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ASSESSMENT OF NITRATE CONTENT IN LETTUCE MARKETED IN SERBIA: INFLUENCE OF CULTIVATION METHOD, SEASON, AND PRODUCTION SYSTEM

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ABSTRACT

Intensive agricultural production and the excessive use of nitrogen fertilizers have led to increased nitrate accumulation in vegetables, particularly leafy greens, which may exert negative effects on human health. The objective of this study was to assess the nitrate content in lettuce marketed in Serbia, considering the influence of cultivation method, season, and production system. A total of 20 lettuce samples were collected from various sources (including supermarkets, independent shops, and farmers' markets), cultivated either in greenhouses or open fields during winter and spring. Nitrate content was determined using the steam distillation method after a 30-minute extraction in hot 1 M NaCl solution. The results were statistically analyzed using the one-way ANOVA, followed by the Tukey HSD test.The results showed that none of the samples exceeded the maximum permissible nitrate concentrations according to the current regulations. The nitrate content ranged from 28 to 663 mg/kg. No statistically significant differences were found between the greenhouse and open-field lettuce or between the winter and spring samples. However, the organically grown lettuce exhibited significantly lower nitrate levels compared to the conventionally produced lettuce.It was concluded that the lettuce marketed in Serbia complies with the food safety standards regarding nitrate content. Nevertheless, variations in nitrate concentration indicate the need for continuous monitoring.

Key words:
food safety, organic and conventional production

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INTRODUCTION

The rapid growth of the global population has necessitated more intensive food production. To achieve high yields that meet increasing food demands, the use of various plant nutrition and protection products has expanded. However, this has often resulted in the irrational application of plant nutrients, particularly nitrogen fertilizers, with the primary focus placed on yield quantity rather than food quality (Miladinov, 2023).

In recent years, greater attention has been directed toward food quality, as consumers have become more discerning regarding the composition, production methods, and potential health effects of the food they consume. One of the major food safety concerns is the presence of nitrates, which enter the human body primarily through the consumption of vegetables and drinking water with elevated nitrate content. Excessive nitrate intake may have adverse effects on human health (Sekulić et al., 2005).

Nitrogen, as a fundamental element in the structure and function of all living organisms, is an essential component of amino acids, proteins, enzymes, and vitamins. It is widely distributed in nature predominantly enters food and drinking water through the soil (Kresović and Blagojević, 2005). The main sources of nitrogen in the soil include atmospheric nitrogen fixation (electrochemical and biological) and the application of organic and mineral fertilizers. The nitrification process, in which ammonia is oxidized to nitrite and subsequently to nitrate by specific soil microorganisms, plays a crucial role in determining nitrogen availability to plants (Jakovljević and Kresović, 2005). Under unfavorable conditions for microbial activity, this process is significantly slowed or even halted. Nitrification proceeds particularly slowly in early spring, when low temperatures and soil water saturation caused by frequent rainfall reduce microbiological activity. Consequently, the amount of nitrate increases during spring and reaches its maximum in the summer months (Džamić and Stevanović, 2007).

Several factors influence nitrate accumulation in plant tissues, including soil nitrate concentration, the form and method of nitrogen application, and environmental conditions such as light intensity, water availability, and temperature. Leafy vegetables, particularly lettuce, exhibit a high affinity for nitrate uptake and accumulation (Sekulić et al., 2005). Previous studies have estimated that 60–70% of dietary nitrates originate from vegetables, especially leafy greens (Antić-Mladenović, 2010).

Once ingested, nitrates can be reduced to nitrites, which pose greater health risks due to their ability to react with hemoglobin, thus forming methemoglobin that impairs oxygen transport. Elevated methemoglobin levels can cause methemoglobinemia, commonly known as "blue baby syndrome", which may be fatal in infants (Antić-Mladenović, 2010). Furthermore, nitrates can interact with secondary amines and amides in an acidic environment, forming nitrosamines and nitrosoamides, many of which possess mutagenic and carcinogenic properties (Jakovljević and Kresović, 2005).

Given these health concerns, it is essential to implement agricultural practices that regulate and minimize nitrate accumulation in vegetables. Fertilization strategies should, particularly in the production of leafy greens, prioritize ammonium- and amide-based nitrogen sources, which may be used in combination with nitrification inhibitors. Additionally, nitrate levels in vegetables should be monitored to ensure compliance with food safety standards (Antić-Mladenović and Kresović, 2018).

In Serbia, the maximum permissible nitrate concentrations in lettuce are legally regulated (Rulebook on maximum concentrations of certain contaminants in food, Official Gazette of RS, No. 73/2024), varying according to production conditions and harvest season (Table 1). The limits are more stringent for lettuce cultivated in open fields and harvested during warmer months due to the influence of environmental factors on nitrate accumulation.

Table 1. Maximum permissible concentrations of nitrates in the fresh weight of lettuce (Rulebook on maximum concentrations of certain contaminants in food, Official Gazette of RS, No. 73/2024)

Lettuce (*Lactuca sativa* L.)		**Maximum concentration ($mg\ NO_3\ kg^{-1}$)**
Lettuce produced indoors	picked from October 1st to March 31st	5000
	picked from April 1st to September 30th	4000
Lettuce produced outdoors	picked from October 1st to March 31st	4000
	picked from April 1st to September 30th	3000

There is a notable lack of published data in Serbia regarding the nitrate concentration of lettuce. Therefore, the present study aimed to address this gap by examining the nitrate content in randomly selected lettuce samples from the Serbian market. The sampling strategy was designed to account for the major factors influencing nitrate accumulation in lettuce, namely cultivation method, seasonal variation, and production system (conventional vs. organic).

MATERIAL AND METHODS

The Material and Method section should provide sufficient information to enable other researchers to reproduce the experiment at a different location. It is essential to include comprehensive information about the materials used, clearly specifying their origin, physical properties, and other relevant characteristics. When instruments or devices are employed to obtain experimental results, the following details must be provided: the name of the device or instrument, model designation, manufacturer, and country of origin. If a scientifically recognized method was applied, it should be cited in the literature rather than described in full. However, if any modifications were introduced to an established method, appropriate references must be provided to support and justify the applied changes. If statistical data processing software was used, its name and version should be stated. Subsections may be included within the Materials and Methods section where appropriate.

Sample Collection

The lettuce (*Lactuca sativa* L.) samples used in this study were collected by random selection in the surroundings of Belgrade, from supermarkets, independent shops, and green city markets, representing both winter and spring growth periods. The sampling encompassed lettuce grown in different seasons (winter and spring), under different cultivation methods (greenhouse and open-field), and production systems (organic and conventional). A total of 20 lettuce samples were collected. During the winter months, all the samples originated from greenhouse production under conventional farming practices. The spring samples included lettuce from both greenhouse and open-field cultivation. While the greenhouse-grown lettuce was produced exclusively using conventional methods, the open-field samples represented both organic and conventional production systems (Table 2).

Table 2. Origin and production characteristics of the lettuce samples

Period of sampling	Production system	Cultivation methods	Number of samples
winter	conventional production	greenhouse	5
spring	conventional production	greenhouse	4
spring	organic production	open field	4
spring	conventional production	open field	7

Determination of nitrogen content in fresh lettuce samples

The nitrate content in the lettuce samples was determined within 24 to a maximum of 48 hours after purchase to ensure freshness and minimize nitrogen transformation.

Each lettuce head was processed by selecting leaves from both the outer and inner parts, which were then shredded to obtain an average sample for analysis. For the nitrate extraction, 0.5 g of the average fresh lettuce sample was mixed with 100 ml of a hot 1 M NaCl solution in extraction bottles. The bottles were placed on a rotary agitator for 30 minutes to facilitate nitrate extraction from the plant tissue. The resulting filtrate was subsequently used for nitrate determination, using the hot steam distillation method described by Bremner and Keeney (1965).

For the distillation, 10 ml of the filtrate was pipetted into a distillation flask. Magnesium oxide (MgO) and calcium chloride ($CaCl_2$) were added to the flask to prevent the decomposition of organic nitrogen compounds. This step was crucial to ensure that the results reflected only the inorganic nitrogen content in the lettuce leaves. In order to determine the nitrate content, the first distillation had to be carried out to eliminate ammonia nitrogen. To quantify ammonia nitrogen, the distillation apparatus was connected, and the distillate was collected in a boric acid solution with an indicator, up to a volume of 35 ml.

The first distillation step was essential to remove ammonia nitrogen present in the plant material. This was done to prevent interference in the determination of nitrate nitrogen, as the procedure relies on the reduction of nitrates to ammonia nitrogen After elimination of ammonia nitrogen, the nitrate nitrogen content was determined in a second distillation. In this step, Devarda's alloy was added to the distillation flask to reduce nitrate to ammonia ions. The distillation was conducted following the same procedure as for ammonia nitrogen. The distillate was then titrated with sulfuric acid of 0.05 $cmol^{++}$/L concentration until a slightly purple color appeared, indicating the endpoint of titration. One milliliter of this sulfuric acid solution is equivalent to 140 µg of nitrogen.

For accuracy, the titration value of the blank (1 M NaCl) was also determined and subtracted during calculations.

The nitrate content in the lettuce samples was calculated using the following formula:

$$NO_3\text{-}\ (mg\ kg^{-1}) = (a - b) \times 140 \times F \times \frac{4{,}43}{1000}$$

where:

a – milliliters of sulfuric acid used for sample titration

b – milliliters of sulfuric acid used for blank titration

F – factor for converting results to the mass of a 1 kg sample (in this case, 20,000)

4.43 – factor for converting nitrogen in nitrate form to nitrate

1000 – conversion factor from µg to mg

For statistical data processing (One Way ANOVA test, Tukey HSD), the Statskingdom online software package was used (https://www.statskingdom.com/).

RESULTS AND DISCUSSION

The analysis of nitrate content in the lettuce samples revealed that none of the examined samples exceeded the maximum permissible levels prescribed by the Rulebook on Maximum Concentrations of Certain Contaminants in Food (Official Gazette of RS, No. 73/2024). The highest nitrate concentration was detected in the lettuce produced in greenhouse during spring, amounting to 663 mg/kg, whereas the lowest concentration was recorded in the lettuce from organic open-field production sampled in spring (28 mg/kg) (Figure 1). A considerable variation in nitrate content across the analyzed samples was observed, indicating potential influences of production methods and environmental conditions.

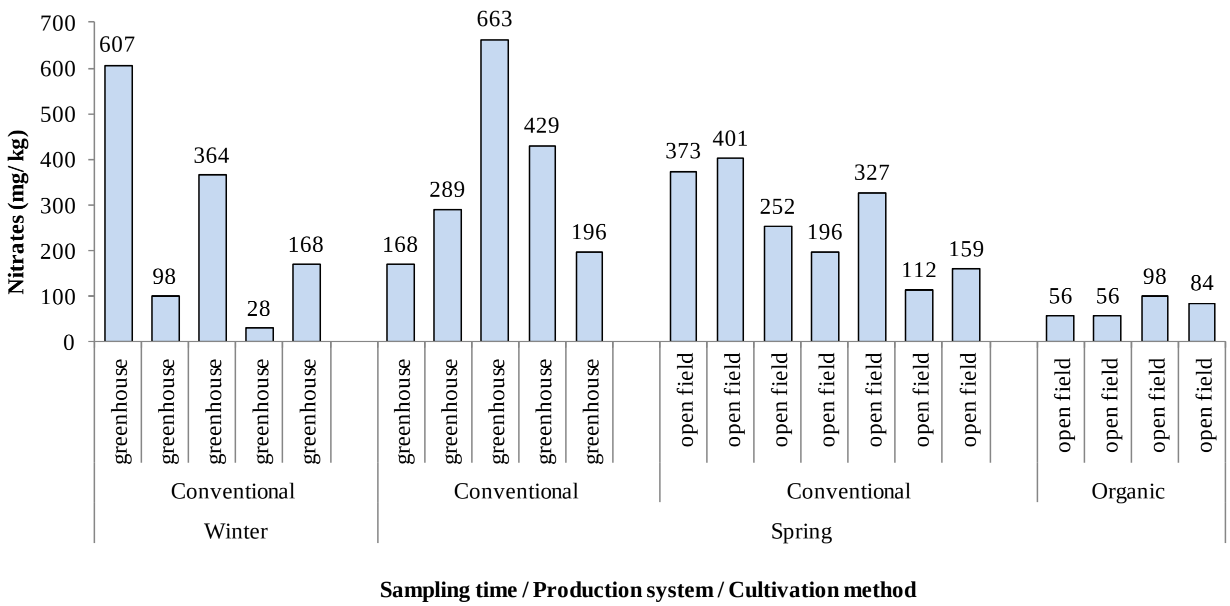

Figure 1. Nitrate concentrations in the lettuce samples

A comparison of the nitrate content in lettuce grown during the spring season under greenhouse and open-field conditions (Figure 2) demonstrated that the nitrate content in the greenhouse-grown lettuce was higher than that in the field-grown lettuce within the same period. However, the statistical analysis showed that this difference was not statistically significant ($p > 0.05$), suggesting that the production method did not have a substantial effect on nitrate accumulation in this survey.

A statistically significant difference ($p < 0.05$) was found in the nitrate levels between the organically and conventionally produced lettuce sampled during the spring cultivation period (Figure 2). The average nitrate content in the conventionally produced lettuce was significantly higher than in the organically produced lettuce, highlighting the influence of agricultural practices on nitrate accumulation. These findings align with previous studies emphasizing the effect of fertilization type/production mode on nitrate content (Manojlović at al., 2017). However, Castro et al. (2009) reported contrasting results, where lettuce cultivated with organic fertilizers exhibited higher nitrate levels compared to lettuce grown with mineral fertilizers. This discrepancy may be attributed to differences in fertilization strategies, soil composition, and climatic conditions across studies.

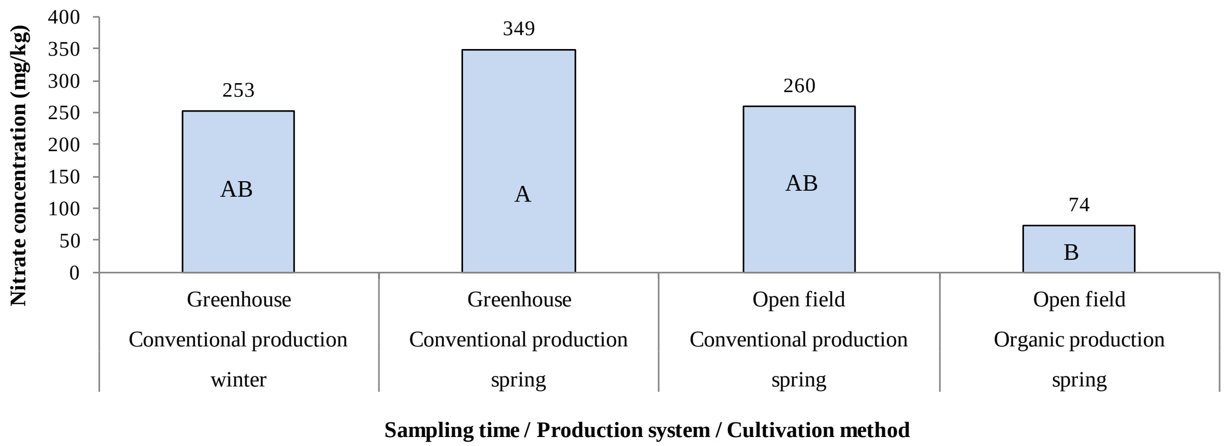

Figure 2. Average nitrate concentrations in the lettuce samples depending on the sampling time, production system and cultivation method (the means that do not share a letter are significantly different at $p < 0.05$)

Contrary to expectations, the nitrate content in the greenhouse-grown lettuce during the spring period was found to be higher than in the greenhouse-grown lettuce during the winter period (Figure 2), with an average nitrate concentration of 349.1 mg kg^{-1} in the spring. This can be explained by the more intensive growth of lettuce under optimal light and temperature conditions in spring, which enhances the nitrate uptake. However, the statistical analysis revealed no significant difference ($p > 0.05$) between these seasonal groups, suggesting that the time of year had no substantial impact on nitrate accumulation in this study. These findings differ from those reported by Zeleňáková et al. (2022), who observed the highest nitrate concentration in lettuce harvested in November (2038.99 mg/kg) and the lowest in May (774.78 mg/kg), indicating a significant seasonal effect on the nitrate level in lettuce. The disparity between these studies underscores the complexity of nitrate accumulation, which is influenced by multiple environmental and agronomic factors.

CONCLUSION

The results obtained indicate that all the lettuce samples tested comply with the quality standards defined by the Rulebook on Maximum Concentrations of Certain Contaminants in Food (Official Gazette of RS, No. 73/2024). All the nitrate values measured were below the maximum permissible limits prescribed by the regulation, confirming that the analyzed lettuce meets food safety requirements. The analysis of various factors influencing nitrate content in lettuce suggests that the cultivation technique was the primary determinant, most probably the type, dosage, and frequency of fertilizer application. In contrast, the production environment (greenhouse vs. open field) and the season (winter vs. spring) did not demonstrate a statistically significant impact on nitrate accumulation in the examined lettuce samples. These findings emphasize the role of fertilization practices in shaping nitrate levels rather than environmental production conditions.

On balance, the study indicates that the lettuce marketed in Serbia is produced in accordance with good agricultural practices regarding the nitrate content. However, given the relatively small sample size in this study and the wide variations in nitrate levels (ranging from 28 to 663 mg/kg), the regular random monitoring of lettuce nitrate content is recommended. Further research with larger sample sizes and a broader range of production conditions is necessary to validate these findings and ensure the continuous compliance with food safety standards.

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