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Detection of genetically modified organisms in bio products on the Croatian market

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Abstract

The production of bio or eco food implies production without the use of mineral fertilizers, hormones, pesticides, and GMOs. In Croatia, organic production has been growing year by year, regulated by legal provisions that set high standards for ecological production, which relies on renewable energy sources, waste recycling, limited pesticide use, and the prohibition of GMO use at any stage of production (food, animal feed, soil enhancers, plant protection). The purpose of organic production is to protect human health, nature, and the environment, as well as consumers. To maintain production consistency, legal regulations aligned with EU regulations on organic agricultural production and food production have been introduced, while the possible presence of GMOs in organic crops and food is regulated by GMO legislation. EU Regulation 1829/2003 regulates monitoring and labeling rules for food containing > 0.9% GMOs, including organic products. The aim of this paper was to determine the presence of GM soybean, corn, wheat, and rice lines in organic products commercially available on the Croatian market, and to determine their share in products containing GMOs. The study detected a low presence (0.1%) of GM soybean line MON 89788 in two organic soy beverages, while no GM corn, wheat, flax, or rice was found. The GMO content in all tested organic products remained below the regulatory labeling threshold (0.9%). These findings indicate that GM contamination in organic products on the Croatian market is minimal and primarily results from unavoidable cross-contamination during production, transport, or storage.

Key words: bioproducts, organic products, eco food, GMO detection, Real-Time PCR, CTAB method

INTRODUCTION

According to the definition of the Ministry of Agriculture and Rural Development, organic production is a comprehensive farm management and food production system that combines the best environmental and climate practices, a high level of biodiversity, and the preservation of natural resources. Due to the increasing spread and cultivation of GMOs for commercial purposes in many countries, the risk of contamination of organic crops is also increasing, thus threatening the fundamental principles of organic farming. As a result of global trade exchange, the EU market offers food and products from various origins. Legally binding provisions regarding the presence of GMOs in food and animal feed also include organic products. In the European Union, the use of genetically modified organisms (GMOs) is regulated by Directive 2001/18/EC on the deliberate release of GMOs into the environment, Regulation 1830/2003 on the traceability and labeling of GMOs, and Regulation 1829/2003, which governs the authorization, supervision, and labeling of genetically modified food and feed containing more than 0.9% GMOs. Since the appearance of the first GM plant, GM crops have become an integral part of agricultural production in many countries. More GM plant varieties are commercially available, especially soy, which is the most widespread in the total production of

transgenic crops (Mannion and Morse, 2013). GM plant species are resistant to herbicides, insects, and some modified plants have altered nutritional compositions, such as increased vitamin content or altered fatty acid composition.

The use of GMOs in organic farming is completely banned, and this area is regulated by the GMO Law (Law on Genetically Modified Organisms, NN 126/19). With the establishment of the legal framework for the cultivation or commercialization of GMOs in the EU, the issue of GMOs has been regulated. Therefore, following the legal provisions, each GMO must undergo a scientific risk assessment by competent authorities before being released into the environment or placed on the market, to exclude any danger to human or animal health and the environment. In Croatia, organic food is produced on an increasing amount of agricultural land, and Croatian soybean seed producers have shown interest in producing non-genetically modified seeds. Seed and reproductive material produced in Croatia must not contain GMOs (zero tolerance). The Regulation on Direct Payments to Agriculture and Rural Development Measures stipulates that soy acceptable for organic farming must be produced from certified non-GMO soy seeds. Due to these provisions, the Ministry of Agriculture has provided funds for laboratory analysis of soybean seed samples to determine the presence of GMOs for domestic producers. At the same time, official inspections by the State Inspectorate are systematically conducted to monitor and control GMOs in seeds and food on the Croatian market.

The most commonly used method for detecting genetic elements characteristic of GMOs is Polymerase Chain Reaction (PCR) (Fraiture, 2015). Currently, Real-Time PCR is considered the most effective and widespread method for detecting and quantifying specific transgenic DNA sequences in both raw material and food (Querci et al., 2010, Mano et al., 2018). Real-Time PCR has proven to be the most accurate method for quantifying transgenic content in food (Bergerová et al., 2011). The most commonly used primers for detecting GMOs are the 35S promoter and the NOS terminator, which amplify parts of the genetic construct introduced in over 60% of GM plants (Amack and Antunes, 2020). The 35S promoter is present in many GM plant species, such as RoundupReady or RRS soybeans, corn MON810, NK603, Bt11 and Bt176, wheat MON71800, and in GM rice lines. Since a large number of modifications have been developed, detection of regulatory elements of promoters and terminators is not sufficient, and more specific reactions must be performed using primers like Cry1Ab/Ac CTP2-CP4-EPSPS, tE-9, and PAT to detect characteristic parts of the genetic construct. Plants possessing CTP2-CP4-EPSPS are glyphosate-resistant, while plants with one of the Cry genes (Cry1Ab, Cry2A...) produce specific Cry proteins toxic to insects (Aljabryn, 2022). The issue of GMOs is regulated by several legal acts, which Croatia fully implemented into its legislation since joining the EU in 2013. Additionally, since 2015, planting genetically modified seeds has been banned in Croatia, in accordance with the national Act on Genetically Modified Organisms (OG 126/2019), and GM food approved in the EU is also approved in all EU member states, including Croatia, but is systematically controlled to implement labeling regulations. However, the increased use of GMOs in conventional agriculture in some countries poses a threat to the development of organic agriculture and potential contamination of organic products in the field, during transport, storage, or food production. According to the currently valid EU regulations, a limit of 0.9% GMO content is allowed in food, which also applies to organic products.

The aim of this paper was to use analytical methods to determine the presence of GM soybean, corn, wheat, and rice lines in organic products commercially available on the Croatian market, and to determine their share in products containing GMOs.

MATERIAL AND METHODS

Testing for the presence of GMOs was carried out on a total of 52 labeled organic food samples collected in 2022 and 2023. The primary raw materials analyzed in the samples were corn, soy, rice, flax, and wheat. Organic samples of soybeans, soy drink, soy tofu, soy flakes and husks, wheat flour and pasta, food rice, flax seeds, corn flour and semolina, and corn flakes were analyzed. Samples were collected from stores and supermarkets in Croatia. Some products were originally sourced from other EU countries but also from non-EU countries.

Table 1. Analysis of bio product samples

Sample Type	Number of Analyzed Samples	Qualitative Analysis	< 0.9%	> 0.9%
Soy: tofu, flakes, drink, husks	29	2	2	0
Corn: flour, grits	6	0	0	0
Wheat: flour, pasta	12	0	0	0
Food rice and flax seeds	5	0	0	0

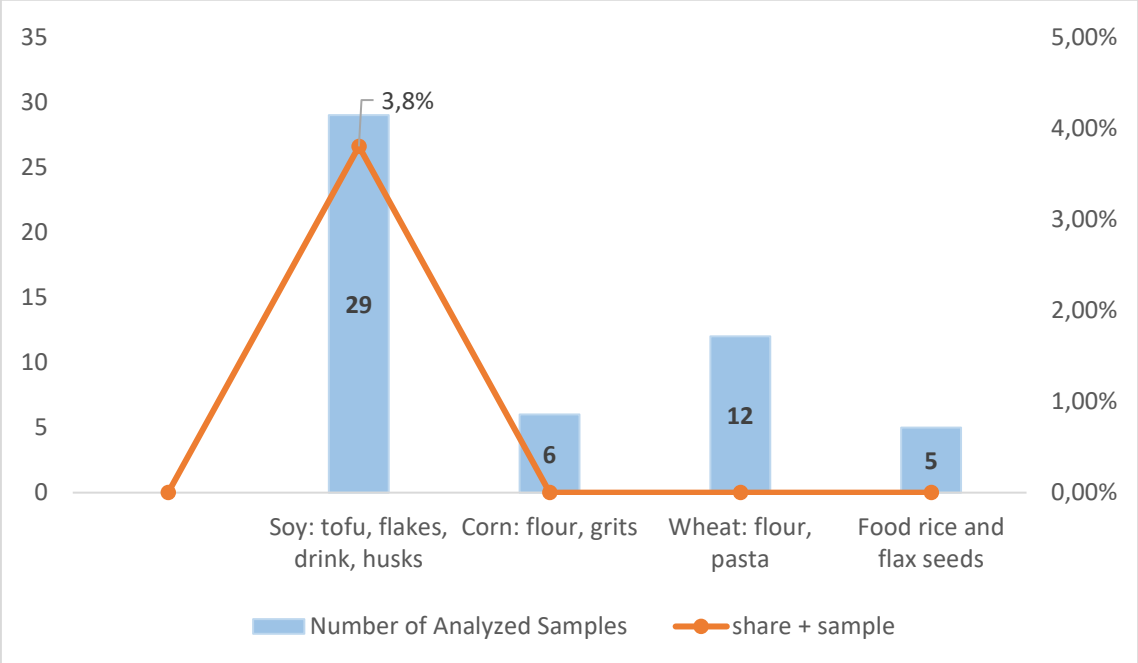
For DNA isolation, the CTAB method was used according to the protocol by Doyle and Doyle (1990). High-quality DNA was extracted from samples using the CTAB method, and its quality and concentration were verified using a spectrophotometer (BioPhotometer, Eppendorf). The results ranged from 1.6 to 2.0, and the DNA was normalized to 50 ng and could be used for Real-Time PCR analysis. In the first step of the analysis, a qualitative analysis was performed using Real-Time PCR (screening), detecting the most common elements characteristic for modifications. All reactions were carried out using the Applied Biosystems 7300 Real-Time PCR device. The analysis was performed using the most commonly used primers P35s and T-nos to amplify regulatory elements of promoters and terminators, as well as Cry1Ab/Ac, CTP2-CP4-EPSPS, T-E9, and PAT primers to amplify parts and binding sites of the introduced gene in plant DNA. Plant DNA was confirmed using primers for soybean and corn (Mazzara et al., 2007), wheat (Grohmann and Maede, 2013), and rice (Grohmann et al., 2009). Using the so-called GMO matrix, specific GM soybean lines were identified in the samples (Bonfini, 2021). The GMO matrix is a computational tool containing information from the GMOMETHODS database (Dongi et al., 2008, Bonfini, 2012) and the Central Information System of Basic DNA Sequences (Fraiture et al., 2015), offering all reference methods for GMO detection analysis. Standard GM soybean, corn, rice, and wheat samples (IRMM, Geel, Belgium) with different GMO content were used as positive controls. The quantification limit was 0.1%. Each reaction included positive controls and a blank reaction solution, with a final reaction volume of 50 µL (40 µL analysis solution mixture, Mastermix + 10 µL 50 ng DNA sample). The reaction was carried out according to the following program: 50°C for 2 min, denaturation at 95°C for 10 min, followed by amplification for 45 cycles at 95°C for 15 seconds, 60°C for 1 minute. Quantitative PCR involves the amplification of a specific target gene and a known quantity of a standard in the same reaction. Primers were used to identify GM soybean lines based on positive screening results and confirmation of the presence of CTP2-CP4-EPSPS, T-E9, which are characteristic of 5 GM soybean lines.

RESULTS AND DISCUSSION

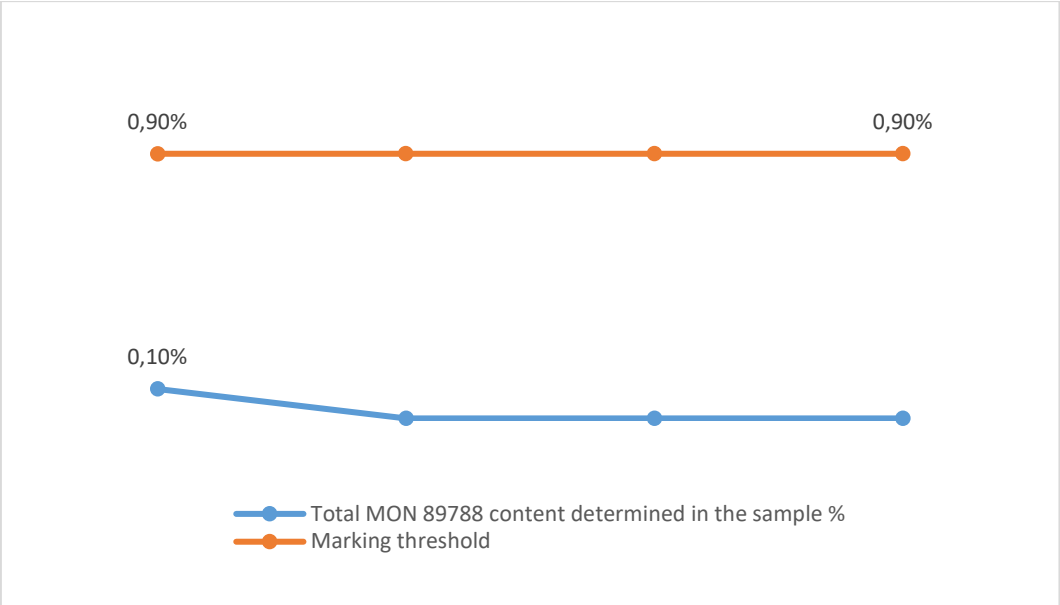
Detection of GMOs was conducted on a total of 52 labeled organic or bio products collected from the Croatian market during official GMO monitoring controls of food from various plant origins (soybeans, corn, rice, wheat). A qualitative analysis of two soy-based beverages confirmed the presence of CTP2-CP4-EPSPS and t-E9 sequences, which are characteristic of several GM soybean lines. In the samples where the presence of CP4-EPSPS and t-E9 sequences was detected, the presence of GM soybean lines MON 89788 was confirmed, and quantification determined the percentage in the tested samples. In positive samples, the identification analysis determined the type of the present GMO using primers for amplifying the entire inserted genetic construct or event-specific sequences. The amplification of specific primers in the samples showed the presence of GM soybean line MON 89788 with a concentration of 0.1% in both samples. None of the analyzed samples contained GM corn, rice, or wheat. The genetically modified soybean detected is approved for use in food production on the EU market. The CTAB method proved to be an effective DNA isolation method and is reliable for isolating quality DNA in processed products (Guertler et al., 2013). For successful PCR analysis, the isolated DNA needs to meet satisfactory purity and concentration levels (Pacheco et al., 2017). The absorbance measurements (A:260/OA:280) of DNA purity ranged from 1.6 to 2.0, indicating that the DNA was of acceptable quality for further analysis, free of inhibitors that could affect the PCR

reaction. Qualitative analysis implies "screening" or screening tests for the most common regulatory elements present in over 90% of GM plants. Since these elements do not cover all soybean modifications, screening tests needed to include more specific reactions for detecting parts of the genetic construct ("construct-specific") or the entire inserted gene ("event-specific"). The genetically modified soybean line MON 89788 found in the products is approved for placement on the EU market (Davison and Ammann, 2017). Generally, GM food, once placed on the EU market, is subject to labeling obligations, as well as to subsequent controls or monitoring. In Croatia, regulations govern the placement of GM food on the market, labeling of GM food, traceability, and control of GM food. The GM soybean line MON 89788 is resistant to glyphosate and contains the CP4-EPSPS gene from the bacterium *Agrobacterium tumefaciens*. Herbicide tolerance is the most common trait in commercial GM plants that have been approved for cultivation or use in food and animal feed production (Tung Nguyen et al., 2008). The most common GM herbicide-resistant line is RRS soybean (Roundup Ready soybean), as indicated by several studies conducted in Hungary and the Czech Republic (Kyrova et al., 2008; Ujhelyi et al., 2008), while the second most common GM soybean line in EU countries is MON 89788 (Stefanova et al., 2016).

In the Croatian market, there is a wide range of products derived from soybeans. Soy is promoted as a healthy raw material rich in plant proteins and is widely used in many healthy and vegetarian food products. A particular demand for healthy food is the use of organic soy, which is grown according to precisely defined rules and involves production that does not use synthetic fertilizers, herbicides, and pesticides, and is neither produced nor consists of GMOs. The presence of GM food on the European Union market is very small compared to the global market. The reason for this is the complex legal framework and the complex area of risk management for GMOs, especially the risks associated with GM food. The approval process (authorization) for placing GM food on the European Union market is very complex and time-consuming, but uniform and consistent for all member states. The procedure for authorizing GM food on the EU market is carried out in accordance with the provisions of Directive 2001/18, Directive 2015/412 (amendment of the 2001 directive), and Regulation (EU) 1829/2003 (Spranger, 2015). In recent years, the EU has seen an increase in the number of producers and areas under organic production and non-modified soybean production, so Croatian producers certainly have their place in the common European market, as Croatia is a country where the cultivation of GM crops for commercial purposes is banned.



Graph 1. Presentation of the number of analyzed bio products by plant type and the proportion of products that contained GM soybeans



Graph 2. Total Content of GM Soybean Line MON 89788
Compared to the 0.9% Labeling Threshold

CONCLUSION

The presence of the GM soybean line MON 89788 at a proportion of 0.1% was detected in two organic soy beverages, while none of the analyzed samples contained GM corn, wheat, flax, or rice. The detected GMO content in organic food products did not exceed the regulatory labeling threshold of 0.9%. Obtained results suggest that GM contamination in organic products on the Croatian market is generally minimal and often due to unavoidable cross-contamination during production, transport, or storage. However, the presence of GMOs in organic products highlights the importance of continuous monitoring and implementation of GMO regulations. To ensure the integrity of organic production and consumer trust, further efforts are required in refining production processes, storage, and transportation to prevent cross-contamination.

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