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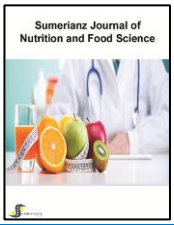

Original Article Open Access

Estimated Daily Intake and Risk of Acrylamide Concentration in Dutched Roasted Cocoa Nibs

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Abstract

Acrylamide, a probable carcinogen, has been detected in many roasted cocoa nibs and this threatens the safety of cocoa products. Two key factors which have been noted to impact the final acrylamide levels in cocoa masse are identified as the degree of alkalization and roasting temperature in a previous study. Thus, the current study sought to optimize these conditions in order to assess the exposure and associated risks in cocoa products. Response Surface D-optimal design was used to design 28 experimental runs with roasting time (20 – 50 min), type of alkali salt ($MgCO_3$, $(NH_4)_2CO_3$, K_2CO_3), roasting temperature (110 – 140 °C) and alkali strength (1%, 3% and 5% w/w) as treatment factors. The acrylamide content for each alkalized cocoa masse was extracted using the QuEChERS method and HPLC analysis was run to determine the various acrylamide concentrations. Subsequently, the data was analyzed and fitted to a linear process order after which diagnostics were done to eliminate outliers. Optimization was done which predicted least acrylamide concentrations at an alkali strength of 2.5%, roasting temperature (140 °C), and a roasting time of 35 min for $MgCO_3$ and 20 min for $(NH_4)_2CO_3$ and K_2CO_3. Though lower concentrations of acrylamide were recorded for these alkalized cocoa beans, it is believed that a potentially carcinogenic furfural could be formed as a by-product. The predicted concentrations were subsequently used to determine the estimated daily intake (EDI), and then used to determine the incremental lifetime cancer risk (ILTCR) and margin of exposure (MoE). These risk estimates were iterated 100,000 times in a Monte Carlo simulation and the central tendencies studied. The average exposure ranged from (3.45×10^{-8} – 4.57×10^{-8}) in adults and (2.01×10^{-7} – 2.72×10^{-7}) in children. Generally, higher exposures were observed in children relative to adults. Significantly, higher MoE far above the threshold (10,000) and lower ILTCR below the *deminimis* ($\times10^{-6}$) were recorded in all 3 alkali salts across all the indices in both adult and children consumers. This indicates negligible risk of developing cancer in both consumer groups. These findings further suggest that although all the 3 alkali salts produced lower risks, K_2CO_3 offered the least exposures and risk estimates. However, since a potentially carcinogenic furfural could be formed as a by-product in alkalized treatment, this is also a cause for concern.

Keywords: Acrylamide; Alkalized cocoa nibs; Cancer risk.

1. Introduction

Known as "food for the gods" in ancient times (Howie *et al.*, 2007), chocolate and other cocoa based products are one of the world's most luxuriously consumed confectionary (Raters and Matissek, 2018). Cocoa bean which is botanically the seed of the fruit of the cacao tree *(Theobroma cacao L.,)* is the primary raw material for processing of cocoa products (Żyżelewicz *et al.*, 2018).

To produce cocoa based products, fermented and dried cocoa beans upon arrival in a cocoa processing plant are cleaned, deshelled and winnowed. The cocoa nibs are then roasted and milled into cocoa liquor or masse which is afterwards pressed into cocoa butter and a solid cake with 10%– 12% fat called cocoa cake which is subsequently pulverized into cocoa powder (Rodríguez *et al.*, 2009).

The roasting is a critical stage during processing that imparts positively on both the physiochemical and the organoleptic properties of the roasted cocoa nibs. This principal stage is also responsible for the development of the characteristic brown colour, texture as well as the typical chocolate flavor of the cocoa bean (Żyżelewicz *et al.*, 2017). Notwithstanding the pleasurable mouthfeel sensation man continues to enjoy from chocolate and other cocoa based products over the decades, scientists have recently reported significant levels of acrylamide in roasted cocoa bean products usually pyrolysed at higher temperatures (>120°C). It appears to be produced during roasting as a by-product of the Maillard reaction – a reaction involving reactive reducing sugars and amino acids particularly asparagine known to drive the formation of desirable colour and flavor of food products (Capuano *et al.*, 2009).

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The growing interest on the subject of dietary acrylamide aroused extensive attention after acrylamide was categorized in Group 2A, meaning it is "probably carcinogenic" by the International Agency for Research on Cancer (IARC) (Farah and Zaibunnisa, 2012; IARC, 1994). From the toxicological viewpoint, enough evidence exists that acrylamide is neurotoxic and its metabolism into glycidamide is genotoxic (Lineback *et al.*, 2012). Further epidemiological studies have also reported likely associations between certain types of cancers and dietary acrylamide intake (Bongers *et al.*, 2012; Hogervorst *et al.*, 2007; Lipunova *et al.*, 2017; Liu *et al.*, 2017) although some studies have concluded that statistically, there is no significant association between dietary acrylamide and human cancers (Kotemori *et al.*, 2018; Mucci *et al.*, 2006; Pelucchi *et al.*, 2016; Wilson *et al.*, 2010). Additionally, some reports have also indicated that, the per capita consumption of chocolate in Switzerland, Belgium and Germany was estimated to be 10 kg/person/year (Rocha *et al.*, 2017). This presupposes that consumers could significantly be exposed chronically to dietary acrylamide (Ofosu *et al.*, 2019) as compared to higher levels reported in fried potatoes, French fries and some breakfast cereals. It is however surprising that some researchers have concluded there is negligible risk associated with consumption of acrylamide in cocoa products (Raters and Matissek, 2018).

Therefore, in response to the deleterious health effects of acrylamide with its present levels in foods, stakeholders in the food industry have suggested some mitigating strategies which include: addition of food ingredients such as antioxidants, amino acids; regulating processing conditions like pH, temperature and time (Friedman and Levin, 2008) as well as addition of metal cations (Kalita and Jayanty, 2013; Kolek *et al.*, 2007; Mestdagh *et al.*, 2008a; Ou *et al.*, 2008). In cocoa bean processing, the addition of metal cations in the form of alkali salts (sodium or potassium carbonate) was proposed by Van Houten, a Dutch chemist, in 1828. He treated the nibs, cocoa masse or cocoa cake (Moser, 2015) with alkali salts in a process known as the Van Houten process otherwise called alkalization or Dutching to produce Dutched cocoa powder which is employed in the confectionary industry, used in the preparation of ice-cream, desserts, baked items and beverages (Rocha *et al.*, 2005). This process also imparts some positive characteristics such as enhancing dark color and flavor notes, reducing bitterness and astringency, as well as improving dispersibility of cocoa powder in liquid (Moser, 2015).

In addition to improving mitigation strategies, researchers constantly discover novel detection techniques to reduce the rate of dietary exposure to acrylamide in order to satisfy the increasing demand for healthy, safe and nutritious foods by consumers world-wide. Several attempts have therefore been made to assess dietary acrylamide exposure using some approaches such as estimated daily intake (EDI). Also, regulatory institutions like United States Environmental Protection Agency (USEPA) together with European Food Safety Authority (EFSA) have established databases for the bench mark dose lower limit ($BMDL_{10}$) and potency factor (PF) which enable researchers to characterize the health risk associated with acrylamide exposure using indices such as the margin of exposure (MoE) and the incremental lifetime cancer risk (ILCR) approach. This is needful as a scientific basis for obtaining awareness of any threat regarding this food toxicant instead of the As-Low-As-Reasonably-Achievable (ALARA) principle.

The EDI approach relies on secondary data mostly from the national food consumption data. In reference to the USEPA standard protocol, the EDI is calculated as the product of the acrylamide concentration by the mass of cocoa product consumed by the proportion of cocoa solids in the products per the body weight (Ofosu *et al.*, 2019). The MoE on the other hand is the ratio of $BMDL_{10}$ to the estimated exposure whereas ILCR is calculated as the product of the exposure and the PF (EFSA, 2015a). In order to better quantify the risk, these regulatory institutions have recommended that the calculated risk values should be compared to the set thresholds in order to conclude whether a potential risk exists or not. Calculated MoE values below 10,000 and 125 signify a public health concern implying risk of tumorigenesis and neurotoxicity respectively. Similarly, ILCR values less than the *de minimis* (10^{-6}) implies no appreciable risk whereas values greater than the *de minimis* suggests risk of developing cancer (EFSA, 2015b; USEPA, 2010).

In spite of the many publications on acrylamide risk assessment and its levels in foods, little attention has focused on cocoa based products. Again, regulatory authorities such as the European Commission (EC) and Food and Drugs Authority (FDA) of the US have set indicative values for acrylamide in biscuits and fries to be 350 µg/kg (Mesías *et al.*, 2019) and 0.077ppm (Farah and Zaibunnisa, 2012) respectively as an interim acceptable level but safe levels in cocoa products are yet to be determined. Therefore, in the interest of consumer health protection, there is an urgent need to find the best processing conditions to substantially reduce acrylamide formation while maintaining the expected nutritional and sensory qualities of roasted Dutched cocoa products. As such, this current study mainly sought to focus on optimizing roasting conditions and degree of alkalization using different alkali salts ($MgCO_3$, $(NH_4)_2CO_3$, K_2CO_3) since they have been demonstrated as two key stages during cocoa bean processing that can impact the final concentration of acrylamide produced. The study further aimed to estimate acrylamide exposure as well as quantify the carcinogenic risks posed through the consumption of projected modeled chocolate and cocoa products.

2. Materials and Methods

2.1. Materials

2.1.1. Sampled Cocoa Beans

Premium quality cocoa beans (well fermented and dried) were sourced from the Produce Buying Company (PBC) in Ghana. The received cocoa beans were dried in an electric oven overnight at 40 °C and afterwards crushed manually to obtain the nibs. The cocoa nibs were kept in a plastic bag and stored at room temperature until further analysis.

2.1.2 Standards and Reagent

Alkali salts (K_2CO_3, $MgCO_3$ and $(NH_4)_2CO_3$) were bought from Qualikems (India). Likewise, hexane, acetic acid and acetonitrile were purchased from Merck (Darmstadt, Germany), Techno Pharmchem (India) and Prolabo VWR International (France) respectively. $MgSO_4$ was bought from Prolabo VWR International (Belgium) and NaCl salt was purchased from Daejung (South Korea). Acrylamide standard and analytical starch were bought from Merck (Darmstadt, Germany). All the reagents and chemicals used were of analytical grade.

2.2 Method

2.2.1 Experimental Design

With reference to other published studies, the Response Surface D-optimal design was used to design the dutching process with 4 independent variables. The roasting temperature (A) and time (B) ranged from 110–140 °C and 20–50 min respectively. K_2CO_3, $MgCO_3$ and $(NH_4)_2CO_3$ were the dutching agents while varying the alkali strength (C) from 1%, 3% to 5% w/w. The design for the model was considered satisfactory with regard to the degrees of freedoms (5) and residual (20), with a pure error of 4 and lack of fit greater than 3.

2.2.2 Dutching Method

A dutching method recommended by the FDA in the US (Moser, 2015) was used with some slight modifications. For each treatment sample, 50 g of cocoa nibs were weighed and dutched with the required concentration (0.5 g, 1.5 g or 2.5 g) of the alkali salt. A duplicate of each dutched sample was made and roasted in a BINDER oven (Tuttlingen, Germany) preheated to the required temperatures 110 °C, 125 °C, and 140 °C for the required time (20, 27.5, 35 and 50 min). To minimize experimental error, the oven was opened to take out the roasted alkalized nibs only when the required times were due. The samples were left to cool at room temperature and then each treatment was milled separately in a Crompton Taura grinder (ACGM- TD 71, India) into cocoa masse, and stored in an airtight bag at 25°C until further analysis.

2.2.3 Extraction and Clean-up

Guided by the QuEChERS protocol, acrylamide was extracted by accurately weighing 2 g of alkalized cocoa masse into a labelled 50 mL centrifuge tube. Five (5 mL) of hexane was added and vortexed for 4 min. Next, 10 mL of 1% acetic acid in acetonitrile was added after which 10 mL of distilled water was added and the solution vortexed for 4 min. A mass of 1.5 g of $MgSO_4$ and 0.5 g of NaCl were weighed and added to the resulting solution and finally vortexed again for 3 min. The mixture was subsequently centrifuged (Heraeus Multifuge X3R 5004515, US) at 4,000 rpm for 6 min and 2 mL of the resulting acetonitrile phase siphoned for High Performance Liquid Chromatography (HPLC) analysis.

2.2.4 Quantification of Acrylamide Using HPLC

This current study employed a previous method described by Gökmen *et al.* (2005) using LC–DAD for quantification to determine the acrylamide concentration. The HPLC analysis was performed using a Cecil-Adept binary pump HPLC together with a Dynamic Absorbance Detector set at 225 nm. The temperature of the column (Agilent eclipse Plus C18 column) with dimensions (4.6 mm × 150 mm, 3.5 µm) was set at 25 °C. A ratio of water/ acetonitrile (80:20 v/v) was used as the mobile phase and the pH was adjusted to 3.5 using orthophosphoric acid. To ensure quality control, analytical starch (2 g) was spiked with varying acrylamide standard concentrations of 100, 50 and 20 µg with a 97% average recovery indicating the accuracy of the method (Chen *et al.*, 2008). The study had a limit of detection (LOD) of 0.03 µg/g, a 0.1 µg/g limit of quantification (LOQ), and a calibration curve with an r^2 of 0.998.

2.2.5 Analysis of Data

Design-Expert (Version 7.1, Minneapolis) was used to analyze the data to fit the summary and evaluate the analysis of variance. The model was found to be significant ($p<0.05$) and the lack-of-fit not significant ($p>0.05$) before the outliers were studied. Subsequently the resulting graphs for each treatment variable was obtained and optimization was done to provide the roasting and dutching conditions that will favored reduced acrylamide levels in the alkalized cocoa masse. The acrylamide content was set to the highest importance (5+) and the optimized conditions for each type of alkali salt, yielded dutching conditions for $MgCO_3$ as 140 °C (roasting temperature), 35 min (roasting time) and 5% (alkali strength) and $(NH_4)_2CO_3$ and K_2CO_3 as 140 °C (roasting temperature), 20 min (roasting time) and 5% (alkali strength).

2.2.6. EDI

The human exposure of acrylamide in the various alkalized cocoa masse projected into different cocoa products for young children and adults were calculated (Equation 1) with reference to the US EPA standard protocols. From Equation 1, C_{acryl} represents the predicted acrylamide concentration (Table 1) from the various alkalized cocoa masse. The other elements from secondary data were used to determine the EDI. The body weight of adults and young children (1–3) were 70 kg and 12 kg respectively (EFSA, 2012a). The fraction of the total cocoa solids (F_{CS}) recommended for developing cocoa products were obtained based on the meaning of chocolate according to Codex Alimentarius, where the value of F_{CS} is the statistical distribution of the fraction of total cocoa solids in developed chocolate products (Ofosu *et al.*, 2019). The mass (M_{choco}) of chocolate products consumed was 8 kg/person/year

(Afoakwa, 2008). The EDI was then determined using Equation 1 and subsequently, the incremental lifetime risk was estimated using a PF of 0.5 mg/kg bw-day based on USEPA (1992).

$$EDI = \frac{Cacryl \times Mchoco \times FCS}{BW} \quad \ldots\ldots\ldots\ldots\ldots (1)$$

$$Risk = PF \times EDI \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

$$MoE = \frac{BMDL10}{EDI} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3)$$

The MoE was calculated using Equation 3 to characterize the carcinogenic risk resulting from exposure to dietary acrylamide. The $BMDL_{10}$ value used was 0.17 mg/kg(bw)-day as recommended by EFSA (2015a). The risk and MoE were further iterated 100,000 times using the Palisade @RISK software (2018).

3. Results and Discussion

3.1. Acrylamide Levels in Cocoa Masse

The various treatment factors for each run of cocoa nibs with their respective acrylamide concentrations are shown in Table 1.

Table-1. Treatment conditions for sampled cocoa nibs and their corresponding acrylamide concentrations

Run	Input variables				Response variables
	A	B	C	D	Acrylamide ($\times 10^{-2}$ mg/g)
1	110	50	5	$MgCO_3$	56.6
2	140	20	1	$MgCO_3$	43.75
3	140	50	1	$MgCO_3$	58.05
4	110	20	5	$MgCO_3$	40.22
5	110	35	1	$MgCO_3$	68.43
6	140	35	5	$MgCO_3$	50.82
7	125	27.5	3	$MgCO_3$	46.91
8	125	50	3	$MgCO_3$	43.24
9	140	20	1	$MgCO_3$	50.92
10	140	20	5	$(NH_4)_2CO_3$	37.59
11	110	50	1	$(NH_4)_2CO_3$	64.89
12	125	20	1	$(NH_4)_2CO_3$	102.86
13	125	50	5	$(NH_4)_2CO_3$	49.11
14	110	20	3	$(NH_4)_2CO_3$	59.66
15	140	50	3	$(NH_4)_2CO_3$	47.18
16	110	35	5	$(NH_4)_2CO_3$	47.41
17	140	20	5	$(NH_4)_2CO_3$	64.57
18	110	50	1	$(NH_4)_2CO_3$	67.89
19	140	50	3	$(NH_4)_2CO_3$	55.49
20	110	20	5	K_2CO_3	44.19
21	110	20	1	K_2CO_3	48.57
22	140	50	5	K_2CO_3	40.36
23	140	20	3	K_2CO_3	50.74
24	110	50	3	K_2CO_3	31.28
25	140	35	1	K_2CO_3	40.91
26	125	50	1	K_2CO_3	47.71
27	125	35	5	K_2CO_3	39.95
28	140	50	5	K_2CO_3	52.7

Input variables are; A= Roasting temperature (°C), B= Roasting time (min), C= Alkaline concentration (%) and D = type of alkali salt

3.2. Effect of the Input Variables on the Acrylamide Concentration

The analysis of variance (ANOVA) table (Table 2) presents the impact of roasting time and temperature, alkali strength and type of base on the concentration of acrylamide levels in the treated cocoa masse.

Table-2. ANOVA of processing conditions of input variables for the treatment of cocoa nibs

Source	Sum of squares	df	Mean square	F-value	p-value
Model	1030.1792	5	206.0358	3.1114	0.0308
A- Roasting temp (°C)	73.4392	1	73.4392	1.1090	0.3049
B- Roasting time (min)	0.1069	1	0.1069	0.0016	0.9683
C- Alkali strength (%)	329.5764	1	329.5764	4.9770	0.0373
D- Type of base	689.3400	2	344.6700	5.2050	0.0151

A= Roasting temperature (°C), B= Roasting time (min), C= Alkaline concentration (%) and D = Type of base

The statistical results summarized in Table 2 indicates that both the roasting time (B) and roasting temperature (A) was not statistically significant ($p>0.05$) though significant differences ($p<0.05$) were observed for the alkali concentration (C) and type of base (D). No interactions between the treatments were observed, thus, each treatment acted independently with only the alkali concentration (C) and type of base (D) influencing the levels of acrylamide in the cocoa masse.

3.3. Predicted Results

Table-3. Prediction of acrylamide concentration of cocoa masse based on optimization under alkalized conditions.

Input variables				**Predicted Response Variables ($\times10^{-2}$ mg/g)**		
D	A	B	C	Prediction	95% Cl low	95% Cl high
$MgCO_3$	140	35	2.5%	44.59	36.65	52.54
$(NH_4)_2CO_3$	140	20	2.5%	49.58	41.14	58.03
K_2CO_3	140	20	2.5%	36.75	28.11	45.39

A= Roasting temperature (°C), B= Roasting time (min), C= Alkaline concentration (%) and D = Type of alkali salt

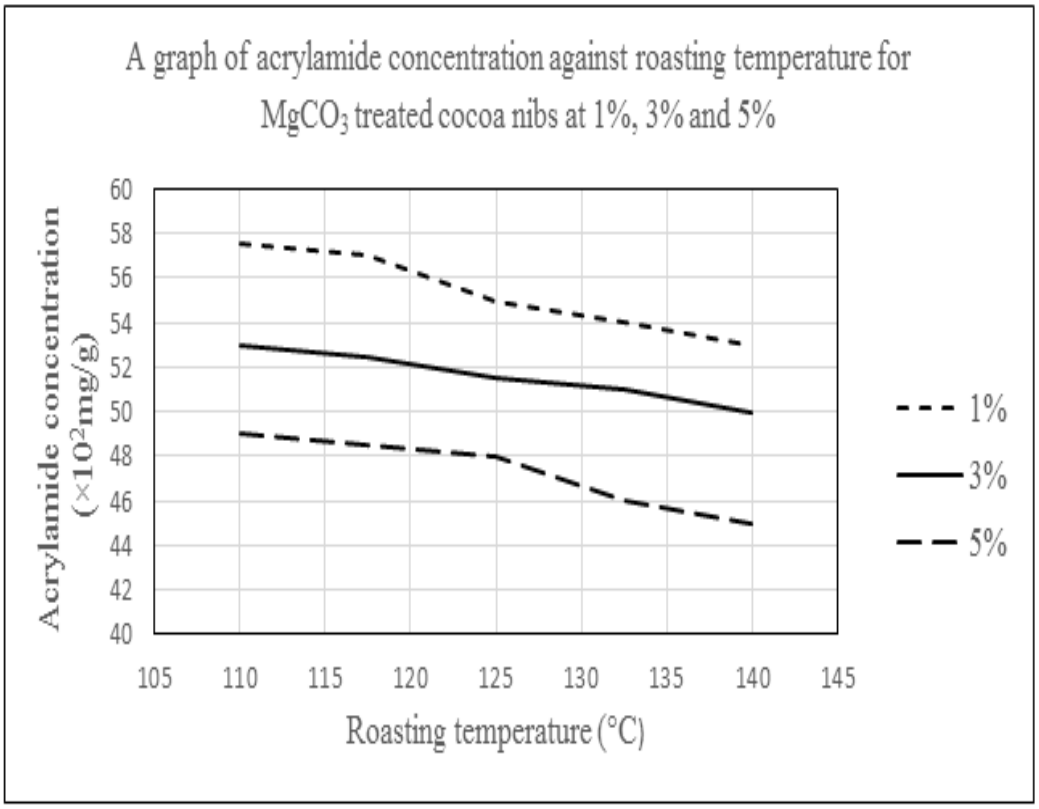
Table 3 shows predicted acrylamide concentrations for alkalized cocoa masse after optimization was done. From the table, the least acrylamide concentration was predicted for K_2CO_3 (36.75×10^{-2} mg/g) followed by $MgCO_3$ (44.59×10^{-2} mg/g) then $(NH_4)_2CO_3$ (40.51×10^{-2} mg/g) at their various optimized conditions. These predicted values are significant and fall within the set minimum (0.2 mg) and maximum limits (0.83 mg) of acrylamide concentrations in food established by the EFSA FAO/WHO (2007).

3.4. Effect of Input Variables

3.4.1: Strength of Alkali Salt

There was a similar trend observed in all three observations (Figure 1, 2 and 3) as the strength of alkali was increased. It was noted that the maximum level of acrylamide formed was at 1% whilst least acrylamide concentrations was recorded at 5%, hence as the strength of alkali salt increased, acrylamide concentration decreased.

Figure-1. Effect of the varying strength of $MgCO_3$ on acrylamide levels in alkalized cocoa masse at roasting between 110 to 140 °C for 50 min



Clearly, from Figure 1, at 110 °C the acrylamide concentration observed for 1% $MgCO_3$ was 57.5×10^{-2} mg/g. When the alkali strength was increased to 3%, the acrylamide concentration decreased to 53.5×10^{-2} mg/g and further decreased to 49×10^{-2} mg/g at 5% for the same roasting temperature. Again, at the end of roasting, the alkali strength at 1 % still presented highest acrylamide concentration (53×10^{-2} mg/g) with the least acrylamide concentration (45×10^{-2} mg/g) realized at 5 %.

It can also be observed in Figure 2 that when the alkali strength was increased from 1% to 3% then to 5%, the acrylamide levels at 110 °C decreased from 50×10^{-2} mg/g to 45×10^{-2} mg/g and 42×10^{-2} mg/g respectively.

Figure-2. Effect of different strengths of K_2CO_3 on acrylamide levels in alkalized cocoa masse at roasting between 110 to 140 °C for 50 min

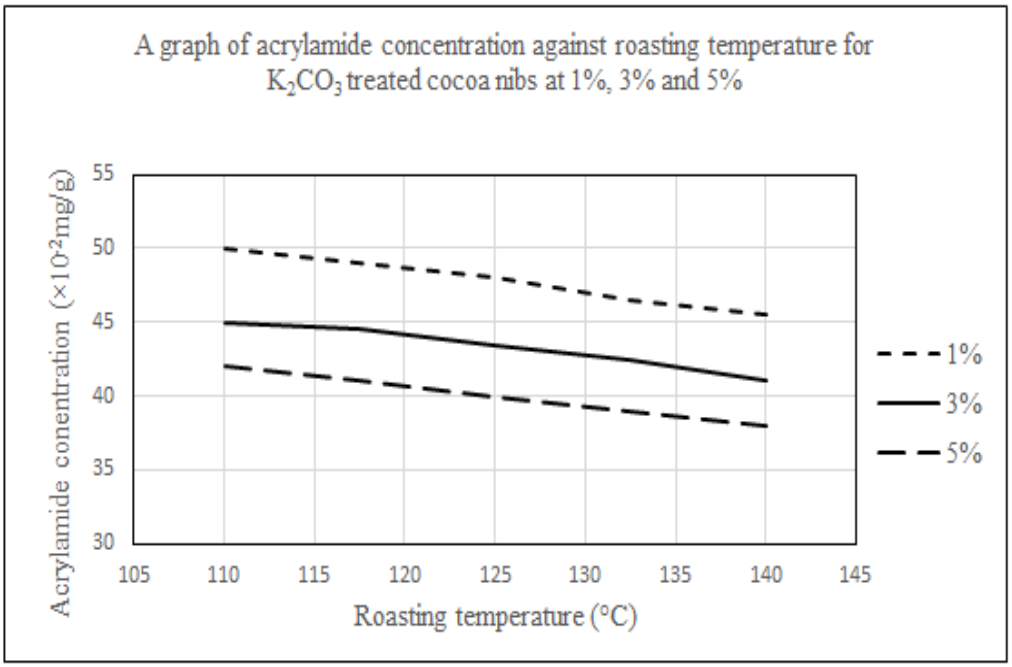
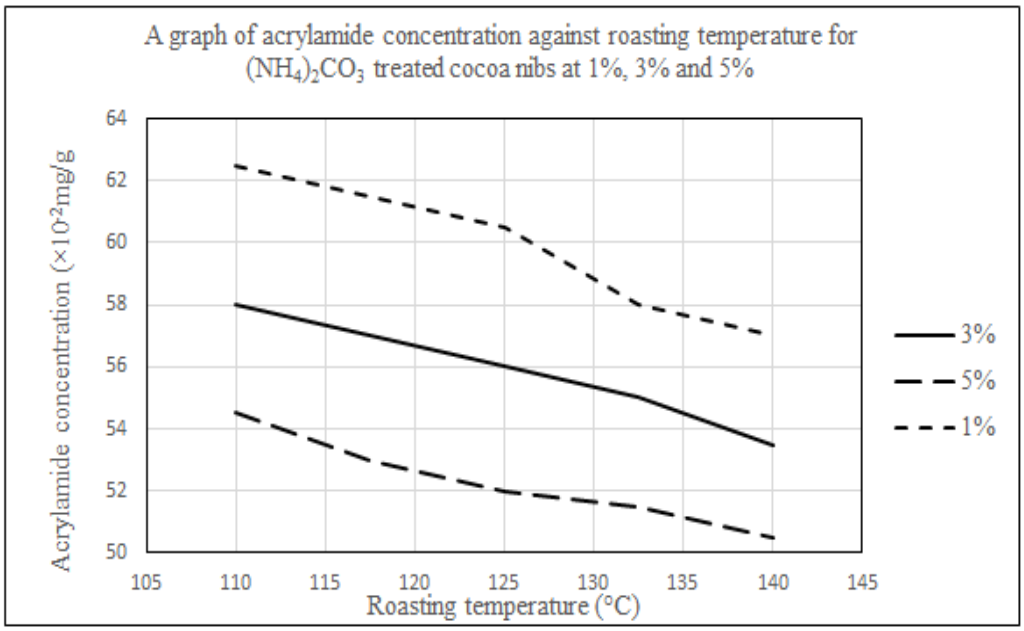


Figure-3. The influence of varying concentrations of $(NH_4)_2CO_3$ on acrylamide levels in alkalized cocoa masse at roasting between 110 to 140 °C for 50 min.



Again, in Figure 3, at 1% alkali strength, acrylamide concentrations were still higher (62.5×10^{-2} mg/g) as compared to acrylamide concentrations at 3% (58×10^{-2} mg/g) and 5% (54.5×10^{-2} mg/g) at 110 °C. These results clearly confirms what was also noted previously by Anese (2010), Levine and Ryan (2009), Ou *et al.* (2008) and Gökmen and Şenyuva (2007b) that acrylamide levels drastically decreased as the concentration of cations increased. Further studies in both glucose-asparagine (Wen *et al.*, 2016) and potato model systems (Mestdagh *et al.*, 2008b) have also observed that first, as the concentration of the alkali salt increase, acrylamide concentration decreases until it reaches a minimum before acrylamide levels rises with increasing alkali salt concentration.

It is therefore possible to assume that as the concentration of cations increase, more cations readily reacts with glucose leaving most of the asparagine unreacted, thus, the rate of asparagine decomposition decreased as the concentration of cation increased, hence, inhibiting acrylamide formation (Gökmen and Şenyuva, 2007b). However, various literature studies has demonstrated that although cations increase the reaction pathway to proceed primarily toward dehydration of glucose to slow down acrylamide formation they also increase hydroxymethylfurfurals (HMF) formation (Gökmen and Şenyuva, 2007b). In fact, HMF is known to be irritating to the eye, upper respiratory tract, skin and mucous membranes after it has been bio transformed to the mutagenic 5-sulphoxymethylfurfural (Monien *et al.*, 2009).

Further studies have also confirmed that the addition of cations resulted in a decrease in acrylamide levels significantly during roasting by influencing the rate of decomposition of the reaction precursors, particularly, increasing the rate of glucose decomposition whilst most of the asparagine remained unreacted (Gökmen and Şenyuva, 2007a). This could be ascribed to some suggested possible mechanisms of how cations change the activity

coefficient and pH of the solution thereby accelerating glucose decomposition to produce more HMF and furfurals (Combs *et al.*, 2013). Another probable mechanism could be due to the chelation reaction between asparagine and the cations hence preventing the Schiff base from forming (Kalita and Jayanty, 2013; Lindsay and Jang, 2005; Tan *et al.*, 2013).

3.4.2. Type of Alkali Salt

Earlier investigations have reported that the type of alkali salt used differed in their effectiveness to reduce acrylamide formation as seen in Figure1, Figure 2 and Figure 3. For instance at an alkali strength of 1%, the acrylamide levels produced at 110 °C for $MgCO_3$, K_2CO_3 and $(NH_4)_2CO_3$ were 57.5×10^{-2} mg/g, 50×10^{-2} mg/g and 62.5×10^{-2} mg/g respectively. Again, at the end of roasting the concentration of acrylamide produced at 5% for $MgCO_3$, K_2CO_3 and $(NH_4)_2CO_3$ were 45×10^{-2} mg/g and 38×10^{-2} mg/g and 50.5×10^{-2} mg/g respectively. Thus, in all cases irrespective of the strength of alkali, K_2CO_3 treated cocoa nibs produced least acrylamide levels as compared to cocoa nibs treated with $MgCO_3$ and $(NH_4)_2CO_3$. However, treatment with $(NH_4)_2CO_3$ produced the highest concentrations, clearly indicating that the type of alkali salt significantly influenced acrylamide levels.

Comparing Figure 1 and Figure 2, indicates that the monovalent potassium (K^+) cation significantly reduced acrylamide concentrations than the divalent magnesium (Mg^{2+}). This is however surprising because from literature it is was expected that Mg^{2+} will effectively reduce the acrylamide levels as compared to K^+. The opposite trend was observed in this study probably due to the different dutching procedures and food matrices hence, our results did not affirm earlier studies of Mestdagh *et al.* (2008a), Gökmen and Şenyuva (2007a), Mestdagh *et al.* (2008b) and Gökmen and Şenyuva (2007b). According to these authors, divalent cations such as Mg^{2+} inhibit acrylamide formation completely whereas monovalent cations such as K^+ partially reduce acrylamide formation. Similar experiments in cereal-based products (Kukurova *et al.*, 2009) and wheat matrices (Levine and Ryan, 2009) also proved that the addition of derivatives of divalent cation in varying forms is able to decrease acrylamide concentration.

Further intensive studies have also demonstrated the inhibitory effects of mono and divalent cations in glucose/fructose asparagine model solutions as well as fried potato model systems (Gökmen and Şenyuva, 2007a; Mestdagh *et al.*, 2008a; Pedreschi *et al.*, 2007). Regardless of the model, the presence of the divalent cations (Mg^{2+}) completely inhibited the formation of acrylamide as compared to mono cations (K^+) which were less effective.

In recent studies, evidence was found that cations such as Mg^{2+}, could change the reaction pathway from Maillard reaction toward dehydration of glucose (Gökmen and Şenyuva, 2007a). However in order for low-valence metal cations to attain maximum inhibitory role higher concentrations were required (Wen *et al.*, 2016). In addition, current findings have also proposed that the use of divalent cations provides high-temperature stability to asparagine, thereby rendering asparagine unavailable for a reaction with reducing sugars to generate acrylamide (Göncüoğlu Taş *et al.*, 2016) . Alternatively, studies from O'brien and Morrissey (1997) and Delgado-Andrade *et al.* (2004) have also suggested that divalent cations probably forms complexes with amine and some intermediates of the Maillard reactions (Stadler *et al.*, 2004; Yaylayan *et al.*, 2004).

Again, comparing Figures 1 and 2 with Figure 3 the acrylamide levels were very high for $(NH_4)_2CO_3$ treated cocoa nibs unlike those treated with $MgCO_3$ and K_2CO_3. Similar to the baking agent_NH_4HCO_3, ammonium salt have been reported to enhance formation of acrylamide in model systems of cereal matrices (Kukurova *et al.*, 2009), bakery products (Biedermann and Grob, 2003; Weisshaar, 2004) and gingerbread (Amrein *et al.*, 2004). The results from this study correlates well with findings from Friedman and Levin (2008), Amrein *et al.* (2004) and Gökmen *et al.* (2008). According to Gökmen *et al.* (2008), addition of a leaving agent like $(NH_4)_2CO_3$ increases the pH thereby boosting acrylamide formation.

An earlier publication by Amrein *et al.* (2006) suggests that ammonium salt promoted the formation of α-dicarbonyls from glucose and fructose via sugar fragmentation leading to sugar fragments like glyoxal, methylglyoxal and glyceraldehyde. These sugar fragments rapidly reacts with asparagine to generate high amounts of acrylamide under milder conditions when compared to fructose and glucose (Amrein *et al.*, 2006; Kukurova *et al.*, 2009). In addition to these sugar fragments, the sum of other α-dicarbonyls and α-hydroxycarbonyls produced from reducing sugars might also contribute to the high yield of acrylamide. Therefore, $(NH_4)_2CO_3$ indirectly boosts more reactive carbonyls arising from the reaction between reducing sugars and ammonia (Amrein *et al.*, 2004).

In a similar research, it has also been proven that ammonium salts enhances acrylamide formation by acting as an additional source of nitrogen and indirectly accelerating breakdown of sugars to generate new reactive carbonyls (Bent *et al.*, 2012; Biedermann and Grob, 2003; Grob, 2005).

3.4.3. Impact of Time and Temperature

Results from the ANOVA in (Table 2), indicates that the roasting time (B) and roasting temperature (A) were not significant ($p>0.05$), and thus, did not influence the levels of acrylamide in the treated cocoa nibs as can be seen in Figure 1, Figure 2 and Figure 3. This observation did not support the hypothesis that roasting time and temperature are the key determinants of the kinetic pathway of acrylamide formation (Amrein *et al.*, 2007; Jackson and Al-Taher, 2005). Previous findings have repeatedly demonstrated the impact of temperature and time on acrylamide formation in various food products (Biedermann *et al.*, 2002; Bråthen and Knutsen, 2005; Farah *et al.*, 2012; Gökmen and Şenyuva, 2007b; Kukurova *et al.*, 2009; Rydberg *et al.*, 2003). These authors established a significant relationship between heating time and temperature and showed that acrylamide formation increased steadily with temperature and time, until it reached a maximum and then decreased slowly afterwards most likely

due to higher rates of degradation or polymerization (Stadler and Scholz, 2004). Besides, acrylamide is an unstable compound and is easily degraded with heating at higher temperatures (Summa *et al.*, 2007).

This observed variation could likely be due to the presence of alkali salts and the extent of different time-temperature profiles of heating procedures. The effect of the latter is known to change the status of some antioxidants known to have poor heat stability and are easily destroyed under high temperature. Subsequently, these antioxidants change completely and are unable to partake in the Maillard reaction (Zhang and Jin, 2015). The influence of the former has been proven to slow down or inhibit acrylamide formation progressively irrespective of the heating time and temperature (Gökmen and Şenyuva, 2007a) though it proceeds to favor the Maillard-driven formation of color and flavour in heat-processed foods (Mottram *et al.*, 2002; Stadler *et al.*, 2002).

Again, the addition of alkali salts is known to have variable effects on acrylamide levels; in some studies a significant reduction was reported whereas in others the effect was minimal (Taeymans *et al.*, 2005). Obviously, in this study, the presence of cations changed the typical kinetic pattern of acrylamide formation with respect to time and temperature.

3.5. EDI and Probable Carcinogenic Risk

3.5.1 Estimated Daily Exposures

Table 4 shows the indices of the estimated exposures of the different alkalized cocoa masse as calculated by Equation 1.

Table-4. Estimated dietary exposures (mg/kg(bw)-day) in $MgCO_3$, $(NH_4)_2CO_3$ and K_2CO_3 alkalized cocoa masse projected into varieties of cocoa products for adult and young consumers

Alkali salt		Central tendency metrics					Percentiles	
		Min	Max	Mean	Mode	Median	5th	95th
K_2CO_3	Adult	2.14×10^{-8}	4.77×10^{-8}	3.45×10^{-8}	4.54×10^{-8}	3.45×10^{-8}	2.27×10^{-8}	4.64×10^{-8}
	Children	1.25×10^{-7}	2.78×10^{-7}	2.01×10^{-7}	2.65×10^{-7}	2.01×10^{-7}	1.32×10^{-7}	2.70×10^{-7}
$MgCO_3$	Adult	2.59×10^{-8}	5.78×10^{-8}	4.19×10^{-8}	3.60×10^{-8}	4.19×10^{-8}	2.75×10^{-8}	5.63×10^{-8}
	Children	1.51×10^{-7}	3.37×10^{-7}	2.44×10^{-7}	2.10×10^{-7}	2.44×10^{-7}	1.61×10^{-7}	3.28×10^{-7}
$(NH_4)_2CO_3$	Adult	2.88×10^{-8}	6.43×10^{-8}	4.57×10^{-8}	4.71×10^{-8}	4.66×10^{-8}	3.06×10^{-8}	6.25×10^{-8}
	Children	1.68×10^{-7}	3.75×10^{-7}	2.72×10^{-7}	2.75×10^{-7}	2.72×10^{-7}	1.79×10^{-7}	3.65×10^{-7}

The exposure to acrylamide in the different alkalized cocoa masse as measured by the EDI generally ranged from 6.43×10^{-8} – 1.25×10^{-7} mg/kg(bw)-day. From the Table it was also observed that minimum exposures ranged from 2.14×10^{-8} – 2.88×10^{-8} mg/kg(bw)-day in adults and 1.25×10^{-7} – 1.68×10^{-7} mg/kg(bw)-day in children. Also, a maximum exposure of 6.43×10^{-8} mg/kg(bw)-day was reported in adults whereas in younger children it was 3.752×10^{-7} mg/kg(bw)-day. The mean (3.45×10^{-8} – 2.72×10^{-7} mg/kg(bw)-day) and 95th percentile (4.64×10^{-8} – 3.65×10^{-7} mg/kg(bw)-day) ranges obtained in this study seemed to be lower than the recommend mean (0.0004 – 0.0019 mg/kg(bw)-day) and 95th percentile (0.0006 – 0.0034 mg/kg(bw)-day) ranges for dietary acrylamide day for consumers across all age groups by EFSA (2015b). This could be attributed to the lower concentrations of acrylamide in the cocoa masse. The highest exposure values across all the central tendency indices in both children and adults were $(NH_4)_2CO_3$ as compared to $MgCO_3$ and K_2CO_3 treated cocoa masse. This trend was also similar in the top 5 and bottom 5 consumers. This could be due the higher acrylamide concentration present in $(NH_4)_2CO_3$ alkalized cocoa masse.

Again, dietary exposure values were found to be higher in children consumers as compared to the adult consumers across all the metrics from the Table. This observation is similar to a study conducted by Jankowska *et al.* (2009) in Poland and Tawila *et al.* (2017) in Saudi Arabia. Findings from their studies concluded that children were highly exposed than adults and the mean acrylamide exposure decreased drastically with increasing age. They attributed this observation to the lower body weight in children and their frequent consumption of sweets such as chocolate and cocoa flavored products.

3.5.2. Margin of Exposure

Table-5. Estimated MoE values based on the various alkalized cocoa masse projected into varieties of cocoa products for adult and young consumers

Alkali salt		Central tendency matrix					Percentiles	
		Min	Max	Mean	Mode	Median	5th	95th
K_2CO_3	Adult	3.57×10^{8}	7.96×10^{8}	5.19×10^{8}	3.58×10^{8}	4.92×10^{8}	3.67×10^{8}	7.49×10^{8}
	Children	6.11×10^{7}	1.36×10^{8}	8.89×10^{7}	6.13×10^{7}	8.44×10^{7}	6.29×10^{7}	1.28×10^{8}
$MgCO_3$	Adult	2.94×10^{6}	6.56×10^{6}	4.27×10^{6}	2.95×10^{6}	4.06×10^{6}	3.02×10^{6}	6.18×10^{6}
	Children	5.04×10^{5}	1.12×10^{6}	7.33×10^{5}	5.05×10^{5}	6.96×10^{5}	5.18×10^{5}	1.06×10^{6}
$(NH_4)_2CO_3$	Adult	2.64×10^{6}	5.90×10^{6}	3.84×10^{6}	2.65×10^{6}	3.65×10^{6}	2.72×10^{6}	5.55×10^{6}
	Children	4.53×10^{5}	1.01×10^{5}	6.59×10^{5}	4.54×10^{5}	6.26×10^{5}	4.66×10^{5}	9.52×10^{5}

Presented in Table 5 are the MoE values for the three alkali salts in both children and adult consumers. Generally, the MoE values for K_2CO_3 in both adult and children consumers presented the lowest risk across all the central tendency matrices followed by $MgCO_3$ then $(NH_4)_2CO_3$. This could probably be due to the low exposures as

mentioned earlier. From Table 5, the minimum and maximum estimate of MoE values ranged from (3.57×10^{8} to 4.53×10^{5}) and (7.96×10^{8} to 1.01×10^{5}) respectively. These values implicate no health risk since they were above the threshold mark (10,000) recommended by EFSA (2015a). Again, the mean, mode, median as well as the 5th and 95th percentile group of adults and children consumers were remarkably above 10,000, thus implicating no health risk. EFSA considers a serious public health concern to be present when the MoE value is below the threshold value (10,000) (EFSA, 2012a). Notwithstanding the negligible risk associated with children consumers, it should be noted that their modal MoE values obtained in $MgCO_3$ (5.05×10^{5}) and $(NH_4)_2CO_3$ (4.54×10^{5}) alkalized samples, were closer to the recommended threshold value. The lower the MoE, with respect to the threshold, the more likely it is for acrylamide concentrations to reach toxic levels.

In other studies, very low modal MoE values of 3 in children and 17 in adults (Ofosu *et al.*, 2019) and low mean (356) and 95th percentile (154) MoE values (Tawila *et al.*, 2017) have been reported indicating serious public health risks contrary to this current study. These differences in MoE values could be due to variable factors such as different exposure estimates from consumers and the different varieties of cocoa products analyzed.

3.5.3. Lifetime Cancer Risk

Table 6 presents the risk profile of the various alkalized cocoa masse projected in different cocoa products for adults and children consumers. From the Table, the modal risk for adult and children consumers ranged from 2.29×10^{-8} to 2.36×10^{-8} and 1.14×10^{-7} to 1.37×10^{-7} respectively. Risk of carcinogenesis have a *de minimis* risk of ($\times10^{-6}$), therefore, risk estimates above the *de minimis* risk implicates a serious health risk, thus, consumers are at risk of developing cancer. These ILTCR values are below the *de minimis* risk ($\times10^{-6}$) implying negligible risk of carcinogenesis in adult and children consumers.

Table-6. Incremental lifetime cancer risk based on the various alkalized cocoa masse projected into varieties of cocoa products for adult and young consumers

Alkali salt		Central tendency metrics					Percentiles	
		Min	Max	Mean	Mode	Median	5th	95th
K_2CO_3	Adult	1.07×10^{-8}	2.38×10^{-8}	1.73×10^{-8}	2.29×10^{-8}	1.73×10^{-8}	1.13×10^{-8}	2.32×10^{-8}
	Children	6.23×10^{-8}	1.39×10^{-8}	1.01×10^{-10}	1.14×10^{-7}	1.01×10^{-7}	6.62×10^{-8}	1.35×10^{-7}
$MgCO_3$	Adult	1.3×10^{-8}	2.89×10^{-8}	2.09×10^{-8}	1.45×10^{-8}	2.09×10^{-8}	1.38×10^{-8}	2.81×10^{-8}
	Children	7.56×10^{-8}	1.69×10^{-7}	1.22×10^{-7}	1.25×10^{-7}	1.22×10^{-7}	8.03×10^{-8}	1.64×10^{-7}
$(NH_4)_2CO_3$	Adult	1.44×10^{-8}	3.22×10^{-8}	2.33×10^{-8}	2.36×10^{-8}	2.33×10^{-8}	1.53×10^{-8}	3.13×10^{-8}
	Children	8.41×10^{-8}	1.88×10^{-7}	1.36×10^{-7}	1.37×10^{-7}	1.36×10^{-7}	8.93×10^{-8}	1.82×10^{-7}

Again, the results also indicate that mean, median as well as the 5th and 95th percentile risks for both adult and children consumers were remarkably below the *de minimis* risk. This further implies that consumers in both the adult and children population will not be at risk of getting cancer for consuming such cocoa products. However, the modal risk obtained this study seems to be much lower in both adult and children consumers compared to the modal risk for adults (4 people out of 1000 consumers) and 2 out of 100 for children consumers reported by Ofosu *et al.* (2019). This disparity could be as a result of the exposure estimates consumers were exposed to. Nonetheless, this current study corroborates findings of Raters and Matissek (2018) who conclude that based on the low acrylamide concentrations in cocoa products on the German market, there is negligible risk associated with its consumption.

4. Conclusion

This study found that the roasting time and temperature did not significantly influence acrylamide formation thus, cocoa processing companies may consider lower roasting temperatures or shorter roasting times to reduce production costs. It was also observed that as the strength of the alkali increased, lower acrylamide levels were formed irrespective of the type of alkali and roasting conditions. After optimatization of the degree of alkalisation and roasting conditions (time and temperature), K_2CO_3 was predicted to produce the least acrylamide concentration followed by $MgCO_3$ and then $(NH_4)_2CO_3$. Since increased alakli concentrations have been linked to the formation of hydroxymethylfurfurals, care must be taken in exploiting this.

The EDI exposures were very low (6.43×10^{-8}–1.25×10^{-7} mg/kg(bw)-day) for both consumer groups. These low exposures subsequently resulted in very high MoE values (7.96×10^{8} – 1.01×10^{5}) and low ILTCR risk estimates (1.07×10^{-8} – 1.22×10^{-7}) indicating neligible risk in both adult and children consumers. Though the exposures and risks seemed negligible in this study, care must be taken in interpreting the results since in this study only the estimated daily intakes were determined and not a tier 4 study involving precise localized food consumption data.

Conflict of Interest

The authors declare that there is no conflict of interest.

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Contribution of Authors

Jemima A. Baidoo wrote the final manuscript; and Naa Kwarley-Aba Quartey contributed significantly in writing the final manuscript. Isaac W. Ofosu, designed the study, worked on the final manuscript and made significant corrections prior to submission.

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