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Bioanalytical Method Validation of Acrylamide and Glycidamide in Dried Blood Spot Using Ultra High Performance Liquid Chromatography Tandem Mass Spectrometry

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ABSTRACT

Acrylamide (AA) is a carcinogenic compound that can be found in food, coffee, and cigarette smoke. When it enters human body, acrylamide will be metabolized by CYP2E1 to glycidamide (GA) which can then react with DNA to form DNA adducts. To analyze acrylamide and glycidamide simultaneously in the blood, commonly used biosampling technique is venipuncture, which is invasive and requires special expertise. The biosampling technique that was used in this study is Dried Blood Spot (DBS) method as it is easy and non-invasive. Methods for analyzing acrylamide and glycidamide simultaneously using DBS have not been carried out in previous studies. Therefore, the aim of this study is to obtain an optimum and validated method of acrylamide and glycidamide simultaneous analysis with propanamide as an internal standard. Sample preparation was done by protein precipitation using a mixture of methanol and water (1:1). Separation of compounds used reversed phase chromatography with the Acquity® UPLC BEH C18 column (1.7 µm, 2.1 mm x 100 mm) and elution flow rate of 0.20 mL/min under gradient conditions with a mobile phase of 0.2% formic acid in water and acetonitrile for 5 minutes. Quantification was performed using triple quadrupole mass spectrometry with positive electrospray ionization and multiple reaction monitoring (MRM) mode set at m/z 72.0> 55.02 for acrylamide, 88.1> 44.0 for glycidamide, and 74.01> 57.1 for propanamide. The lower limit of quantification was obtained at 1 µg/ml for both acrylamide and glycidamide. The range of linear concentration was 1 - 40 µg/ml. The analysis method was valid according to FDA 2018 guidelines.

Keywords: Acrylamide, Glycidamide, Propanamide, Dried Blood Spot, KCKUT-SM/SM, Validation

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INTRODUCTION

One of the chronic diseases found in Indonesia is cancer. The International Agency for Research on Cancer (IARC) reports that in 2018, around 18.1 million new cancer cases were found and 9.6 million die because of cancer (IARC, 2018). One cause of cancer is continuous exposure to carcinogens. An easily found carcinogens are acrylamide (AA) and its active metabolite, glycidamide (GA). Acrylamide can be found in cigarette smoke, coffee, and carbohydrate-rich foods such as potatoes and cereals. In foods that contain a lot of carbohydrates, acrylamide is formed when heating to high temperatures, as a result of the reaction between reducing sugars and the amino acid asparagine. This reaction is known as the Maillard reaction. Acrylamide is also neurotoxic, immunotoxic, and toxic to the reproductive system. Glycidamide, an active metabolite of acrylamide, can be found in patients with lung cancer, liver cancer, kidney cancer, bile duct cancer, cervical cancer, and other cancers at lower glycidamide concentrations. Acrylamide levels needed to cause damage to rat DNA are 10, 20, and 30 mg/kg body weight (Zamani, Shokrzadeh, Fallah, & Shaki, 2017; Zhivagui et al., 2019).

To predict the risk of exposure to acrylamide and glycidamide in humans can be done by quantification the levels in the blood. The usual way to take biological samples (biosampling) is venipuncture. Venipuncture is defined as taking blood from a vein that is used for testing in the laboratory (Harbert, 2007). However, the implementation of these methods requires special expertise and adherence to guidelines in the collection, storage, and delivery of blood samples that have been determined. An alternative way of collecting blood that can be used is by Dried Blood Spot (DBS). The collection of DBS is relatively painless, simple, less invasive than venipuncture, and easier to store (Ostler, Porter, & Buxton, 2014).

In food, acrylamide can be analyzed using LC-MS/MS or high performance liquid chromatography with UV detectors (Jozinović et al., 2019; Wang, Feng, Guo, Shuang, & Choi, 2013). Yet, the analysis of acrylamide which is carried out in a biological matrix generally uses LC-MS/MS (Harahap, Elysia, Starlin, & Jayusman, 2020). In previous studies, research on acrylamide and glycidamide has been carried out in various biological matrices (Kim et al., 2015) as well as toxicokinetic tests of acrylamide and glycidamide in mice (Doerge, Young, McDaniel, Twaddle, & Churchwell, 2005). However, there has not been any simultaneous validated method of analyzing acrylamide and glycidamide in human DBS samples yet. The aim of this study is to develop and validate the analytical method of acrylamide and glycidamide in human DBS simultaneously using the UPLC-MS/MS method. Validation parameters to be tested were selectivity, carry over, the lower limit of quantification (LLOQ), linearity, accuracy, precision, dilution integrity, matrix effects, and stability (FDA, 2018).

MATERIALS AND METHOD

Stock and Sample Solution: Acrylamide, Glycidamide, and Propanamide were obtainez from Sigma-Aldrich (Singapore). Formic Acid, HPLC-Grade Acetonitrile and methanol were acquired from Merck (Darmstadt, Germany). Whatman® 903 Protein Saver Card was obtained from Sigma-Aldrich.

Blank and Matrix Preparations: Rabbit whole blood was used for blank and biological matrix. The blood was collected from 6 rabbits that weight not less than 2 kg. The collection site used for collecting blood was the central ear artery. Ketamine from Hameln Pharmaceuticals (Germany) was used for anesthesia. The method used was ethically approved by The Ethics Committee of The Faculty of Medicine, Universitas Indonesia (protocol number 20-02-0124).

LC-MS/MS: The analysis was carried out with liquid chromatography tandem mass spectrometry using the Acquity® UPLC BEH C18 column (1.7 µm; 2.1 x 100 mm). Detection was carried out at mass spectrometer using positive type ESI and MRM transition m/z values for each acrylamide, glycidamide, and propanamide (PA) were 72.0> 55.02, 88.1> 45.0, and 74.0> 57.1 respectively. The capillary voltage used is 3.50kV with 22V cone voltage for acrylamide and propanamide, and 16V for glycidamide. Desolvation temperature, gas flow rate, and gas cone source flow rate were set at 400°C, 650L/h, and 1L/h. The voltage in the collision chamber for AA, GA, and PA was 8V, 9V, and 9V respectively. The amount of sample injected into LC-MS/MS was 5 µl.

Stock and Standard Preparation: 10 mg of acrylamide, propanamide, and glycidamide stock solution were prepared by dissolving each substance in 10 ml of ultrapure water. The acquired concentration is 1000 µg/ml. The stock solution of AA and GA is diluted with rabbit blood until a concentration range of 1-40 µg/ml to obtain the working solution. Acrylamide and glycidamide quality control samples were prepared separately by the same procedure the working solution at a concentration of 3 µg/mL, 30 µg/mL, and 40 µg/mL for QCL, QCM, and QCH respectively. To prepare the internal standard solution, propenamide stock solution was diluted to 10 µg/ml.

Chromatographic Condition Optimization: The optimized parameters were mobile phase combination, mobile phase composition, and flow rate. Lastly, system suitability test was carried out. The mobile phase combination tested was 0.1% formic acid in water – acetonitrile, 0.2 % formic acid in water – acetonitrile, and 0,5% formic acid in water – 0,5% formic acid in acetonitrile. The composition of mobile phase tested was 90:10 v/v, 80:20 v/v, 70:30 v/v, 60:40 v/v, and 50:50 v/v. The variation of flow rate that was tested was 0.1 ml/min, 0.2 ml/min, and 0.3 ml/min.

Sample Preparations Optimization: Working solutions of AA and GA with appropriate concentration were spotted on the DBS paper. Then, the DBS paper was dried for 2 hours at room temperature. The spotting volume was optimized with a variation of 30 µl, 40 µl, and 50 µl. Then, as much as 100 µl internal standard solution was added into a sample cup containing the dried blood spot. Then, extraction solvent was subsequently added. The extracting solution used for optimization was 100% methanol, methanol-water mixture (1: 1), methanol-water mixture (4: 1), methanol-water mixture (1: 4), and 100% water. Variation of extraction solvent volume for optimization were 500 µL, 800 µL, and 1000 µL. The mixture was then vortexed, sonicated, and centrifuged. The variations of vortex time tested for optimization were 30, 60, and 180 seconds. Sonication time variations for optimization were 5, 15, and 20 minutes. The variations of centrifugation time for optimization were 1; 3; and 5 minutes.

Method Validation: The method validation was carried out according to FDA (2018) Bioanalytical Method Validation Guidance for Industry. Parameters validated in this study are as followed.

Selectivity: The selectivity of this method was evaluated by using 6 different blood sources at LLOQ concentrations and blanks of 2 replicas each. Then, interference in the blank was observed. Blank and zero should be free from interference. The resulting interference value should not exceed 20% of the peak area at the LLOQ concentration for the analyte and should not exceed 5% for the internal standard.

Carry Over: Carry over was tested for five replicas with concentrations of acrylamide and glycidamide on ULOQ, blank, and LLOQ. interference from blanks was observed. The resulting interference value must be within ± 20% of the peak area at the LLOQ concentration for the analyte.

Sensitivity: Determination of the lower limit of quantification (LLOQ) carried out in 5 replicas. then, the value of accuracy and %CV was calculated with the requirements that do not exceed ± 20%.

Linearity: calibration curves made with a concentration range of 1-50 µg/ml for both AA and GA. The linear regression equation of the curve and the regression coefficient obtained was calculated. The calculation results meet the requirements if the calibration concentration other than zero has a concentration of ± 15% of the theoretical nominal concentration, except for the LLOQ where the calibrator must be ± 20% of the nominal concentration in each validation process. Also, 75% of the concentration points other than zero, and a minimum of six concentration points other than zero must meet the above criteria in each validation process.

Accuracy and Precision: Accuracy and precision tests were carried out as many as five replicas for each analysis (within-run) and as many as three runs for analysis in a period of at least two different days (between-run) at concentrations of LLOQ, QCL, QCM, and QCH for acrylamide and glycidamide respectively. The resulting area was observed, and the nominal and theoretical concentration comparison values were calculated accuracy and %CV value for precision value. The concentration ratio value and %CV produced should not exceed ± 15% for all concentration levels except for LLOQ, which should not exceed ± 20%.

Recovery: The recovery test was carried out by comparing the extracted sample area with the blank area that was spiked post-extraction. The concentrations used were QCL, QCM, and QCH.

Dilution Integrity: Dilution integrity test was carried out on five replicas of 5000 ng / mL acrylamide and glycidamide blood samples for each dilution factor. The dilution must not affect accuracy and precision with the requirement of nominal concentration values and %CV must not exceed ± 15%.

Matrix Effect: The matrix effect was observed by comparing the acrylamide, glycidamide, and propanamide peak area in blood and standard solution. The matrix effect test meets the requirements if the %CV of matrix factor normalized by internal standard is not more than 15%.

Stability: Stability of stock solutions and DBS samples at 25˚C and 4˚C were determined. Stability was assessed from the ratio between the peak area of the measurement results of the sample after being stored under certain conditions for a certain time to the peak area of the measurement results of the sample prepared shortly before injection. The value obtained should not exceed 15% except for stock solution should not exceed 10%.

RESULT

Chromatographic Conditions: The optimum mobile phase was 0.2% formic acid in water - acetonitrile with a composition of 60:40 v/v with a gradient elution for 5.00 minutes. The optimum flow rate was 0.2 mL/min. The gradient elution profile is shown in table 1.

Sample Preparation: The optimum sample preparation method used in this study was 30 µl of sample was spotted on the DBS paper and then dried for 2 hours at room temperature. Then, as much as 100 µl internal standard solution was added into a sample cup containing the dried blood spot. Next, 500 µl of methanol and ultrapure water (1:1) were subsequently added. The mixture was vortexed for 30 seconds, sonicated for 5 minutes, and centrifuged for 1 minute at 7000 rpm. Then, 400 µl of supernatant was evaporated in at 45°C with 5 psi of pressure for an hour with vacuum assisted evaporation with the help of nitrogen gas. The residue was then reconstituted by the mobile phase and sonicated for 15 minutes. The mixture was vortexed for 30 seconds, centrifuged for 5 minutes at 3000 rpm. Then, 70 µl of supernatant fluid was inserted into a vial. 5 µl of final mixture was injected into LC-MS/MS.

Method Validation: selectivity test results showed that the range of response interference during acrylamide retention times was 7.02% to 19.65%, 5.23% to 19.00% at the retention time of glycidamide, and 0.24% to 3.27% at the retention time of propanamide. The chromatogram of blank, LLOQ, and QC samples are shown in figure 1.

Carry over test results showed that the interference value for acrylamide ranged from 8.62% to 10.23%. Whereas, for glycidamide and propanamide, respectively were 7.27% to 14.14% and 0.80% to 1.22%.

Analysis conducted at a concentration of 0.5 µg/ml gave a value of% diff for acrylamide that ranged from -397.12% to -313.83% with a CV value of 7.26%. As for glycidamide, the value of% diff ranged from 12.33% to 110.8% with a CV value of 111.82%. Whilst analysis at 1µg/ml gives a value of %diff range, which is between -5.98% to 11.95% with a CV value of 7.20% for acrylamide and -4.79% to 17.29% with a CV value of 7.43% for glycidamide.

A calibration curve was made with 8 points for acrylamide and 9 points for glycidamide (including blank and zero). The concentrations for acrylamide and glycidamide are 1; 1,5; 10; 20; 30; and 40 ppm and 1; 2; 2.5; 10; 20; 30; and 40 ppm, respectively. The average correlation coefficient for each acrylamide and glycidamide was 0.9975 and 0.9976. The results as shown in table 2.

The within day accuracy and precision test showed that the value of %diff for acrylamide ranges from -15.96% to 10.04% at LLOQ concentrations, while at other concentrations it ranges from -12.44% to 13.25%. Then, the CV value for acrylamide ranges from 0.91% to 10.64%. Furthermore, for glycidamide, the value of %diff is in the range of -13.24% and 13.05%, and the CV value ranges between 4.39% and 8.61%. The results as shown in table 3.

The average recovery value for acrylamide was 96.72%, 95.62%, and 98.2% respectively at the concentrations of QCL, QCM, and QCH. Whereas, for glycidamide was 96.43%, 97.27%, and 98.77%. Lastly, for propanamide, the average recovery value obtained was 94.56%.

Based on the results obtained from the dilution integrity test, the value of %diff was in the range of -13.68% to 3.75% for acrylamide and -14.08% to 13.29% for glycidamide, whereas the CV value was in the range of 0.95% to 7.29% for acrylamide and 2.66% to 8.83% for glycidamide.

The average value of the matrix effect for acrylamide and glycidamide at QCL concentrations was 103.86% and 95.46%. Then, for the concentration of QCH was 99.80% and 101.04%. The matrix factor value obtained for internal standards is 102.97%. Moreover, the average value of the internal standard normalized matrix factor at the concentrations of QCL and QCH for acrylamide is 1.01 and 0.97. Meanwhile, the average for glycidamide is 0.92 and 0.98.

The results of the stability test showed that the value of %diff for samples stored in the refrigerator (4˚C) ranged from -12.18% to 14.4% for acrylamide and -5.25% to 11.97% for glycidamide. Also, the value of % diff for samples stored at room temperature (25˚C) did not exceed 15%.

DISCUSSION

Chromatographic Conditions: Analysis using LC-MS/MS relies on the detection of analytes with mass spectrometry. The process begins with the ionization of the analyte compound. Ionization was carried out with positive Electrospray Ionization (ESI). Analytes that enter the mass spectrometer ion source through capillary tubes are given a high-voltage electric current that causes the liquid to be dispersed into small, charged droplets due to the Coulomb force. The solvents contained in the droplets are then evaporated using N_2 gas and heating thereby increasing the charge density on the droplet surface. Then, the gas phase ion is formed from a high-charged droplet. Furthermore, the ions formed are transferred through vacuum pressure into the mass analyzer. The mode used for analyzing mass is the MRM mode. This mode analyzes ion pairs with specific m/z. Precursor ions which are called parent ions enter into Q1 and then fragmented in Q2 that formed daughter ions which then are selected to enter the detector in Q3 (Van der Gugten, 2020).

Due to its structure, acrylamide, glycidamide, and propanamide have primary amine groups. These compounds will change into ionic form utilizing protonation which is assisted by the use of formic acid in the mobile phase (Liigand, Laaniste, & Kruve, 2017).

As a source of H^+ needed in the protonation process, the mobile phase used has acid, in this case, formic acid. From the optimization results, 0.2% formic acid in water - acetonitrile was chosen to be the mobile phase because the resulting chromatogram has a sharp peak and has the largest area compared to the others with consistent retention time. The retention time of this combination is not the fastest in terms of retention time but does not differ much from the other combinations tested.

The optimization results showed that the composition of 60:40, 70:30, 80:20, and 95:5 produced a chromatogram with a single peak and almost the same retention time. However, the composition of 60:40 has the largest area compared to the composition of 70:30, 80:20, and 95: 5.

Whereas, the 50:50 composition gives a single peak result with a much larger area than the 60:40 composition with relatively similar retention times. However, the large area formed is obtained from the widening peak. This is caused by the type of elution carried out, namely isocratic elution. Compared to isocratic elution, gradient elution gives a relatively sharper peak. This is caused by differences in elution strength in gradient elution (Cabo-Calvet, Ortiz-Bolsico, Baeza-Baeza, & García-Alvarez-Coque, 2014).

A flow rate of 0.2 mL/min gives the best results, which are good chromatogram peaks and a fairly large area. Meanwhile, a flow rate of 0.1 indicates a poor chromatogram with twin peaks. Then, a flow rate of 0.3 gives a chromatogram shape similar to a flow rate of 0.2 ml/min, but a very small area. The system suitability test shows the results of the CV value (%) for acrylamide, glycidamide, and propanamide respectively, 1.51%; 5.04%; and 2.73%. The results are eligible when the CV value is not above 6% (Briscoe, 2013).

Sample Preparations: The spotting volume of 30 µl provides a large area for all analytes and internal standards, whereas the spotting volume of 40 µl gives a larger area than 30 µl in acrylamide, but it is smaller in the peak area of glycidamide. Since glycidamide is a metabolite of acrylamide, the amount in the blood is smaller than acrylamide. Thus, 30 µl is chosen as the optimum spotting volume and is used in further optimization.

Based on the type of solvent tested, namely 100% methanol, methanol-water mixture (1: 1), methanol-water mixture (4: 1), methanol-water mixture (1: 4), and 100% water, solvents that produce best results is methanol-water mixture with a ratio of 1: 1. The volume of the extracting solvent of 500 µl gives the largest area. In a series of sample preparations, mixing with vortex is an important process that aims to make it easier for the extracting solvent to enter into the DBS paper fiber. The vortexing time tested was 30, 60, and 90 seconds. Vortexing time optimization results show 30 seconds is the most effective and efficient time because it provides the largest area. A vortexing time which is too long can cause an emulsion to form in the sample. Therefore, the most optimum vortexing time is 30 seconds and is used for further processing. When sonication, microbubbles are formed which then burst almost instantaneously. This process is called cavitation that is caused by high-frequency waves that resulted in intense pressure and vibration so that it helps draw analytes from the DBS paper (Smith & Xu, 2012). Based on the optimization results, the optimum sonication time is 5 minutes because it provides the largest area. In the centrifugation process, the impurity is separated from the sample liquid through the centrifugal force applied by rotating the sample tube very quickly. From there, residues and supernatants are formed. The purpose of centrifugation is to get cleaner samples to maintain column quality. The optimization results show that the best centrifugation time is 1 minute because it provides the largest area.

Method Validation: Selectivity tests are carried out to ensure the analytical method can distinguish between analytes and internal standards with impurities to minimize interference. The selectivity test result shows that, the analytical method used meets the FDA guidelines that the interference value does not exceed 20% for each analyte and does not exceed 5% for internal standard.

The carryover test is carried out to see whether there is an analyte detection in the sample being analyzed from the previous sample. This is done by injecting the analyte in the ULOQ, blank, and LLOQ concentrations in a sequence and then calculating the ratio of peak area on blank to peak area at LLOQ concentration. Based on the requirements stated in the FDA guidelines, i.e, the interference value does not exceed 20% for each analyte, then this method can be declared eligible.

In this study, the LLOQ of acrylamide and glycidamide was 1 µg/ml each. Based on the acquired data, a concentration of 0.5 µg/ml does not meet the requirements so the previous concentration, which is 1 µg/ ml, is determined as the LLOQ. The calibration curve that has been made meets the FDA guidelines, that is, every point on the curve has a concentration in the range of ± 15% of the theoretical nominal concentration, except for the LLOQ where the calibrator must be within the range of ± 20% of the nominal concentration in each validation process. Besides, 75% of the concentration points other than zero, and a minimum of six concentration points other than zero must meet the above criteria in each validation process.

Accuracy and precision tests consist of two types of tests i.e within-run and between-run. Both of these tests were conducted to determine the value of the concentration of the analysis result and the original concentration, furthermore, to determine the repeatability of the analysis method used. According to FDA guidelines, the requirements of the accuracy and precision test are %diff and CV does not exceed 15% except at LLOQ concentrations not exceeding 20%. After conducting the accuracy and precision test, the results showed that the method used meets the requirements.

Recovery tests were carried out to determine the effectiveness of the sample extraction process. This test was carried out by calculating the ratio of the analyte response in the extracted sample to the response of the analyte on the spiked blank post-extraction. A high recovery value (close to 100%) indicates an effective extraction method.

When analyzing a sample, there are times when the analytical response contained in the sample exceeds the range of the calibration curve (above the ULOQ concentration). To mitigate this, a dilution integrity test is performed. This test is carried out by analyzing analytes with concentrations above the calibration curve range then diluted to enter the range. the analytical method used meets the requirements of the dilution integrity test.

The matrix effect test is carried out to find out whether the endogenous components contained in the matrix can influence the analysis. In LC-MS/MS with ESI ionization, the most commonly encountered matrix effect is ion suppression, which decreases the intensity of the analyte response. This is caused by the interaction between the endogenous and exogenous components of the matrix with the ionization process of interactions that occur including the addition of charge to the analyte in the mobile phase, evaporation of ions from the surface of the droplet, and the competition of the matrix component with the analyte on the charge in the liquid phase (Panuwet et al., 2016).

Both the stock solution and DBS samples of all analytes and internal standard are stable in a refrigerator (4˚C) for 21 days, and stable in room (25˚C) temperature for 24 hours.

CONCLUSION

The developed method was valid in accordance with FDA's (2018) guidelines with parameters of selectivity, carry over, sensitivity, calibration curves, accuracy, precision, recovery, dilution integrity, matrix effect, and stability. The method was linear with the range concentration of 1 - 40 µg/ml

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Appendix 1. Table

Table 1. Gradient Elution Profile

Table 2. Data of Inter-day Calibration Curve of Acrylamide and Glycidamide

Replicates	Acrylamide			Glycidamide		
	Slope (b)	Intercept (a)	Correlation Coefficient (R)	Slope (b)	Intercept (a)	Correlation Coefficient (r)
1	0.0290	0.0995	0.9989	0.0078	0.0084	0.9970
2	0.0291	0.0946	0.9978	0.0084	0.0083	0.9982
3	0.0288	0.0894	0.9957	0.0066	0.0083	0.9977
Average	0.0290	0.0945	0.9975	0.0076	0.0083	0.9976
SD	0.0002	0.0051	0.0016	0.0009	0.0001	0.0006
CV (%)	0.5273	5.3447	0.1629	12.0594	0.6928	0.0579

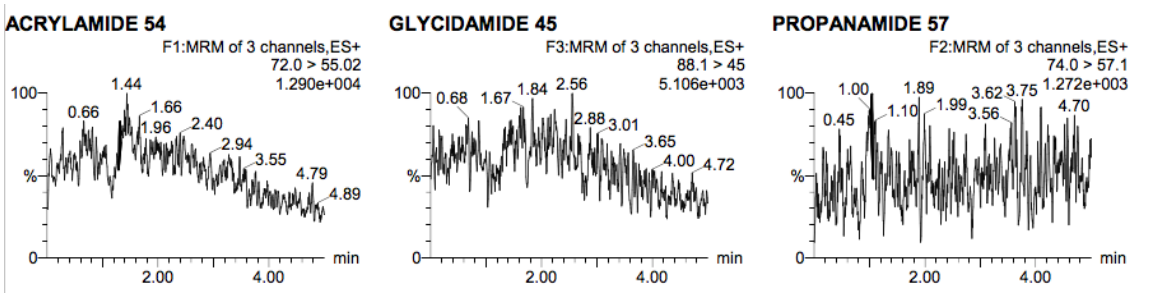
Table 3. Data of Within-run and Between-run Accuracy and Precision Test

Analyte	Concentration	Accuracy (%diff)		Precision (%CV)	
		Within-run	Between-run	Within-run	Between-run
Acrylamide	LLOQ	-6.677	-0.689	10.646	10.071
	QCL	9.093	7.949	5.241	6.617
	QCM	-6.938	-5.389	4.347	6.134
	QCH	4.031	-1.970	0.910	5.985
Glycidamide	LLOQ	2.780	0.950	7.163	5.834
	QCL	-5.720	-6.213	8.589	8.061
	QCM	-5.323	-2.743	8.618	6.621
	QCH	-5.211	-5.628	4.395	6.795

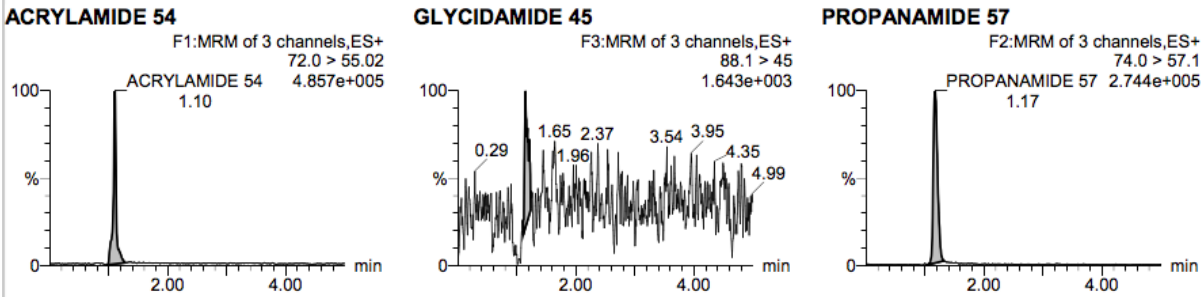
LLOQ: Lower Limit of Quantification, QC: Quality Control, L: Low, M: Medium, H: High

Appendix 2. Figures

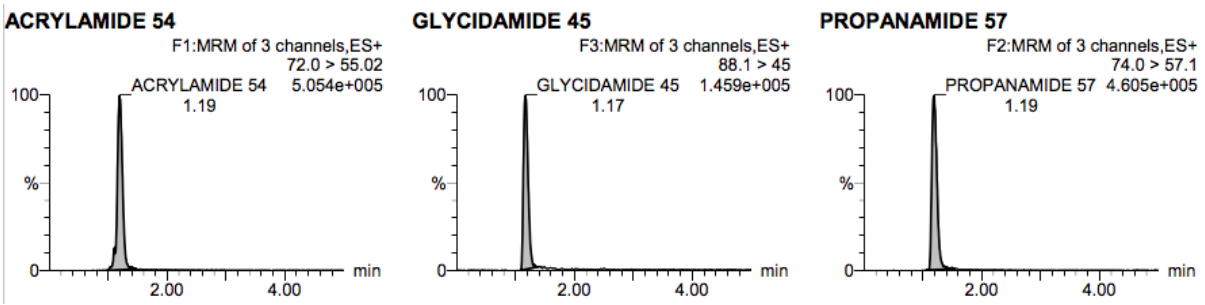
Figure 1. Chromatogram of (a) Blank, (b) Zero, (c) LLOQ, (d) QCL, (e) QCM, and (f) QCH.



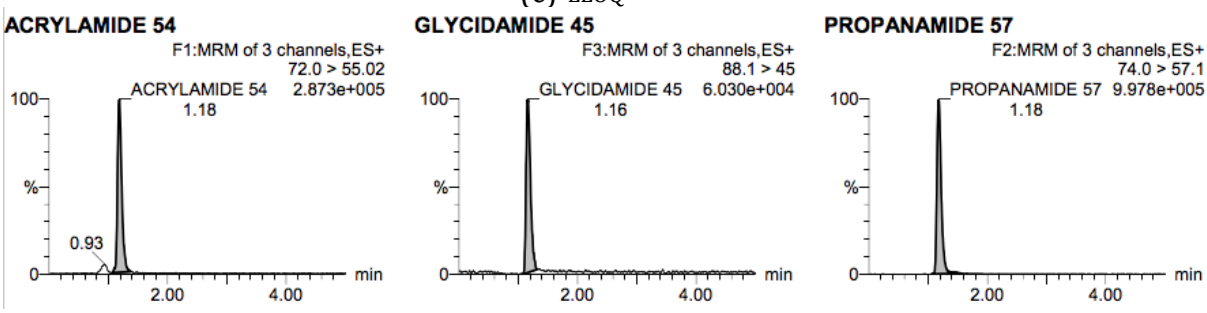
(a) Blank



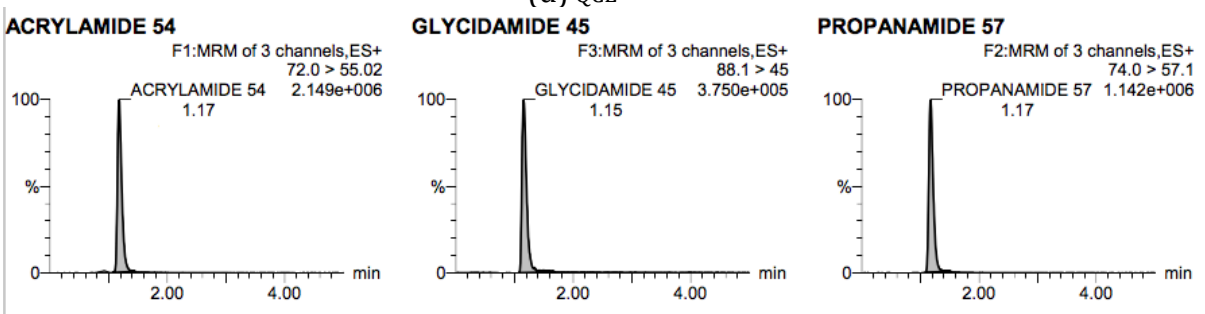
(b) Zero



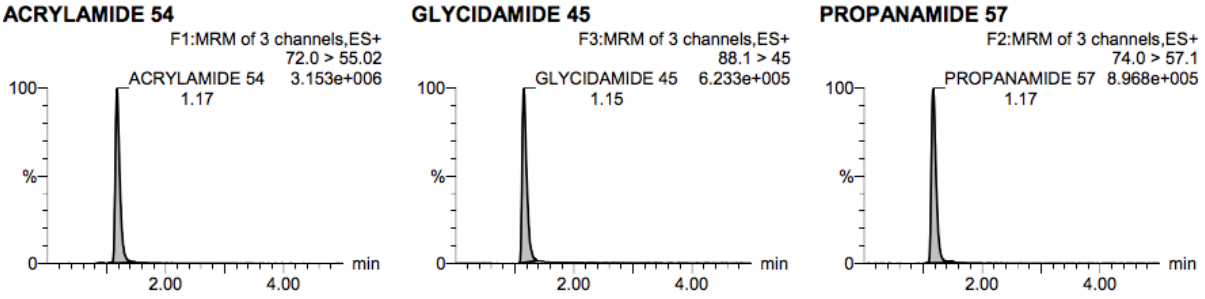
(c) LLOQ



(d) QCL



(e) QCM



(f) QCH