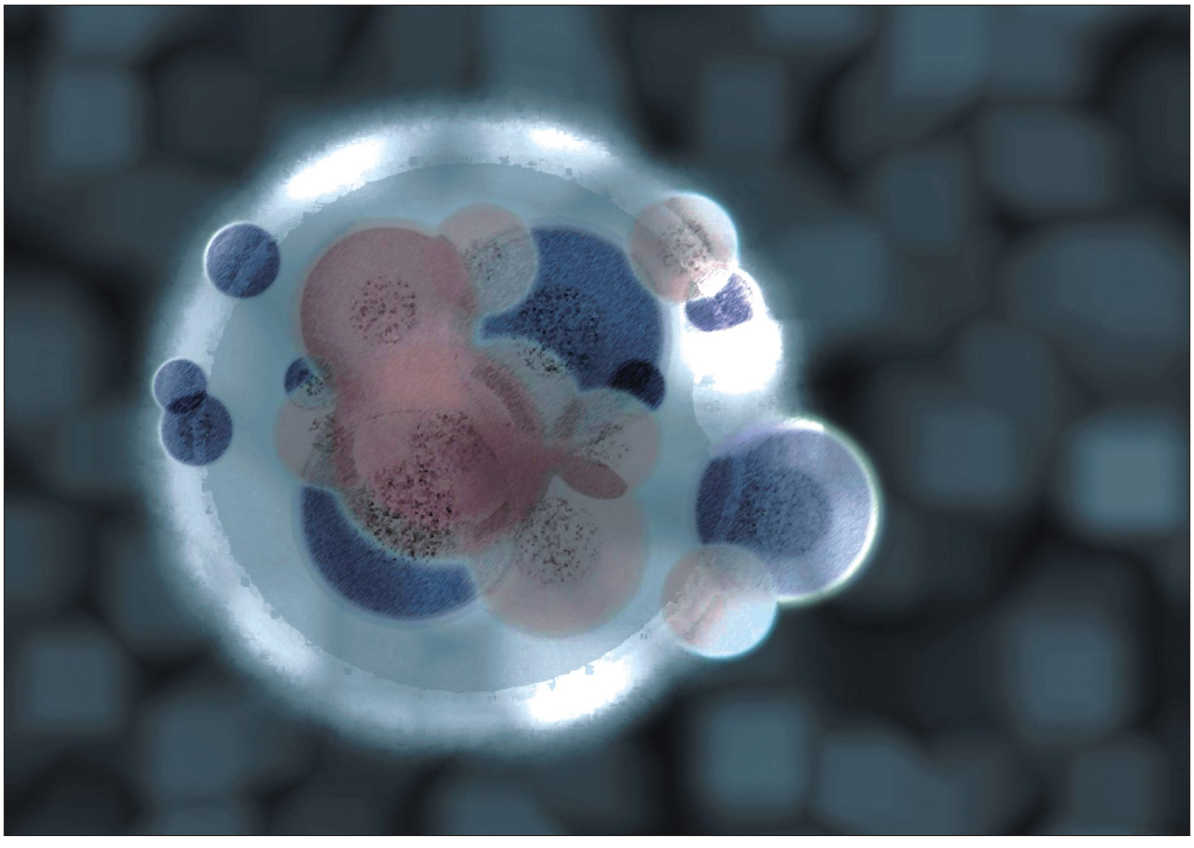

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# Research Article
# Analysis of Acrylamide in Dried Blood Spot of Students by Liquid Chromatography-Tandem Mass Spectrometry

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## Abstract

**Background and Objective:** Acrylamide is a chemical which is genotoxic, potentially carcinogenic to humans. The aim of this study was to know whether there is a significant difference in the concentration of acrylamide between the DBS samples of the students and the negative control subjects. **Materials and Methods:** In this research, analysis of acrylamide was performed in dried blood spot (DBS) samples of 15 students, who consume a lot of acrylamide-containing foods and 15 subjects of a negative control group. DBS samples were extracted by a method of ultrasound-assisted liquid extraction and analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). A reversed-phase chromatography separation was performed on an Acquity UPLC BEH $C_{18}$ column eluted at a flow rate of 0.2 mL $min^{-1}$ under a gradient of the mobile phase of 0.1% formic acid and acetonitrile within 3 min. The bioanalytical method of acrylamide in DBS samples of students with propranolol as the internal standard using LC-MS/MS had been validated in this study. **Results:** The calibration curve range for acrylamide obtained was 2.5-100 μg $mL^{-1}$ with the coefficient of correlation of more than 0.99. The analysis results of 15 students showed acrylamide levels were within the range of 5.87-14.17 μg $mL^{-1}$. The DBS samples of the negative control group presented the presence of acrylamide in concentration ranging from 2.72-3.51 μg $mL^{-1}$. **Conclusion:** The results indicated that DBS could be used to determine acrylamide exposure in humans and there was a significant difference in acrylamide concentration between DBS of students and negative control subjects.

Key words: Acrylamide, dried blood spot, LC-MS/MS, exposure, food, calibration curve, gradient

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Acrylamide had been used as an industrial chemical in paper, plastic and dye industries, water treatment and others for years but it was firstly discovered in thermally treated foods[1]. In Swedish National Food Administration (NFA) published that acrylamide was found in different thermally treated foods including potato chips, french fries, popcorn, biscuits, cereals, coffee and bakery products[2].

Shortly after acrylamide was firstly discovered in foods containing high content of carbohydrate and heated at high temperature, which is above 120°C, thermal degradation of free asparagine in the presence of reducing sugars in Maillard reaction is thought to be a method of acrylamide formation[3-5]. Protein-containing foods, such as beef and chicken, also produce acrylamide in smaller amounts. Maillard reaction does not occur in foods that are processed at a low temperature[6].

Acrylamide is definitely genotoxic and carcinogenic in animals. International Agency for Research on Cancer (IARC) has classified acrylamide into group 2A, which is a compound that is probably carcinogenic to humans. Acrylamide causes health problems due to its genotoxic and carcinogenic effects[7,8]. Acrylamide is mutagenic and damages DNA by interacting with DNA and forming DNA adducts[9]. Acrylamide has the potential to cause damage to nerve cells and reproductive disorders in experimental animals and long-term administration of acrylamide can cause tumors[10].

The research conducted by Kim *et al.*[11] used liquid chromatography-tandem mass spectrometry (LC-MS/MS) to quantify acrylamide in plasma, urine and 14 different tissues in mice. The sample preparation method applied was protein precipitation and the lower limit of quantification (LLOQ) obtained was 5 ng $mL^{-1}$ for the biological matrix of plasma. In this study, the subjects used were students because they were one of the potential populations consuming a lot of acrylamide-containing foods so this research wanted to know how much acrylamide exposure they received from foods[12-15]. Almost all school-aged children (95-96%) do snack. The expenditure on children's pocket money for snacks (50-80%), which is classified as high, shows that snacks are a priority. Snacks contribute significantly to 20-31.1% of the daily energy need of children. Research in Semarang showed that the average frequency of snacking in students was 2-3 times every day[16,17,7].

Analysis of acrylamide in dried blood spot (DBS) has never been conducted yet. Therefore, this research conducted an analysis of acrylamide and propranolol as the internal standard in DBS of students using LC-MS/MS which applied a bioanalytical method that had been developed and fully validated. This study was an application of the bioanalytical method in determining the human exposure of acrylamide deriving from foods. The aim of this study was to obtain the data of acrylamide concentration in DBS of students by using LC-MS/MS and to obtain the comparison data of acrylamide concentration between DBS of students and negative control subjects by performing a statistical test.

## MATERIALS AND METHODS

**Study area:** The study was conducted from April-July, 2019. The study was carried out at the Laboratory of Bioavailability-Bioequivalence, Faculty of Pharmacy, Universitas Indonesia, Depok, Indonesia.

**Chemicals and materials:** Acrylamide and propranolol hydrochloride were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Formic acid HPLC grade and methanol HPLC grade were obtained from Merck (Darmstadt, Germany). Ultrapure water generated via Sartorius Water Filter system (Göttingen, Germany) was used throughout the experiment. Whole blood was from Palang Merah Indonesia. PerkinElmer 226 papers were from PerkinElmer (Waltham, Massachusetts, USA).

**Preparation of stock solutions, calibration samples and quality control samples:** Acrylamide and propranolol as the internal standard were prepared by diluting them in ultrapure water and methanol respectively to obtain a concentration of 1 mg $mL^{-1}$ for each compound. Acrylamide stock solution was used to prepare a working solution containing 10 µg $mL^{-1}$ acrylamide in ultrapure water.

All calibration curves consisted of at least 6 calibrator concentrations, a blank sample (without IS) and a zero sample (with IS). Calibration samples were prepared by diluting working solution using whole blood to obtain a calibration range of 2.5-100 µg $mL^{-1}$ at a minimum of six concentration levels. Quality control solutions were prepared at 2.5 (LLOQ), 7.5 (QCL), 37.5 (QCM) and 80 µg $mL^{-1}$ (QCH) by diluting the working solution in whole blood.

**Sample preparation:** Calibration and quality control samples were prepared by pipetting 30 µL aliquots from appropriately spiked whole blood onto the PerkinElmer 226 papers. This was allowed to dry at room temperature for 3 h. DBS discs were made by cutting the blood spot from the PerkinElmer 226 papers and putting it into a sample cup. One hundred µL of 10 µg $mL^{-1}$ propranolol was added to the sample cup. The analyte was then extracted using 500 µL methanol.

The mixture was shaken using vortex for one min and sonicated for 5 min. It was then centrifuged for 1 min at 4,006 g. Four hundred µL supernatant was transferred to a sample cup and evaporated at 40°C for 25 min under the gentle stream of nitrogen. The dried extract was reconstituted with 100 µL of 0.1% of formic acid-acetonitrile (60:40) as the mobile phase and then sonicated for 20 min and shaken using vortex for 30 sec. It was then centrifuged for 3 min at 736 g. Seventy µL aliquot was put in an insert vial and 10 µL aliquot was injected into the LC-MS/MS system.

**LC-MS/MS equipment and conditions:** Samples were analyzed using Waters Xevo TQD Triple Quadrupole with Acquity UPLC $C_{18}$ BEH (2.1×100 mm), 1.7 µm column at 30°C, controlled by MassLynx Software from Waters (Milford, USA). The mobile phase consisted of 0.1% of formic acid (eluent A) and acetonitrile (eluent B) at 0.2 mL $min^{-1}$. A gradient program was performed for the elution. The initial composition of eluent was A-B (40:60). Eluent A was maintained for half a min and then followed by increasing its composition to 60% A for the next 0.70 min and continuing to 50% A at 1.20 min. The volume of injection was 10 µLand the total analytical time was 3 min.

The MS condition was using electrospray ionization positive for acrylamide and propranolol with m/z values of 71.99>55.23 and 260.2>116.2, respectively. The samples were detected by using the mode of analysis of multiple reaction monitoring (MRM). The capillary voltage used was 3.5 kV. The temperature and flow rate of nitrogen was controlled at 400°C and 650 L $h^{-1}$. Argon was used as the collision gas. The cone and collision voltage for acrylamide and propranolol were 26 V, 35 V and 8 V, 20 V, respectively.

**Validation of analytical method:** Lower Limit of Quantification (LLOQ):LLOQ is the lowest concentration of the calibration curve, which was 2.5 µg $mL^{-1}$ for acrylamide, which still fulfills the precision and accuracy requirement. It is tested using 5 replicates. It claimed to fulfill the requirement if the percentage difference and percentage CV values are within 20%.

**Linearity:** The working solution containing acrylamide which was diluted by whole blood to get a minimum of 6 concentration levels: 2.5, 5, 7.5, 25, 37.5, 50, 75, 80 and 100 µg $mL^{-1}$. Calibration samples were spotted onto the PerkinElmer 226 paper according to the procedure explained above. Calibration curve measure was based on the ratio of acrylamide area to propranolol area.

**Accuracy and precision:** Quality control samples were prepared at a minimum of 4 concentration levels which were 2.5 (LLOQ), 7.5 (QCL), 37.5 (QCM) and 80 µg $mL^{-1}$ (QCH) by diluting the working solution in whole blood. Each concentration was tested using a minimum of five replicates by within-run and between-run. It fulfills the requirement if % difference and percentage CV obtained within 20% for LLOQ and within 15% for other concentration levels besides LLOQ.

**Recovery:** Recovery was performed to observe the extraction efficiency. Quality control samples were prepared at three concentration levels: QCL, QCM and QCH. Each concentration level was tested using 3 replicates.

**Selectivity:** Blank samples obtained from 6 different human sources were prepared according to the procedure explained above to assure that there is no interference response that can disrupt analyte and internal standard detection. The presence of interference can be tolerated if the response is not higher than 20% of analyte area at LLOQ concentration and not higher than 5% of internal standard area.

**Carry over:** Working solutions were diluted to obtain the upper limit of quantification (ULOQ), then it was spotted onto DBS paper and prepared according to the procedure explained above. Then, blank samples were prepared by the same procedure. The blank sample was injected after ULOQ. Each concentration level was tested using five replicates. Blank sample area should be within 20% of analyte area at LLOQ concentration and within 5% of internal standard area.

**Matrix effect:** Matrix effect was observed by measuring the matrix factor, which compares the acrylamide and propranolol area that were added after the extraction process to acrylamide and propranolol area in the standard solution. Then, measure the internal standard normalized matrix factor, which divides the analyte matrix factor to internal standard matrix factor. It fulfills the requirement if the percentage CV value is not higher than 15%.

**Stability:** Stability was tested using acrylamide and propranolol stock solution which were stored at room temperature for 0, 6 and 24 h and stored at 20°C for 0, 20 and 45 days before being analyzed. Its percentage difference value was measured toward the response at time 0 and day 0 and should not be higher than 10%. It was tested using 2 replicates. The stability test was also performed to observe the analyte in DBS matrix at 2 concentration levels: QCL and

QCH, which were stored at room temperature for 0, 6 and 24 h and for 0, 20 and 45 days. It was tested using 3 replicates. Besides, the stability of the analyte in the matrix that was stored in autosampler was also tested for 0 and 24 h. It was also tested using three replicates.

**Application of the method:** After approval (KET-109/UN2.F1/ETIK/PPM.00.02/2019) by the Human Research Ethics Committee of Universitas Indonesia and Cipto Mangunkusumo Hospital (HREC-FMUI/CMH), a total of 15 students and 15 negative control subjects were enrolled in the study. They signed the informed consent prior to participating in this study.

The study inclusion criteria of the student group were a student who was 6-18 years old, was not an active and passive smoker and consumed a lot of acrylamide-containing foods such as french fries, potato chips, popcorn, biscuits, coffee and cereals. The study inclusion criteria of the negative control group were a subject who was 6-75 years old, was not an active and passive smoker, consumed little acrylamide-containing foods and was willing not to consume those foods during 96 h before and during the process of blood sampling. Information about the habits of smoking and consuming acrylamide-containing foods in both groups of subjects was obtained by using a questionnaire.

Finger prick blood samples were collected from 30 subjects. Around 100 µL blood samples were collected from the fingertips. Blood was taken by finger prick technique using lancet and the first drop of blood from fingertip was thrown away by rubbing it with an alcohol swab and then the blood drops were collected in a 0.5 mL K3 EDTA microtube. After that, 30 µL aliquot blood was immediately transferred onto the DBS paper using a calibrated pipette. Next, the DBS paper was dried at room temperature for 3 h. After drying, the DBS paper was stored in a sealed bag in which the silica gel was inserted into it[14].

**Statistical analysis:** A statistical test was performed on acrylamide concentration obtained from both groups of subjects to find out whether there was a significant difference between the 2 groups. The statistical test performed was independent samples t-test. Based on the results of the normality test performed, the data in each group of data was normally distributed. The p value of the t-test was 0.000 so this showed that there was a significant difference in the acrylamide concentration between DBS of students, namely subjects who consumed a lot of acrylamide-containing foods and negative control, namely subjects who consumed little of those foods and were willing not to consume them for 96 h before blood sampling.

## RESULTS

**Chromatography and sample preparation:** Sample preparation was done by spotted 30 µL aliquot blood on PerkinElmer 226 paper. The extraction process was performed in a short time which was assisted by vortex for a min and sonication for 5 min. DBS technique needs a sensitive and selective method because it has low concentration and it uses whole blood which still contains many interferences hence, LC-MS/MS is suitable to analyze it. This study was performed by using Acquity UPLC $C_{18}$ BEH (2.1×100 mm), 1.7 µm to separate compound of interest with a total analytical time of 3.0 min. The retention time of acrylamide and propranolol were 1.15 min and 1.29 min, respectively.

**Validation of analytical method:** The calibration curve was linear in the range of 2.5-100 µg $mL^{-1}$ with an r-value of 0.99378. The results of accuracy and precision fulfilled the requirements. They were determined by the value of % diff and % CV respectively as shown in Table 1.

Although LC-MS/MS is a highly selective instrument, there is still a probability of the presence of another component besides analyte and internal standard that can interfere ionization process. Hence, when using a mass spectrometer, we have to test the matrix effect to assure that it does not affect accuracy and precision.

The matrix factors of acrylamide were 91% and 98% at QCL and QCH concentrations. This showed that ion suppression occurred but that did not affect the repeatability

Table 1: Results of the validation of the analytical method

| | | | Within-run | | Between-run | | | |
|---|---|---|---|---|---|---|---|---|
| Analyte | Linear regression | Quality control | Accuracy (%) | Precision (%) | Accuracy (%) | Precision (%) | Extraction yield (%) | Matrix factor (%) |
| Acrylamide | y = 0.00463 | LLOQ | 110.40 | 3.37 | 102.77 | 9.51 | - | - |
| | + 0.00293x | QCL | 104.27 | 7.12 | 101.93 | 4.12 | 95.51 | 91 |
| | r = 0.99378 | QCM | 91.28 | 1.94 | 106.48 | 4.48 | 84.61 | - |
| | | QCH | 92.78 | 2.72 | 101.46 | 5.41 | 90.61 | 98 |

LLOQ: Lower limit of quantification, QCL: Low quality control, QCM: Medium quality control, QCH: High quality control

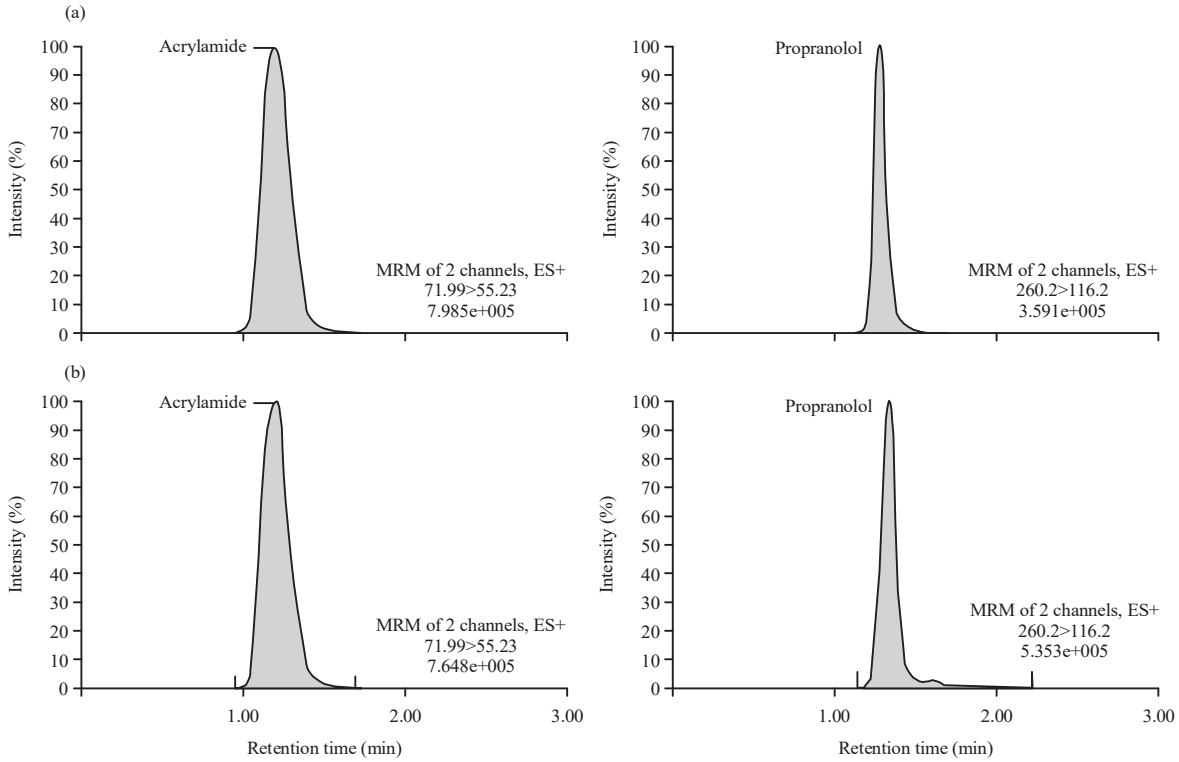


Fig. 1(a-b): Chromatograms obtained from DBS, (a) Sample of subject SP 13 and (b) Sample of subject SP 15

Table 2: Data of acrylamide concentration based on the order of the amount of consumption of acrylamide-containing foods in students

| Subject codes | Food and beverage consumption habits | | | | | | | | | | Acrylamide concentration (μg mL$^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Potato chips | French fries | Coffee | Fried starchy foods | Popcorn | Cereals | Biscuits | Crackers | Bread | Fast food | |
| SP 13 | 5 | 5 | 5 | 4 | 2 | 5 | 5 | 3 | 5 | 4 | 14.17 |
| SP 08 | 4 | 5 | 5 | 3 | 1 | 5 | 5 | 4 | 4 | 3 | 10.92 |
| SP 05 | 4 | 3 | 3 | 5 | 3 | 4 | 3 | 4 | 5 | 3 | 9.22 |
| SP 09 | 3 | 4 | 4 | 5 | 2 | 4 | 4 | 3 | 4 | 3 | 9.87 |
| SP 10 | 3 | 4 | 4 | 3 | 1 | 5 | 3 | 3 | 5 | 5 | 9.80 |
| SP 14 | 5 | 3 | 3 | 4 | 2 | 3 | 4 | 3 | 3 | 4 | 9.83 |
| SP 02 | 3 | 5 | 4 | 3 | 2 | 4 | 3 | 3 | 4 | 3 | 8.91 |
| SP 06 | 3 | 5 | 3 | 3 | 1 | 3 | 3 | 4 | 4 | 3 | 8.12 |
| SP 03 | 3 | 4 | 5 | 3 | 2 | 2 | 3 | 2 | 3 | 3 | 7.68 |
| SP 01 | 3 | 3 | 2 | 3 | 1 | 5 | 3 | 3 | 5 | 2 | 6.89 |
| SP 04 | 4 | 3 | 1 | 3 | 1 | 4 | 3 | 2 | 3 | 5 | 7.10 |
| SP 11 | 3 | 4 | 1 | 5 | 1 | 2 | 3 | 3 | 5 | 2 | 7.06 |
| SP 07 | 4 | 3 | 2 | 3 | 2 | 2 | 5 | 2 | 3 | 3 | 6.00 |
| SP 12 | 3 | 3 | 2 | 3 | 1 | 3 | 3 | 5 | 3 | 2 | 6.35 |
| SP 15 | 2 | 2 | 1 | 2 | 1 | 1 | 5 | 3 | 4 | 2 | 5.87 |

SP: Subject code for the subject group of students, 5: More than 4 servings a week, 4: 3-4 servings a week, 3: 1-2 servings a week, 2: <4 servings a month, 1: Not at all

of the method which resulted in % CV value that was not higher than 14.11%. On the other side, endogen component in whole blood can also interfere with the analysis. The test showed a minimum response interference in the range of 0.70-1.06% for acrylamide and 0.06-1.92% for propranolol.

During analysis, carry over could happen from the previous injection. The result showed an acceptable percentage of carry-over which was in the range of 11.89-18.83% for acrylamide and 0.40-0.46% for propranolol. Another aspect that should be concerned in the development of an analytical method is to assure that dilution does not affect accuracy and precision. The result of dilution integrity test showed percentage difference value was not lower than -14.81% and was not higher than 10.87%. The % CV value was

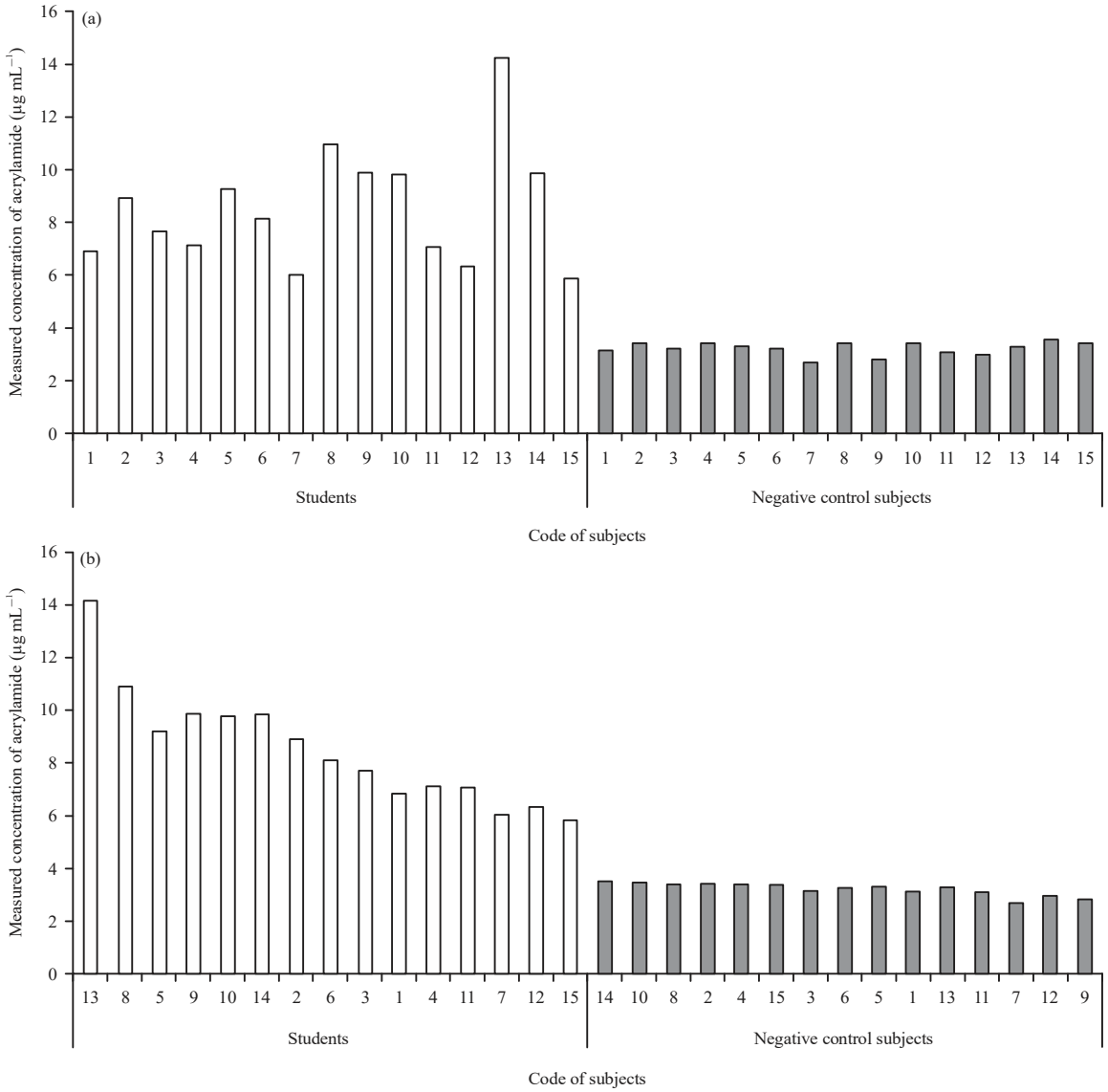


Fig. 2(a-b): Graph of the result of the analysis of acrylamide in DBS of (a) Students and negative control subjects and (b) Based on the order of the amount of consumption of acrylamide-containing foods

not higher than 7.46%. Extract from DBS that contained acrylamide at QCL and QCH concentrations were stable for 24 h in the autosampler. For the storage condition, DBS samples were stable at 25 and 4°C for 24 h and 28 days with the maximum %CV value of 10.10%. The stock solutions of acrylamide and propranolol were stable for at least 28 days at 4°C.

**Application of the method:** The analysis results of DBS of 15 students showed that all samples contained acrylamide in certain concentrations. Table 2 shows that the lowest acrylamide concentration was found in subject code of SP 15-5.87 µg $mL^{-1}$ and the highest concentration was found in SP 13 of 14.17 µg $mL^{-1}$. The data of acrylamide concentration based on the order of the amount of consumption of acrylamide-containing foods in students can be seen on Table 2. The chromatograms from DBS samples of SP 13 and SP 15 are shown in Fig. 1. The average acrylamide concentration in DBS of students was 8.52 µg $mL^{-1}$ with a standard deviation of 2.22 µg $mL^{-1}$ and a coefficient of variation of 26.10%. Based on the data obtained, it could be concluded that the acrylamide concentration in DBS of students had a wide range. The variation of acrylamide concentration was not due to the differences in acrylamide exposure deriving from cigarette smoke or drinking water but due to the differences in the amount of consumption of acrylamide-containing foods in each subject.

The analysis results of DBS of 15 negative control subjects showed that all samples contained acrylamide in smaller concentration compared to those of students. The lowest acrylamide concentration was found in subject code of SN 07 of 2.72 μg $mL^{-1}$ and the highest concentration was found in SN 14 of 3.51 μg $mL^{-1}$. The average acrylamide concentration in DBS of negative control subjects was 3.23 μg $mL^{-1}$ with a standard deviation of 0.24 μg $mL^{-1}$ and a coefficient of variation of 7.55%. The graph of the result of the analysis of acrylamide in DBS of students and negative control subjects can be seen in Fig. 2a. The graphs of the result of the analysis of acrylamide in DBS of students and negative control subjects based on the order of the amount of consumption of acrylamide-containing foods can be seen in Fig. 2b.

## DISCUSSION

This study found that the more acrylamide-containing foods that the subjects consumed, the higher concentration of acrylamide that was detected in the DBS samples. DBS of SP 13 contained acrylamide in the highest concentration of 14.17 μg $mL^{-1}$. Table 2 shows the data of acrylamide concentration based on the order of the amount of consumption of acrylamide-containing foods in students. Based on data obtained from the questionnaire, SP 13 was the student who consumed acrylamide-containing foods the most, namely potato chips, french fries, cereals, coffee, biscuits, bread, fast food and fried starchy foods. the average acrylamide concentration in potato chips, french fries, cereals, coffee, biscuits and bread are 1939, 1764, 163, 1123, 289 and 30 μg $kg^{-1}$, respectively[8,9,10]. In addition, fried starchy foods also contain acrylamide.

DBS of SP 15 contained acrylamide in the lowest concentration of 5.87 μg $mL^{-1}$. This was in accordance with the data obtained from the questionnaire that SP 15 was the student who consumed acrylamide-containing foods the least. SP 15 consumed little french fries, potato chips, fried starchy foods and fast foods, i.e., less than four servings a month for each type of those foods. SP 15 never consumed coffee, popcorn and cereals.

Acrylamide was still detected in small concentration in DBS of negative control subjects even though they had not been consuming acrylamide-containing foods for 96 h before blood sampling. This might be due to consuming the foods during the 96 h might still contain acrylamide in small concentration but there was no data in journals so the researchers did not include the foods in the list of foods that were prohibited to eat during 96 h before blood sampling. Besides that, the subjects might still consume little foods that were prohibited to eat during the 96 h.

There has been no study on the quantification of acrylamide in human blood samples. Therefore, it is not yet known the normal acrylamide concentration contained in human blood. In addition, the Food and Drug Administration (FDA) has not set limits on acrylamide-containing foods that can be consumed by humans and the maximum acrylamide concentration in human blood that is safe so as not to cause cancer. Therefore, it is not yet known how much acrylamide concentration in human blood that is considered as low or high. However, a comparison of acrylamide concentration could be done between the blood samples of subjects who consumed a lot of acrylamide-containing foods, such as students and those of subjects who consumed little of those foods and were willing not to consume them for a certain period of time before blood sampling. After being exposed orally, acrylamide is absorbed well, is distributed to the tissues and is thought to cause various types of cancer[8] so it is important to reduce the consumption of acrylamide-containing foods and to keep its concentration in the blood as low as possible.

## CONCLUSION

In this study, it was found that there was a significant difference in acrylamide concentration between DBS of students and negative control subjects. These results indicated that DBS could be used to determine acrylamide exposure in humans to reduce exposure to acrylamide.

## SIGNIFICANCE STATEMENT

This study discovered that there was a significant difference in acrylamide concentration between DBS of students and negative control subjects. These results indicate that DBS can be beneficial for determining acrylamide exposure in humans to reduce exposure to acrylamide. This study will help the researchers to uncover the critical areas of analyzing acrylamide in DBS that many researchers were not able to explore. Thus a new theory on method of analysis of acrylamide in DBS may be arrived at.

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