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Content of heterocyclic aromatic amines and D-enantiomers of amino acids in sausage products

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Abstract. The scientific community has recently paid attention to the formation and content of heterocyclic aromatic amines in food products. This is because of the carcinogenic and mutagenic properties of HAAs, which can have a carcinogenic effect on the human body. A similar situation is with the D-enantiomers of amino acids - stereoisomers of L-forms of amino acids, which are often found in tissues for pathological diseases such as diabetes, Alzheimer's disease, etc. The present work examines sausages sold in Moscow, Russia, for their HAA and D-enantiomer amino acid content. The results showed that the accumulation of HAA and D-enantiomers of amino acids occurred in each of the selected species during the manufacturing process. The authors found the least amount of HAA in boiled sausage products, with an average content of about the same (0.36-2.87 μg/kg), except for one sample (7.24 μg/kg). Smoked sausage products contained HAA ranging from 3.05 to 7.53 μg/kg, except for one sample, which contained 0.49 μg/kg of HAA. Boiled-smoked sausages had the highest HAA content, these samples also had the highest range, from 1.54 to 40.68 μg/kg. The lowest content of D-enantiomers of amino acids was in the samples of boiled sausages, with an average content of ≈13 mg/100g. The next highest content of D-enantiomers of amino acids - products of sausage boiled-smoked with an average content of ≈21 mg/100g. Next comes smoked sausage products, the content of analytes (D-enantiomers of amino acids) at ≈27 mg/100g. The highest number of D-enantiomers of amino acids was in the samples of smoked sausage products, which were made with starter cultures, and ranged from 52.36 to 72.13 mg/100g.

1 Introduction

1.1 HAA content in ready-to-eat foods

Scientists now classify heterocyclic aromatic amines (HAA) as 2a and 2b carcinogens. Their mutagenic and carcinogenic properties were the basis of this classification. This led to a significant increase in the number of publications on HAA content in food. According to statistics from the website sciencedirect.com, the number of published studies on HAA in food increased from 210 in 2010 to 971 in 2022. The most active increase in publications is from 2020, with 632 publications in 2020, 755 in 2021, and 971 in 2022. 382 papers have

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already been published in 2023 (as of March 2023) [1]. These statistics are for Elsevier-owned journals only, excluding other publications and databases.

Almost these papers present results from studies of food prepared "at home," i.e., by frying, baking, barbecuing, etc. Incorporating ingredients with chemical compounds that inhibit the HAA formation reaction or changing the cooking regime can reduce HAA in most of these studies. Few studies have monitored culinary, fast-food, and industrial ready-to-eat products such as sausages, and meat products. But the results of such studies show that HAA accumulation also occurs in the manufacture of such products.

The results of [2] showed that smoked cheese forms HAAs such as Trp-P-2 in amounts from 0.7 to 1.1 μg/kg, PhIP - 4.2-6.2 μg/kg, Trp-P-1 - 3.7-5.6 μg/kg, AαC - 5.1-7.6 μg/kg, MeAαC 5.2-7.8 μg/kg. The values got showed that a large amount of HAA is formed in cheese during smoking. Work [3] contains monitoring studies on the products of fast-food restaurants, namely burgers. Some samples showed much lower amounts of HAA as these products were subjected to a much higher temperature treatment than smoking, namely frying. The authors [4] examined soft cheeses after electron-beam irradiation. The results showed HAAs such as MeIQx, Harmine, Harman and Norharman at levels of 11-13 μg/kg.

A study [5] investigated the content of HAAs in sujuk and the effect of different spices on their amount. MeAαC was not detected in any of the samples. The study detected IQx up to 0.54 μg/kg, IQ up to 5.96 μg/kg, MeIQx up to 0, 21 μg/kg, MeIQ to 0.34 μg/kg, 7.8-DiMeIQx to 0.32 μg/kg, 4.8-DiMeIQx to 0.19 μg/kg, PhIP to 1.94 μg/kg and AαC to 0.98 μg/kg.

The authors [6] examined 12 samples of sausages and bacon sold in shops in Guangzhou, China. They detected Harman and Norharman in amounts from 2.8 to 12.4 μg/kg in all 12 samples. According to [7], a study of smoked sausages sold in a shop in Mahasarakam province, Thailand, the amounts of MeIQ, 4,8-DiMeIQx, Harman and PhIP ranged from 100 to 350 μg/kg. Such values show a very high content of HAA in the product. The amounts of some analytes in some samples were higher than in meat samples roasted at 230ºC for 20 minutes [8]. A study [9] investigated samples of meat products from shops in Reading, UK, in ready-to-eat poultry (chicken) and pork sausages. They found the highest amount of IQ in charcoal-cooked chicken, 22.68 μg/kg. In barbecue chicken and Tikka chicken, 9.16 and 9.74 μg/kg, respectively. In pork sausage products, MeIQ and 4,8-DiMeIQx are in amounts of 2.87 and 2.32 μg/kg respectively. Generally the samples in work [9] were tested for the content of 5 HAAs - IQ, MeIQ, MeIQx, 4,8-DiMeIQx and PhIP. At least 2 of the 5 investigated analytes were in each of the samples tested, and their total content ranged from 0.57 to 37.45 μg/kg.

Russian scientists also conducted studies on HAA content in ready-to-eat products in the Russian Federation. An article [3] examined samples of burgers available in fast-food restaurants in the Russian Federation. They found HAAs in at least one product from each selected restaurant. But it is important to note that there were also samples in which they detected no HAAs at all. Values in samples with positive results ranged from 0.84 to 11.00 μg/kg.

In another study [10] on HAA content in ready-to-eat products, the authors investigated chilled second dinner dishes with garnish - 16 samples of culinary products from Moscow (Russia) shops. The authors examined the samples for MeIQx and PhIP content. The authors found HAA in amounts from 0.17 to 47.40 μg/kg in each of the samples. The total amount of HAA detected was MeIQx only, while they did not detect PhIP in any of the samples.

Among ready-to-eat products, HAA was not detected only in sterilized canned meat [11]. HAA was not detected in any of the samples examined, and samples produced under both standard sterilization conditions and over-sterilized conditions were examined in this work. We assume that the absence of HAA in canned food is due to insufficiently high processing temperatures or to a lack of air (oxygen) access to the product during processing. Considering the works described above, in particular [10], where HAA was detected in a sample subjected to prolonged simmering at a temperature not exceeding 95ºC, the statement that the sterilization temperature of canned meat is insufficient for the formation of HAA is incorrect.

1.2 Content of D-enantiomers of amino acids in ready-to-eat foods

The presence of L-amino acids in foodstuffs attracts attention because of their taste, nutritional and nutraceutical properties. The presence of D-enantiomers because of natural or artificial interventions (microbial action, preservation) is a frequent sign of microbial contamination, making these compounds an indicator of food quality. D-alanine, D-aspartic acid, and D-glutamic acid are present in peptidoglycan, which is a major component of the bacterial cell wall [12].

D-enantiomers are marker compounds in various pathologies. For example, scientists have found D-phenylalanine in a patient affected by gestational diabetes mellitus and together with D-tyrosine in high concentrations in patients with chronic renal insufficiency. Researchers discovered an accumulation of D-glutamic acid, D-valine, leucine, and lysine in cancerous tumors and Alzheimer's disease [13].

Scientists have been considering the problem of D-enantiomers in food since the 1990s. A growing number of scientific publications dedicated to this issue each year testify to this. Vegetables and fruits, and products derived from fermentation processes such as fermented dairy products, alcoholic and non-alcoholic beverages have been of major interest.

Work [14] investigated the effect of microorganisms on the enantiomers content of dairy products. The researchers introduced different amounts of microorganisms into the milk and then used it to produce cheeses. The results showed that as the total number of microorganisms increased, the concentration of D-enantiomers increased. The percentage of D-aspartic acid to total unbound amino acids was 30%, the percentage of D-glutamic acid was between 18-27% and the proportion of D-alanine was about 40%.

A similar study [12] investigated the increase in D-enantiomers in ripening cheese over 6 months. D-alanine increased from 20.5% to 83%, D-aspartic acid increased by 15.5% and D-glutamic acid by 7.2%.

Other dairy products also contain enantiomers. Pasteurization did not significantly increase the content of these compounds, but ultrapasteurization of milk increased the enantiomers from 2-4% to 4-6%. Using yeast and lactic acid bacteria sources resulted in up to 38% unbound D-alanine and up to 34% unbound D-glutamic acid in the batter. Roasted coffee contained between 9% and 41% D-phenylalanine, D-aspartic acid and D-glutamic acid, whereas green coffee beans contained <0.2% enantiomers. Fermented cabbage contained 25 times more enantiomers (alanine, aspraginic acid, glutamic acid, glycine, leucine and lysine) compared to fresh white cabbage [15].

Commercially produced fruit juices contained D-alanine 10-42% of the total alanine content. Such high concentrations show bacterial contamination of the product at the initial preparation stage. Since bacteria do not survive after heat treatment, we can consider D-alanine remaining after pasteurization as a marker of bacterial contamination of the starting material [16].

The concentration of enantiomers in fruits and vegetables is low, not exceeding 3.4% (fruits) and 0.7% (vegetables). D-serine in clementine is 1.7% and D-glutamic acid is 1.3%. D-aspartic acid was found in grapefruit and grapes, and D-alanine in apples was 2.7% [16].

According to the foregoing, the purpose of this work was to conduct monitoring studies of sausage products for the content of GAA and D-enantiomers of amino acids.

2 Materials and methods

The objects of research were 10 samples of boiled sausages (5 samples of boiled sausage "Doktorskaya" and 5 samples of boiled sausage "Molochnaya"), 10 samples of smoked sausages (5 samples of smoked sausage "Braunshveigskaya" 5 samples of smoked sausage "Moskovskaya"), and 10 samples boiled-smoked sausage products (5 samples smoked sausage "Moskovskaya", 5 samples smoked sausage product "Servelat"). We bought all the samples from a shops in Moscow, Russia. All samples, according to the label, were produced according to the state standard (GOST).

To determine amino acid D-enantiomers, we also selected 10 samples of smoked sausage products produced using starter cultures.

The content of HAA in samples was determined according to [17]. The content of D-enantiomers in the samples was determined according to [18].

3 Results

We carried out the study in two selections of 3 parallel measurements in each selection. The result was the arithmetic mean of the three parallels in the first selection, provided that the Student's test of the two selections for each analyte in each sample did not exceed the table value at n=3 and confidence probability p=0.95, i.e. the differences between the samples were not statistically significant.

Tables 1-3 show the results of the HAA content of boiled, smoked, and boiled-smoked sausages. Tables 4-7 show the results of the amino acid D-enantiomers in boiled, boiled-smoked, smoked, and smoked sausages made with starter cultures, respectively.

Table 1. HAA content in boiled sausage products.

Sample No.	Amount of MeIQx, µg/kg	Amount of PhIP, µg/kg	Amount, mcg/kg
1	2,68	0,19	2,87
2	0,13	0,23	0,36
3	0,59	0,25	0,84
4	0,16	0,63	0,78
5	0,94	Less than 0,1	0,94
6	6,83	0,41	7,24
7	0,84	0,22	1,06
8	0,12	0,30	0,42
9	0,68	0,31	0,99
10	0,92	0,29	1,21

Table 2. HAA content in smoked sausage products.

Sample No.	Amount of MeIQx, µg/kg	Amount of PhIP, µg/kg	Amount, mcg/kg
1	2,68	3,02	5,70
2	2,54	3,34	5,88
3	1,19	3,22	4,41
4	0,49	Less than 0,1	0,49
5	3,66	2,87	6,53
6	3,92	3,61	7,53
7	2,73	4,71	7,44
8	3,01	4,00	7,01
9	1,63	3,90	5,53
10	0,95	2,10	3,05

Table 3. HAA content in boiled-smoked sausages.

Sample No	Amount of MeIQx, µg/kg	Amount of PhIP, µg/kg	Amount, mcg/kg
1	11,80	Less than 0,1	11,80
2	29,14	Less than 0,1	29,14
3	39,51	1,17	40,68
4	8,20	Less than 0,1	8,20
5	1,54	Less than 0,1	1,54
6	4,64	Less than 0,1	4,64
7	37,52	0,98	38,49
8	15,64	Less than 0,1	15,64
9	14,69	Less than 0,1	14,69
10	7,31	Less than 0,1	7,31

Table 4. Results for D-enantiomers in boiled sausage products, mg/100 g.

Amino acid name	1	2	3	4	5	6	7	8	9	10
D-Alanine	3,15	3,28	3,12	3,25	2,84	3,46	3,16	2,71	3,45	3,14
D-Phenylalanine	3,24	3,16	3,14	3,61	3,08	3,73	3,45	2,59	3,06	2,91
D-Methionine	Less than 1.00	1,14	1,08	Less than 1.00	1,03	1,00	Less than 1.00	1,15	Less than 1.00	Less than 1.00
D-Threonine	2,18	2,05	3,01	2,86	3,12	2,21	2,12	2,61	2,09	3,05
D-Isoleucine	1,24	1,37	1,19	1,15	2,05	1,74	1,25	2,34	2,08	1,63
D-Leucine	2,54	2,91	1,97	2,07	1,98	2,58	1,09	2,04	1,67	1,49
Sum	12,35	13,91	13,51	12,94	14,10	12,65	11,07	13,44	12,35	12,22

Table 5. Results for D-enantiomers in boiled-smoked sausages, mg/100 g.

Amino acid name	1	2	3	4	5	6	7	8	9	10
D-Alanine	4,15	5,05	4,12	4,59	4,45	4,85	4,42	5,04	5,05	5,49
D-Phenylalanine	4,25	5,01	4,84	5,07	4,81	4,26	4,31	5,26	4,64	4,56
D-Methionine	1,24	1,62	2,09	1,18	1,98	1,72	1,11	1,19	2,09	2,07
D-Threonine	3,08	3,28	4,26	4,25	3,50	4,21	4,72	5,06	4,15	4,12
D-Isoleucine	2,72	2,46	3,08	3,27	2,26	3,08	2,44	2,59	2,16	3,56
D-Leucine	3,26	3,24	4,03	4,26	4,61	4,75	3,31	4,16	3,04	4,25
Sum	18,70	20,66	22,42	22,62	21,61	22,87	20,31	23,30	21,13	24,05

Table 6. Results for D-enantiomers in smoked sausage products without the addition of starter cultures, mg/100 g.

Amino acid name	1	2	3	4	5	6	7	8	9	10
D-Alanine	Less than 1.00	1,11	Less than 1.00	Less than 1.00	Less than 1.00	1,56	Less than 1.00	Less than 1.00	Less than 1.00	Less than 1.00
D-Phenylalanine	10,15	10,26	11,87	12,09	10,59	10,23	12,12	11,43	10,64	10,17
D-Methionine	Less than 1.00	1,02	Less than 1.00	Less than 1.00	Less than 1.00	1,06	Less than 1.00	Less than 1.00	Less than 1.00	Less than 1.00
D-Threonine	4,36	3,25	5,11	5,18	3,49	3,58	4,26	3,58	4,08	5,01
D-Isoleucine	1,05	1,25	1,07	1,25	1,16	1,14	1,09	1,07	1,02	1,14
D-Leucine	10,23	10,54	11,54	11,04	9,56	9,89	10,22	11,01	11,14	10,56
Sum	25,79	27,43	29,59	29,56	24,8	27,46	27,69	27,09	26,88	26,88

Table 7. Results of D-enantiomer content in smoked sausage products with added starter cultures, mg/100 g.

Amino acid name	1	2	3	4	5	6	7	8	9	10
D-Alanine	10,23	10,58	9,25	10,36	11,15	11,78	10,24	10,15	9,87	9,64
D-Phenylalanine	13,45	12,23	12,54	14,63	11,87	15,26	12,12	15,01	13,45	13,79
D-Methionine	5,13	5,19	6,08	5,45	5,67	6,01	6,23	5,23	5,14	5,01
D-Threonine	11,43	10,56	10,25	12,03	10,23	11,54	10,26	12,23	11,12	11,15
D-Isoleucine	12,12	12,45	13,06	13,10	13,16	12,48	12,72	13,15	13,16	12,87
D-Leucine	14,58	14,73	15,11	14,02	14,12	15,06	15,10	15,00	14,41	14,40
Sum	52,36	65,74	66,29	69,59	66,20	72,13	66,67	70,77	67,15	66,86

4 Discussion

4.1 HAA in sausage products

The results of the monitoring studies of boiled sausage products showed HAA in each of the samples. The amount of PhIP did not exceed 1 μg/kg, and in sample 5 it was below the limit of quantitative detection. In sample 1 the total amount of HAA was 2.87 μg/kg and in sample 6 it was 7.23 μg/kg, which is the highest amount of HAA detected in all tested samples of boiled sausage products. The HAA content in samples 1 and 6 is extremely high relative to other samples, where the total amount of HAA does not exceed 1.21 μg/kg, and in 6 of the 10 samples examined, it does not exceed 1 μg/kg at all. This HAA content in the sample could be because of a difference (or maybe a violation) of the technological process: increased temperature or excessive heat treatment duration. Whatever the reason, this amount of HAA is too high for boiled sausage compared to other samples. The amount of HAA in sample 6 is close to, and sometimes even higher than, the HAA content of burgers from fast-food restaurants [3], which are cooked at higher temperatures than traditionally boiled sausages.

The results of a study of smoked sausages showed PhIP is formed in higher quantities during the smoking process than during boiling. The total amount of HAA in most samples ranges from 5.53 to 7.53 μg/kg. Sample 3 with 4.41 μg/kg does not differ much from most samples, as well sample 10, with an HAA content of 3.05 μg/kg. Sample 4 differs the most in HAA content - it has no PhIP at all and MeIQx is amounting to 0.49 μg/kg.

Comparing the values of HAA content in smoked and boiled sausages, we can see that, if we exclude sample 6 from the boiled sausages, smoking leads to a higher formation of HAA in the product. The values of heterocyclic aromatic amines were higher in 9 samples of smoked sausages than in boiled sausages. We can explain the higher values of heterocyclic aromatic amines in smoked sausages because smoked sausages are subjected to a fairly long heat treatment (which can last up to two days), although not at high temperatures.

Comparing the results of studies on the HAA content in smoked sausages with those of [2], which investigated smoked cheese, the results are comparable. The HAA content, both in the current work and in papers [2], is low when compared to work [7], which also examined smoked products.

The results of the study of boiled-smoked sausages showed that, of the three selected meat products, this species produced the greatest amount of HAA. We cannot discern any average range in which most of the samples tested fell. It is important to note that 8 of 10 samples did not contain PhIP, and in the remaining 2 samples, the amount was low relative to the MeIQx. We suppose that the highest quantity of HAA in samples of boiled and smoked sausages among all investigated types of meat products is because products in this case undergo two types of heat treatment - boiling and smoking. And smoking is hot, unlike smoked sausages, i.e. the temperature is higher than smoked sausages, but the duration is incomparably shorter.

4.2 D-enantiomers of amino acids in sausage products

We observe D-enantiomers according to the results of monitoring studies of boiled sausage products. D-phenylalanine and D-alanine in the samples are, on average, at 3.20 mg/100g, which is 2 times higher than D-leucine and D-isoleucine. D-methionine in the samples is at or below 1 mg/100g. This enantiomer in samples 1, 4, 7, 9 and 10 is less than 1 mg/100g. The number of enantiomers in boiled sausage products is 2 times lower than in smoked products and 5 times lower than in boiled-smoked products.

The low cooking temperature and the lack of enzyme preparations or micro-organisms in the production process likely caused the low content of D-enantiomers in boiled sausages. Enantiomers were likely in the raw material from the beginning. Dried milk may have come from pasteurized milk. Meat raw materials have D enantiomers in their peptide chain structure because of nutrition, as the diet of pigs and cows includes feed and plants, which have high enantiomeric values in their structure [19-21].

The results of these studies have shown that boiled-smoked sausages have an average D-alanine content 5 times higher than boiled sausages. This difference in the results could be because of the additional heat treatment by smoking in the manufacture of boiled-smoked sausages.

By comparing the results of smoked sausage products without and with the addition of starter cultures for rapid maturation, we see a clear increase of isomers such as D-alanine by a factor of 10-13 and D-methionine by a factor of 5. The enantiomers D-phenylalanine and D-leucine increased by an average of 2.6 times, but the content of D-threonine was found to be about the same in samples with and without starter cultures. The results once again show the powerful influence of microorganisms on the formation of D-enantiomers of amino acids in food.

5 Conclusion

These studies have shown that the formation of HAA and D-enantiomers occurs in sausages (from grocery stores in Moscow) during the technological process of their production. The HAA content could theoretically be much higher than the presented results show. HAA are probable carcinogens and products containing them can have carcinogenic effects on the human body. Unfortunately, it is not possible to eliminate HAAs, as they are the reaction products of a large variety of ingredients in any food product (e.g. even in cheese subjected to electron-beam processing). Many studies on the reduction of HAA production suggest that it is possible to reduce the amount of HAA by using a recipe component with strong inhibitory properties. Such a peculiarity of the sausage production process as the most careful homogenization of the raw materials will make it much easier to reduce the number of HAAs if the scientific community ever faced such a task. The only question remains the right formulation ingredient, which will achieve both the maximum reduction of HAAs and have little effect on the organoleptic properties of the finished product.

The number of D-enantiomers, unlike HAAs, is almost impossible to reduce by introducing additional components during the food manufacturing process. Any ingredient used in the manufacturing process can, on the contrary, only lead to an increase. Microbiological influences and temperature regimes influence the increase of D-enantiomers. And while it is possible to control temperature regimes in the production process of some products, it is not possible to eliminate the use of micro-organisms in the production of some products.

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