

# Analysis of Acrylamide and Glycidamide In Dried Blood Spot After Food Exposure and Its Carcinogenicity Potential: An Article Review

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## ABSTRACT

Acrylamide is a chemical compound formed when carbohydrate-rich food is placed in the heating process with temperatures above 120°C. Many studies have discussed the toxicity and carcinogenicity effects of acrylamide producing neurotoxin, genotoxin, and cytotoxin. After ingestion, acrylamide undergoes metabolism which is catalyzed by the CYP2E1 enzyme into its epoxide compounds, glycidamide. Both acrylamide and glycidamide are very reactive to DNA and can form DNA-adducts, which are known to be genotoxic and cytotoxic. Glycidamide is known to have a higher affinity for DNA compared to its precursors, so it can be said that glycidamide is the ultimate carcinogen of acrylamide. Human exposure to acrylamide can be obtained from a few factors, such as occupational exposure, food exposure, and cigarette smoke. However, studies found that dietary intake is the major source of acrylamide and glycidamide exposure. To determine the risk of acrylamide and glycidamide exposure to humans from dietary intake, it is necessary to analyze its concentration levels in the blood. One of the biosampling methods that can be used is Dried Blood Spot (DBS). The quantitative analysis was conducted using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). This article review aims to analyze the bioanalytical method that is most suitable for the analysis of acrylamide and glycidamide in DBS using LC-MS/MS. Furthermore, it is necessary to examine the relationship between dietary intake with acrylamide and glycidamide levels in the blood, as well as knowing the potential carcinogenicity of both analytes to humans, especially glycidamides.

**Keywords:** Acrylamide, Glycidamide, Dried Blood Spot, LC-MS/MS

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## INTRODUCTION

Acrylamide is one of the organic materials used in the paper, paint, plastic manufacturing industry, as well as in drinking water handling.<sup>[1]</sup> This substance also can be found naturally in food. Formation of acrylamide in food occurs when food containing high carbohydrates such as potato, corn, and wheat undergo high temperatures heating process (above 120°C). Nowadays, food is known to be one of the main sources of acrylamide exposure in humans through the daily intake. Besides food, the other main sources of exposure are from cigarette smoke.<sup>[2-4]</sup>

Acrylamide exposure can cause toxic effects such as neurotoxic, reproductive toxicity, and carcinogenic in experimental animals.<sup>[5]</sup> Its toxicity is known to be mediated by glycidamide, which is the results of acrylamide metabolism with the help of the enzyme CYP2E1. Glycidamide can bind with DNA and will form DNA-adducts that are associated as carcinogen. Therefore, it is important to evaluate the risk of effects caused by acrylamide exposure from either food or work.<sup>[6]</sup> The International Agency for Research on Cancer (IARC) has classified acrylamide into group 2A which mean it is a compound that might cause cancer in humans.<sup>[7]</sup>

Up until now, the Food and Drug Administration (FDA) has not issued an appeal to not consume acrylamide-contained foods. This is due to the lack of research on how far the effect of acrylamide causes cancer in humans.<sup>[1,4]</sup> The latest experimental research conducted by Zhivagui et al. (2019) succeeded in identifying the presence of glycidamide gene mutations that are found in one-third of 1600 tumor genomes which correspond to 19 types of tumors in humans from 14 different organs.<sup>[8]</sup>

Acrylamide is contained in many daily foods such as potato chips, french fries, biscuits, cereals, and coffee. Therefore, it is necessary to analyse the relation of eating habits with acrylamide and glycidamide levels in the human body to determine the potential risk of exposure to human health. Nowadays, there is alternative biosampling method that is simpler and more comfortable for the subject, namely the Dried Blood Spot (DBS) method. DBS is a biosampling technique where blood samples are blotted and dried on filter paper.

In 2020, an analysis of acrylamide in students' blood was carried out using the DBS biosampling method with Ultra High-Performance Liquid Chromatography Tandem Spectrometry (UHPLC-MS/MS). The obtained data from this study shows that the more acrylamide-contained food consumed by the subject, the higher the acrylamide concentration detected in the DBS sample. This study also proves that the DBS biosampling method can be used to analyse acrylamide in the blood.<sup>[9]</sup>

Based on the matter stated above, the authors are going to evaluate the simultaneous analysis of acrylamide and glycidamide in the blood with the DBS biosampling method. Previously, there had never been a study about the analysis of these two compounds simultaneously in DBS. Moreover, the authors aim to see the relationship between diet with acrylamide and glycidamide levels in the blood, as well as the potential carcinogenicity produced by glycidamide obtained from food.

## ACRYLAMIDE FORMATION AND ITS EXPOSURE TO HUMANS

### *Acrylamide from Maillard Reaction*

Maillard reaction is a reaction that produces taste and aroma in foods that are cooked by roasting, frying, or baking, and is often called the non-enzymatic browning reaction because it takes place in the absence of enzymes. This reaction occurs between naturally occurring amino acids and reducing sugars (such as glucose or fructose) when the foods are exposed to high temperatures above 120°C in the manufacturing process. Many different types of sugars and amino acids can experience this reaction. However, asparagine has a much greater tendency to interact with sugars and form acrylamide than other amino acids. The reducing sugars are naturally contained in plant material such as cereal seeds, vegetables (such as potatoes), cocoa, and coffee bean.<sup>[10-14]</sup>

### *Acrylamide Exposure in Humans*

Acrylamide has widely been used as an industrial material for the production of polyacrylamide used in soil conditioning, wastewater treatment, cosmetics, paper, and textile industries since the 1950s, and subsequently found in cigarette smoke. Acrylamide can be swallowed together with drinking water as a result of the drinking water treatment process. Skin exposure to acrylamide can be caused by the use of cosmetic products, gardening products, paper and pulp products, coatings, and textiles due to the use of polyacrylamide in the manufacture of products. Occupational exposure to acrylamide is mainly due to skin contact with solid monomers and inhalation of dust and steam during the production of acrylamide and polyacrylamide.<sup>[15]</sup>

Acrylamide can also be found in food. In 2002, a study from Sweden conducted by Tareke *et al.* reported that acrylamide can be formed from heated foodstuffs, especially potato products and other rich-carbohydrate foods that are baked or fried.<sup>[16]</sup> Based on reported data, 50% of acrylamide exposure in humans comes from potato-based products. About 20% of the exposure comes from cakes, bread, and baking products. According to the latest data, the average human intake is estimated to range between 0.3-0.6 µg/kg body weight/day for adults. Meanwhile, in children, the average intake of acrylamide is greater, which is 0.4-0.6 µg / kg. This may be caused by higher calorie intake in children, and many of the foods that children consume are foods rich in acrylamide, such as french fries and potato chips. However, according to The Joint FAO / WHO Expert Committee on Food Additives, acrylamide intake can vary from 0.2 to 1 µg / kg body weight/day.<sup>[2,17-18]</sup>

In 2010, Tardiff *et al.* researched the safe level estimation of acrylamide from food intake in humans. This estimation was obtained by extrapolating the administration of high doses to animals to low doses in humans. The estimated results of these levels are the tolerable daily intake (TDI) for the neurotoxicity of acrylamide estimated at 40 µg/kg/day. Whereas TDI for cancer is estimated to be 2.6 and 16 µg / kg/day for acrylamide and glycidamide respectively.<sup>[19]</sup>

A research carried out by Harahap *et al.* in 2020, analyzed the acrylamide level in students' dried blood spots using LC-MS/MS. The purpose of this study is to observe the relationship between dietary intake and acrylamide levels in the blood. Students were chosen as subjects because they are one of the populations whose consuming acrylamide-containing foods. Before samples are taken, subjects were required to fill out a questionnaire regarding their daily eating habits. From the results of the research, it can be seen that there is a relationship between diet and acrylamide levels in the blood. In subjects who consumed a lot of acrylamide-containing foods such as french fries, potato chips, coffee,

fried foods, popcorn, cereals, biscuits, pastries, bread, and fast food, acrylamide levels were found to be much greater than negative subjects who rarely ate food containing acrylamide.<sup>[9]</sup>

## ACRYLAMIDE METABOLISM IN THE HUMAN BODY

### *Pharmacokinetics of Acrylamide*

Absorption of acrylamide in the body can be through the respiratory tract, digestive tract, and skin.<sup>[1]</sup> In studies conducted on mice, it is known that acrylamide is absorbed quickly and thoroughly in the digestive tract of rats. Meanwhile, absorption in rat skin is only 25% of the dose given.<sup>[20]</sup> Acrylamide consists of small hydrophilic molecules and can diffuse passively within the body. This compound can be found throughout the body compartment system. It is also known that acrylamide can penetrate the placental membrane in mouse and in human, as well as can be found in human breast milk.<sup>[21-22]</sup> Acrylamide can react with sulfhydryl groups present in hemoglobin, which can result in its accumulation (about 12% of acrylamide) in red blood cells for at least 10 days.<sup>[23]</sup> In the body, acrylamide will be metabolized into glycidamide, which is an epoxide that is suspected to cause cancer. At least 6% of the dose of acrylamide ingested will be converted to glycidamide.<sup>[3]</sup> The average exposure to glycidamide in humans is known to be 2 times lower than in rats when given at the same dose and route. Acrylamide and glycidamide are mostly excreted in urine and bile. In studies on rats, acrylamide and glycidamide metabolism resulted in the form of mercapturic acid in the urine.<sup>[24]</sup>

In 2014, Kim *et al.* conducted a pharmacokinetic study in mice by giving acrylamide at a dose of 2 mg/kg BW via the oral and intravenous routes. In oral administration, the metabolism of acrylamide to glycidamide is faster when compared with intravenous administration, this occurs due to the presence of the first-pass metabolism. After intravenous injection, plasma levels of acrylamide and glycidamide decrease mono-exponentially. While the elimination half-life ( $t_{1/2}$ ) of acrylamide and glycidamide does not depend on the administration route,  $t_{1/2}$  glycidamide is significantly longer than acrylamide for both administration routes.<sup>[5,24]</sup>

### *Biotransformation of Acrylamide*

In both humans and animals, acrylamide is metabolized through two pathways namely (1) direct conjugation by glutathione(GSH) reduction which is catalyzed by glutathione-S-transferase (GST) or (2) enzymatically by oxidation to form glycidamide epoxide which later on will also be conjugated. There are 2 phases of enzymatic metabolism of acrylamide. In the first phase of metabolism, some of the acrylamide is converted to glycidamide epoxide due to oxidation with the help of the CYP2E1. Furthermore, glycidamide can be further metabolized due to hydrolysis by the enzyme epoxide hydrolase to form glyceramide (2,3-dihydroxypropionamide), a non-toxic compound. Both acrylamide and glycidamide can bind covalently to the nucleophilic sites of biological macromolecules (such as -SH, -NH<sub>2</sub>, or -OH). The main target of this compound is nucleophilic nitrogen in DNA, which is also susceptible to form adducts especially with glycidamide, so glycidamide is considered as the main compound that causes genotoxicity.<sup>[10,25-26]</sup>

In the second phase metabolism, acrylamide and glycidamide can be conjugated with GSH to form GSH-adduct. After degradation and acetylation of the conjugate, acrylamide and glycidamide are excreted in the urine in the form of mercapturic acid. Mercapturic acid is produced from acrylamide is N-acetyl-S-(2-carbamoyl-ethyl)-l-cysteine (AAMA) mercapturic acid, whereas glycidamide will produce

N-acetyl-S-(2-carbamoyl-2-hydroxyethyl)-l-cysteine (GAMA) acid and N-acetyl-S-(1-carbamoyl-2-hydroxyethyl)-l-cysteine (iso-GAMA). Glutathione conjugation is important to reduce the acrylamide reactivity to cellular components. Another important aspect of conjugating acrylamide with glutathione is that it can reduce the conversion of acrylamide to glycidamide.<sup>[10,27]</sup>

In 2016, Wang *et al.* conducted a toxicokinetic study on acrylamide to examine mercapturic acid metabolites as biomarkers for short-term acrylamide exposure. This test was conducted on rats and adolescent populations in China. AAMA, GAMA, and iso-GAMA mercapturic acid metabolites were detected in all rats' urine samples. The three metabolites are quickly produced from the reaction between GSH and acrylamide or glycidamide within 3 hours. The cumulative excretion of all these metabolites reaches peak levels within the next 3 hours. The amount of AAMA excretion was higher than GAMA and iso-GAMA in the urine of all animal groups. Then, AAMA excretion decreased sharply while the number of GAMA and iso-GAMA decreased steadily with a slow elimination rate. In the adolescent population, it shows that AAMA is the main urine metabolite ( $51.8 \pm 9.8\%$ ) of the total number of four metabolites, followed by AAMA-sul, GAMA, and iso-GAMA. AAMA-sul or N-acetyl-S-(2-carbamoyl-2-hydroxyethyl)-l-cysteine-sulfoxide is the result of oxidation from AAMA, which has not been detected in rat urine.<sup>[28]</sup>

## ACRYLAMIDE AND GLYCIDAMIDE TOXICITY

### *Acrylamide Toxicity in Humans*

Acrylamide has been shown to cause various tumors in experimental animals. Carcinogenicity of acrylamide has been widely studied and the increased incidence of tumors in the mammary, thyroid, and skin glands has been documented. The carcinogenic nature of acrylamide is mediated by its epoxide metabolite, glycidamide. Acrylamide and glycidamide can modify DNA to form acrylamide-DNA adduct and glycidamide-DNA adduct. Glycidamide is believed to have mutagenic and genotoxic effects in animals, whereas acrylamide is considered to be neurotoxic in both animals and humans. Neurotoxicity is the only toxic effect of acrylamide that has been proven in humans (due to occupational exposure) and animals. Acrylamide can also cause several other toxicities, such as reproduction toxicity in rodents and mutagenicity in somatic cells *in vitro* and *in vivo*, as well as in germ cell *in vivo*.<sup>[5,29-34]</sup>

The toxicity of acrylamide occurs when there is an imbalance in the ratio between biological oxidants and antioxidants. This can cause oxidative stress. Several studies have shown that acrylamide can induce oxidative stress. Oxidative stress occurs when there is an imbalance between free radical production and the body's ability to neutralize its damaging effects through neutralization with antioxidants. To neutralize the effects of free radicals, the body has protectors such as antioxidant enzymes (superoxide dismutase (SOD), catalase, glutathione S-transferase, glutathione peroxidase) and proteins such as GSH. If the body cannot neutralize the effects of oxidative stress, it can cause cellular macromolecular damage and ultimately cell death through apoptosis.<sup>[2,35-38]</sup>

### *Glycidamide Carcinogenicity in Humans*

Glycidamides interacts more easily with DNA, about 100-1000 times compared to acrylamide. This is because the structure of glycidamide, which has an electrophilic epoxide group, is more reactive. The main adducts that are formed from glycidamide are N7-(2-carbamoyl-2-hydroxyethyl)-guanine (N7-GA-Gua) and N3-(2-carbamoyl-2-hydroxyethyl)-adenine (N3-GA-Ade).<sup>[10,39-40]</sup>

Although both acrylamide and glycidamide DNA-adduct are formed *in vitro*, only glycidamide-DNA adduct is found after giving acrylamide or glycidamide *in vivo*. This supports the important role of glycidamide over its precursor, acrylamide, as a genotoxic effect of acrylamide. Also, it was found that N7-GA-Gua can be detected in human urine. AAMA levels in urine were found to have a significant relationship with N7-GA-Gua levels. Therefore, it can be said that N7-GA-Gua in the urine can be used as a biomarker for mutagenicity and in molecular epidemiological studies in humans.<sup>[10,24]</sup>

In experimental studies in which acrylamide was given to mice, it showed that interference was occurring in the purine bases of liver, lung, and kidney DNA due to exposure to acrylamide and glycidamide, especially glycidamide. It has also been found that at the same administration dose as acrylamide, glycidamide is more mutagenic in experimental animals. Recent research shows that the effects of genotoxic (mutagenic) acrylamide and glycidamide on cell DNA produce very serious damage such as damage to the DNA chain, cross-chain linkages, base shifts, and base changes. This genotoxic effect on DNA is caused by glycidamide.<sup>[41]</sup>

In 2019, Zhivagui *et al.* conducted an experimental study to identify the presence of DNA mutations due to acrylamide through its metabolite, glycidamide. The results showed the discovery of signs of mutations that are typically due to glycidamide in one-third of 1600 tumor genomes that correspond to 19 types of human tumors originating from 14 organs. The spread of these typical mutations due to glycidamide is observed in lung cancer (88% of observed tumors), liver (73%), kidneys (> 70%), bile ducts (57%), cervix (50%), and several types other cancers at a lower rate. Overall, this study revealed an unexpected contribution of acrylamide (via its metabolite, glycidamide) to DNA mutagenesis in human cancer cells.<sup>[8]</sup>

Based on several studies as mentioned above, the authors found the potential carcinogenicity of acrylamide and glycidamide. However, there has not been found a direct relationship between the levels of acrylamide and glycidamide in human blood obtained from food exposure with its carcinogenicity potential.

## DRIED BLOOD SPOT

In recent years, the Dried Blood Spot (DBS) technique is often used as a biosampling method in qualitative and quantitative bioanalysis. This technique was first introduced in 1913 to estimate blood glucose levels. Since the 1960s, DBS techniques have been developed for screening neonates to detect metabolic disorders and also for monitoring HIV disease. At present, DBS is not only used in bioanalysis but is also used in various fields of research and analysis such as the measurement of antibody viruses, the development of new drugs, pharmacokinetics, and toxicokinetics.<sup>[42-46]</sup>

DBS sampling is a simple and non-invasive method. Blood samples are taken from the fingertip or heel using a lancet. Then the blood will be blotted on filter paper made specifically for DBS. DBS cards are composed of non-cellulose or cellulose matrices (filter paper) with a specific pore size and thickness. The obtained blood spots must be allowed to dry at room temperature for several hours, depending on the type of DBS card. Then the DBS card can be stored in a container that contains a drying package (such as silica gel) or can be prepared directly for analysis.<sup>[44-46]</sup>

DBS usage has several advantages when compared to other biosampling techniques. Some of the advantages are: DBS only required a small amount of blood when compared to venipuncture techniques, less invasive and more comfortable for patients, compatible with various types of bioanalysis

methods, has a high level of stability during storage and delivery, and also more economical.<sup>[46]</sup>

Although DBS has many advantages in bioanalysis, this method has several limitations. DBS only requires a very small blood volume, so analysis with DBS can only be done for highly sensitive substances. DBS also cannot be used for the analysis of compounds that are sensitive to air or are volatile. Also, blood taken for DBS is derived from capillaries. There are several possibilities that the concentration of capillary blood analyte can vary from venous blood. It has been reported that paracetamol has a significantly higher concentration in blood taken from fingertips (capillaries) than venous blood.<sup>[44]</sup>

## DISCUSSION

### *The Proposed Method of Acrylamide and Glycidamide Analysis in Dried Blood Spot*

Analysis of acrylamide and glycidamide in the blood has been done before on experimental animals and also in humans. The samples used in these studies also varied, such as blood samples, various body tissues, urine, and placenta. For this review article, the authors choose two methods that have been developed by Harahap *et al.* (2020) and Kim *et al.* (2014). The methods chosen is based on the discussion that will be stated below. The combination of these two methods is expected to develop a new method.

The first method is developed by Harahap *et al.* in 2020, in their research on acrylamide analysis in DBS. This study used blood samples from student as subjects. The DBS biosampling technique is used because it have some advantages when compared to venepuncture technique, namely, increase comfort for the subject because it does not require large blood draws, only about 100  $\mu$ L (approximately five drops). Harahap *et al.* use propranolol as the internal standard. In the sample preparation, the samples are extracted by protein precipitation method using methanol as the solvent. The extracted samples then are injected to the LC-MS/MS. This instrument has analytical capabilities with high selectivity and sensitivity, making it suitable for analyzing analytes that have small amounts in the blood such as acrylamide and glycidamide.

The analytical condition is:

- The UPLC BEH C<sub>18</sub> Acquity<sup>®</sup> column (2.1 x 100 mm; 1.7  $\mu$ m)
- The mobile phase is 0.1% formic acid and acetonitrile (60:40)
- The modes of detection were positive ESI and MRM with m/z values 71.99 > 55.23 for acrylamide and 260.2 > 116.2 for propranolol.
- And the flow rate of 0.2 ml/min.

This condition has been optimized by Harahap *et al.* (2020). Lower Limit of Quantification (LLOQ) value obtained is 2.5  $\mu$ g/ml for acrylamide.

The second method is developed by Kim *et al.* (2014), in his research on the determination of acrylamide and glycidamide levels in various body tissues of rats and their pharmacokinetic studies. The samples used in this study were plasma and some rat body tissue. Kim *et al.* (2014) used Stabled Isotope Labeled (SIL) Acrylamide-D<sub>3</sub> as internal standard. The method used in sample preparation is protein precipitation with acetonitrile as the solvent. Then, the sample is injected to the LC-MS/MS instrument with the analytical condition:

- dC<sub>18</sub> column (2.10 x 150 mm; 3  $\mu$ m)
- The mobile phase is 0.05% formic acid and acetonitrile (90:10)

- The modes of detection were positive ESI and MRM with m/z values for acrylamide and glycidamide were 71.90 > 55.00, 87.9 > 44.2 respectively
- And the flow rate of 0.1 ml/min.

The LLOQ value obtained are 5 ng/mL for acrylamide and 10 ng/mL for glycidamide. Compared with the LLOQ value obtained in the Harahap's research in 2020, Kim has smaller value of LLOQ. This may be is the effect shown because of different biosampling technique. The difference in LLOQ values in plasma and DBS can also be influenced by drug distribution in plasma and cellular blood components. Also, the value difference could be caused by the different internal standards used in these two studies. Nonetheless, both LLOQ obtained by Harahap and Kim have met the requirements of Bioanalytical Method Validation from 2018 FDA Guideline and also 2011 EMEA Guideline.

The development of new methods requires a combination of the two methods above. The method carried out by Harahap *et al.* (2020) has been applied to 15 students who were potentially consumed a lot of acrylamide-contains foods. This research indicates that DBS can be used to determine acrylamide exposure in humans so that it is expected to reduce acrylamide exposure. However, this research only carried out the analysis of acrylamide. Therefore, it is necessary to have a combination with other methods that carried out a simultaneous analysis such as in Kim's research.

After the results are obtained, then it can be seen the relation between diet intake and acrylamide and glycidamide levels in the blood. The obtained data of levels are expected to be used for further studies regarding the potential carcinogenicity of acrylamide and glycidamide for humans related to diet. This further study can refer to research conducted by Tardiff *et al.* in 2010 regarding the estimation of safe levels of acrylamide from food intake in humans which has been discussed before in this article.

## CONCLUSION

Based on the articles review in the aforementioned, higher levels of acrylamide were found in subjects, who frequently consumed foods containing a lot of acrylamide such as french fries, potato chips, coffee, fried foods, popcorn, cereals, biscuits, pastries, bread, and fast food. Acrylamide and glycidamide, especially glycidamide, can bind to DNA to form DNA-adducts and can trigger gene mutations, which can lead to cancer in humans. A new methods for the analysis of acrylamide and glycidamide in DBS with LC-MS/MS, namely the analysis conditions in the form of: column Aquity<sup>®</sup> BEH C<sub>18</sub>; mobile phase acetonitrile-formic acid 0.1% (40:60); flow rate of 0.2 mL/min; ESI positive detection methods and MRM with m/z values for acrylamide, glycidamide, and d3-acrylamide, respectively 71.99 > 55.23, 87.90 > 44.20, and 75.0 > 58.0. The sample preparation method is protein precipitation using methanol as the solvent.

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