact on the roasted coffee colour. The vacuum infusion method was supposed to achieve a better contact of enzyme and substrate with the extra physical force without causing the adverse effect of steam & soak method. As the results indicated, without prior steam process, the acrylamide results suggested that the vacuum power was not strong enough to deliver the enzyme to the core part of the coffee beans. Though the acrylamide content in enzyme treated samples was significantly different from the control sample, the reduction rate was not as good as the steam and soaking method. Besides, the vacuum infusion method caused higher impacts on the colour differences between control and treated groups. long incubation experiment was also carry out to overcome the diffusion hindrance as other researcher suggested. However, the long-time incubated samples still showed low acrylamide reduction rate compared to the steam & soak sample. Adversely, the darker colour of longer time treated samples was observed.

In conclusion, in this experiment, the vacuum infusion enzyme delivery method had a lower acrylamide reduction rate and similar or higher colour difference compared to the steam & soak method. Though vacuum infusion method in this experiment did not showed great acrylamide reduction rate as expected, it still provided another approach on enzyme delivery. With the optimised parameters, vacuum infusion method may achieve better acrylamide reduction rate as it showed in the literature.

Chapter 6 General Discussion

6.1 Introduction

This chapter is a summary of the research work that was carried out and an overview of potential future studies. It includes the key findings, the limitations and the implications of the results, suggestions for future work and the conclusions for this research.

6.2 Key Findings

Commercial asparaginase, PreventASe® from DSM (Heerlen, The Netherlands) and Acrylaway® from Novozymes A/S (Bagsvaerd, Denmark), successfully hydrolysed asparagine in green coffee beans. 3 Arabica and 2 Robusta Brazilian coffee was utilized in the experiment. Amino acid determination of enzyme-processed (2000 - 20000ASNU/kg coffee bean) green coffee beans showed a significant increase of aspartic acid (167% - 267%) and a significant decrease of asparagine (43% - 91%). Enzyme-treated coffee beans had significant lower acrylamide content (40% - 91%) compared to the untreated control group and it was observed that the percentage of acrylamide reduction was dependant on the factors of pre-treatment (steaming), enzyme dosage and roast level. In untreated beans, acrylamide contents decreased as roast level increased, confirming previously published results (Guenther et al., 2007; Lantz et al., 2006).The colour determination test showed that the enzyme-processed coffee was significantly darker. This could possibly be explained by the fact that the silver skin of green coffee beans was partially lost in the steaming and soaking process. Hence, the subsequent heat treatment resulted in the enzyme-treated coffee beans being comparatively darker than the control samples. Also, the sugar content loss in the enzyme treated green coffee beans showed another adverse effect of the steam & soak enzyme treatment. Regarding the differences between coffee species, Robusta coffee had higher asparagine content, hence more acrylamide was generated in the roasted coffee. Both enzyme had showed great acrylamide reduction in all coffee species. However, different enzymes had showed variations on the impact on the specific sensory attribute of the roasted coffee samples.

One of the aims of this study was to determine whether this enzymatic treatment affected the

organoleptic properties of coffee and therefore volatile compounds determination and sensory tests were carried out. Volatile compound analysis showed that the enzyme-processed coffee had similar aroma profile to the untreated coffee, with less than 10% of major volatile compounds (aldehydes, ketones, furans, phenols and pyrazines) being significantly different.

Furthermore, sensory evaluation of the enzyme treated coffee by means of discrimination tests found significant differences between enzyme-treated beans and untreated beans. In descriptive test, for 39 attributes tested in each roast level, 12 attributes were found to be significant different in light roast whereas 17 attributes were found to be significant different in medium roast. Preference tests, however showed that control and treated samples were of similar acceptability, with no unpleasant flavours or aromas associated with the treated beans.

As a result of the silver skin loss by the steam & soak method and in order to minimize the observed adverse effects, vacuum infusion method was tested. It was observed that although the new method could significantly reduce acrylamide, the reduction rate was significantly lower compared to the conventional method. In addition, vacuum infusion processed roasted coffee beans showed a higher colour difference between the control and treated group.

6.3 Limitations & Implications

For enzyme treatment, the major limitation comes from the internal structure of the coffee bean. Inside the green coffee bean, there is a nest-like microstructure with pores which have a diameter of around 10 nm. The microstructure of the coffee bean makes it very hard for enzyme solution to fully penetrate to the core part of the bean. In addition, asparaginase is a medium to large size enzyme with a diameter of 5-10 nm. Considering these two aspects, ensuring sufficient contact between enzyme and substrate was the major issue in the enzyme treatment.

Two enzyme treatment methods were used in this research. The initial method, which used a steaming and soaking process, achieved an acrylamide reduction rate ranging from 40% to 91%. This method was aiming at increasing the surface area of green coffee beans. However, adversely, aroma precursors like sugars were lost in the process. Furthermore, the silver skin was partially lost in the process thus the roasted enzyme-treated coffee was darker. Therefore, although steam & soak process achieved

high acrylamide reduction rate, it had an impact on the aroma precursors and silver skin which ultimately affected the volatile profile and sensory properties of the enzyme-treated coffee.

The two side effects of steam & soak process were beneficial in terms of acrylamide reduction. However, these side effects were harmful for maintaining the original sensory properties. Precursor loss which leads to the lower amount of asparagine in the enzyme treated samples. So, asparagine in the steamed green coffee bean was comparably lower than the control group. The roast level shift caused the treated samples to be roasted darker. As roast level was the dominant factor for acrylamide generation, higher roast level implied lower acrylamide content. So, in terms of acrylamide reduction, these two side effects had worked jointly on enhance acrylamide reduction efficiency.

However, for sensory properties and aroma profiles of the roasted coffee, the two factors all caused adverse effects. The precursor loss caused less precursor content in the enzyme processed green coffee bean which cause the significant difference for aroma profile in roasted coffee. Then, the roast level shift factor caused the treated coffee had sensory properties of a darker roast coffee. So, in terms of maintain the original flavour and taste, these two side effects made treated coffee significantly different from the control group.

In terms of the acrylamide reduction alone, current experiment design suited perfectly. Treated group compared with process control group showed the effect of enzyme alone while control group compared to the treated group showed the impact of whole enzyme treatment, include the impact from enzyme alone and impact from the process. Statistically analysis was carried out throughout the thesis using this logic. However, it had vital theoretical basis that every groups in this experiment was roasted to the same extent (no colour difference). Otherwise, the roast level factor had to be taken into consideration and the interaction between roast level factor and enzyme treatment (enzyme & process) factor needed to be considered. For acrylamide experiment, the roast levels (light, medium and high) was purposely selected to minimise the impact of roast level.

However, volatile compounds showed different variation at the different selected roast level. So, roast process control group needed to be introduced in the future study to illustrate the significant impact of roast level factor on volatile compounds. This roast process control group should use control green coffee then roast it to the exact same colour of the enzyme treated sample with minimal ΔE value. In

volatile compounds determination, comparing roast process control group to the enzyme treated group, statistical analysis would reveal the sole effect of enzyme treatment (steam & soak process) factor with minimum interference of roast level factor.

An alternative method to treat the coffee beans, vacuum infusion, was tested to overcome the adverse impacts of the steam & soak process. This method aimed to push the asparaginase inside the coffee beans by a vacuum force rather than opening the pore of the coffee bean (as seen with the steaming treatment). By delivering the enzyme this way, aroma precursors in coffee beans were unlikely to be lost in the process. The vacuum infusion method also aimed to reduce the loss of silver skin while maintaining the high acrylamide reduction rate. This method achieved a significant acrylamide reduction rate (12%-56%) but not to the extent of the steam & soak method (40%-91%). In addition, after roasting, the colour of the treated beans was still darker than that of the untreated beans. The minimal difference of acrylamide reduction rate between each enzyme dosage in the vacuum infusion method (comparing to steam & soak method) implied that the enzyme did not have sufficient contact with asparagine present in the coffee beans.

Regarding the interactions of sensory test results and volatile compounds determination experiment results, data analysis suggested the results were cross-referenced. For instance, in the discrimination test, the enzyme processed coffee was commented by the consumers as more acidic than the control. Then in the descriptive test, sour was a significant different attribute with treated coffee had higher scores. In volatile determination experiments, statistical data analysis showed acetic acid had significant differences. However, there were limitations for the data cross analysis. For instance, sensory attributes may not simply relate to one compound odour impression. The compounds that contribute to an attribute, some may increase while the roast level increase and some may decrease while the roast level increased. So, the GC-O test were recommended for future study. After the odour impression and the potency of the specific volatile compounds were identified, more connections between the volatile compounds and sensory properties of the coffee samples would be revealed.

6.4 Recommendations for Future Study

To summarize the experimental work, it can be concluded that asparaginase performed well at reducing the acrylamide content in coffee without causing major adverse effects. However how the enzymatic treatment is performed still needs some consideration.

In order to improve the enzyme delivery efficiency while reducing the adverse effects, several approaches could be investigated in the future. Modifying the current method would be the first approach. Admittedly, vacuum infusion experiment failed to achieve high acrylamide reduction rate like the steam & soak experiment. However, the vacuum infusion or pressure infusion method still might be an efficient approach if the asparaginase could be successfully pushed into the coffee beans. The second option would be crack the coffee beans before enzyme treatment, this approach aimed to increase the surface area of coffee beans. Because of the unique internal structure, if the coffee beans were ground to pieces, the surface area would be increased. However, this approach requires roast kinetic modification to generate the similar desirable flavour and taste like the conventional roast method for complete coffee beans.

Considering from the angle of the coffee manufacturer, coffee cultivar selection with lower asparagine and blend of Arabica/Robusta would be the simplest way to control the acrylamide content presented in the roasted coffee. However, for asparaginase approach, if only the steam & soak method had to be utilized to meet the acrylamide reduction target, reducing the roast time and temperature for the enzyme treated coffee would be the most cost-effective solution. In this way, the roast shift for the enzyme treatment would be mitigated. However, the difference caused by the enzyme treatment would still be sensed by the consumers.

6.5 Conclusion

The enzymatic approach had shown great acrylamide mitigation rate in coffee. However, the enzyme delivery method physically damaged the coffee beans and this change caused the enzyme-treated coffee beans to be roasted to a higher degree under the same heat treatment which impacted in the colour of the enzyme treated beans becoming darker as well as significantly altering the aroma volatile

profile. Furthermore, the organoleptic properties of the roasted coffee beans showed significant differences when compared to the control. Though no unpleasant flavour was reported from the preference test, future studies should aim at minimizing the adverse effect of enzyme treatment process. To achieve this goal, more efficient enzyme delivery method needs to be developed.

Regarding the future of acrylamide research, legislation has played an important role in forcing food manufacturers to look for methods to reduce the acrylamide content in foods including the enzymatic treatment. In Feb of 2017, the European Commission announced plans to set the maximum levels for acrylamide in ready-to-eat foods (Michail, 2017) with the proposal being submitted in June that year. As a result, in July 2017 new benchmark levels were set for the acrylamide in a variety of foods. For coffee, the benchmark level in roast coffee was set to 400 µg/kg while the value in instant coffee had been set to 850 µg/kg (EFSA, 2017). Comparing to the indicative value established in 2013, 450 µg/kg and 800 µg/kg for roast coffee and instant coffee, respectively, both values had reduced 50 µg/kg. As the government and consumers have started to pay more attention to the risks of dietary acrylamide, asparaginase could play a vital role for mitigating acrylamide in a variety of foods in the future.

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Appendix

Appendix I Published Paper

Appendix II Title of oral and poster presentations in conferences

- **The use of asparaginase to reduce acrylamide levels in cooked food**

  Poster Presentation at 14th Weurman Flavour Research Symposium
  Queen`s College, Cambridge
  2014

- **Supported Liquid Extraction-New, Rapid Technique for the Analysis of Acrylamide in Roasted Coffee**

  Poster Presentation at 2ND Nursten Symposium
  University of Nottingham, Nottingham
  2014

- **Chemical and Sensory Analysis of Asparaginase-Treated Brazilian Coffee**

  Oral Presentation at 4th Nursten Symposium
  University of Reading, Reading
  2016

Appendix III University of Reading Research Ethics Committee Study Evaluation-Study Number 41/15 for Preliminary Discrimination Test

SCHOOL OF CHEMISTRY, FOOD AND NUTRITIONAL SCIENCES & PHARMACY

ETHICS APPLICATION INTERNAL REVIEW (FORM SFBE2 version 4**)**

HEAD OF DEPARTMENT APPROVAL

Approval for projects which are exempt from requirement for full submission to the University Ethics and Research Committee

Title of Project: **Tasting of enzyme treated coffee.**

Investigator(s): **Dr Lisa Methven, Dr Jose Oruna Concha, Dr Steve Elmore, Fei Xu**

Department: **Food and Nutritional Sciences**

1. Has this application been read by your Supervisor/PI? (where applicable)

YES ☑

NO ☐

NOT APPLICABLE ☐

2. Has your Group Internal Reviewer read the application and any suggested revisions been undertaken?

YES ☐

NO ☐

This project has been subject to the SFB Internal Ethics Review procedure and is allowed to proceed.

Internal Reviewer Signature ……. Date …….

Head of Department Signature …… Date……

Ethics Administrator Signature …… Date……

SFBE2/v3/29.10.08

Once this form is signed, a copy must be given to C. Ashford (FBS 1.05) for record keeping

School of Chemistry, Food and Nutritional Sciences and Pharmacy

Research Ethics Committee

Application Form

SECTION 1: APPLICATION DETAILS

1.1

Project Title: Tasting of enzyme treated coffee

Date of Submission: 26th November 2015 Proposed start date: 7th December 2015 Proposed End Date: 1st November 2016

1.2

Principal Investigator: Dr Lisa Methven

Food and Nutritional Sciences, office room number: 2-65

Internal telephone: 8714 / alternative contact telephone:

Email address:

Other Investigators:

Dr Steve Elmore

Food and Nutritional Sciences, office room number: 4-26

Email:j.s. Telephone:

Dr Maria Jose Oruna-Concha

Food and Nutritional Sciences, office room number: 2-16

Email:m k Telephone:

Fei Xu Email

Mateusz Gertchen Email:

….

1.3**Project Submission Declaration**

I confirm that to the best of my knowledge I have made known all information relevant to the Research Ethics Committee and I undertake to inform the Committee of any such information which subsequently becomes available whether before or after the research has begun.

I understand that it is a legal requirement that both staff and students undergo Criminal Records Checks when in a position of trust (i.e. when working with children or vulnerable adults).

I confirm that a list of the names and addresses of the subjects in this project will be compiled and that this, together with a copy of the Consent Form, will be retained within the School for a minimum of five years after the date that the project is completed.

Signed…… (Principal Investigator) Date…

…… (Student) Date…

…… (Other named investigators) Date…

…… (Other named investigators) Date…

1.4

University Research Ethics Committee Applications

Projects expected to require review by the University Research Ethics Committee must be reviewed by a member of the School research ethics committee and the Head of School before submission.

Signed…… (Chair/Deputy Chair of School Committee) Date…

Signed…… (Head of Department) Date…

Signed…… (SCFP Ethics Administrator) Date…

SECTION 2: PROJECT DETAILS

2.1

Lay summary

Acrylamide is an industrial chemical used in the production of polymers; it is categorised as a potential carcinogen. On 24th April 2002, the University of Stockholm and Swedish National Food Administration published a report showing levels of acrylamide in certain fried and baked foods at levels up to 3000 ppb which is six times of the max limit set by WHO (Stadler et al., 2002). Later that year, the result was confirmed by the UK Food Standards Agency. Work carried out at University of Reading in the same year showed that acrylamide was formed naturally (Mottram, Wedzicha, & Dodson, 2002) when the free amino acid asparagine reacts with reducing sugars via the Maillard reaction, a well-known reaction responsible for the aroma, colour and taste of heated products. Asparaginase, which hydrolyses asparagine into aspartic acid and ammonia, has been used to mitigate acrylamide in food products with good results (Hendriksen, Kornbrust, Ostergaard, & Stringer, 2009). Coffee undergoes a roasting process which generates Maillard products, including acrylamide. In this study, Brazillian coffee has been treated by different dosages of asparaginase enzyme and then roasted to three different levels (Light, Medium and High).

Project Objectives:

The aims of this project includes to: (1) determine whether there is a discriminable difference in sensory perception between the control coffee and enzyme-treated coffee samples and (2) determine the threshold at which the enzyme dosage is effective in the coffee.

Reference:

Hendriksen, H. V., Kornbrust, B. A., Ostergaard, P. R., & Stringer, M. A. (2009). Evaluating the Potential for Enzymatic Acrylamide Mitigation in a Range of Food Products Using an Asparaginase from Aspergillus oryzae. *J Agric Food Chem, 57*(10), 4168-4176. doi:10.1021/jf900174q

Mottram, D. S., Wedzicha, B. L., & Dodson, A. T. (2002). Acrylamide is formed in the Maillard reaction. *Nature, 419*(6906), 448-449. doi:10.1038/419448a

Stadler, R. H., Blank, I., Varga, N., Robert, F., Hau, J., Guy, P. A., Riediker, S. (2002). Acrylamide from Maillard reaction products. *Nature, 419*(6906), 449-450. doi:10.1038/419449a

2.2

Procedure

Upon arrival at the Food and Nutritional Sciences Department in the University of Reading, informed consent (Appendix C) will be taken from participants by a trained researcher before any measurements are taken. All sensory testing will be performed in individual sensory booths within an environmentally-controlled sensory laboratory. After taken the demographic information of consumers, the smelling test and tasting tests will begin.

1. Smelling evaluation using Tetrad Discrimination Tests

Coffee will be brewed (200g 100℃ water mix with 15g of coffee powder, 5 minutes) then stored in the thermo insulated flasks. The brewed coffee will be poured to brown glass bottles with lids. The coffee will be prepared without milk or sugar. In each evaluation set four samples of coffee will be given and the consumers will be asked to put them into two groups of identical samples. In total 6 sets of coffee will be given to the consumers as follows. Consumers will be asked to open the lids, smell the headspace and then close the lid.

Control	Control	Dosage 1	Dosage 1
Control	Control	Dosage 2	Dosage 2
Control	Control	Dosage 3	Dosage 3
Dosage 1	Dosage 1	Dosage 2	Dosage 2
Dosage 2	Dosage 2	Dosage 3	Dosage 3
Dosage 3	Dosage 3	Dosage 1	Dosage 1

Presentation orders will be balanced.

The tetrad methodology has been chosen as it has more statistical power than other discrimination tests; less assessors are needed to find a small perceptual difference compared to other non-directional (ie no attribute) discrimination tests (Ennis & Jesionka, 2011). Results from the tetrad tests will be analysed using Thurstonian modelling using V-power software (Jesionka, Rousseau, & Ennis, 2014) which considers the probability of guessing correctly in each individual test (1:3) as well as both type I and type II error, it also calculates the proportion of discriminators and uses this to calculate d' (a value indicating the extent of difference between samples which can be used to compare between tests).

2. Tasting evaluation using Same-Different Tests

As coffee samples have a strong bitter taste, which causes sensory adaptation, it is not possible to carry out the same tetrad methodology as above for the tasting test. Therefore a simpler same/different discrimination test will be used with a surety index. In tasting session, coffee will also be brewed (200g 100℃ water mix with 15g of coffee powder, 5 minutes) then stored in the thermo insulated flasks. The brewed coffee will be poured to black tasting cups. The coffee will be prepared without milk or sugar.

After the smelling test, consumers will be given only two sets of coffee to taste test, each containing two samples; control versus dosage 1 (Lowest dosage) and control versus dosage 3 (Highest dosage).. Milk will be used as palate cleanser between each test. The order of the pairs presented as well as the order within the pairs will be balanced. Consumers will be asked if

the two coffee samples taste the same or different. After each set, consumers will be asked to score their sureness of their decision :

1	Very Unsure
2	Unsure
3	Sure
4	Very Sure

The data will be analysed using Thurstonian modelling as above, however the surety index will be incorporated within the analysis as previously used by Delime (Delime, 2015).

Each consumer will attend the Sensory Unit for one session, they will first complete the smelling test followed by the tasting test where they will consume a maximum of 8g coffee. In order to minimise carryover effects, milk will be provided as palate cleansers. All the researchers have Level 2 Food Hygiene Certificates. All coffee bean samples were prepared in Brazil by the researcher (Fei Xu) under conditions of good manufacturing practise, preparation of the liquid coffee will be done by the researcher within the Sensory Science Centre here in Reading, again under conditions of good manufacturing practise. The enzyme used in the experiment is Acrylaway. It is a highly specific commercial food grade asparaginase enzyme produced by Novozymes. Acrylaway has received the "generally recognized as safe" status from the US government and has been given a favorable evaluation by the Joint FAO/WHO Expert Committee on Food Additives (JEFCA, 2007). It has also been given a favourable evaluation as a food additive by the Joint FAO/WHO Expert committee (JEFCA, 2007). It is currently used in several countries, including United States, Australia, New Zealand, China, Russia, Mexico and several European countries.

Asparaginase hydrolyses asparagine into aspartic acid and ammonia. Traceable amounts of product will be generated from the reaction, however, neither compounds hasan adverse effect on human health. The optimum temperature for Acrylaway is 60℃. Above 90 ℃, the enzyme will be completely inactivated. The coffee has undergone a roasting procedure after enzymatic treatment where all the enzyme has been completely inactivated and therefore it is safe to use it in food.

Coffee sourced from local premium dealer in Brazil have been processed in a clean food pilot plant in Embrapa, Brazil. Roasted coffee was packaged in sealed plastic bags and transported to the UK by plane. After arrival, the samples have been stored in the processing plant in the Food and Nutritional Science Department at 4℃. Samples will be prepared one hour before the sensory test in the sensory kitchen. They will be kept in the thermo flasks before serving.

2.3

Where will the project take place?

Sensory Science Centre, Food and Nutritional Sciences Department, Whiteknights Campus, University of Reading.

2.4

Funding

Is the research supported by funding from a research council or other *external* sources (e.g. charities, business)? No

2.5

Ethical Issues

Could this research lead to any risk of harm or distress to the researcher, participant or immediate others? Please explain why this is necessary and how any risk will be managed.

Potential consumers with relevant food allergies will be excluded from study entry. All of the food samples that will be provided were produced under good manufacturing practise in Embrapa, Brazil then safely transported and stored. Food grade enzyme has been completely inactivated during the coffee roasting procedure. Traceable product of enzyme reaction will cause no harm to human health. These coffee samples have already been tested in sensory test in Brazil. All samples will be prepared under food-safe conditions. Consumers are free to withdraw from the study at any time.

2.6

Deception

Will the research involve any element of intentional deception at any stage (i.e. providing false or misleading information about the study, or omitting information)?

NO

2.7

Payment

Will you be paying your participants for their involvement in the study?

No

2.8

Data protection and confidentiality

What steps will be taken to ensure participant confidentiality? How will the data be stored?

Volunteers will be given a participant number, and throughout data collecting will be referred to by this number. Data linking the name of the participant to their participant number will be kept in a locked filing cabinet in a locked office of the Department of Food and Nutritional Sciences.

2.9

Consent

Please describe the process by which participants will be informed about the nature of the study and the process by which you will obtain consent

Once potential participants have responded to a study advertisement, or if they have already consented to be on our volunteer database, then they will be contacted by email, phone or post. They will be given an information sheet about the study. Informed consent will be taken by a trained researcher prior to study entry (Appendix C). The signed consent forms will be stored in a locked cabinet for a 5-year period.

2.10

Genotyping

Are you intending to genotype the participants? Which genotypes will be determined?

N/A

SECTION 3: PARTICIPANT DETAILS

3.1

Sample Size

How many participants do you plan to recruit? Please provide a suitable power calculation demonstrating how the sample size has been arrived at or a suitable justification explaining why this is not possible/appropriate for the study.

The aim is to recruit 120 volunteers into the study. This is based on power calculations by V-power (Ennis & Jesionka, 2011) where for a relatively small sensory difference between two samples (d'=1) 81 assessors are needed to find a significant difference using a tetrad test at 90% power. Assuming that the same-different test with the surety index has similar power to a directional 2AFC test then only 31 assessors would be needed to find this difference (at 90% power) in the tasting tests.

3.2

Will the research involve children or vulnerable adults (e.g. adults with mental health problems or neurological conditions)?

No.

3.3

	Will your research involve children under the age of 18 years? No Will your research involve children under the age of 5 years? No
3.4	Will your research involve NHS patients, Clients of Social Services or will GP or NHS databases be used for recruitment purposes? Yes/No (delete) No
3.5	**Recruitment** Adults aged 18 to 65 years will be recruited via posters (Appendix E) placed around the University of Reading and the local area. Within the university, email circulation lists will be used to circulate the study email. Potential volunteers already consented to our volunteer database will be contacted directly via email or telephone. Social media websites focusing on the local area (eg Facebook groups) will also be used to place the study poster.

Important Notes

1. The Principal Investigator must complete the Checklist in Appendix A to ensure that all the relevant steps and have been taken and all the appropriate documentation has been appended.

2. If you expect that your application will need to be reviewed by the University Research Ethics Committee you must also complete the Form in Appendix B.

3. For template consent forms, please see Appendices C.

Appendix A: Application checklist

This must be completed by an academic staff member (e.g. supervisor)

Please tick to confirm that the following information has been included and is correct.

Indicate (N/A) if not applicable:

Information Sheet

Is on headed notepaper ☑

Includes Investigator's name and email / telephone number ☑

Includes Supervisor's name and email / telephone number ☑

Statement that participation is voluntary ☑

Statement that participants are free to withdraw their co-operation ☑

Reference to the ethical process ☑

Reference to Disclosure
N/A ☑

Reference to confidentiality, storage and disposal of
personal information collected ☑

Consent form(s) ☑

Questionnaires
☐ N/A ☑

Advertisement/leaflets ☑
N/A ☐

Letters
☐ N/A ☑

Other (please specify) ☐
N/A ☑

Expected duration of the project 12

(months)

Name (print) ……Lisa Methven…… Signature ……

Appendix C

COFFEE TASTING STUDY

Consent Form

Investigators:

Dr Maria Jose Oruna-Concha, Dr Steve Elmore, Dr Lisa Methven

Study researchers:

PhD Student: Fei Xu Email:f.xu@pgr.reading.ac.uk

Visiting Student: Mateusz Gertchen Email: M.G.Gertchen@reading.ac.uk

Department Address:

Department of Food and Nutritional Sciences, University of Reading, PO BOX 226, Whiteknights, Reading, RG6 6AP.

Information Regarding Products Used in this study:

In this study, you will be asked to taste a number of Brazilian coffees. Please inform the researcher if you are suffering from any medical conditions that may affect your smell and taste. Besides, as coffee and milk will be used in the test, consumers that have allergy to coffee and milk are not eligible for this test.

All data generated in this study are anonymous. You will not be identified from your answers and you are free to withdraw from this study at any time. If you wish to withdraw from the study, please let any of our researchers know. You can withdraw by using any of the contact details listed on this information sheet, such as telephone, email or personal communication.

All information and data will be handled in a confidential manner. Please note that this study has been ethic reviewed by the school ethic committee and conduct under the permission of the committees.

Consent

Please tick the box below if you agree with the statement below

I have read the information above relating to this study. I agree to the arrangements described in the Information Sheet so far as they relate to my participation.

☐

I understand that participation is entirely voluntary and that I have the right to withdraw from the project any time.

☐

Name:

Signed:

Date:

I have had explained to me that consent for my contact details and personal information to be added to the Sensory Science Centre Database of Tasting volunteers is entirely voluntary. Accordingly I consent as indicated below:

I consent to my contact details being stored on the Sensory Science Centre Volunteer Database :
Yes / No (delete either Yes or No)

Screening Questionnaire

Please answer the following questions and return to Fei Xu as soon as possible. You will be notified directly on your suitability to take part in the practical sessions. All information will be treated in a confidential manner.

Name: Age: Gender:

Contact details (address or email):

Telephone Number:_____

Do you have any food allergy? For example milk etc. if YES, please specify.

YES/NO

If Yes, please state type of allergy:.....

Do you have any food intolerance? For example lactose.

YES/NO

If Yes, please state type of allergy:.....

Do you have any coffee (e.g. caffeine) allergies?

YES/NO

Do you have any medical condition(s) which may affect your food intake?

YES/NO

If Yes, please specify:.....

Are you taking any medication or do you have any medical condition(s) which may affect your sense of smell?

YES/NO

If Yes, please specify:.....

Are you taking any medication or do you have any medical condition(s) which may affect your sense of taste?

YES/NO

If Yes, please specify:.....

Thank you very much for your time.

Name:

Signed:

Date:

Appendix D

Volunteer Information Sheet

Coffee Tasting

Investigating the difference in sensory properties of coffee that has been roasted with and without enzyme treatment

You have been invited to take part in a short study at the Sensory Science Centre, Department of Food and Nutritional Sciences, School of Chemistry, Food and Pharmacy, University of Reading. Before you decide whether you wish to take part in the study, please read the following information carefully. If you want to know anything about the study, which is not written here, please ask the investigator/researcher.

Introduction

This study aims to find out whether coffee tastes differently depending on whether it has been treated with an enzyme before roasting. Acrylamide is a carcinogen that widely exists in coffee, potato crisps and chips and other heat treated foods. The aim within food manufacturing is to minimise levels of this compound in food. Acrylamide is formed when the naturally occurring amino acid, asparagine, is heated. If the enzyme, asparaginase, is applied to green coffee beans before they are roasted, this converts asparagine into harmless aspartic acid and ammonia. Therefore, acrylamide formation is reduced.

In this study you will be presented with different coffee samples to smell and taste and asked questions such as "are these samples the same or different".

Am I suitable to take part?

Please read the following exclusion criteria to identify if you are able to participate in the trial.

Exclusion Criteria - If any of the following condition apply to you, you will be unable to participate in the trial:

- You have had surgery or suffered from a medical condition in the previous 12 months affecting your ability to taste or smell food
- You have a known and relevant food allergy
    1. (e.g. milk protein allergies) or intolerance (e.g. lactose)
    2. Coffee allergy (caffeine allergy)
- You use a medication likely to affect taste, smell or appetite
- Your age is below 18 or above 65 years

If you are eligible based on the above criteria you will be asked to sign a Consent Form.

What will the study involve?

The study will involve tasting and smelling coffee samples. You will be asked whether you think samples are different from each other or not.

Do I have to modify my diet or other activities in any way?

No changes to your normal diet or everyday activities will be imposed by the study

Are there any risks of being a volunteer in this study?

No

Confidentiality

Your records will be kept strictly confidential and all information and data will be kept in locked cabinets and on password-protected computers. Information obtained from the study may be published in scientific journals but in the form of average group values. No identifiable individual results will be used, published or presented in scientific meetings. Your records will be kept by the investigators for five years. However, these records will

only contain an identification number and code while information matching volunteer names with identification numbers/codes will be kept separately from these files within the School of Food, Chemistry and Pharmacy.

General

- This study has been subject to ethical review by the University Research Ethics Committee and has been given a favourable ethical opinion for conduct.
- Your participation in this study is purely voluntary. You may leave the study at any time without giving a reason. You are free to ask the investigator for more information about this study before you give your consent to take part.

Investigators

Dr Maria Jose Oruna-Concha

Email:

Dr Steve Elmore

Telephone:

Email

Dr Lisa Methven

Telephone:

Email:l.

PhD Student: Fei Xu

Department of Food & Nutritional Sciences

University of Reading E-mail: f.xu@pgr.reading.ac.uk

P.O. Box 226, Reading

RG6 6AP

If you have any queries please do not hesitate to contact us.

Appendix E

Advertisement

We are looking for healthy, non-smoking, men and women aged 18-65 years to **taste Brazilian Coffee**

The study involves attending the Sensory Science Centre at the University of Reading for a 30 minute visit on one occasion. You will be asked to taste and smell a range of coffee samples and answer questions asking whether the coffee samples are the same or different.

Please note that sufferers of **milk or coffee allergies** are not eligible for this study

If you would like more information, please contact:

Fei Xu

E-mail:

Are you a coffee-lover?

Fancy tasting some authentic Brazilian coffee?

Why not join our test?

Are you aged 18-65, a non- smoker and available for half an hour?

If yes, then come have a taste of different types of Brazilian coffee at:

Sensory Science Centre, Room 2.23, the Department of Food and Nutritional Sciences, University of Reading. (15:00-18:00 Mon & Wed)

For more information, please contact us by email:

Appendix IV University of Reading Research Ethics Committee Study Evaluation-Study Number 20/16 for Major Discrimination Test, Descriptive Test, Preference Test.

SCHOOL OF CHEMISTRY, FOOD AND NUTRITIONAL SCIENCES & PHARMACY

ETHICS APPLICATION INTERNAL REVIEW (FORM SFBE2 version 4**)**

HEAD OF DEPARTMENT APPROVAL

Approval for projects which are exempt from requirement for full submission to the University Ethics and Research Committee

Title of Project: **Tasting of enzyme treated coffee by Trained Panel and Consumers.**

Investigator(s): **Dr Lisa Methven, Dr Jose Oruna Concha, Dr Steve Elmore, Fei Xu**

Department: **Food and Nutritional Sciences**

1. Has this application been read by your Supervisor/PI? (where applicable)

YES ☑

NO ☐

NOT APPLICABLE ☐

2. Has your Group Internal Reviewer read the application and any suggested revisions been undertaken?

YES ☐

NO ☐

This project has been subject to the SFB Internal Ethics Review procedure and is allowed to proceed.

Internal Reviewer Signature ……. Date ……..

Head of Department Signature …… Date……

Ethics Administrator Signature …… Date……

SFBE2/v3/29.10.08

Once this form is signed, a copy must be given to C. Ashford (FBS 1.05) for record keeping

School of Chemistry, Food and Nutritional Sciences and Pharmacy

Research Ethics Committee

Application Form

SECTION 1: APPLICATION DETAILS

1.2

Project Title: Tasting of enzyme treated coffee

Date of Submission: 8th September 2016 Proposed start date: 14th September 2016 Proposed End Date: 1st November 2017

1.2

Principal Investigator: Dr Lisa Methven

Food and Nutritional Sciences, office room number: 2-65

Internal telephone: 8714 / alternative contact telephone:

Email address:

Other Investigators:

Dr Steve Elmore

Food and Nutritional Sciences, office room number: 4-26

Email: Telephone:

Dr Maria Jose Oruna-Concha

Food and Nutritional Sciences, office room number: 2-16

Email Telephone:

Fei Xu Email:

….

1.3

Project Submission Declaration

I confirm that to the best of my knowledge I have made known all information relevant to the Research Ethics Committee and I undertake to inform the Committee of any such information which subsequently becomes available whether before or after the research has begun.

I understand that it is a legal requirement that both staff and students undergo Criminal Records Checks when in a position of trust (i.e. when working with children or vulnerable adults).

I confirm that a list of the names and addresses of the subjects in this project will be compiled and that this, together with a copy of the Consent Form, will be retained within the School for a minimum of five years after the date that the project is completed.

Signed…… (Principal Investigator) Date…

…… (Student) Date…

…… (Other named investigators) Date…

…… (Other named investigators) Date…

1.4

University Research Ethics Committee Applications

Projects expected to require review by the University Research Ethics Committee must be reviewed by a member of the School research ethics committee and the Head of School before submission.

Signed…… (Chair/Deputy Chair of School Committee) Date…

Signed…… (Head of Department) Date…

Signed…… (SCFP Ethics Administrator) Date…

SECTION 2: PROJECT DETAILS

2.1

Lay summary

Acrylamide is an industrial chemical used in the production of polymers; it is categorised as a potential carcinogen. On 24th April 2002, the University of Stockholm and Swedish National Food Administration published a report showing levels of acrylamide in certain fried and baked foods at levels up to 3000 ppb which is six times of the max limit set by WHO (Stadler et al., 2002). Later that year, the result was confirmed by the UK Food Standards Agency. Work carried out at University of Reading in the same year showed that acrylamide was formed naturally (Mottram, Wedzicha, & Dodson, 2002) when the free amino acid asparagine reacts with reducing sugars via the Maillard reaction, a well-known reaction responsible for the aroma, colour and taste of heated products. Asparaginase, which hydrolyses asparagine into aspartic acid and ammonia, has been used to mitigate acrylamide in food products with good results (Hendriksen, Kornbrust, Ostergaard, & Stringer, 2009). Coffee undergoes a roasting process which generates Maillard products, including acrylamide. In this study, Brazillian coffee has been treated by different dosages of asparaginase enzyme and then roasted to three different levels (Light, Medium and High).

Project Objectives:

The aims of this project includes to: (1) determine whether there is a discriminable difference in sensory perception between the control coffee and enzyme-treated coffee samples

2.2

Procedure

Upon arrival at the Food and Nutritional Sciences Department in the University of Reading, informed consent (Appendix C) will be taken from participants by a trained researcher before any measurements are taken. All sensory testing will be performed in individual sensory booths within an environmentally-controlled sensory laboratory. After taken the demographic information of consumers, the smelling test and tasting tests will begin.

The coffee used in this study has been obtained as green coffee beans from Rave Coffee (an UK green coffee beans dealer in Cirencester). All the researchers have Level 2 Food Hygiene Certificates. All coffee bean samples were prepared in Pilot Plant by the researcher (Fei Xu) under conditions of good manufacturing practise, preparation of the liquid coffee will be done by the researcher within the Sensory Science Centre here in Reading, again under conditions of good manufacturing practise. The HACCP plan for coffee processing is also attached (Appendix E). The enzymes used in the experiment are Acrylaway & Preventase. Acrylaway is a highly specific commercial food grade asparaginase enzyme produced by Novozymes. Acrylaway has received the “generally recognized as safe” status from the US government and has been given a favorable evaluation by the Joint FAO/WHO Expert Committee on Food Additives (JEFCA, 2007). It has also been given a favourable evaluation as a food additive by the Joint FAO/WHO Expert committee (JEFCA, 2007). It is currently used in several countries, including United States, Australia, New Zealand, China, Russia, Mexico and several European countries. Preventase is also an extracellular asparaginase produced by DSM that also been cloned from GRAS (generally recognized as safe) fungi which are commonly used for industrial enzyme production.

Asparaginase hydrolyses asparagine into aspartic acid and ammonia. Traceable amounts of product will be generated from the reaction, however, neither compounds has an adverse effect on human health. The optimum temperature for Acrylaway is 60℃. Above 90 ℃, the enzyme will be completely inactivated. The coffee has undergone a roasting procedure (light roast 230℃-12mins & Medium Roast 240℃-14mins) after enzymatic treatment where all the enzyme has been completely inactivated and therefore it is safe to use it in food.After processing, the samples have been vacuum packed, then stored in the processing plant in the Food and Nutritional Science Department at 4℃. Samples will be prepared one hour before the sensory test in the sensory kitchen. They will be kept in the thermos flasks before serving.

Stage 1: Sensory Profiling with Trained Expert Panel

Coffee will be brewed (200g 100℃ water mix with 15g of coffee powder, 5 minutes) then stored in the thermo insulated flasks. The trained expert panel employed by MMR will taste (and swallow) the coffee samples in order to create a sensory profile of the products. They will taste up to 6 products per day and up to a maximum of 50 mL of each product.

The procedure is our standard tasting procedure. Panelists first develop a vocabulary as a consensus by tasting the product and comparing it to reference standards. Once they have agreed a vocabulary then the score the products (up to 5 per day) in individual isolated tasting booths and rate the intensity of the attributes on unstructured line scales.

Stage 2: Discrimination Testing and Liking Tests with Consumers

Each consumer will attend the Sensory Unit for two tasting sessions.

Following the sensory profiling we will carry out sniffing and tasting experiments with untrained consumers. This will be to determine whether consumers can detect differences between the control and treated products, and to see which products they prefer.

The Discrimination methods are the same as used in our previous approved study on enzyme treated coffee (Study number 41/15), so we have only briefly described these methods again here.

1. Smelling evaluation using Tetrad Discrimination Tests

Coffee will be brewed as above. The brewed coffee will be poured to brown glass bottles with lids. In each evaluation set four samples of coffee will be given and the consumers will be asked to put them into two groups of identical samples. In total 6 sets of coffee will be given to the consumers. Consumers will be asked to open the lids, smell the headspace and then close the lid. Presentation orders will be balanced.

The tetrad methodology has been chosen as it has more statistical power than other discrimination tests; less assessors are needed to find a small perceptual difference compared to other non-directional (ie no attribute) discrimination tests (Ennis & Jesionka, 2011).

2. Tasting evaluation using Same-Different Tests

As coffee samples have a strong bitter taste, which causes sensory adaptation, it is not possible to carry out the same tetrad methodology as above for the tasting test. Therefore a simpler same/different discrimination test will be used with a surety index. After the smelling test, consumers will be given 4 sets of coffee to taste test, each containing two samples. Milk will be used as palate cleanser between each test. The order of the pairs presented as well as the order within the pairs will be balanced. Consumers will be asked if the two coffee samples taste the same or different. After each set, consumers will be

asked to score their sureness of their decision. The data will be analysed using Thurstonian modelling as above, however the surety index will be incorporated within the analysis as previously used by Delime (Delime, 2015).

3. **Liking Tests**

Depending on the results of the sensory profiling experiment, we will either carry out a liking rating on a series of coffee samples, or carry out a paired preference test. If there are a range of significant differences in attributes between coffee samples then liking ratings will be carried out. If the differences between sample attributes are mostly non-significant then the two processing extremes (control vs highest dosage and highest roasting condition) will be compared in a preference test.

In a liking test: Consumers will be presented with up 6 coffee samples in a balanced presentation order and asked to rate their liking on a 9 point category scale from "Dislike Extremely" (1) to "Like Extremely" (9).

In a paired preference test: Consumers will be presented with two samples together and asked which they prefer.

2.3

Where will the project take place?

Sensory Science Centre, Food and Nutritional Sciences Department, Whiteknights Campus, University of Reading.

2.4

Funding

Is the research supported by funding from a research council or other *external* sources (e.g. charities, business)? No

2.5

Ethical Issues

Could this research lead to any risk of harm or distress to the researcher, participant or immediate others? Please explain why this is necessary and how any risk will be managed.

Potential consumers with relevant food allergies will be excluded from study entry. All of the food samples that will be provided were produced under good manufacturing practise in Pilot Plant then safely vacuumed and stored in fridge. Food grade enzyme has been completely inactivated during the coffee roasting procedure. Traceable product of enzyme reaction will cause no harm to human health. All samples will be prepared under food-safe conditions. Consumers are free to withdraw from the study at any time.

2.6

Deception

Will the research involve any element of intentional deception at any stage (i.e. providing false or misleading information about the study, or omitting information)?

NO

2.7

Payment

Will you be paying your participants for their involvement in the study?

The sensory panel are paid employees of MMR, they will not receive additional payment for this study. The Consumers will be paid £5 on attending their second visit.

2.8

Data protection and confidentiality

What steps will be taken to ensure participant confidentiality? How will the data be stored?

Volunteers will be given a participant number, and throughout data collecting will be referred to by this number. Data linking the name of the participant to their participant number will be kept in a locked filing cabinet in a locked office of the Department of Food and Nutritional Sciences.

2.9

Consent

Please describe the process by which participants will be informed about the nature of the study and the process by which you will obtain consent

The trained panel will be asked to sign the consent form (Appendix C)

Consumers : Once potential participants have responded to a study advertisement, or if they have already consented to be on our volunteer database, then they will be contacted by email, phone or post. They will be given an information sheet about the study. Informed consent will be taken by a trained researcher prior to study entry (Appendix C). The signed consent forms will be stored in a locked cabinet for a 5-year period.

2.10

Genotyping

Are you intending to genotype the participants? Which genotypes will be determined?

N/A

SECTION 3: PARTICIPANT DETAILS

3.1

Sample Size

How many participants do you plan to recruit? Please provide a suitable power calculation demonstrating how the sample size has been arrived at or a suitable justification explaining why this is not possible/appropriate for the study.

The trained sensory panel has 12 members (the ISO minimum for profiling is 12).

Consumers: The aim is to recruit 120 volunteers into the study. This is based on power calculations by V-power (Ennis & Jesionka, 2011) where for a relatively small sensory difference between two samples (d'=1) 81 assessors are needed to find a significant difference using a tetrad test at 90% power. Assuming that the same-different test with the surety index has similar power to a directional 2AFC test then only 31 assessors would be needed to find this difference (at 90% power) in the tasting tests.

3.2

Will the research involve children or vulnerable adults (e.g. adults with mental health problems or neurological conditions)?

No.

3.3

Will your research involve children under the age of 18 years? No

Will your research involve children under the age of 5 years? No

3.4

Will your research involve NHS patients, Clients of Social Services or will GP or NHS databases be used for recruitment purposes? Yes/No (delete)

No

3.5

Recruitment

Consumers : Adults aged 18 to 65 years will be recruited via posters (Appendix D) placed around the University of Reading and the local area. Within the university, email circulation lists will be used to circulate the study email. Potential volunteers

already consented to our volunteer database will be contacted directly via email or telephone. Social media websites focusing on the local area (eg Facebook groups) will also be used to place the study poster.

Important Notes

4. The Principal Investigator must complete the Checklist in Appendix A to ensure that all the relevant steps and have been taken and all the appropriate documentation has been appended.

5. If you expect that your application will need to be reviewed by the University Research Ethics Committee you must also complete the Form in Appendix B.

6. For template consent forms, please see Appendices C.

Appendix A: Application checklist

This must be completed by an academic staff member (e.g. supervisor)

Please tick to confirm that the following information has been included and is correct.

Indicate (N/A) if not applicable:

Information Sheet

Is on headed notepaper ☑

Includes Investigator's name and email / telephone number ☑

Includes Supervisor's name and email / telephone number ☑

Statement that participation is voluntary ☐

Statement that participants are free to withdraw their co-operation ☑

Reference to the ethical process ☑

Reference to Disclosure
N/A ☑

Reference to confidentiality, storage and disposal of personal information collected ☑

Consent form(s) ☑

Other relevant material

Questionnaires
☐ N/A ☑

Advertisement/leaflets ☑
N/A ☐

Letters
☐ N/A ☑

Other (please specify) ☐
N/A ☑

Expected duration of the project 12

Appendix B

Volunteer Information Sheet

Coffee Tasting

Investigating the difference in sensory properties of coffee that has been roasted with and without enzyme treatment

You have been invited to take part in a short study at the Sensory Science Centre, Department of Food and Nutritional Sciences, School of Chemistry, Food and Pharmacy, University of Reading. Before you decide whether you wish to take part in the study, please read the following information carefully. If you want to know anything about the study, which is not written here, please ask the investigator/researcher.

Introduction

This study aims to find out whether coffee tastes differently depending on whether it has been treated with an enzyme before roasting. Acrylamide is a carcinogen that widely exists in coffee, potato crisps and chips and other heat treated foods. The aim within food manufacturing is to minimise levels of this compound in food. Acrylamide is formed when the naturally occurring amino acid, asparagine, is heated. If the enzyme, asparaginase, is applied to green coffee beans before they are roasted, this converts asparagine into harmless aspartic acid and ammonia. Therefore, acrylamide formation is reduced.

In this study you will be presented with different coffee samples to smell and taste and asked questions such as "are these samples the same or different".

Am I suitable to take part?

Please read the following exclusion criteria to identify if you are able to participate in the trial.

Exclusion Criteria - If any of the following condition apply to you, you will be unable to participate in the trial:

- You have had surgery or suffered from a medical condition in the previous 12 months affecting your ability to taste or smell food
- You have a known and relevant food allergy
  3. (e.g. milk protein allergies) or intolerance (e.g. lactose)
  4. Coffee allergy (caffeine allergy)
- You use a medication likely to affect taste, smell or appetite
- Your age is below 18 or above 65 years

If you are eligible based on the above criteria you will be asked to sign a Consent Form.

What will the study involve?

The study will involve tasting and smelling coffee samples. You will be asked whether you think samples are different from each other or not.

Do I have to modify my diet or other activities in any way?

No changes to your normal diet or everyday activities will be imposed by the study

Are there any risks of being a volunteer in this study?

No

Confidentiality

Your records will be kept strictly confidential and all information and data will be kept in locked cabinets and on password-protected computers. Information obtained from the study may be published in scientific journals but in the form of average group values. No identifiable individual results will be used, published or presented in scientific meetings. Your records will be kept by the investigators for five years. However, these records will only contain an identification number and code while information matching volunteer names with identification numbers/codes will be kept separately from these files within the School of Food, Chemistry and Pharmacy.

General

- This study has been subject to ethical review by the University Research Ethics Committee and has been given a favourable ethical opinion for conduct.
- Your participation in this study is purely voluntary. You may leave the study at any time without giving a reason. You are free to ask the investigator for more information about this study before you give your consent to take part.

Investigators

Dr Maria Jose Oruna-Concha
Telephone:

Email:

Dr Steve Elmore

Telephone:

Email:

Dr Lisa Methven

Telephone:

Email

PhD Student: Fei Xu

Department of Food & Nutritional Sciences
University of Reading E-mail:
P.O. Box 226, Reading
RG6 6AP

If you have any queries please do not hesitate to contact us.

Appendix C

COFFEE TASTING STUDY

Consent Form

Investigators:

Dr Maria Jose Oruna-Concha, Dr Steve Elmore, Dr Lisa Methven

Study researchers:

PhD Student: Fei Xu Email:f.xu@pgr.reading.ac.uk

Visiting Student: Mateusz Gertchen Email: M.G.Gertchen@reading.ac.uk

Department Address:

Department of Food and Nutritional Sciences, University of Reading, PO BOX 226, Whiteknights, Reading, RG6 6AP.

Information Regarding Products Used in this study:

In this study, you will be asked to taste a number of Brazilian coffees. Please inform the researcher if you are suffering from any medical conditions that may affect your smell and taste. Besides, as coffee and milk will be used in the test, consumers that have allergy to coffee and milk are not eligible for this test.

All data generated in this study are anonymous. You will not be identified from your answers and you are free to withdraw from this study at any time. If you wish to withdraw from the study, please let any of our researchers know. You can withdraw by using any of the contact details listed on this information sheet, such as telephone, email or personal communication.

All information and data will be handled in a confidential manner. Please note that this study has been ethic reviewed by the school ethic committee and conduct under the permission of the committees.

Consent

Please tick the box below if you agree with the statement below

I have read the information above relating to this study. I agree to the arrangements described in the Information Sheet so far as they relate to my participation.

☐

I understand that participation is entirely voluntary and that I have the right to withdraw from the project any time.

☐

Name:

Signed:

Date:

I have had explained to me that consent for my contact details and personal information to be added to the Sensory Science Centre Database of Tasting volunteers is entirely voluntary. Accordingly I consent as indicated below:

I consent to my contact details being stored on the Sensory Science Centre Volunteer Database : Yes / No (delete either Yes or No)

Screening Questionnaire

Please answer the following questions and return to Fei Xu as soon as possible. You will be notified directly on your suitability to take part in the practical sessions. All information will be treated in a confidential manner.

Name: Age: Gender:

Contact details (address or email):

Telephone Number:_____

Do you have any food allergy? For example milk etc. if YES, please specify.

YES/NO

If Yes, please state type of allergy:.....

Do you have any food intolerance? For example lactose.

YES/NO

If Yes, please state type of allergy:.....

Do you have any coffee (e.g. caffeine) allergies?

YES/NO

Do you have any medical condition(s) which may affect your food intake?

YES/NO

If Yes, please specify:.....

Are you taking any medication or do you have any medical condition(s) which may affect your sense of smell?

YES/NO

If Yes, please specify:.....

Are you taking any medication or do you have any medical condition(s) which may affect your sense of taste?

YES/NO

If Yes, please specify:.....

Thank you very much for your time.

Name:

Signed:

Date:

Appendix D

Advertisement

We are looking for healthy, non-smoking, men and women aged 18-65 years to **taste Brazilian Coffee**

The study involves attending the Sensory Science Centre at the University of Reading for a 30 minute visit on one occasion. You will be asked to taste and smell a range of coffee samples and answer questions asking whether the coffee samples are the same or different.

Please note that sufferers of **milk or coffee allergies** are not eligible for this study

If you would like more information, please contact:

Fei Xu

E-mail: f.xu@pgr.reading.ac.uk

Are you a coffee-lover?

Fancy tasting some authentic Brazilian coffee?

Why not join our test?

Are you aged 18-65, a non- smoker and available for half an hour?

If yes, then come have a taste of different types of Brazilian coffee at:

Sensory Science Centre, Room 2.23, the Department of Food and Nutritional Sciences, University of Reading. (15:00-18:00 Mon & Wed)

For more information, please contact us by email:

Appendix E

HACCP plan for Coffee Processing

Introduction

Acrylamide is an industrial chemical used in the production of polymers; it is categorised as a potential carcinogen. On 24th April 2002, the University of Stockholm and Swedish National Food Administration published a report showing levels of acrylamide in certain fried and baked foods at levels up to 3000 ppb, which exceeds six times of the maximum limit set by WHO. Later that year, UK Food Standards Agency confirmed the result. Work carried out at University of Reading in the same year showed that acrylamide was formed naturally when the free amino acid asparagine reacts with reducing sugars via the Maillard reaction, a well-known reaction responsible for the aroma, colour and taste of heated products. Several methods have been developed in the past decade to solve acrylamide problem. The main objective of my PhD is to use asparaginase to mitigate acrylamide in coffee without altering the coffee flavour attributes. This will include a review of the strategies taken to reduce acrylamide in food stuffs, the characterisation of the acrylamide content in coffee beans as a result of the coffee processing (pre- and post-enzymatic treatment), the impact of such process on the aroma profile and sensory properties of coffee and the investigation of the relationship between mitigation strategies and changes in nutritional (e.g chlorogenic acids) and flavour precursors present in coffee (sugar, caffeine and other amino acids).

Hypothesis

1. By implanting enzyme in green coffee, asparagine will be hydrolysed to aspartic acid and ammonia. As the asparagine content is reduced, acrylamide content in the final food product will also be reduced.
2. Enzyme will be inactivated in the late heat treatment. The traceable amount of aspartic acid and ammonia generated in the treatment will pose a minimum effect on the sensory properties of the food products.

Aims and Objectives

Profilling: to determine the differences between the sensory attributes of the treated coffee and controlled one

Materials and Methods

Materials

Brazilian Santos coffee sourced from Rave coffee UK.

Commercial asparaginase: Acrylaway sourced from Novozymes and Preventase sourced from DSM

Sample Production

	Steaming	Enzyme treatment	Drying	Roasting	Quantity
Control	No	No	No	Three roast levels	150g per sample, triplicate for each treatment
Process Control	100 °C, 45 minutes	Soak in water at 60°C for 60 min	60°C, 4 to 6 hours	Three roast levels	150g per sample, triplicate for each treatment
Enzyme treated by Acrylaway	100 °C, 45 minutes	Soak in water at 60°C for 60 min	60°C, 4 to 6 hours	Three roast levels	150g per sample, triplicate for each treatment
Enzyme treated by Preventase	100 °C, 45 minutes	Soak in water at 60°C for 60 min	60°C, 4 to 6 hours	Three roast levels	150g per sample, triplicate for each treatment

Operating Procedure

1Steaming

Coffee samples were steamed (100 °C, 45 minutes) before enzyme treatment to open the pore and enhance contact of enzyme in next step.

2Enzyme Treatment

150g of coffee sample were mixed with 103.5mL of water containing Acrylaway® L. Samples were incubated at 60°C for 60 min with stirring.

3Drying

Samples were dried to original weight (150g) in tray drier (60°C, 4 to 6 hours).

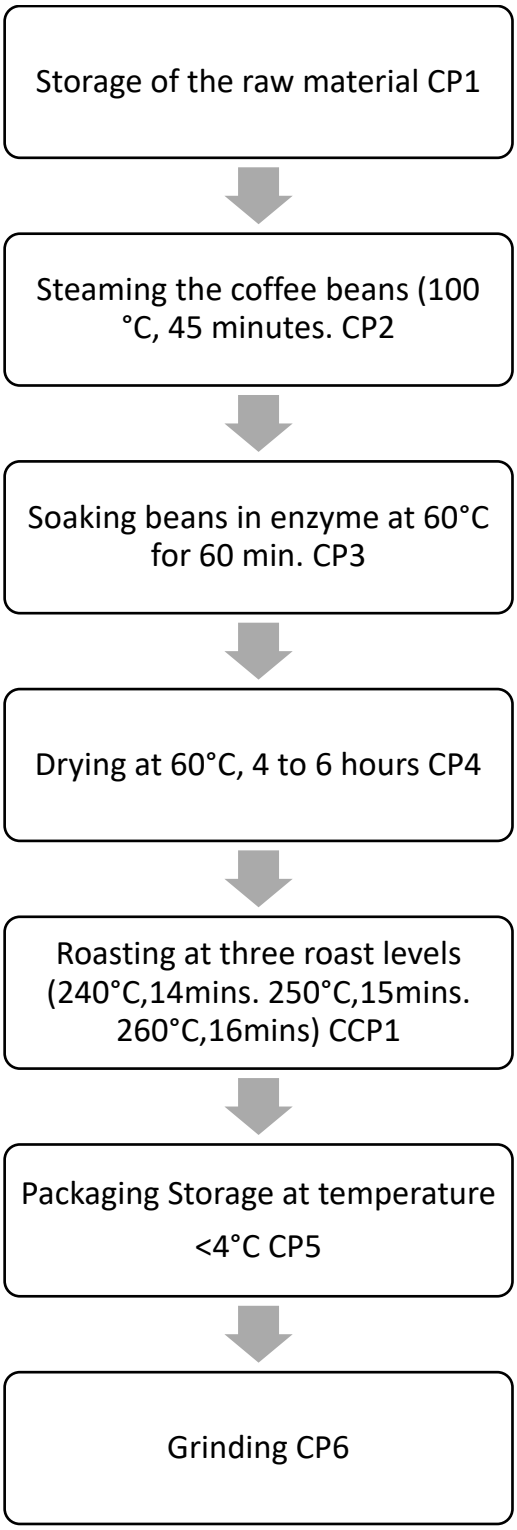
4Roasting

Dried coffee samples were roasted (roast machine sourced from Gene Coffee) to three roast levels.

5Packaging

Vacuum packed the roasted coffee beans then stored in 4℃

HACCP Plan for Coffee Enzyme Treatment:


Storage of the raw material CP1
Steaming the coffee beans (100 °C, 45 minutes. CP2
Soaking beans in enzyme at 60°C for 60 min. CP3
Drying at 60°C, 4 to 6 hours CP4
Roasting at three roast levels (240°C,14mins. 250°C,15mins. 260°C,16mins) CCP1
Packaging Storage at temperature <4°C CP5
Grinding CP6

HACCP Plan for Coffee Enzyme Treatment:

Step	Process	Harzards	Control Points	Prevention measurement	Critical Limits	Monitoring Procedure		Corrective Action
						Procedure	Frequency	
1	Storage of the Raw Material	Physical: Contamination from foreign material Chemical: Contamination with toxic compounds and odours	CP1	Dry Conditions of storage away from chemical and strong odours. Handlers to wear gloves, coats and hair nets.	All controls adhered to	Checks if all handlers are adhering	Every Batch	Reject sample
2	Steaming the coffee beans	Physical: Contamination from foreign material	CP2	Use distilled water and high hygiene procedure	Coffee steamed at100 °C for 45 minutes	Checks temperature every 15 minutes and hygiene procedure	Every Batch	Reject Sample
3	Soaking beans in enzyme	Physical: Contamination from foreign material Microbial: Enzyme contamination	CP3	Checks temperature and high hygiene procedure for food handlers	Coffee beans soaked in enzyme at 60°C for 60 min	Checks all beans are equally soaked and rotate the container	Every Batch	Adjusting Temperature
4	Drying	Microbial: less extent of drying leads to mould	CP4	Sufficient drying and weight measurement	Coffee beans dried at 60°C for 4 to 6 hours	Shake the tray drier to ensure the equal drying	Every Batch	Adjusting Temperature and time
5	Roasting	Physical: Contamination from foreign material Microbial: Survival of microorganisms	CCP1	Clean the machine between each roast and high hygiene procedure	240°C,14mins. 250°C,15mins. 260°C,16mins	Checks if the roasting chamber have fully cool down	Every Batch	Reject Samples
6	Packaging	Physical: Contamination from foreign material Microbial: Food spoilage or compromise of food safety	CP5	Handler wear gloves, hair nets and lab coats.	Storage at temperature <4°C	Checks storage temperature and environment	Every Batch	Reject Samples
7	Grinding	Physical: Contamination from foreign material	CP6	High hygiene procedure	All controls adhered to	Checks if handlers are adhering	Every Batch	Reject Samples

Appendix V Photos of vacuum infusion method processed coffee

Transverse cut of the enzyme treated green coffee beans by vacuum infusion method

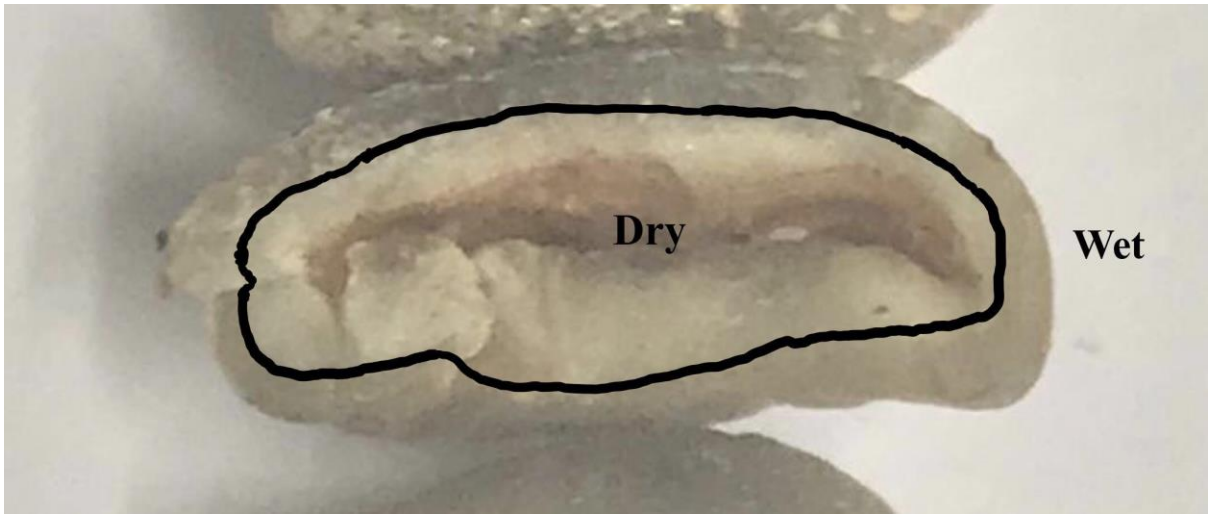


Illustration for the penetrated (wet) part and unpenetrated (dry) part of processed bean.