Methodology for measuring the mass fraction of monosodium glutamate in meat matrices

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Abstract. Over the past century, human lifestyles and eating habits have changed dramatically as people in developed countries resort to fast food, they are indiscriminate and get used to frequent snacking. Production of dietary dishes and increase in the range of food products lead to the fact that the manufacturer has to use a large number of functional ingredients, such as those that improve the flavor. One widely used additive is monosodium glutamate. Monosodium L-glutamate (E621) is the sodium salt of glutamic acid present in all protein products which is used worldwide as a food flavor enhancer. The legislation of the Russian Federation sets the level of introduction of monosodium glutamate, or additive E621, into a food product. In connection with the above, there was a need to develop a method for quantitative determination of the mass fraction of introduced monosodium glutamate in the production of food products. A new method for identification of added monosodium glutamate in food products is proposed within the framework of the work under consideration. The authors have developed a technique for the determination of the mass fraction of sodium glutamate in food products by high-performance liquid chromatography (HPLC) with precolumn derivatization. The metrological evaluation of the developed methodology is presented, accuracy and reproducibility indices in two concentration ranges are established.

1 introduction

The consumption of food is an important process for the vital activity of a living organism. However, a person wants to eat not only correctly, but also quickly and deliciously. The rhythm of life of modern man is very fast, and snacking is an integral part of everyday life. Food additives used in the food industry serve to ensure safety and improve the quality of products. They perform certain functions, imparting the raw materials and the finished product the desired properties. In many cases, they are necessary for the manufacture and preservation of the product. With the increasing need of food industry enterprises to improve the economic performance of products, there have been significant changes in the composition and quantity of recipe ingredients used in the direction of increasing additionally introduced food additives stabilizing effect. Unfortunately, in the modern biomedical field, not all ingredients and used food additives have been thoroughly studied: there is no hundred percent certainty in their safety when constantly used in food and in combinations of different components. Scientists do not currently have complete information about their possible effects on the body and on the next generations. At present, people suffer from mutagenic and carcinogenic effects of many genotoxic agents in everyday life and in the workplace due to changing lifestyles. These changes include the rapid increase in the use of chemicals, such as drugs, food additives, pesticides and nanomaterials. Therefore, elucidating the adverse effects of these chemicals on the human genome has become of great importance [1].

Of course, not all food additives are a priori harmful. However, in large quantities or when consumed in combination from different products, one of the ingredients may have a negative effect on the body. Studying the effects of food additives is complicated by the lack of data on the actual content of the claimed ingredient in a particular product. And one of the main problems remains the lack of required methodologies for identifying the composition of food products. It is worth noting that the methodology should be easily and highly reproducible for further application in various fields and food laboratories.

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Returning to the topic of this paper, monosodium glutamate (E621), or the sodium salt of L-glutamic acid in proteins, is a widely used flavor enhancer worldwide. It is used to enhance the natural flavor of most food products [2,3]. Glutamate, a typical umami ligand, is often added to Asian cuisine to enhance the flavor of food [4]. Umami is abundant in various foods including vegetables, seafood, meat and cheese and it contributes significantly to their characteristic flavor [1,5,6]. It is what explains the distinct flavor and aroma of a food product. During storage, or technological processing, the amount of glutamic acid and glutamates decreases, which leads to a decrease in flavor and aroma in food products. Hence, the addition of glutamic acid and its salts allows enterprises to restore the quality of the product and compensate for the decrease in organoleptic characteristics during any technological processing.

However, there are norms of introduction of the food additive E621, as its excess will have a negative effect on the organoleptic properties of the food product. For example, if glutamates are added in excess of 0.20%, the taste and aroma of the product will be excessively distorted, i.e. it may become over-salted, rancid or oxidized.

Glutamate is the most abundant excitatory neurotransmitter [7] in the central nervous system (CNS). It plays a key role in long-term potentialization and is also involved in the metabolism and regulation of basic physicochemical and biochemical processes of the nervous system [1,8,9].

Glutamate is biosynthesized in mitochondria from the tricarboxylic acid (TCA) cycle intermediate α-ketoglutarate by transaminase [10]. It does not cross the blood-brain barrier (BBB) easily but is transported by a high-affinity transport system. It can also be converted to glutamine, which is able to cross the GEB and then can be converted to glutamate by the action of phosphate-activated glutaminase [11,12].

Monosodium glutamate was first isolated by extraction and crystallization in 1908 by Japanese biochemist Kikunae Ikeda during research on kombu (edible seaweed often used in Japanese cuisine to make broths). After that, the scientist investigated various salts of glutamic acid to reproduce its flavor, and monosodium glutamate showed the best result: it can be dissolved well in water and the flavor was the most vivid [13].

Since its discovery, monosodium glutamate has been industrially produced by three methods:

1. hydrolysis of plant proteins with peptide bond breaking using hydrochloric acid. This method was used in 1909-1962 [14,15,16];

2. direct chemical synthesis from acrylonitrile: the method was used in 1962-1973, but due to the difficulty of separating glutamic acid isomers, it is no longer relevant [14,15,16];

3. bacterial fermentation is the method still in use today. During fermentation, bacteria of Corynebacterium species are cultured with ammonia and carbohydrates from sugar beet, sugar cane; tapioca or molasses release amino acids into the culture broth from which L-glutamic acid is separated [14,15,16].

Since the early twentieth century, scientists have studied the metabolic functions and health effects of glutamate in animals [16]. The use of monosodium glutamate was thought to cause several health effects, including headache and nausea, in addition to a risk factor for obesity [17].

A scientist from Hong Kong, Kwoka, described a possible link between monosodium glutamate and the "Chinese restaurant" syndrome. His research describes the effects of this salt on the development of diseases such as asthma, diabetes, obesity and allergic rhinitis, as well as on high blood pressure [18,19].

Due to changes in human taste preferences, the incidence of non-communicable diseases (NCDs), such as hypertension, has increased. Data from the 2002 China National Nutrition Survey show that one in six people suffer from hypertension. Only 19% of people with hypertension can control their blood pressure. However, even at this time, work is still underway to investigate the association between monosodium glutamate consumption and increased blood pressure [17].

Turkish scientists from Erciyes University conducted a study of the influence of a food supplement on anxiety, panic, and memory levels. The experiment was conducted on rats. Monosodium glutamate was introduced into the body through drinking water. The results presented by the scientists proved the effect of glutamate on the disruption of the nervous system. Negative effects on the body were observed already after 12 weeks [5].

However, a review of clinical trials examining the causal relationship between monosodium glutamate intake and adverse health outcomes has not found support due to a lack of adequate blinded experiments and consistent results [1].

Monosodium glutamate (E621) is a food additive authorized for use by international, European intergovernmental, national legislative and regulatory documents in 12 countries [20].

Despite all the above-mentioned negative effects of glutamate on the living organism, this supplement has positive properties. Scientists have proven that monosodium glutamate enhances the action of cations, using iron as an example. The combined presence of E621 and the metal cation significantly increased the hemoglobin level in the blood [21].

When added to food, monosodium glutamate dissociates in the neutral region and is free glutamate [3,4]. Glutamate in a free form alone activates umami taste receptors, such as T1R1 and T1R3, and this function is thought to mediate responses to protein-rich foods [5]. According to the EFSA Food Safety Authority, the average intake of glutamatamic acid (both free and protein-bound) based on protein intake was 18 g/day and that of monosodium glutamate was 0.55 g/day [4]. This intake level is based on the highest dose at which scientists have not observed adverse effects on experimental animals in toxicity studies.

Nowadays, when choosing food products, rarely anyone pays attention to their composition. Therefore, it

is necessary to control the content of glutamate to ensure quality control of products.

Several techniques have been developed for the determination of glutamic acid and its salts, but only one has been standardized.

Such techniques include a capillary electrophoresis, potentiometric method, HPLC [20], an HPLC method with post-column derivatization.

In the HPLC method, linearity is observed in the concentration range of 0.4-1.0 μg/mL. A diode matrix detector was used to register the analyte [22, 24].

Wollenberger's work in 1989 proposed a biosensor method. The biosensor response depends linearly on the concentration of L-glutamate in the range of 0.001-1.0 mmol/L. The measurement time is 2 min. [23].

The electrophoresis method has been validated by LUMEX. The range of measured masses by capillary electrophoresis and diode-induced fluorescence detection is 1.0-100 g/kg for foodstuffs and food raw materials, and 2.5-100% for food additives.

Many of the methods described above are either not standardized, require special equipment and reagents, or are not sufficiently reproducible and sensitive.

In view of the above, the aim of the present work was to develop a technique for the determination of the mass fraction of monosodium glutamate using an HPLC system with pre-column derivatization.

2 Methodology and materials

The method is based on extraction of the introduced monosodium glutamate from the samples and further analysis by HPLC with pre-column derivatization.

2.1 Reagents

The following reagents were used: acetonitrile for HPLC (Panreac, France), hexane (Panreac, France), ethyl acetate (Sigma Aldrich, USA), formic acid (Merck, USA), hydrochloric acid, x. h. trichloroacetic acid ≥ 99.0%, 3-mercaptopropionic acid ≥ 99.0% (Sigma), sodium hydroxide ≥ 99.0. There was also sodium hydrophosphate ≥ 99.0, sodium tetraborate b/w ≥ 99.0, sodium tetraborate decahydrate ≥ 99.5%. Deionized water was obtained on a Milli Q Direct 8 system (France). Acrylamide with the content of the main substance not less than 99.0% produced by Sigma-Aldrich (USA) was used as a standard sample.

The following biological matrices (b/m) were chosen as the objects of study:

1 b/m - sausage according to GOST 23670-2019;

2 b/m - pate.

The convergence of the method was evaluated by samples prepared in the laboratory with the addition of monosodium glutamate. To assess the convergence prepared laboratory samples with the introduction of monosodium glutamate in the formulation of cooked sausage, they were produced according to GOST 23670-2019 in real production conditions in the amount of 9 samples. The reproducibility of liver pate was assessed according to GOST R 55336-2012.

For chromatographic analysis, the samples were ground using a homogenizer beforehand. The homogenized sample weighing 5 g was placed in a centrifuge tube. 4 cm^3 of 20% trichloroacetic acid (TCA) was added to the weighed sample and brought to 30 cm^3 with saline buffer having pH of 2.2. 5 cm^3 of hexane was added to the resulting solution. The mixture was mixed thoroughly and incubated for 1 h at 18 ºC up to 25 ºC. The extract was then centrifuged for 5 min at 2000 g and the aqueous layer was filtered through a membrane filter with a pore diameter of 0.45 μm. The filtrate was then transferred to a vial.

Derivatization was carried out in automatic mode using a sample introduction device. 10 mm^3 of the orthophthalic aldehyde solution and 2 mm^3 of the sample solution were introduced into the chromatograph. The volume of the injected sample was 12 mm^3.

The measurement results were determined at a wavelength of 338 nm including the following parameters:

- column temperature of 40 °C;
- mobile phase A: acetonitrile:carbinol:water (45:45:10);
- mobile phase B: 10 mM $Na_2 HPO_4$, 10 mM Na B O_{247}, pH 8.2;
- flow rate of 1 ml/min;
- for the gradient elution mode, see Table 1.

Table 1. Parameters of gradient elution mode.

Time, min	Volume fraction of eluent A, %	Volume fraction of eluent B, %
0	2	98
0,5	2	98
20	57	43
20.1	100	0
23.5	100	0
23.6	2	98
25	2	98

3 Results and discussion

In order to control the information on the product label and compliance with monosodium glutamate standards, regulatory authorities and accredited laboratories need a highly reliable method that is metrologically validated and easily reproducible.

There are several methods for determining both protein bound and unbound amino acids, but none of the techniques involve detection of the added salt of glutamic acid. In addition, background thresholds for glutamic acid or monosodium glutamate for different

biological matrices are not prescribed, and there is no cut-off threshold for natural concentrations.

One of the popular techniques in analytical laboratories recently is the methodology based on HPLC, and therefore it was chosen for the study. The most difficult process was that of selection of chromatographic separation conditions and choice of detection methods. The methodology for the determination of L-(+)-glutamic acid according to GOST 34448-2018, proposed by the authors and previously standardized, was taken as a basis.

The method is based on extraction of free sodium glutamate from the sample followed by pre-column derivatization and analysis by high-performance liquid chromatography (HPLC) with a spectrophotometric or diode matrix detector.

Quantification is carried out by the peak area of monosodium glutamate relative to the calibration relationship obtained using calibration solutions of the pure substance under similar conditions.

The development of the methodology took place in several stages:

- selecting conditions for the separation and detection of monosodium glutamate in a standard mixture;
- constructing a calibration curve;
- checking the stability of the calibration characteristic;
- setting LOQ and LOD limits of the method;
- controlling convergence and reproducibility of the method;
- establishing the limits of relative error of the method.

Having selected the detection conditions, the calibration relationships were established and the detection and detection limits of the method were determined.

For the determination of monosodium glutamate, calibration solutions of the mass concentration of 50 μg/cm^3, 100 μg/cm^3, 200 μg/cm^3, 400 μg/cm^3 were prepared.

Glutamic acid can also be used to construct a calibration curve, in which case a conversion factor of 1.15 is applied.

The linear correlation coefficient of the obtained calibration dependence should be at least 0.99. If this condition is not fulfilled, it is necessary to find out and eliminate the reasons leading to unsatisfactory results. If necessary, new calibration solutions should be prepared.

To calculate the lower limits of detection for quantitative (Limit of Detection - LOD) and qualitative (Limit of Quantification - LOQ) determination of the method, 16 unadded muscle tissue samples were collected in which the analyte under study was added into concentrations of 0.01; 0.05; 0.1 and 0.15%. The LOQ was 0.01% and LOD was 0.1%, respectively.

During metrological certification of this method, the values of repeatability and reproducibility limits at confidence probability P = 0.95 were calculated (Table 2) for two concentration ranges.

Table 2. Values of the limits of repeatability, reproducibility and a critical range at a confidence probability P = 0.95.

Measuring range of the mass fraction of monosodium glutamate, %	**Repeatability limit (allowable relative differences between two results of parallel determinations), r, %**	**Accuracy index (limits of relative error at a confidence probability P = 0.95), ± δ**
0.1 to 1.0 on.	17	30
1.0 to 10.0 incl.	6	10

The convergence and reproducibility conditions of the method were then checked.

For this purpose, convergence and reproducibility were carried out on a special experiment with the method of additions of standard substances of monosodium glutamate in different concentration ranges. Of interest was the evaluation both at the detection limit of the method and at a sufficiently weighted addition of the monosodium glutamate additive under consideration. The results are presented in Table 3.

Table 3. Results in a blank matrix when administering monosodium glutamate at two concentration levels covering the lower and upper ranges of the methodology

Sample no.	Convergence		Reproducibility	
	Concentration of added monosodium glutamate, %			
	Dobob 0.14.	Dobob 10:00.	Dobob 0.14.	Dobob 10:00.
x1	0.15	10.00	0.15	10.00
x2	0.15	10.01	0.16	9.89
x3	0.14	10.05	0.17	9.91
x4	0.14	10.01	0.17	9.95
x5	0.13	10.03	0.14	10.01
x6	0.14	10.00	0.15	9.87
x7	0.14	10.00	0.16	9.92
x8	0.15	10.05	0.17	9.97
x9	0.15	10.04	0.18	9.90
x10	0.15	10.02	0.18	9.90
x11	0.14	10.03	0.16	9.98
x12	0.14	10.01	0.18	9.99
x13	0.15	10.02	0.15	10.02
x14	0.14	10.00	0.16	9.98
x15	0.15	10.00	0.18	9.94
x16	0.14	10.03	0.15	10.01
x cf	**0.14**	**10.02**	**0.16**	**9.95**

To calculate the conditions of convergence and reproducibility of the method, it is recommended to use at least 16 single measurement results in accordance with GOST R ISO 21748-2021. Good convergence and reproducibility of the developed method were obtained when analyzing working samples. Then, in order to evaluate the suitability of the method in routine laboratory conditions, the verification of the developed methodology was carried out on real matrices of meat products.

For this purpose, we have developed products, according to the recipe sausages by GOST 23670-2019 and pate by GOST R 55336-2012, with the introduction at the stage of production of monosodium glutamate in the amount of 0.3 to 1.2 g/100g. Taking into account the factors of loss during technological operations, an additional 30% of monosodium glutamate was introduced. Therefore, the calculated values should be 0.39% for cooked sausage samples and 1.56% for pates. Samples 1 b/m and 2 b/m, reflecting native concentrations of monosodium glutamate in the sample, served as controls. The purpose of the canning study was to evaluate the native sodium glutamate content to provide information on the effect of high temperatures (on the order of 120 °C) on the breakage of peptide bonds in meat protein. The native content of the sodium salt of glutamic acid in both samples was determined to be 0.03-0.07%. Figure 1 below shows the evaluation of convergence of the developed methodology by the example of the experiment of sodium glutamate determination when added to the formulation of cooked sausages.

Control Glutamate concentration Na, %

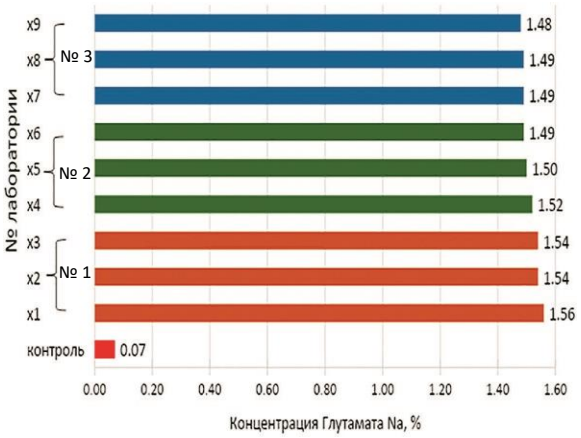
Fig. 1. Method convergence evaluation diagram (N = 10)

The number of single parallel determinations for each working sample was n = 3.

Hence, in the conditions of routine laboratory, during verification of the developed method of determination of monosodium glutamate in samples with a pre-added concentration of the desired analyte, equal to C = 0.39%, the dispersion of variation of 0.03-0.04% of the mean value was established. It is included in the conditions of the method error of 0.35 ± 0.11%.

A similar experiment was also conducted for another range of concentrations. The experiment involved 3 accredited laboratories, on the basis of which the adequacy of the developed methodology was initially assessed, and subsequently they also took part in the interlaboratory experiment.

Lab #1 - experiment x1, x2, x3;
Lab #2 - experiment x3, x4, x5;
Lab #3 - experiment x6, x7, x8.
Figure 2 shows the data from the experiment.



No. of a laboratory Glutamate concentration Na, %

Fig. 2: Method reproducibility diagram by the example of liver pate.

In the study of pâté samples with the addition of monosodium glutamate, a dispersion of 0.03-0.05% of the mean value was obtained, which is included in the conditions of the method error of 1.51±0.45%. The concentration of the desired analyte was 1.56%.

The developed and standardized method described above was used to monitor the quality of sausages (produced according to GOST, in industrial conditions No. 1-16, No. 17-27 produced according to TU) with different methods of technological processing: boiled, boiled-smoked and raw-smoked. Therefore, during the monitoring study, it was found that almost all sausages contained the normative level in TR TS 029 of monosodium glutamate in the food product of 10 g/kg, or 1%. All meat products of the monitoring study do not exceed the established value.

Pre-column derivatization has obvious advantages, namely time stability of product yield, high sensitivity, selectivity and a unique derivatizing agent compared to post-column derivatization.

At the same time, methods based on post-column derivatization show, as a rule, less variability of analysis results than methods based on pre-column derivatization do. It should also be noted that the reagent consumption in post-column derivatization will be significantly higher.

The above advantages allow us to recommend precolumn derivatization for the identification and quantification of monosodium glutamate in the food industry.

4 Conclusions

As a result of the conducted work, the technique of determination of the mass fraction of monosodium glutamate using the HPLC system with pre-column derivatization in meat products was developed. Metrological parameters, such as convergence and

reproducibility, lower limits of the qualitative and quantitative detection limit for two concentration ranges (from 0.1 to 1.0% and from 1.1 to 10.0% inclusive) were substantiated and established. The limits of relative error at a 95% confidence level were also calculated. For the first range, the limit of 30% was set, and for the second range, it was 10%.

The introduction and use of this method for the determination of monosodium glutamate in a chemical-analytical laboratory will allow the use of any HPLC system with UV or a diode matrix detector, the only modification may be a customizable autosampler, but also in a manual mode, it can be derivatized.

Also the method with pre-column derivatization has high sensitivity and selectivity.

Acknowledgments

The developed methodology was certified on the basis of FGBNU "FSC Food Systems named after V. M. Gorbatov" RAS (certificate of certification of measurement methodology No. 241.0224/RA.RU.311866/2019 from November 2019 and implemented in the laboratory.

More than 700 samples of meat products were successfully analyzed using the developed methodology. The developed methodology was also validated on other matrices containing monosodium glutamate.

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