Research Article

Determination of acrylamide using the immuno-enzymatic method in commercial dog and cat foods

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Abstract: Pet animals raised by humans do not have the chance to choose their own food. Therefore, mistakes made by humans in feeding dogs and cats will adversely affect the health of the animal. There are applications at high temperatures at various stages during the production of dog and cat foods. It is predicted that acrylamide (AA) can be formed as a result of a possible Maillard reaction since the cat and dog foods contain starch in its structure. The aim of this study is to determine the AA residue immuno-enzymatically in dry cat and dog feeds from different companies available on the market. Thus, possible threats to cat and dog health, if any, will be determined, and the applicability of the method will be tested by determining the AA level in foods for the first time with this method. For this purpose, 42 cat and dog foods belonging to various companies in the market were randomly collected from different pet clinics in Aydın and made ready for testing by passing through various stages. AA residues in foods were tested in an immuno-enzymatic way and as a result, AA residue was found in approximately 33% (14 samples) of the analyzed samples in dry cat and dog foods, with an average AA concentration of 87.35 ppb in these samples. The AA concentration in the samples was determined as 45 ppb at the lowest and 155 ppb at the highest. AA could not be detected in 67% of the investigated samples. In conclusion, the Maillard reaction observed during the preparation of food for pets is an important cause of AA formation. Therefore, improvement of formulation and process needs to be regulated carefully. In addition, it is recommended that the immuno-enzymatic method used in the study must be supported by more future studies.

Key words: Cat, dog, dry feed, acrylamide, immuno-enzymatic method

1. Introduction

Acrylamide was first synthesized in 1893 by the German scientist Charles Moureu by adding ammonia to a saturated solution of acryl chloride in benzene [1]. The importance of AA, which was limited in use until the 1930s, has increased with the widespread use of acrylic fibers in textile and artificial rubber production. With the commercial production of AA by the American Cyanide company in 1954, industrial consumption increased, and new methods were developed for the polymerization and synthesis of AA [2,3].

AA is a versatile organic compound that takes place in many products that we use in our daily life in different ways and has two forms, monomeric and polymeric. Monomer AA has a toxic effect on the nervous system, and as a result of studies performed on laboratory animals, it has been reported that it also has carcinogenic effects for animals and maybe a carcinogenic substance for humans (group 2A). Although the monomeric form of AA is more harmful than its polymeric form, it is stated that polyacrylamide has harmful effects if it is contaminated with food from the outside [4].

Clinical reports of AA toxicity are few in the veterinary literature. On the other hand, experimentally, AA toxicity has been thoroughly investigated in rats, primates, and dogs. Greyhound dogs exposed to oral AA for eight weeks developed sensorimotor peripheral neuropathy with features similar to AA neuropathy in other breeds. Most animals developed clinical and radiological signs of megaesophagus. The relationship between neuropathy and megaesophagus has revealed that vagus axonopathy may be the etiological factor in this disorder [5]. In another report [6], three healthy Labrador puppies developed ataxia, hypermetria, and convulsions shortly after eating burnt corn porridge. The nervous symptoms of three puppies with signs of poisoning after eating starch-based foods prepared at high temperatures suggested AA toxicity. Following this, the death of two puppies increased the severity of the situation and it was reported that deaths could be caused by distal axonopathy. In the experimental

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studies in cats, intense tonic-clonic convulsions and diffuse central nervous system excitation were reported when AA monomer was administered intravenously at lethal doses (100 mg/kg adult cat). Dosage application of AA below lethal doses triggers ataxia and tremors [7].

There are also studies on other animal species related to AA. When adult male Beagle dogs and miniature pigs were fed 1 mg of acrylamide/kg/day for 3–4 weeks, neurotoxic signs were not revealed, but AA was detected in muscle tissue collected after postmortem examination of experimental animals. Although the primary target for AA monomer is the nervous system, only less than 1% of this substance was detected in the brain. When AA 5 mg/kg/day (for a period of 30–60 days) was administered to dogs and pigs, neuropathy was observed [8]. In dogs, chronic exposure to AA leads to typical progressive sensorimotor peripheral neuropathy, including ataxia, weakness, and especially megaesophagus due to vagal nerve axonopathy [9]. When sublethal doses are discontinued, the developing neuropathy might gradually resolve. More severe disorders such as spasticity and cerebellar ataxia have been reported to be irreversible and permanent [10-11].

There are high temperature applications at various stages during the production of cat and dog foods. It is estimated that AA may be formed as a result of a possible Maillard reaction due to the presence of protein and starch in the structure of cat and dog foods. The aim of this study is to determine the AA residue in 42 dry cat and dog foods from different companies on the market by immuno-enzymatic method. Thus, a possible threat to the health of cats and dogs, if any, will be detected and the applicability of this method in determining the AA level in foods will be tested.

2. Materials and methods

2.1. Collection of pet feed samples and preparation for the experiment

a. A total of 42 pet foods, including 21 cat and 21 dog feeds belonging to 22 different companies, were obtained from different pet clinics in Aydın and its surroundings. Feed samples were collected between January and April 2015 by contacting veterinarians and stored under appropriate conditions until the analysis.

b. The sample samples were weighed 2 g each and solvent was added at a ratio of 5:1. In the study, 10 mL of solvent (70% methanol) was used for 2 g of sample. The mixture is prepared for 5 min by quickly mixing in a vortex (Heidolph Reaxtop).

c. Separate test tubes for each sample were placed on the appropriate floor. The mixture obtained from the vortex was filtered. For this process, the funnel was placed in the test tube. Circular folded blotter paper was placed inside the funnel in such a way that it completely covered

the inner wall of the funnel. Some glass cotton was added to the paper and some sodium sulfate was added on top of it, and the mixture was filtered through this apparatus.

d. Each of the filtrates taken into the test tube was weighed on a precision scale, and the samples with close weights were placed opposite each other in the centrifuge device (Nüve) at 3000 rpm for 5 min.

e. After centrifugation, each of the supernatants was transferred to a separate tube and dilution was started. In the dilution process, 100 μL was taken from the upper part of the test tube and 900 μL of methanol was added to it.

f. For the derivatization process, the steps below were followed:

- 250 μL of the sample extract was taken into a glass bottle with a screw cap.

- 50 μL of derivatization reagent was added for each sample.

- It was vigorously vortexed for 10–15 s.

- It was incubated at 47–53 ℃ for 60 min. The samples were allowed to cool for 15 min.

- 2.0 mL of AA Test Buffer was added to the refrigerated derived samples.

- Derivatization and analysis of the samples were carried out on the same day.

2.2. EIA analysis method

a. All reagents (Akrilamid EIA kit (Abraxis) were brought to room temperature before use. Fifty microliters of standard solution, control, and derived samples were placed in each well of the antibody-coated plate.

b. 50 μL of AA HRP enzyme conjugate solution was added.

c. 50 μL of AA antibody solution was added. It was covered with paraffin film or tape, then the contents were mixed by moving the tabletop in circular motions for about 60 s.

d. The plate was incubated for 60 min at 4 ℃.

e. After the incubation, the parafilm was removed and the contents of the wells emptied. It was then washed three times with wash buffer solution with each wash buffer (250 μL volume) in the EIA Washer. The remaining buffer liquid in the wells was removed by drying the plate on a stack of paper towels.

f. 150 μL of coloring reagent was added to each white box and incubated for 20–30 min at room temperature.

g. 100 μL of stop solution was added to each white box. The standard curve of optical density corresponding to AA content was generated. Samples were measured by reading the standard curve. The absorbance signal was read in a plate reader at 450 nm. When the samples were higher than the highest standard, they were diluted with 70% methanol and retested, and the added solution factor was taken into account when stating the result (Table1).

Table 1. Optimum test values given for the commercial EIA (ABRAXIS) kit.

Validation values	7.5 ng/mL	30 ng/mL	100 ng/mL
Tested	5	5	5
N (Samples)	25	25	25
Median value	7.9	30.4	102.8
Recovery value (%)	106.1	101.2	102.8

2.3. Statistical analysis

Research data were presented as arithmetic mean and standard deviation. Calculations were performed in the SPSS 20 Windows package program.

3. Results

3.1. Acrylamide EIA analysis findings

3.1.1. Linearity

For the linearity value, a 7-point (0-2.5-5-10-25-50 and 200 ppb) and EIA linearity calibration chart were drawn and the correlation coefficient (r^2) was found to be 0.999 (Figure)

The presence of acrylamide was found in approximately 33% (14 samples) of the analyzed samples, and the average AA concentration in these samples was determined as 87.35 ppb (Table 2). According to the findings, the highest AA concentration was 155 ppb, the lowest was 45 ppb, and AA could not be detected in 67% of the total number of samples. According to the AA distribution ranges detected in pet food samples, 2 samples above 100 ppb were detected, 11 between 50 and 100 ppb, and only 1 sample between 0 and 50. In Table 3, arithmetic mean and standard deviation values are given according to AA levels in pet food samples.

4. Discussion

AA, which is produced commercially as a chemical product, can also occur naturally in starch-rich foods at high temperatures. The main pathway in AA formation is the reduction of sugar between the amino acid and the carbonyl group, known as the Maillard reaction [12-13]. AA is present in many types of food, particularly in potato chips, biscuits, bread, cereal, and coffee.

Animals and humans can be exposed to AA via ingestion, inhalation, or skin. Regardless of the route taken, AA spreads rapidly to all tissues. It passes into
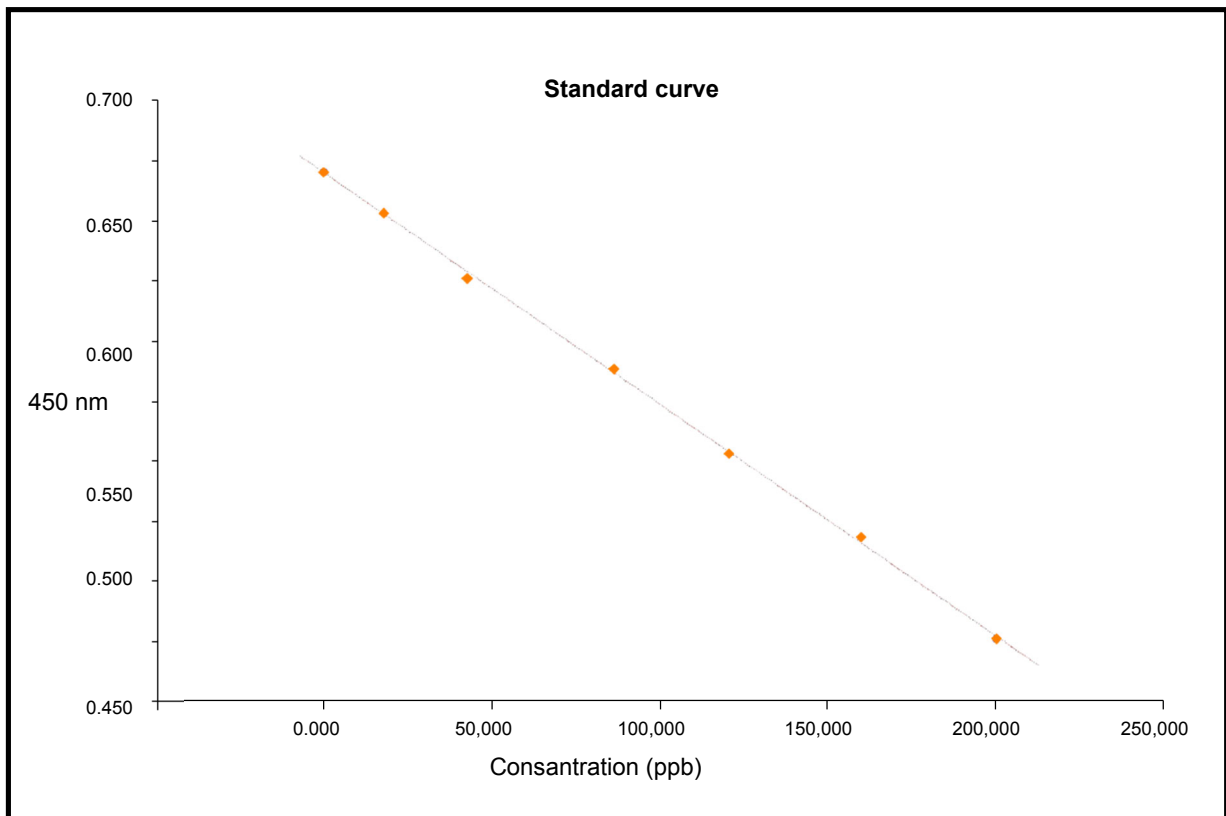

Figure. Acrylamide standard curve.

Table 2. Acrylamide (AKR, ppb) levels in pet food samples according to EIA method.

n	AKR (ppb)	n	AKR (ppb)	n	AKR (ppb)
1	TED	15	TED	29	TED
2	TED	16	TED	30	TED
3	TED	17	TED	31	TED
4	TED	18	TED	32	TED
5	TED	19	**93**	33	**85**
6	**148**	20	**89**	34	TED
7	**155**	21	**96**	35	TED
8	TED	22	**45**	36	TED
9	**62**	23	TED	37	**95**
10	**85**	24	**65**	38	TED
11	TED	25	TED	39	TED
12	**55**	26	**85**	40	TED
13	**65**	27	TED	41	TED
14	TED	28	TED	42	TED

n: Pet food samples TED: not detected.

the liver, heart, brain, kidneys, placenta and breast milk, and especially into the thymus. AA is metabolized to glycidamide by the oxidation of cytochrome P450 [14]. AA and glycidamide can bind to hemoglobin, serum albumins, DNA, and enzymes in vivo [15-16]. It has neurotoxic effects on humans and animals. Typical toxic effects include ataxia and skeletal muscle weakness. The International Agency for Research on Cancer (IARC) has included AA in group 2A [17]. AA has been confirmed to cause cancer in mice and rats. It has carcinogenic effects on many organs such as the lungs, uterus, and mammary glands [18-19]. It has been proven to have genetic and reproductive toxicity in various biological studies [20].

As a result of the studies conducted in Europe, it has been seen that especially coffee, potato products, and bread are in the first place in the intake of food-borne AA, taking into account the high amount of daily consumption [20]. In order to evaluate the problem of AA contamination in foods in our country on the subject, extensive research has been carried out on human foods, especially in children's foods, milk, and dairy products. In this study, for the first time, the level of AA in pet feeds for animals was evaluated by testing samples collected from different companies in Türkiye.

Experimental studies on acute or chronic AA toxicity began in the 1960s and its effects in animals were well understood. In a study conducted for this purpose, very rapid and extensive absorption of AA was detected in dogs following oral ingestion. In conclusion, the intensity of the syndrome following neuropathy and intoxication is independent of the size of the dose, the rate of administration, and the duration of exposure to the toxin [21]. In another study conducted on dogs, it was reported by the authors that especially the peripheral (motor and sensory) and central nervous systems were affected as a result of acute AA toxicity, and higher doses of toxin would be needed to maintain the severity of the effect [22]. There are more recent studies giving information that terminal degenerations are the primary pathology area and primarily cause axonopathy. As a result of acute exposure to high levels of AA, encephalopathy is typically associated with pain and ataxia in clinical presentation and then neuropathy is encountered. In addition, these findings were found to be accompanied by widespread autonomic dysfunction [23]. Unlike other animal species, megaesophagus was detected in dogs due to the toxicity of AA administered orally for an average of 2 months at 410 mg/kg. Regurgitation is the most typical clinical finding in the last stage of the disease. The observation of foamy white liquids on the floor of the room where the animal is located in an early sign that leads to suspicion of the disease. Later, the animals are seen to regurgitate the foods they eat in a characteristic way. The regurgitated material initially appears undigested and is covered with mucus. It is difficult to clearly define the onset of regurgitation, and it is reported that it starts on the 54–68 days following the AA application [24].

On the other hand, studies conducted on this subject in cats revealed that when AA monomer is administered intravenously at a lethal dose (100 mg/kg adult cat), intense tonic-clonic convulsions and other diffuse central nervous system excitation are observed. Administration of doses below lethal doses triggers ataxia and tremors [7]. If adult male Beagle dogs and miniature pigs were fed 1 mg AA/kg/day for 3–4 weeks, neurotoxic signs were not revealed, but AA was present in muscle tissue collected at postmortem examination. Although the primary target for AA monomer is the nervous system, only less than 1% of this substance was detected in the brain. When AA 5 mg/kg/day (for a period of 30-60 days) was administered to dogs and pigs, neuropathy was observed [8]. Chronic exposure in dogs leads to typical progressive sensorimotor peripheral neuropathy, including ataxia, weakness, and megaesophagus, particularly due to vagal nerve axonopathy [9]. When sublethal doses are discontinued, the developing neuropathy may gradually resolve. More intense disorders such as spasticity and cerebellar ataxia are thought to be permanent [10-11].

The presence of AA was found in approximately 33% (14 samples) of the samples analyzed in our study, and the average AA concentration was determined as 87.35 ppb

Table 3. Arithmetic mean and standard deviation values of acrylamide levels in pet food samples.

Pet food	n	Minimum	Maximum	Median		Standard deviation	Variance
				Value	Standard error		
	14	45.00	155.00	87.3571	8.42589	31.52681	993.940

in these samples. According to the findings, the highest AA concentration was 155 ppb, the lowest was 45 ppb, and AA could not be detected in 67% of the total number of samples. Although the limits determined in the study do not pose a normal acute toxicity risk, the continuous intake of high-temperature feeds during the production stages may pose a chronic toxicity risk in cats and dogs. AA toxicity should also be considered, especially in cats and dogs with neurological disorders. There is very little literature available on this subject. In a clinical report [6], it was reported that ataxia, hypermetria and convulsions developed in three healthy Labrador puppies shortly after eating burnt corn porridge. The nervous symptoms of three puppies showing signs of poisoning after eating starch-based foods prepared at high temperatures suggested AA toxicity. Following this, the death of two puppies increased the severity of the situation and it was reported that deaths could be caused especially by distal axonopathy. Porridge made from cornmeal is the main diet for people in South Africa, and pet animals are often fed with burn debris. The possibility of AA toxicity in these animals is an issue that should be carefully considered after neurological signs appear, especially in young animals. Although the scarcity of clinical reports on the subject causes the subject to be overlooked, it reveals that cats and dogs, especially those fed with starch-rich foods exposed to high temperatures and showing neurological disorders, should also be examined in terms of AA toxicity.

When research on the AA concentrations of dog and cat foods is examined, it is observed that there are very few sources on the subject. Veselá and Šucman [24] studied the AA level in dry dog and cat food from various companies by the voltammetric method. To test the accuracy of the method, the authors used rusk and crispbread in the control group. In conclusion, in this study, in which calculations were made with the voltammetric method, AA level was found between 106 and 358 µg/kg in dog foods and between 66 and 269 µg/kg in cat foods. A correlation was found between the measured and calculated AA levels. In our study, AA was determined by the immuno-enzymatic method, and results similar to the study performed on cat and dog food were found with the voltammetric method. The measured AA level was found in the range of 45–155 ppb in cat and dog foods.

Gas chromatography (GC), high-performance liquid chromatography (HPLC), and capillary electrophoresis (CE) were used for the determination of AA. In addition, only 2 publications with similar methods were found by the same authors on the determination of AA in cat and dog foods. In both of these studies, AA levels were measured voltammetrically and similar results were reported [24-25]. The authors stated that the use of voltammetric methods in the determination of AA in dry cat and dog foods is reliable, sensitive, fast, and cost-effective; they also calculated the total time spent for analysis, including extraction and evaluation of measurements, as approximately 2 h. In this study, AA levels were determined immuno-enzymatically in cat and dog foods. This method can be preferred because it requires less equipment than the voltammetric method, it is cheaper, it is sensitive, and it requires a short time such as a few hours.

In accordance with the SML = ND (DL = 0.01 mg/kg) values given in the Communiqué on Plastics and Materials in Contact with Foodstuffs [26] of the Turkish Food Codex (Communiqué No: 2005/31), the Specific Migration Limit (SML) of AA is equal to that which cannot be determined (ND) and the detection limit of the (DL) analysis method was determined as 0.01 mg/kg. The Specific Migration limit is defined as the highest legal level of AA that can pass into food from plastic materials or materials that come into contact with food. Accordingly, when compared with our findings in the study, the results found are below the mentioned value.

There are no official limit values determined for AA levels in foods, neither in the European Union nor in our country yet. However, the European Commission (EC) published a list called 'Indicative Values' for some commonly consumed foods, based on the data of the 'AA Monitoring Program in Food' carried out by the European Food Safety Agency (EFSA) between 2007 and 2008 [27]. The 'Indicative Values' are not the maximum limit values for AA but are a guide to help the competent authority find out where the problem is when these values are exceeded. In this study, the results were interpreted according to the 'Indicative Values' list. According to these indicative values, it is shown as 1000 µg/kg for potato chips, 150 µg/kg for bread, and 80 µg /kg for baby food. It is seen that the AA values determined in the findings are well below

the food indicator values of the European Food Safety Administration (EFSA). Compared to human food, the result was well below the toxicity limit.

The absence of sufficient epidemiological data to quantify the carcinogenic effect of AA on humans makes it impossible to make a definitive and reliable assessment and reach a clear conclusion. Risk assessment committees recommend that AA risk be reassessed in the light of ongoing carcinogenicity and long-term neurotoxicity studies and that studies to reduce AA formation in foods should be continued [28]. When this is evaluated in terms of Türkiye, no attempt was found by the official authorities on this issue. This means that a risk assessment for individuals living in our country has not been made yet. Although studies on AA analyses are carried out within TÜBİTAK, no report has been published on AA-related risks as far as is known. While the effects of AA on human health have not been fully elucidated as stated above, its possible effects on animals are completely unclear. Moreover, no domestic source or report on the subject has been found. However, the presence of AA, a potentially carcinogenic substance, in feeds is a fact. For this reason, AA concentration levels should be determined from samples taken from all animal feeds at regular intervals and kept under constant observation.

5. Conclusion

In this study, AA levels determined by the immuno-enzymatic method were found below the levels that would affect cats and dogs. Our results showed parallelism with AA levels previously determined only by the voltammetric method [24], and it was concluded that it could be an alternative method. In order for the immuno-enzymatic method to be used as a standard technique in cat and dog foods, it needs to be supported by more extensive studies. Again, in order to limit the Maillard reaction that causes AA formation during the preparation of the feed of pet animals that do not have the chance to choose their food as a preservative, it can be recommended to change the formulation and process parameters, especially extrusion and other heat-requiring processes are recommended to be done with care.

Conflict of interests

The authors do not have any interest-based financial or personal relationships.

Acknowledgment

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