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Optimization, Validation, and Application of a Quantitative GC/NPD Method for Acrylamide Determination in Yemeni Fried Fish Samples

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ABSTRACT

The matrix's complexity makes measuring acrylamide in food items challenging, requiring a time-consuming extraction technique and high method sensitivity. A homemade solid phase extraction (SPE) device and a gas chromatography/nitrogen phosphorus detector (GC/NPD) were used to establish a simple and inexpensive acrylamide detection method in fried fish. Optimization and validation were used to measure acrylamide concentrations in twelve fried fish samples, four of which were home-fried and eight from Sana'a, Yemen, restaurants. The validated technique has high linearity R^2 (0.995) in the 0.02–4 ppm range of acrylamide standards and good sensitivity with LOD (0.0062 ppm), substantially below the authorized level. The twelve samples had acrylamide values of 0.0583–0.4643 ppm. Due to variations in thermal treatments during frying, market-fried fish samples had greater acrylamide concentrations than home-fried fish samples. In the current study, the improved simple extraction process and GC/NPD protocol were useful for acrylamide measurement and may be employed as a prescreening tool for food sector acrylamide quantification.

CONTENTS

1. Introduction:

In 1912, L.C. Maillard identified acrylamide as the byproduct of the Maillard reaction [1] between asparagine or other amino acids that can create acrylic acid and reduce carbohydrates like glucose. More specifically, acrylamide is formed in foods when asparagine reacts with carbonyl-containing compounds like reducing sugars at high temperatures and low moisture levels, forming a Schiff base, and then decarboxylating and eliminating the imine resulting in the formation of unsaturated amide [2]. When ingested, acrylamide, a tiny molecule, is quickly absorbed and dispersed throughout several

organs, including the thymus, liver, heart, brain, and kidneys [3,4]. Also, the skin and mucosa absorb acrylamide easily, and it diffuses strongly into tissues and the fetus [5].

Acrylamide has been discovered in a wide range of food items. The most common sources of this acrylamide are potato chips, french fries, fried food, roasted cereals, coffee, tea, and baked goods [6–11]. A significant source of neurotoxic, genotoxic, and carcinogenic acrylamide is grilled food [12]. The amount of acrylamide precursors in the raw material, the chemical makeup of the food, additives, and baking or frying procedures are just a few of the reasons why there were such stark variances in acrylamide levels within each food category [13-15].

Because of its genotoxicity and carcinogenicity, the European Union classified acrylamide as a Category 2 carcinogen and a Category 2 mutagen [16, 17] and the International Agency for Research on Cancer (IARC) labeled it a neurotoxin and Group 2A probable carcinogen [16] in 1994. Thus, acrylamide-based polymeric food packaging containers are now banned from being used in direct contact with food or drinkable water in the United States [16, 18, 19]. In 2018, the European Union (EU) established regulations restricting the amount of acrylamide allowed in food to 0.75 ppm [20], and the state of California has passed legislation requiring food product labels to incorporate acrylamide disclosure [21].

The trend in acrylamide determination in food products seems to focus on the use of established techniques such as high-performance liquid chromatography (HPLC) [22], gas chromatography (GC) [23], GC-MS [24], GC/ECD [25], GC/NPD [26], HPLC/UV [27], Ultra HPLC/MS [28], and liquid chromatography-tandem mass spectrometry (LC-MS/MS) [29]. The use of GC/MS and LC/MS/MS which are widely adopted by many laboratories worldwide are too expensive for small labs with limited resources. Other techniques have been reported for acrylamide determinations such as immunoassay [30], electrochemical biosensors [31], fluorescent methods [4], and quartz microbalance sensors [32]. These techniques are either commercially unavailable or require tedious extraction and clean-up procedures.

Thus, the current research contribution to understanding the exposure level of consumers to acrylamide from fried fish. This is an important contributes in a country like Yemen which has been in a civil war for the last nine years where consumer protection agencies are not functioning properly. Based on this, the present work aimed to optimize and validate a cost-effective and easy-to-use solid-phase extraction and clean-up procedure coupled with GC/NPD to quantify acrylamide in fried fish samples. The optimized procedure should be helpful for acrylamide prescreening in fried fish products and could easily be adapted by labs that can not afford expensive equipment.

2. Materials and methods

2.1. Chemicals and materials:

We used ultra-pure acrylamide from Sigma-Aldrich (> 99 percent). Hydrochloric acid, formic acid, and n-hexane were from Sigma-Aldrich, whereas GC-grade solvents (acetonitrile, acetone, ethyl acetate, methanol) were purchased from Scharlau (Spain). The water utilized was purified using a Direct-Q3 (Millipore, Bedford, MA, USA) water purification system. The home-made SPE cartridges were fabricated using activated charcoal from Merck, Germany, and medical syringes from China.

After measuring out 10^3 mg of acrylamide standard, one litter of acetone was used to dissolve the solid, yielding a stock solution with a known concentration of acrylamide (10^3 ppm). For both low and high-concentration spiking, standard solutions of the analyte (10, 100 ppm) were employed respectively.

2.2 GC/NPD analysis:

Burker 450 GC gas chromatography/Nitrogen Phosphorus Detector (GC/NPD) system (Agilent, Palo Alto, CA, USA) with CP-8410

Auto-injector, DB-Wax capillary column (30 m x 0.2 mm I. D), and film thickness of 0.25 µm was used to analyze the samples. High-purity (>99.99%) nitrogen was employed at a flow rate of 1.2 mL min^{-1} as the carrier gas. At 250 ^{o}C injection temperature and 280 ^{o}C detection temperature, 2 µL of sample was injected in split less mode.

The following temperature program was used in the study: The column temperature was initially set at 40 ^{o}C with a holding time of 1 min. From there, the temperature was raised to 140 ^{o}C at a rate of 10 ^{o}C min^{-1}, and finally to 250 ^{o}C at a rate of 30 ^{o}C min^{-1}. The heat was maintained at 250 ^{o}C for 2 minutes. The total analysis time was 16.67 minutes.

2.3 Fried Fish Samples Preparation:

Eight samples of fried fish were acquired from several neighborhood eateries in Sana'a, Yemen, while four samples were fried at home. The materials were sub-sampled, homogenized for 10 min., and then kept in a glass beaker that had been cleaned with acetone. The backers were kept at 0 °C in the dark while being wrapped in aluminum foil. The sample (10 g) was properly homogenized after being carefully spiked with standard acrylamide.

Before extracting, the spiked sample was allowed to stand for the entire night. A 100 mL beaker containing 10 g of homogenized sample was precisely weighed, and 20 mL of 0.1 v/v% formic acid/water was then added. For 60 min., the material was stirred to aid in extraction. The sample was chilled for 15 min. before the greasy layer was removed. The extract was filtered through 4.5 µm filter paper after the sample was centrifuged at 1000 rpm for 10 min. Clean-up/preconcentration operations were then performed on the extract.

2.4. Samples Clean-up using Solid Phase Extraction Cartridge (c-SPE):

Alnedhary, A. A. et al. [33**]** reported that the sample clean-up procedure was conducted**.** In summary, medical syringes were filled with varied masses (0.2-0.8 g) of previously purified activated charcoal and then treated with 3 M HCl at 40 ^{o}C to create clean-up c-SPE. Thin discs of medical cotton that had already been cleaned were put into the bottom and top of an activated charcoal layer. Acetone was used to rinse the cartridges, and deionized water was used to condition them.

3. Results and discussion:

3.1. Method Optimization:

Using acrylamide-spiked fried fish samples, we investigated several extraction/clean-up performance factors to determine the optimum settings for acceptable linearity, repeatability, and high extraction recovery. The experimental methodologies of c-SPE extraction were refined by comparing the GC response of the spiked sample effluent to that of the reference solution

3.1.1. Selection of Extraction Solvent:

The extraction solvent is a key variable since it affects the extraction rate, the procedure's cost, and the toxicity of the final product. Therefore, different solvents, such as deionized water, acidified deionized water, methanol, ethyl acetate, and acetonitrile, were tested for their ability to extract analytes effectively. Fig. 1 shows the acrylamide extraction efficiencies (peak areas) for a variety of solvents.

Based on the solubilities (in grams/100 mL of solvent at 25 ^{o}C), we found that acidified deionized water is the most effective solvent for the extraction of acrylamide from fried fish. The high efficiency of acidified distilled water may be due to the high hydrophilic nature of acrylamide, which makes it highly soluble in water but hinders the solubility of hydrophobic molecules in food. Matrix contaminants (such as proteins and carbohydrates) can impede detection and degrade chromatographic efficiency if not eliminated [34]. Previous work indicated that acidified distilled water was highly effective as well in the extraction of acrylamide from potato ships. [33].

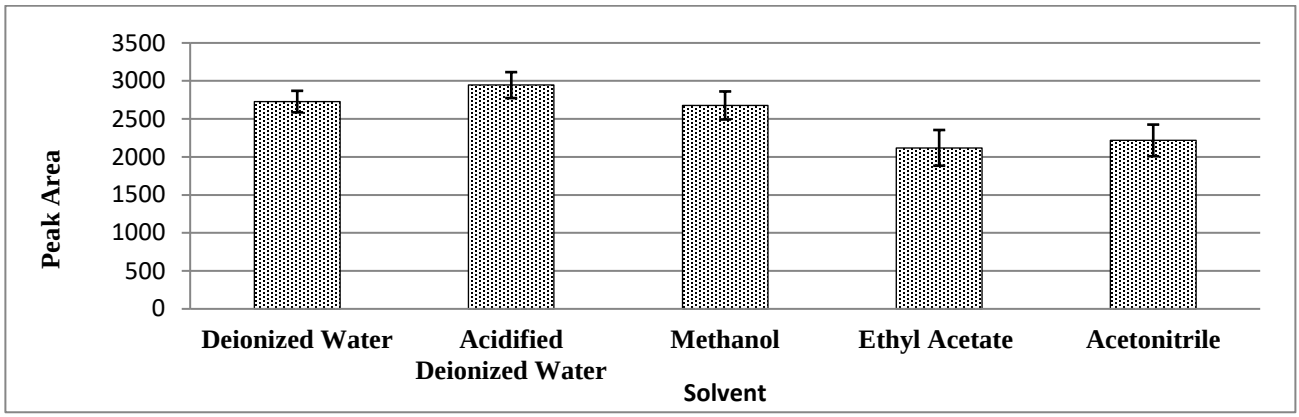

Figure. 1: Effect of Solvents on Extraction Efficiency of Fried Fish Sample Spiked with 4 ppm.

3.1.2. Stirring / Extraction Time:

To determine the best stirring time for acrylamide extraction from fried fish samples, the influence of the extraction time parameter was studied. An acrylamide concentration of 4 ppm was added to fried fish samples at room temperature, and the contents were agitated for different periods ranging from 10 to 90 minutes. The analytical signal reached a plateau after approximately 60 minutes which was considered the optimal extraction time as depicted in Fig. 2.

3.2. Optimization of c-SPE:

We looked at the c-SPE parameters that were necessary for effective acrylamide extraction from fried fish, including the type and volume of the eluting solvent, the volume of the cartridge, and the quantity of activated charcoal**.** Each parameter was separately optimized as follows**:**

3.2.1 Type of Solvent:

Acetone, acetonitrile, methanol, and n-hexane were only a few of the eluting solvents used. To achieve this, several 5 mL-capacity cartridges have 0.4 g of charcoal added to them. The charcoal was cleaned with acetone and then conditioned with D.I.W. A 4-ppm acrylamide standard solution was put through each cartridge in triplicate at a flow rate of 3 mL/min. The acrylamide in each cartridge was eluted using each of the aforementioned solvents after the cartridges were dried. Our results (Table 1) showed that acetone had the highest elution efficiency, with an average recovery of 97.4%. Acetone was chosen as the best eluting solvent and employed in further research as a result.

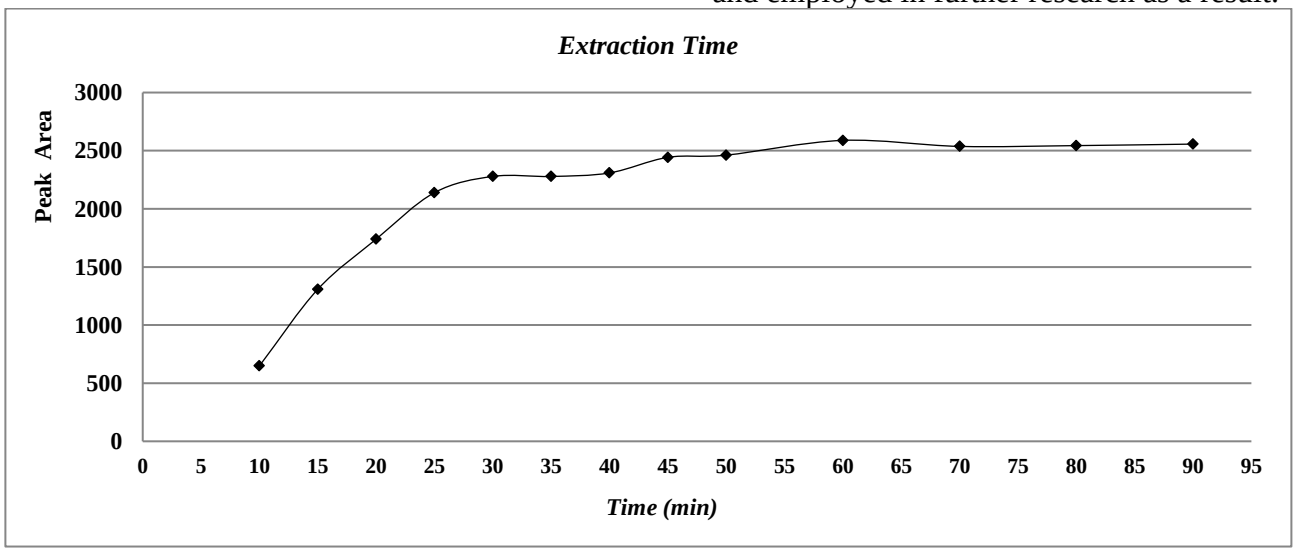

Figure. 2: Stirring/Extraction Time of Fried Fish Sample Spiked with 4 ppm at 1000 rpm

Table 1: Eluting solvents efficiency

Eluting Solvent	Recovery %
Acetone	97.4
Acetonitrile	94.1
Methanol	89.9
n-Hexane	82.7

Conditions: Acrylamide Standard: 4 ppm, Activated Charcoal mass: 0.4 g, Solvent Flow Rate: 3mL/min, Injection Volume into the GC/NPD: 2µL.

3.2.2 Volume of the Eluting Solvent:

To guarantee optimal elution efficiency and recovery, the amount of acetone used as the eluting solvent was tuned through a series of studies. One, two, three, four, and five milliliters of acetone were used to elute the acrylamide adsorbed in the cartridge. For chromatographic analysis and to find the ideal amount of acetone to utilize to get the greatest recovery of adsorbed acrylamide, 2 µL of the effluent was injected into the GC/NPD system. It may be assumed that the preconcentration factor that results from using 1 mL of acetone to elute 2 mL of the acrylamide with a 4 ppm concentration will yield the maximum recovery. When 1 mL of acetone was utilized, the recovery only reached 83.4% with %RSD of 12.1%, according to data in Fig. 3. The best recovery was attained when 4 mL of acetone was utilized, according to a scan of the recovery findings shown in Fig. 3. The dilution factor for this elution volume, however, was larger than it was for the situation when 2 mL of acetone was used. This dilution factor resulted in a LOD of 0.025 ppm. Due to this, the 2 mL volume of acetone was chosen as the ideal elution volume since it provided a respectable recovery (99.1% and 2.6 % RSD) and the lowest LOD (0.012 ppm).

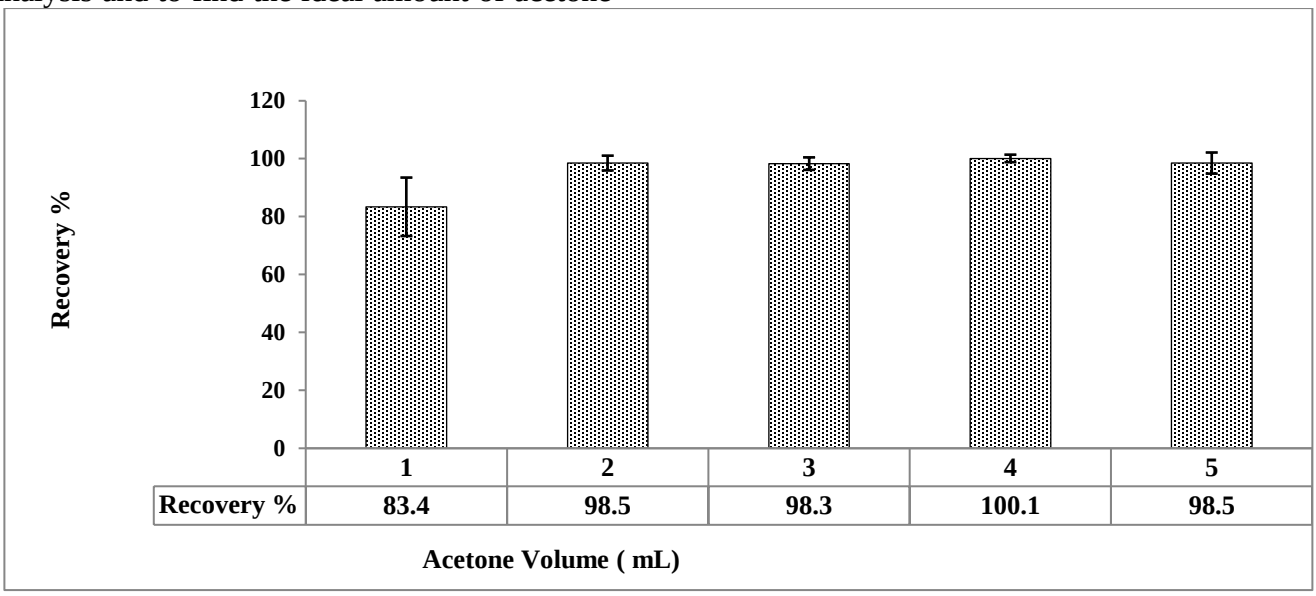

Figure. 3: Effect of Eluting Solvent Volume on Recovery Percentage of 4 ppm Acrylamide

3.2.3 The volume of the Cartridge:

Further experimentation was carried out to optimize the volume of the cartridge. Three medical syringes of varying capacities (3 mL, 5 mL, and 10 mL) were used as cartridges in this experiment. The cartridges were filled with 0.4 g of charcoal. Each cartridge was cleaned and conditioned as mentioned in section 3.2 before having 2 mL of 4 ppm acrylamide standard solution pumped through it (in triplicate). After drying the cartridges, 2 mL acetone was used to release the acrylamide that had been captured by the cartridge. Based on the results depicted in Fig. 4, a 3 mL cartridge volume was selected for further studies since it provided the highest average recovery percentage (98.5%) under the conditions tested.

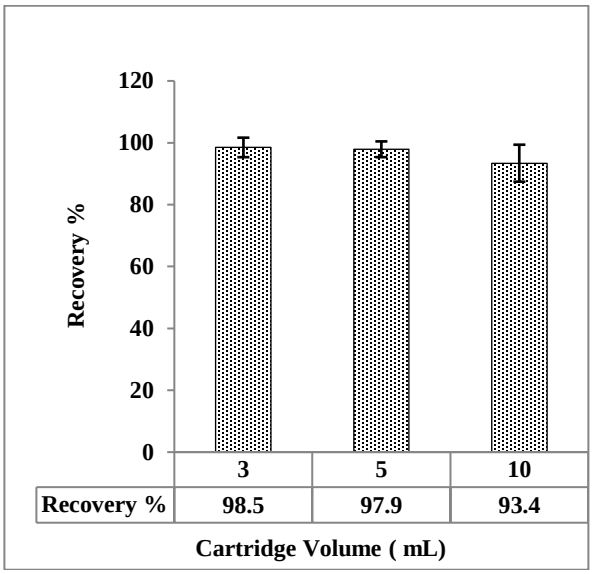

Figure. 4: Effect of Cartridge Volume on the Extraction Efficiency of 4 ppm Acrylamide

3.2.4 Mass of the Adsorbent:

The results of our investigation into the impact of activated carbon (charcoal) mass (0.2 g, 0.4 g, 0.6 g, and 0.8 g) on acrylamide elution recovery are shown in Fig. 5. Following the proper conditioning processes, two milliliters of a 4-ppm standard solution were passed through, dried, and eluted with two milliliters of acetone. A 99.3% recovery was achieved with 0.4 g of activated charcoal, however, only an 85.2% recovery was achieved with 0.2 g of activated charcoal. This should lead us to the conclusion that using charcoal with a greater mass (0.6 g or 0.8 g) would enable us to capture more acrylamide and increase recovery after elution. Indeed, a higher charcoal mass will effectively capture more acrylamide, but doing so necessitates using more elution solvent, which raises the dilution factor and reduces the method's sensitivity. This is why we thought the 0.4 g charcoal was the best.

3.3. Method's Analytical Performance:

The chromatographic analysis of a sample of spiked fried fish that was performed under ideal experimental circumstances is shown in Fig. 6. The chromatogram demonstrated sufficient separation efficiency for the acrylamide peak

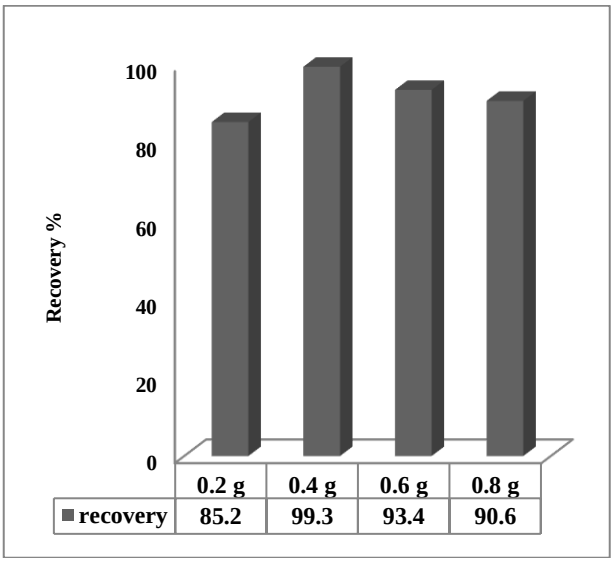

Figure. 5: Effect of the Mass of Activated Charcoal on Recovery of 4 ppm Acrylamide Standard

(N= 221600) without matrix interference. We have also evaluated the analytical performance of the developed method in terms of linearity range, precision, accuracy, and LOD under optimized conditions. To ensure the method was linear, we spiked fried fish with six different acrylamide concentrations ranging from 0.02 to 4 ppm. The concentration of the spikes was correlated to the area beneath the peak of the spiked sample, and this relationship is shown graphically in Fig. 7. Over the relevant concentration range, a correlation value of 0.9946 indicates satisfactory linearity.

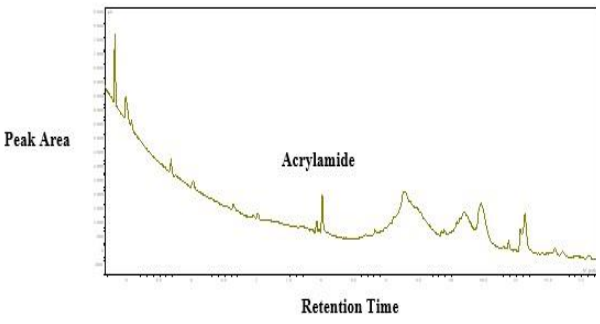

Figure. 6: GC/NPD Chromatogram of the Acrylamide in Real Fried Fish Sample
Chromatographic Conditions: Column type: polyethylene glycol (PEG), Injection volume: 2µL, Column temp: 250 °C, Injection temp: 280 °C, Detector temp: 280 °C, Flow Rate 1.2 mL/min..

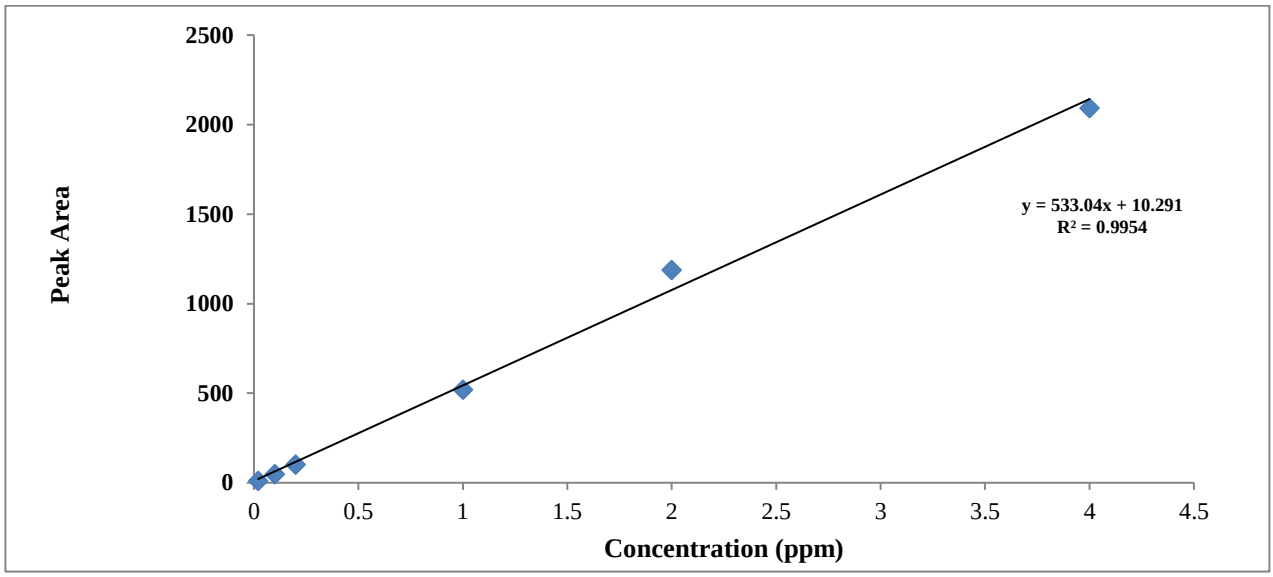

Figure. 7: Calibration Curve of the Acrylamide Standards in Spiked Fried Fish Samples

The method's reproducibility was also examined. Three separate experiments were conducted on fried fish samples with varying amounts of acrylamide to evaluate reproducibility. There was a wide variation in RSDs, from 5.69 to 12.96% (n = 3 samples). In all calculations, we utilized the same information to compute the recovery rate by applying the following formula [35]:

% R = (A_1-A_2/A_3)*100

Where A_1 is the spiked sample's peak area, A_2 is the sample's peak area before spiking, and A_3 is the standard's peak area.

Table 2 showed that recoveries ranged from 70.13% to 79.5%. Experimentally, a signal-to-noise ratio of 3:1 was used to determine the limit of detection (LOD) [36]. LOD for our approach was determined to be 0.0062 ppm, making it perfectly applicable to the assessment of acrylamide levels in fried fish samples.

Table 2: Spiked Fried Fish Precision and Accuracy Results

Conc. (ppm)	Peak Area			Average Area	% RSD	% R	LOD (ppm)
	Replicate 1	Replicate 2	Replicate 3				
0.02	10.2	12.4	9.8	10.8	12.96	70.13	
0.1	45.6	51.1	48.3	48.3	5.69	71.32	
0.2	110.3	104.2	92.8	102.4	8.67	77.62	0.0062
1	476.8	516.4	567.4	520.2	8.73	78.03	
2	1276.3	1192.4	1096.9	1188.5	7.55	79.04	
4	2246.3	1996.3	2037.4	2093.3	6.40	74.73	

3.4. Quantification of Acrylamide in Fried Fish Samples:

The optimal method was used to analyze acrylamide levels in twelve varieties of fried fish, four of which were prepared at home and eight of which were purchased from restaurants in Sana'a, Yemen's capital. Each sample was analyzed three times, and the results were presented in Table 3.

The measured acrylamide concentrations ranged from 0.0583 to 0.4643 ppm, with a relative standard deviation (RSD) between 4.2 and 11.6 %, as shown in Table 3.

Higher amounts were found in eight of the twelve samples (RF1-RF8), with values ranging between 0.1918 ppm and 0.4643 ppm when compared to earlier studies [12]. These eight samples were all purchased from restaurants.

Home-fried fish samples had lower acrylamide levels between 0.0583 to 0.1147 ppm with an average value of 85.8 ± 6.15 %.
Previous research suggested that acrylamide levels in food items may be influenced by a variety of factors including the differences in cooking and processing temperature, cooking method, and type and quality of raw materials [37]. This could explain the variation in acrylamide levels among the test samples in our experiments. Even though the highest level of the acrylamide was 0.4643 ppm, this concentration is still below the permissible level of the acrylamide 0.750 ppm [20].

Table 3: Fried Fish Real Samples Results

Sample No.	Sample Code	Average Concentration ppm (n=3)	Mean Average	Standard Deviation SD	RSD %	Mean RSD %
1	HF1	0.0583	0.0858	0.0024	4.2	6.15
2	HF2	0.0785		0.004	5.1	
3	HF3	0.1147		0.0083	7.2	
4	HF4	0.0918		0.0074	8.1	
5	RF1	0.2462	0.2744	0.0135	5.5	7.05
6	RF2	0.2309		0.0206	8.9	
7	RF3	0.3707		0.0193	5.2	
8	RF4	0.1431		0.0132	9.2	
9	RF5	0.4643		0.0246	5.3	
10	RF6	0.1918		0.0119	6.2	
11	RF7	0.3277		0.0147	4.5	
12	RF8	0.2206		0.0256	11.6	

HF: Home Fried Fish
RF: Restaurants Fried Fish

4. Conclusion

In the present study, a simple, user-friendly, accurate, and cost-effective method for the quantitative determination of acrylamide was developed. The study offers highly intriguing analytical advantages, as the final extract could be injected directly into GC/NPD without any additional derivatization treatment. Compared to other chromatographic methods (GC/MS, GC/ECD), the sensitivity and accuracy of this method were sufficient to monitor the levels of acrylamide in fried fish samples**.**

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Conflicts of Interest:
The authors declare no conflicts of interest regarding the publication of this paper.

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