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MONITORING AND DETECTION OF GENETICALLY MODIFIED SOYBEAN IN ANIMAL FEED USING REAL-TIME PCR (2019–2024)

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Abstract: The detection and monitoring of genetically modified organisms (GMOs) in animal feed is becoming increasingly important due to legal requirements related to labeling and control of authorization for placing GM products on the EU market. This study focuses on the presence and diversity of GM sequences in soybean meal samples collected between 2019 and 2024. A total of 94 samples were analyzed using sensitive molecular methods, including qualitative and quantitative PCR (Real-time PCR), in accordance with validated international protocols. DNA extraction was carried out using the CTAB method, and GM sequences were detected using specific primers for the most common promoters, terminators, and transgenes, including P-35S, T-nos, GTS 40-3-2, and A5547-127. The analysis was further improved by the use of the GMO Matrix tool and reference materials for result validation. The results showed a clear trend of increasing numbers of analyzed samples and greater diversity of detected GM sequences during the observed period, especially in 2023 and 2024. Although the GTS 40-3-2 sequence remained the most frequently detected, more complex combinations of transgenes such as Cry1Ab/Ac and CTP2-CP4-EPSPS, along with Pat and Bar genes, were also identified. These findings indicate the presence of modified soybean lines MON87701, MON877051-7, DAS44406, DAS81419-2, and MON87708-9, pointing to a growing diversity of GM traits in animal feed. These results highlight the importance of continuous GMO monitoring in animal feed, as well as the necessity of maintaining high standards of analytical accuracy. Systematic testing contributes to the safety of the food chain, the protection of animal and human health, and sustainable food production.

Key words: GMO, soybean meal, real-time PCR, animal feed, GMO monitoring

Introduction

Genetically modified organisms (GMOs) have become an integral component of modern agriculture, offering benefits such as improved crop yields, pest resistance, and herbicide tolerance. However, their widespread adoption has raised significant concerns regarding food and feed safety, environmental impacts, and consumer acceptance. In the European Union (EU), the regulation of GMOs is particularly stringent, requiring rigorous risk assessments and traceability systems to ensure transparency and safeguard public health (European Commission, 2021; EFSA, 2018).

Soybean (*Glycine max*) is one of the most commonly genetically modified crops globally, with more than 75% of global soybean cultivation attributed to GM varieties, predominantly those expressing herbicide tolerance traits such as CP4 EPSPS or stacked events combining multiple traits. Due to its high protein content, soybean meal is widely used as a primary protein source in livestock diets (OECD/FAO, 2021). Consequently, the presence of genetically modified soybean in animal feed has become a focal point for food and feed control authorities, particularly in regions where strict labelling and authorization requirements apply, such as the EU (Holst-Jensen et al., 2012; Mazzara et al., 2007).

The accurate detection and identification of GMOs in food and feed products are essential for regulatory compliance, risk management, and meeting consumer demand for transparency (Bawa and Anilakumar, 2013; Fraiture et al., 2015). Molecular techniques such as the polymerase chain reaction (PCR), including Real-Time PCR and quantitative PCR (qPCR), have become standard tools in GMO testing due to their high specificity, sensitivity, and reproducibility (Mazzara et al., 2007). These techniques enable the detection of common genetic elements (e.g., promoter P-35S, terminator T-nos), event-specific sequences, and quantification of GM material in complex food and feed matrices (Holst-Jensen et al., 2012).

Despite the routine use of PCR-based methods in official control laboratories, limited data are available on the longitudinal trends of GMO presence in feed ingredients, particularly in the context of temporal changes in the market, regulatory approvals, and trade patterns. Monitoring these trends is vital for assessing compliance with legal thresholds, tracking the introduction of new GM events, and informing risk-based sampling strategies (Grohmann et al., 2019).

In this study, PCR-based methods were applied to detect and identify GM sequences in soybean meal samples collected over a six-year period (2019–2024). Using validated detection protocols, certified reference materials (CRMs), and up-to-date sequence databases. Particular attention was given to the accuracy and sensitivity of the analytical procedures, as well as to characterizing the diversity of GM events identified in the tested samples. The primary objective was to evaluate the frequency and diversity of GM sequences in soybean meal entering the feed

supply chain, with a focus on temporal trends and changes in the composition of detected genetic modifications over the study period.

Materials and Methods

For the detection and confirmation of the presence of genetically modified organisms (GMOs) in food, seed materials and animal feed various analytical methods are employed, with Real Time polymerase chain reaction (RT-PCR) being the most commonly used. This technique enables the specific identification of transgenic DNA sequences and, in the case of a quantitative approach, the determination of the proportion of genetically modified lines within a sample (Dong et al., 2008). Samples of animal feed intended for analysis were collected between early 2019 and the end of 2024. Animal feed samples were received at the Central Laboratory for Seed Testing and Biotechnological Analysis, in the Department for Biotechnological Analysis of the Croatian Agency for Agriculture and Food (CSR, HAPIH) as part of official controls. DNA extraction was performed following the CTAB protocol (Lipp et al., 1999; Querci et al., 2020). After homogenization, 200 mg of each sample was weighed and mixed with 1000 µL of CTAB extraction buffer preheated to 65 °C. Following cell lysis, DNA was purified using 500 µL of chloroform. After centrifugation and phase separation, two volumes of CTAB precipitation solution were added to the aqueous phase. The DNA pellet obtained from the initial precipitation was dissolved in 350 µL of 1.2 M NaCl solution and further purified with 350 µL of chloroform. In accordance with the protocol, two precipitation steps were conducted, with the second step involving isopropanol in a volume ranging from 0.6 to 1× the sample volume. Finally, the purified DNA was washed with 70% ethanol and resuspended in 100 µL of sterile deionized water. The concentration and purity of the isolated DNA were determined spectrophotometrically (BioPhotometer, Eppendorf) by measuring absorbance at 260 nm and 280 nm (A260/A280 ratio). All DNA samples used for PCR analysis were normalized to a concentration of 50 ng/µL. This protocol is widely recognized for its reliability and efficiency in molecular detection of GMOs, particularly in complex matrices such as feed products (European Commission, 2015). The initial phase of the analysis involved qualitative screening using Real-Time PCR to detect commonly occurring genetic elements indicative of modification. All PCR reactions were conducted on the Applied Biosystems 7300 Real-Time PCR system. Detection of GMO-specific DNA sequences was performed using quantitative PCR (qPCR) with TaqMan chemistry, following the methodology described by Navarro et al. (2015). For the identification of genetic modifications, widely adopted primer sets such as P35S and T-nos were employed to target regulatory regions like promoters and terminators. Additionally, primers specific to Cry1Ab/Ac, CTP2-CP4-EPSPS, T-E9, and PAT were utilized to amplify regions associated with inserted transgenes. The integrity of DNA samples was verified using soybean-specific primers, as

described by Mazzara et al. (2007). The robustness and sensitivity of PCR-based methods have made them the gold standard in the regulatory monitoring of genetically modified materials (Grohmann et al., 2009). To identify specific genetically modified soybean events within the samples, the GMO Matrix tool was applied (Bonfini, 2023). This computational system integrates data from the GMOMETHODS database (Dong et al., 2008; Bonfini, 2012) and the Central Information System for Basic DNA Sequences (Fraiture et al., 2015), and provides validated reference methods for GMO detection and identification. The use of such databases significantly enhances accuracy and traceability in official GMO testing laboratories (Holst-Jensen et al., 2012). Certified reference materials (CRMs) of genetically modified soybean (IRMM, Geel, Belgium), with defined GMO content, were used as positive controls. The established limit of quantification for the assay was 0.1%. Each qPCR reaction was carried out in a final volume of 50 µL, comprising 40 µL of reaction mix (including Mastermix) and 10 µL of DNA sample at a concentration of 50 ng. The thermal cycling conditions were as follows: 2 minutes at 50 °C, initial denaturation at 95 °C for 10 minutes, and 45 amplification cycles of 95 °C for 15 seconds followed by 60 °C for 1 minute, where the extension step occurs. Quantification was based on co-amplification of the target GMO-specific sequence along with a standard of known concentration within the same reaction. Based on positive screening results, specific GM soybean lines were confirmed through the detection of characteristic genetic elements such as CTP2-CP4-EPSPS and T-E9, which are associated with five authorized GM soybean events.

Results and Discussion

The Table 1 presents the frequency and percentage of analysed soybean meal samples by sampling year (from the year 2019 till the year 2024), including cumulative values. The data show that the number of samples analysed was relatively low in the period from 2019 to 2021. During these three years, annual sample shares ranged from only 4.26% in 2021 to 11.70% in 2020. Altogether, only 26.60% of all samples were analysed in this initial period, indicating a limited scope of analysis, possibly due to organizational constraints, reduced demand, or limited laboratory capacity during those years.

From 2022 onwards, there is a noticeable increase in the number of analysed samples. In 2022, the number rose to 15 samples (15.96%), followed by a further increase in 2023, with 20 samples (21.28%), and reaching a peak in 2024 with 34 samples analysed, which represents the largest share at 36.17% of the total. This rising trend strongly suggests a growing interest in the quality control and monitoring of soybean meal in recent years. The increased sample frequency may be attributed to the expanded use of soybean meal as a protein-rich feed ingredient, stricter quality assurance requirements, or improvements in laboratory infrastructure and analytical procedures.

Table 1. Frequency and percentage of analysed soybeans meal depending on the sampling year

Sampling year	Frequency, N	Percent, %	Cumulative frequency, N	Cumulative percent, %
2019	10	10.64	10	10.64
2020	11	11.70	21	22.34
2021	4	4.26	25	26.60
2022	15	15.96	40	42.44
2023	20	21.28	60	63.83
2024	34	36.17	94	100.00

Cumulative data reinforce this pattern. By the end of the year 2022, a total of 42.44% of all samples had been analyzed, while in just the last two years, 2023 and 2024, more than half of the total samples (57.56%) were examined. This distribution clearly highlights the importance of recent years in terms of data volume and underlines the increased relevance of soybean meal analysis in current livestock nutrition and feed quality assurance practices.

Furthermore, in the context of animal nutrition, such an increase in soybean meal analysis is particularly relevant. Soybean meal is one of the most important plant-based protein sources used in livestock feeding, and its composition can significantly influence animal performance, nitrogen utilization efficiency, and overall farm sustainability. The observed rise in analytical frequency likely corresponds with an increased need for precise formulation of rations, especially in systems aiming to optimize protein efficiency and reduce nitrogen emissions.

Also, the increase in recent years reflects not only growing scientific and industry interest in feed quality but also broader shifts toward more data-driven, sustainable, and quality-assured animal nutrition practices. This trend underscores the importance of ongoing monitoring and reinforces the role of laboratory analysis in supporting evidence-based decision-making in livestock production systems.

Table 2 presents the identification of genetically modified (GM) sequences in analysed soybean meal samples, with a breakdown by year of sampling. The data include the number of analysed samples, the sequences that were requested for detection, the sequences that were actually detected, and the number of samples in which these sequences were found.

In 2019, 10 samples were analysed, and several GM sequences were requested, including P-35S, t-NOS, GTS 40-3-2, and others. A total of 8 samples (80%) tested positive for the sequence P-35S, T-nos, GTS 40-3-2, A5547-127, and two samples (20%) were found to contain the sequence P-35S, T-nos, GTS 40-3-2. This indicates a significant presence of genetically modified material in the samples, with the most frequent sequences being related to the GTS 40-3-2 event.

In 2020, a similar number of samples were analysed (10), with multiple GM sequences tested, including the same ones as in 2019. Five samples (50%) were detected with the sequence P-35S, T-nos, GTS 40-3-2, A5547-127, while the remaining samples tested positive for P-35S, T-nos, GTS 40-3-2. This shows a continued presence of GM material, with a higher detection rate for one specific sequence, P-35S, T-nos, GTS 40-3-2, A5547-127. In addition, a single sample in 2020 (from a different set) was tested for a broader set of sequences, including CTP2-CP4-EPSPS, Cry1Ab/Ac, and other GM markers. However, no sequences were detected in this sample, indicating that not all samples contain detectable GM material.

In 2021, only four samples were analysed, and all of them contained the sequence P-35S, T-nos, GTS 40-3-2, suggesting a high consistency in the presence of this specific GM sequence. This result shows that, although fewer samples were analysed, the prevalence of the GM sequence was still significant.

By 2022, the number of analysed samples increased to 15. The majority of the samples (14 out of 15) tested positive for the P-35S, T-nos, GTS 40-3-2, sequence, while 1 sample contained P-35S, T-nos, GTS 40-3-2, A5547-127. This highlights the continued presence of genetically modified sequences, with a slight increase in the diversity of sequences detected in 2022 compared to previous years.

In 2023, 18 samples were analysed, one sample (approximately 5%) tested positive for P-35S, T-nos, GTS 40-3-2, A5547-127, while 17 samples (94%) contained P-35S, T-nos, GTS 40-3-2. Additionally, two more samples were analysed with a broader range of GM sequences, including CTP2-CP4-EPSPS, Cry1Ab/Ac, and others, and both tested positive for several GM markers. These results demonstrate an even higher frequency of genetically modified material in the samples, with an increasing diversity of sequences detected in 2023.

The data for 2024 shows a continued trend with 32 analysed samples. Among these, 22 samples (68.75%) tested positive for P-35S, T-nos, GTS 40-3-2, and 10 samples (31.25%) were found to contain the sequence P-35S, T-nos, GTS 40-3-2, A5547-127. Additionally, two samples analysed in 2024 tested positive for a more complex set of GM sequences, including CP4-EPSPS, Cry1Ab/Ac, and others, indicating that the genetic modification in these soybean meal samples is becoming increasingly diversified.

Finally, the results from Table 2 reveal a consistent presence of genetically modified sequences across all years, with a notable increase in the detection of more diverse GM sequences over time. While the most common sequences remained related to the GTS 40-3-2 event (P-35S, T-nos, GTS 40-3-2), newer sequences and more complex combinations of genetic modifications started to appear, particularly from 2023 onwards. This trend suggests that the genetic modification in soybean meal is becoming more varied, possibly reflecting changes in the sources of the soybean meal or in the adoption of new GM crops.

Table 2. Presentation of identified genetically modified sequences in analysed soybeans meal depending on the sampling year

Sampling year	N, analysed samples	Requested sequences	Detected sequences	N, detected samples
2019	10	P-35S, t-NOS, GTS 40-3-2, DP-356043-5, A2704-12, A5547-127, FG 72	P-35S, T-nos, GTS 40-3-2, A5547-127,	8
			P-35S, T-nos, GTS 40-3-2,	2
2020	10	P-35S, t-NOS, GTS 40-3-2, DP-356043-5, A2704-12, A5547-127, FG 72	P-35S, T-nos, GTS 40-3-2, A5547-127,	5
			P-35S, T-nos, GTS 40-3-2,	5
2020	1	P-35S, t-NOS, CP4-EPSPS, Cry1Ab/Ac, PAT, MON89788, GTS 40-3-2, MON87701, MON 87705, 356043, A5547-127, A2704-12, DAS-44406-6	/	0
2021	4	P-35S, t-NOS, GTS 40-3-2, DP-356043-5, A2704-12, A5547-127, FG 72	P-35S, T-nos, GTS 40-3-2,	4
2022	15	P-35S, t-NOS, GTS 40-3-2, DP-356043-5, A2704-12, A5547-127, FG 72	P-35S, T-nos, GTS 40-3-2,	14
			P-35S, T-nos, GTS 40-3-2, A5547-127,	1
2023	18	P-35S, t-NOS, GTS 40-3-2, DP-356043-5, A2704-12, A5547-127, FG 72	P-35S, T-nos, GTS 40-3-2, A5547-127,	1
			P-35S, T-nos, GTS 40-3-2,	17
	2	P-35S, t-NOS, CTP2-CP4-EPSPS, Cry1Ab/Ac, PAT, Bar, tE9, MON89788, GTS 40-3-2, DP-356043-5, DAS 44406, MON87701, DAS 81419-2, DAS-68416-4, MON87705, FG72, MON87751-7, MON87708-9, MON87769-7	MON89788, DAS 44406, MON87701, DAS 81419-2, MON87751-7, MON87708-9	1
			CTP2-CP4 EPSPS, Cry1Ab/Ac, PAT, tE9, MON89788, MON87701, DAS 81419-2, MON87751-7, MON87708-9	1
2024	32	P-35S, t-NOS, GTS 40-3-2, DP-356043-5, A2704-12, A5547-127, FG 72	P-35S, T-nos, GTS 40-3-2,	22
			P-35S, T-nos, GTS 40-3-2, A5547-127,	10
	2	P-35S, t-NOS, CTP2-CP4 EPSPS, Cry1Ab/Ac, PAT, Bar, tE9, MON89788, GTS 40-3-2, DP-356043-5, DAS 44406, MON87701, A2704-12, DAS 81419-2, DAS-68416-4, MON87705, A5547-127, FG72, MON87751-7, MON87708-9, BPS-CV127-9, MON87769-7	CTP2-CP4 EPSPS, Cry1Ab/Ac, tE9, MON89788, MON87701, MON87751-7, MON87708-9	1
			CTP2-CP4 EPSPS, Cry1Ab/Ac, PAT, tE9, MON89788, DAS 44406, MON87701, MON87751-7, MON87708-9	1

Conclusion

The obtained results indicate a clear trend toward increased monitoring of soybean meal quality and genetic composition over the years. The frequency of analysed samples has risen significantly, especially in the last two years, reflecting a growing focus on feed safety, sustainability, and regulatory compliance in animal nutrition. This shift suggests a heightened awareness of the importance of feed quality, driven by evolving industry standards and consumer demands.

Simultaneously, the detection of genetically modified (GM) sequences in soybean meal has become more widespread, with a marked increase in the variety of GM traits identified over time. While the GTS 40-3-2 event was the most commonly detected sequence, new and more complex GM sequences have emerged, indicating that genetically modified soybeans are becoming more diverse in their traits. This diversification is likely a result of ongoing developments in biotechnology and the increasing use of genetically modified crops in agricultural production.

These findings highlight the need for continued monitoring and regulation of genetically modified materials in animal feed. As the presence and diversity of GM traits in soybean meal grow, it is essential to assess their potential impact on animal health, nutrition, and food safety. The results also underscore the importance of adapting regulatory frameworks and testing protocols to ensure the safe and responsible use of genetically modified ingredients in livestock feed, supporting sustainable and traceable food production systems.

Praćenje i otkrivanje genetski modifikovane soje u hrani za životinje pomoću Real time PCR-a (2019 – 2024)

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Rezime

Otkrivanje i praćenje genetski modifikovanih organizama (GMO) u stočnoj hrani postaje sve važnije zbog zakonskih zahteva u pogledu označavanja i kontrole autorizacije modifikacija za stavljanje na tržište EU. Ovo istraživanje usmereno je na prisutnost i raznolikost GM sekvenci u uzorcima sojine sačme prikupljenima u periodu od 2019. do 2024. godine. Ukupno je analizirano 94 uzorka primenom osetljivih molekularnih metoda, uključujući kvalitativni i kvantitativni RT-PCR (Real-time PCR), u skladu s validiranim međunarodnim protokolima. Ekstrakcija DNK sprovedena je prema CTAB metodi, a detekcija GM sekvenci izvršena je

pomoću specifičnih prajmera za najčešće promotore, terminatore i transgene, uključujući P-35S, T-nos, GTS 40-3-2 i A5547-127. Analiza je dodatno unapređena korišćenjem alata GMO Matrix i referentnih materijala za validaciju rezultata. Rezultati su pokazali jasan trend porasta broja analiziranih uzoraka te raznolikosti detektovanih GM sekvenci tokom posmatranog perioda, naročito u 2023. i 2024. godini. Iako je sekvenca GTS 40-3-2 ostala najčešće detektovana, pojavile su se i kompleksnije kombinacije transgena poput Cry1Ab/Ac i CTP2-CP4-EPSPS, Pat i Bar gena koji ukazuju na prisutnost modifikovanih linija soje MON87701, MON877051-7, DAS44406, DAS81419-2, MON87708-9 i upućuje na raznolikosti GM svojstava u stočnoj hrani. Ovi rezultati naglašavaju važnost kontinuiranog praćenja GMO-a u stočnoj hrani, kao i nužnost održavanja visokih standarda analitičke tačnosti. Sistematsko testiranje doprinosi sigurnosti hranidbenog lanca, zaštiti zdravlja životinja i ljudi, te održivoj proizvodnji hrane.

Ključne reči: GMO, sojina sačma, real-time PCR, stočna hrana, GMO monitoring

Conflict of interest
The authors declare that they have no conflict of interest.

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