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Original Article

Presence of Atrazine in the Biological Samples of Cattle and Its Consequence Adversity in Human Health

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Abstract
Background: Cattle can be considered as an important source for herbicides through nutrition. Therefore, herbicide residue in animal products is a potential human exposure to herbicides causing public health problems in human life. Triazines are a group of herbicides primarily used to control broadleaf weeds in corn and other feed ingredients and are considered as possible human carcinogens. To evaluate trace residue of these pollutants molecular imprinted solid phase extraction (MISPE) method has been developed, using biological samples.
Methods: Blood samples were taken from the jugular vein of 45 Holstein cows in 3 commercial dairy farms in Khuzestan Province, Iran. Urine samples were also taken from the cows.
Results: The mean ± SD concentrations of atrazine in serum and urine samples of the study group (0.739 ± 0.567 ppm and 1.389 ± 0.633 ppm, respectively) were higher ($P < 0.05$) than the concentrations in serum and urine samples of the control group (0.002 ± 0.005 ppm and 0.012 ± 0.026 ppm, respectively).
Conclusion: Atrazine in the feed ingredients ingested by cattle could be transferred into the biological samples and consequently can be considered as a potential hazard for the public health.

Keywords: Atrazine, Molecular imprinted polymers, High performance liquid chromatography, Cattle

Introduction

"Food and Agriculture organization (FAO) has defined the term of pesticide as: any substance or mixture of substances intended for preventing, destroying or 3controlling any pest, including vectors of human or animal disease, unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage, transport or marketing of food, agricultural commodities, wood and wood products or animal feedstuffs, or substances which may be administered to animals for the control of insects, arachnids or other pests in or on their bodies. The term includes substances intended for use as a plant growth regulator, defoliant, desiccant or agent for thinning fruit or preventing the premature fall of fruit. Also used as substances applied to crops either before or after harvest to protect the commodity from deterioration during storage and transport" (1).

These substances can be classed as "insecticides (insect killers), fungicides (fungus killers), herbicides (weed killers), rodenticides (rodent killers), repellent (substances used to deter vermin from cultivated land) and fumigants (gaseous chemicals used for clearing planta-

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tions of microbes or insects)" (2). Although pesticides can improve quality and quantity of crops, they are reported to cause occupational diseases in farmers (2).

Triazines, a group of herbicides including atrazine, simazine, propazine, cyanazine, sebuthylazine, are most effective on broadleaf weeds primarily corn, sorghum, sugarcane, cotton, macadamia orchards, pineapple, asparagus, other crops, and landscape vegetation, to some extent (3). Very low biodegradability (risk for drinking water) and xenostrognic effects are among the serious risks of triazines.

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1, 3, 5-teriazine) ($C_8H_{14}ClN_5$) Atrazine is the most widely used S-triazine. Other S-triazines used as herbicides are Symazine and cyromazine. Atrazine is not very volatile, reactive or flammable but dissolves readily in water and has been heavily used throughout the world especially applied to corn, sorghum and sugar cane (4). Atrazine usage have been increasing steadily since the 1960's to the level of about 64 to 80 million 1bs each year in the United States, making it one of the two most widely used pesticides in that country (3).

Atrazine is also used in Iran in corn and sugarcane areas. The amount of the pesticide which has been used in Iran from 2003 to 2006 has been reported as 224, 409, 221 and 240 tones, respectively which mostly used in Khuzestan province (114, 295, 125, and 125 tones, respectively) (5).

Based on reports indicating increased mammary gland tumors in female laboratory animals triazines are considered as possible human carcinogens (group C) (3).

Hopenhayn-rich et al., (2001) reported that the highest percent of ovarian cancers were found among the mice which received the highest atrazine dose (6). Low doses of atrazine, and s-triazine herbicides are reported to have toxic effects on ovaries, liver, kidneys, and myocardium and lymphatic organs of gilts (7).

Metabolites of atrazine were measured in human urine after dermal exposure and also in saliva and plasma in rats (which shows salivary monitoring of atrazine in humans may prove useful and practical (8, 9). Some researchers have evaluated the children's exposure to pesticides (chlorpyrifos, diazinon, malathion, and atrazine) and their metabolites in urine (10,11).

Nowadays, considerable amount of method developments is spent on optimizing modern strategies of sample preparation that deals with the trace level determination of occupational and environmental pollutants such as herbicides (12).

Since the number of environmental pollutants, drugs and their metabolites, and additives used in the food and feed industry is growing, the need for efficient and accurate analytical methods to detect such compounds is increasing, especially for compounds affecting human health (13). Advanced instrumentation and their detectors are able to detect and identify trace levels of analytes in complex samples (13).

Although sample preparation is the major step in overall time needed for trace residue analysis and much research seems to focus on the final separation and detection steps, however, less attention is paid to the development of faster, more selective clean-up methods (13).

Sample preparation using solid phase are commonly used as a purification methods for evaluating environmental and occupational exposures. Nevertheless, because of the presence of different interferences in the samples, sometimes it is necessary to develop methods, having more selectivity, sensitivity and detectability to overcome co-extraction of interferences in the final solution (14-16).

In comparison to liquid-liquid extraction (LLE), reduced sample preparation time required, especially for automated methods, smaller sample volumes, and amount of used solvent, are the major advantages of SPE in the whole analysis scheme (17, 18).

A relatively new development in the area of SPE is the use of molecularly imprinted polymers (MIPs) for the sample treatment (19-21). MIPs are synthetic polymers with specific cavities considered as a selective adsorbent designed for a target molecule (22). Molecular imprinting technology has received much attention during the last decade because of its high selectivity, and seems to be a promising technique for preparation of biologicl samples such as urine and plasma (23-26).

Since biological samples are complex matrices with interferences from the sample, an efficient purification step is needed before analysis. Using this step, it is possible to remove the interferences easily and a purified sample would be available for the analytical stages (13).

Cattle can accumulate herbicides in their body through ingestion plants infested with these compounds and one of the ways, by which, human beings are exposed to atrazine is through cattle meat and milk consumption.

This study was aimed to monitor presence of atrazine in the cattle biological samples, using molecular imprinted solid phase extraction followed by high performance liquid chromatography.

Material and Methods

Sampling procedure: blood samples were taken from the jugular vein of 45 Holstein cows in 3 commercial dairy farms in Khuzestan province, Iran. The cows were fed with corn silage from farms in which Atrazine was used at 3 kg/hectare. Urine samples were also taken from the cows. All samples were transferred to the Clinical Laboratory of Veterinary Department, Islamic Azad University, Shooshtar Branch, Khuzestan, Iran. Serum samples were harvested and kept at -20$^{\circ c}$. Urine samples were centrifuged at 4000 rpm for 5 minutes. Then supernatants were removed and kept at -20$^{\circ c}$ until analysis. Blood and urine samples were also taken from 5 normal cows with no corn silage in their ration.

MISPE procedure: The SupelMIP Triazine 10 (Sigma-Aldrich Company, Germany) was used as a SPE media in this study. To set up the method for measurement of atrazine in serum and urine samples, the calibration curve was prepared. The known concentrations (1, 2, 4, 6, 8 and 10 ppm) of atrazine standard (Sigma-Aldrich Company, Germany) were prepared and injected to the HPLC (Knauver, Germany, C8). A reversed phase-HPLC-UV was employed with an isocratic solvent delivery system [acetonitrile: mixture of H_2O and ammonium acetate (1×10-3 M), 50:50], a flow-rate of 1.4 mL min $^{-1}$, and a UV-wavelength of 226 nm and then calibration curve was constructed (Fig. 1-4). Materials used and extraction procedures were as follows:

SupelMIP SPE –Triazine10: 25 mg/10 mL (LRC) (Cat. No.53208-U from Sigma-Aldrich), with no sample pre-treatment.

1. Conditioning/equilibrating the cartridge was done with:
   - 1 mL methanol
   - 1 mL ultra-pure water
   - 1 mL 25 mM ammonium phosphate (NH4H2PO4), pH 3

2. Loading sample:

1 mL serum/urine sample was applied to the cartridge. The recommended flow rate was about 0.5 mL/min.

3. Washing (interference elution) was done by:
   - 1 mL 0.1 M HCl
   - 1 mL ultra-pure water

Gentle vacuum was applied between each wash step and at the end, full vacuum through cartridge for 20 min to remove residual moisture from cartridge.

4. Analyte elution was done by running 3 × 1 mL methanol. A gentle vacuum was applied between each fraction of this step and recommended flow rate was about 0.2 mL/min.

In order to determine the percentage of atrazine isolation in each of the above-mentioned steps, 10 ppm concentration was loaded on the MIP cartridge. 90% of atrazine was isolated in the first step of elution procedure (running 1 ml methanol).

Other standard concentrations (1, 2, 4, 6, 8 and 10 ppm) were also loaded on cartridges and the related calibration curves were prepared after extraction procedure using SUPELMIP (Fig. 2). Recovery percentage was calculated by comparison of atrazine concentrations before and after extraction step. The mean recovery was 94.8%.

To evaluate the matrix effect on the results, bovine serum control was spiked with increasing amounts (1, 2, 4, 6, 8 and 10 ppm) of atrzine and then extracted using MIP cartridges and calibration curves were constructed. For urine samples the same protocol was applied.

After setting up the method, the real samples were analyzed to measure atrazine concentrations.

Results

In the present study, the use of MISPE of atrazine with regard to qualitative and quantitative parameters for biological samples was described. The atrazine chromatogram was detected at 226 nm (Fig. 5).

For the validation of the present method, the biological spiked samples of 1 mL of atrazine were used for extraction followed by HPLC-UV determination. Linear standard curve (for extracted samples) were obtained by different concentrations of standard sample, before and after extraction (n=6), the correlation coefficient was 0.997.

The calibration curves of standards, before and after extraction and also of serum and urine spiked samples are shown in Fig. 1-4. The detection limit of the method was 0.001µg/ml and the recovery rate of the method was estimated about 94.8%. The mass chromatograms of standard samples (before and after extraction) and a spiked serum sample are shown in Figures 5, 6 and 7.

The mean ± SD concentrations of atrazine in serum and urine samples of the study group (the group which were fed with corn silage) were 0.739 ± 0.567 ppm and 1.389 ± 0.633 ppm, respectively while the atrazine mean ± SD concentrations in serum and urine samples of the control group were 0.002 ± 0.005 ppm and 0.012 ± 0.026 ppm, respectively (Table 1). The difference between atrazine concentrations in serum and urine of control and study group were statistically significant (P<0.001) (Figs. 8-10)

The mean ± SD concentrations of atrazine in the serum samples of male and female cattle were 0.774 ± 0.519 ppm and 0.728 ± 0.587 ppm, respectively.

The mean ± SD concentrations of atrazine in urine samples of male and female cattle were 1.432 ± 0.460 ppm and 1.375 ± 0.683 ppm, respectively (Table 2).

In study group atrazine concentrations in serum samples of male cattle was higher than female. The difference was not statistically significant ($P > 0.05$).

In the study group atrazine concentrations in urine of male cattle was higher than female but the difference was not significant ($P > 0.05$).

The mean ± SD concentrations of atrazine in serum samples of cows at different age groups (6 months and 18 months of age) were 0.720± 0.553 ppm and 0.716 ± 0.806 ppm, respectively. The mean ± SD concentrations of atrazine in the urine samples of cows at different age groups (6 months and 18 months of age) were 1.402 ± 0.521 ppm and 1.397 ± 0.738 ppm, respectively (Table 3).

The mean concentrations of atrazine in serum samples of 6-month cattle were higher than the concentrations of atrazine in serum samples of 18-month-cattle but the difference was not statistically significant ($P > 0.05$).

The mean concentrations of atrazine in urine samples of 6-month cattle were higher than that of atrazine in serum samples of 18-month cattle. The difference was not significant.

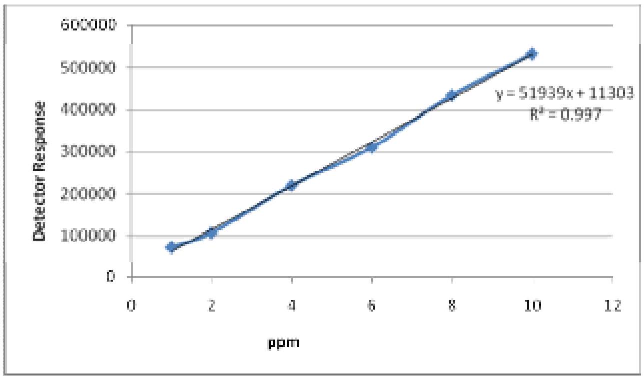

Fig. 1: Calibration curve of standard samples at different concentrations before extraction

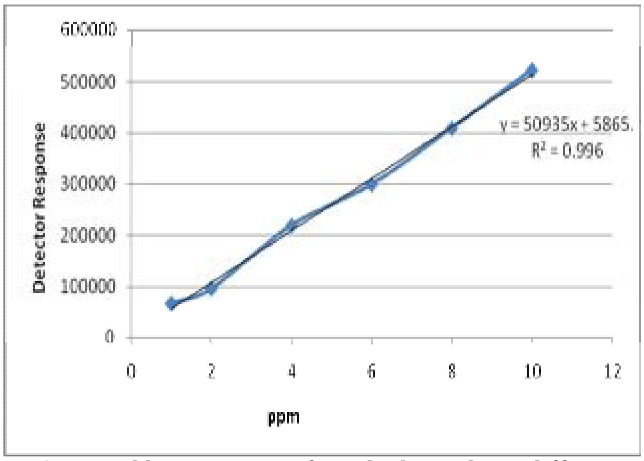

Fig. 2: Calibration curve of standard samples at different concentrations after extraction

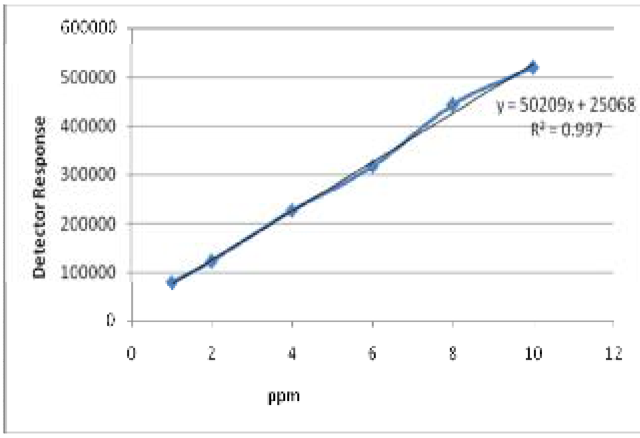

Fig. 3: Calibration curve of spiked serum samples after extraction

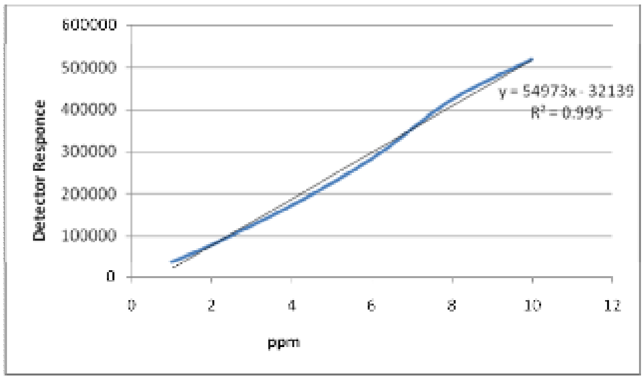

Fig. 4: Calibration curve of spiked urine samples after extraction

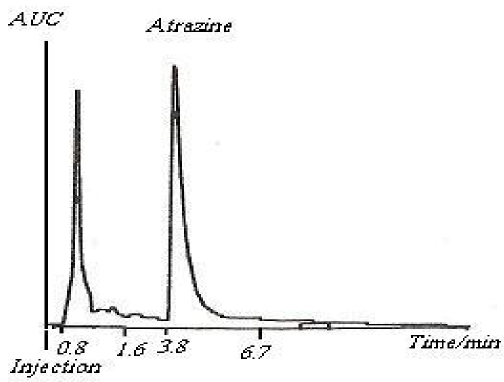

Fig. 5: The HPLC chromatogram of standard spiked atrazine at the concentration of 10 ppm before extraction. AUC: Area Under Curve

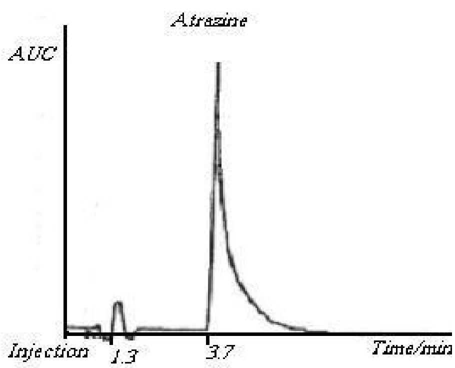

Fig. 6: The HPLC chromatogram of standard spiked atrazine at the concentration of 10 ppm after extraction AUC: Area Under Curve

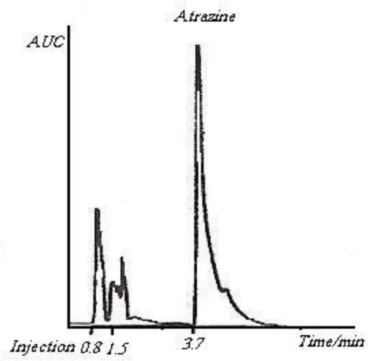

Fig. 7: The HPLC chromatogram of serum spiked atrazine at the concentration of 10 ppm after extraction. AUC: Area Under Curve

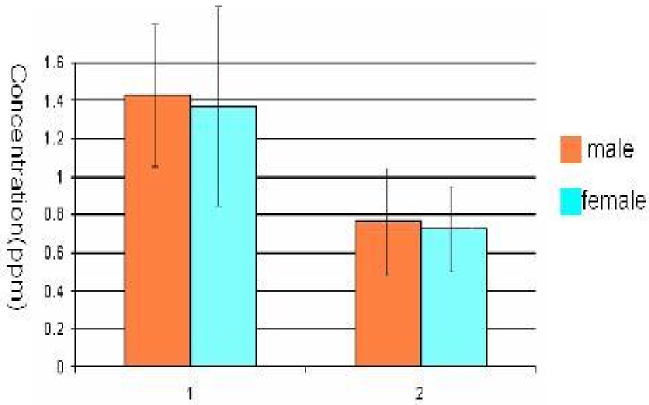

Fig. 9: Comparison of atrazine concentration in serum (2) and urine (1) samples of male and female groups

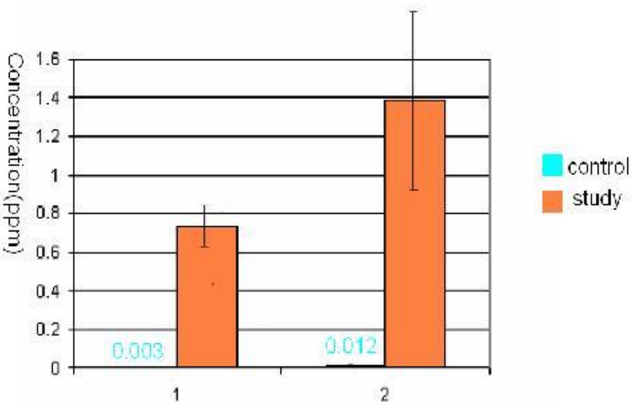

Fig. 8: Comparison of atrazine concentration in serum (1) and urine (2) samples of control and study groups

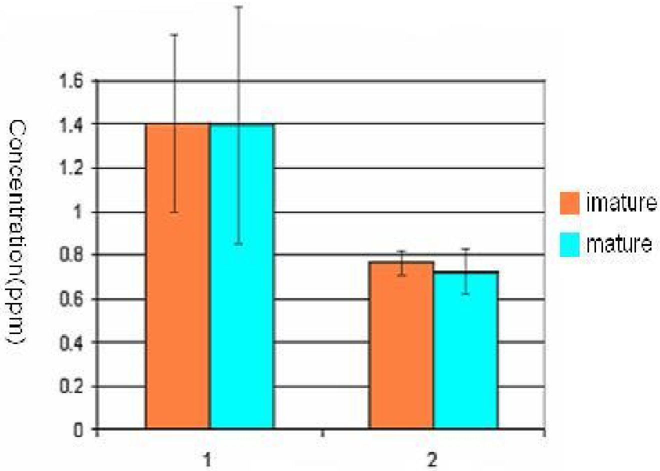

Fig. 10: Comparison of atrazine concentrations in serum (2) and urine (1) samples of 6-month and 18 month age groups

Table 1: Comparison of Atrazine concentrations in serum and urine samples of the study and control groups

		n	Mean (ppm)	SD	Min (ppm)	Max (ppm)
Study group	Serum	45	0.739	0.567	< LOD	1.96
	Urine	45	1.389	0.633	0.59	2.93
Control group	Serum	5	0.002	0.005	< LOD	0.013
	urine	5	0.012	0.027	< LOD	0.60

Table 2: Comparison of Atrazine concentrations in serum and urine samples of male and female cattle in the treatment group

		n	Mean (ppm)	SD	Min (ppm)	Max (ppm)
Male	Serum	10	0.774	0.519	<LOD	1.51
	Urine	10	1.432	0.460	<LOD	1.88
Female	Serum	35	0.728	0.587	<LOD	1.96
	urine	35	1.375	0.683	0.59	2.59

Table 3: Comparison of Atrazine concentrations in serum and urine samples of different ages in the treatment group

Age groups		n	Mean (ppm)	SD	Min (ppm)	Max (ppm)
6 months	Serum	22	0.720	0.553	< LOD	*1.70*
	Urine	22	1.402	0.521	0.59	*2.59*
18 months	Serum	23	0.716	0.806	< LOD	2.84
	urine	23	1.397	0.738	0.59	*2.93*

Discussion

Atrazine is a major herbicide of s-triazine family which has been heavily used throughout the world (27) and also in Iran, especially in corn-growing areas. Since Atrazine would be a potential hazard for environment and consequently for human health, a lot of studies investigated the amount of atrazine in environmental samples and its toxic effects on different organs of animal and human.

One of the commonly used pesticide in ground and surface waters is atrazine and its metabolites (28). High amounts of atrazine were found in the urine of farmers and their spouses and children on the day after atrazine application (28).

Atrazine has more acute toxic effects on ruminants than rodents. In one study, two doses of 250 mg/kg caused death in both sheep and cattle (29).

To assess the environmental impact and human exposure to triazine herbicides, sensitive and selective analytical methods such as gas chromatography, mass spectrometry, ELISA, HPLC and enzyme-linked immunosorbent assay were developed and introduced for their determination in environmental water and soil samples as well as in human urine (8,30-33). MISPE allows a sensitive, simple and inexpensive extraction and detection of the analyte in biological samples. MIP was also used to extract the herbicide from organic extracts like beef liver samples (32).

Since the main objective of the present study was to detect atrazine in cattle biological samples, MIP cartridge was set up in agreement with the other studies (34). Achieving this purpose, serum and urine were spiked and loaded on the cartridges. The next parameter studied was the concentration of atrazine in serum and urine samples of the studied cattle. Atrazine concentrations in the serum and urine samples of the study group were higher ($P < 0.001$) than those of the control group, which indicates that atrazine in the feed ingredients, ingested by cattle, could be transferred in to the biological samples and would be a potential hazard for human health. Ballantine and Simoneaux (1991) have reported that metabolites of atrazine in plants have a little tendency to be transferred to meat, milk or eggs (35). This result is against of our findings.

In the study group atrazine concentrations in serum and urine samples of the male cattle was higher than in female but the difference was not statistically significant. Therefore the obtained results indicate that gender has no effects on up taking the atrazine. No study was found concerning gender as a factor in atrazine accumulation in body fluids or organs.

The mean concentrations of atrazine in serum and urine samples of 6-month cattle were higher ($P > 0.05$) than the concentrations of atrazine in serum samples of 18-month cattle. So it seems that age is not an effecting factor for atrazine up take in cattle. No study was found to show the effect of age on atrazine uptake by animals.

It should be mentioned that in all cases (both control and study groups), the concentrations of atrazine in urine samples were higher those in serum ones. This finding is in accordance with another study in which the kinetics of atrazine in the plasma of rats was described (36).

In the present study it was concluded that the amount of atrazine in urine samples of all cases were higher than the amount of atrazine in serum samples. This can be attributed to excretion of atrazine by urinary system after it is metabolized in liver.

It seems that the present study is the first one which reports atrazine residues in biological samples of cattle. The statistically significant difference between atrazine concentration in the serum and urine samples of the study and control groups indicated that atrazine in the feed ingredients ingested by cattle could be transferred in to the biological samples and could be a potential hazard for human health.

It is suggested that determination of pesticides in meat, milk and other products of animal, which can be harmful for human health, could be the subject of further studies.

Ethical Considerations

Ethical issue principles including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc. have been completely observed by the authors.

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