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Detection and Quantification of Genetically Modified Soybean in Some Food and Feed Products. A Case Study on Products Available on Romanian Market

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Abstract: The aim of this paper is to trace genetically modified soybean in food and feed products present on the Romanian market by using molecular extraction, identification and quantification methodologies. Nine samples (3 food samples, 5 soybean samples and 1 soybean meal) were analysed using the classical and real-time polymerase chain reaction (PCR) method. DNA-genetically modified organism (GMO) was not detected in two of the three analysed samples (food products). However, it could be found in four samples ranging below the limit of 0.9%, and in three samples, above the limit of 0.9%. The results obtained through real-time PCR quantification show that DNA-RRS was detectable in different amounts in different samples: ranging between 0.27% and 9.36% in soy beans, and reaching 50.98% in soybean meal. The current research focuses on how products containing GMO above the limit (it is common knowledge that it is necessary to label the products containing more than 0.9% Genetically Modified DNA) are differentiated on the market with a view to labeling food and feed products in terms of the accidental presence of approved genetically modified plants. The benefits brought by genetic engineering in obtaining genetically modified organisms can be balanced with their public acceptance and with certain known or unknown risks that they can bring.

Keywords: detection; GMO; PCR; risks; quantification; soybean

1. Introduction

1.1. Genetically Modified Organisms—General Considerations

In agriculture, the importance of genetically modified organisms (GMOs) is attached to the characteristics deriving from the presence of new genes or gene fragments in their genome, which give them certain advantages: resistance to disease and pests, tolerance to herbicides or adaptation to stress (extreme temperatures, drought, salinity or acidity of the soil). Soybean tolerance is due to the transfer of a gene (cp4epsps) from a soil bacterium (*Agrobacterium tumefaciens* L.) that determines the synthesis of a glyphosate-insensitive protein (CP4EPSP).

As a European Union Member State, Romania was forced to give up transgenic soybean because the cultivation of this product is not approved in the EU. In fact, there are not many countries in the EU which cultivate soybean, not genetically modified, and we have to acknowledge that there is a high demand. Consequently, this genetically modified plant (GMP) is not cultivated, and soy beans and

grains are heavily imported from Argentina, Brazil, the USA and so on, that is, from major transgenic soybean countries.

To our best knowledge, currently only one GM crop is cultivated in the EU, namely the insect resistant maize event MON810, in the Czech Republic, Poland, Portugal, Slovakia, Romania and Spain; however, coexistence regulations referred to in this study also refer to those countries that do not cultivate GM crops but might do so in the future [1].

The World Food and Agriculture Organization (FAO) recognizes the potential of these new technologies, but at the same time, awareness of the problems that they may generate has been raised. GMOs have recently given rise to fierce debates globally, including in Romania [2].

Proponents of transgenic products believe that, however, during digestion, food DNA is destroyed; at the opposite end, there are many scientists who oppose genetic changes of any kind, arguing that this kind of foods can cause allergies of different types in humans and can affect their immune system [3]. On the other hand, some researchers suggest that, in fact, there is no complete picture of the toxicity (and safety) of transgenic products consumed by humans and animals. In the same vein, better communication is needed to inform producers and consumers about detection methods and labeling legislation [4,5].

Many consumers feel that labeling should be mandatory and that they have a "right-to-know" if the food they consume has been made using GM ingredients; labeled non-GM food is not available on the market, presumably because there is no requirement to pay for the costs of labeling [6].

Costs could differ even depending on the type of labeling [7,8]. Therefore, while voluntary labeling might increase costs for only a subset of foods, mandatory labeling will largely increase the costs and decrease the availability of all food. Moreover, mandatory labeling could be seen as a regressive policy that largely impacts on low-income consumers [9].

According to the Directive no. 18/2001/ECA [10], food based on GMOs must be labeled as a food containing genes, proteins or foreign DNA, or as food in which new genes have been removed by processing technology, but showing some compositional changes such that they cannot be considered natural food.

Detection underpins novel GMO-specific proteins or foreign genetic material. Detection is difficult because there are many interfering compounds, such as polyglucides, which can inhibit the polymerase chain reaction (PCR), leading to false positive results. Food processing causes protein denaturation, and subsequently of DNA, which can no longer be recognized by primers and antibodies, being present in a very small amount [11].

However, the screening strategies for identifying genetically modified (GM) elements in food have been developed significantly lately. For example, during the routine monitoring of GMOs in food products in the Netherlands (to identify unknown GM papaya events) a screening strategy was applied using additional GM screening elements, including a newly developed PRSV coat protein PCR [12]. Also, because of the importance of the P35S promoter in screening detection of GMOs, a large variety of GMO screening tests have been established [13].

1.2. GMO—Between Risk and Sustainability

Problems related to the detection and traceability of GMOs are of worldwide interest due to the trade relations between states and the economic and social implications of this. The availability of reliable traceability strategies is very important and this may increase public trust in transparency in GMO related issues [14].

Legislation requiring mandatory labeling of food products containing GMO stems from the need and right of consumers to know about the technologies used to produce the food they consume [15,16]. Compulsory labeling of these products may be ineffective because a large proportion of consumers do not understand the exact problem [17]. Although many consumers claim to oppose genetically modified food, there is a general lack of knowledge about genetically modified foods. Certain sociological studies have established that the briefing of consumers on the safety of genetically modified food does not

bring major benefits, meaning that there is an almost equal number of consumers preferring or not, the mandatory labeling of genetically modified foods [18].

The legal obligations to disclose information on environmental risks exist at four distinct levels:

- disclosure to governments, through environmental impact statements for planning or licensing of specific projects, dangerous activities for the environment, risk-prone industries or "dangerous goods";
- disclosure to citizens in "right to know" systems for specified work environments or beneficiary communities adjacent to industrial facilities;
- disclosure to investors as part of the business registration schemes;
- disclosure to consumers ranging from warnings to certification of content or origin related to a product or process [19].

Food labeling has been a concern for a long time regarding consumers' information and a source of legal controversy, mainly because of its implications for public health and trade. However, when it comes to regulations on human nutrition, health and trade are not the only arguments.

According to some authors, genetically modified plants are nutritionally equivalent with their genetically unmodified correspondents and can be used safely in food and feed [20].

Although the assessment of the risks and benefits of GMOs must be a scientific exercise, many debates on this issue seem to remain impermeable to scientific evidence [21].

Food containing GMOs raise wider socio-cultural and ethical issues at national and international levels [10,22] and the legislation in this area must take into account multiple policy objectives that may sometimes be conflicting; to protect the environment and biological diversity against the irreversible side effects of biotechnology; to protect the various cultural and religious traditions of food production and consumption; optimizing the biotechnological potential for improving food supply and their nutritional quality; and ensuring a fair social distribution of the benefits and biotech risks among farmers on global food markets [23].

Although these perceived risks are scientifically unreasonable [24], and the research undertaken so far has not detected any significant health risk directly linked to the use of genetically modified crops [25,26], without perceptible benefits, consumer decisions could nevertheless be inclined to avoid genetically modified foods.

Often, scientists have said they are forbidden to carry out fully independent research on the efficacy and environmental impact of genetically modified crops [27].

The report from the Advisory Committee on Releases to the Environment (ACRE) [28] examines how the current system could be more effective when considering applications to grow GM crops or release GM products.

Previous ACRE reports on EU regulations on genetically modified organisms have recommended the creation of a new framework. However, on short term, further improvements could be made to the current framework. The final report concludes that there is potential for improvement within the current framework by developing a better understanding of what is meant by "environmental damage" using "risk assumptions" and looking for options for managing environmental risk through better use of the existing information.

NGOs have traditionally played a less important role in the US, but have been very successful in Europe in terms of harnessing GMOs as a threat to biodiversity, farmers' self-sufficiency and food safety [29,30]. These groups have strongly focused on the potential risks and possible negative effects of genetically modified foods and feeds. Their often-sensational campaigns have been retrieved and multiplied by press articles [31].

There seems to be a regional difference in the relative perception of the risks and benefits of genetically modified foods. While at the European level consumers have a higher risk perception than those in North America and Asia, perceptions of benefits are opposed [32]. The perception of risks and benefits plays an important role in the consumers' behaviour [33,34].

2. Materials and Methods

The quantification method of the genetically modified DNA is based on amplification and gene detection by real-time PCR, of both the plant-specific DNA sequence and a sequence specific to the GTS 40-3-2 (Roundup Ready) line. For this procedure, primers and probes specific to the target sequences, which emit fluorescence only after hybridisation with amplicons, were used.

The legislation regulating the activities related to GMOs has as main objective the management of the risks to human, animal and environmental health, associated with their use.

The need to enforce such legislation is at the core of this research, which seeks to ensure that GM products are differentiated on the market, given that the legal limit of 0.9% has been established for food and feed products labelling, with respect to the accidental presence of approved GMPs [10,22].

Verifying compliance with EU legislation on GMOs involves detecting them, assessing the introduced genetic constructors and determining the need for labeling, which is hampered by the insufficient harmonization of global regulations [35]. Recent policy and society developments show a strengthening of the negative environment for agricultural biotechnology in Europe as a fairly recent issue, calls for GM food labeling in the US, and a particular focus on a possible authorization of genetically modified rice in China.

The overall objective of this study was to analyze GM soybean in food products (raw materials, processed food) and feed products (meals) by applying DNA extraction, identification and quantification methods specific to GTS 40-3-2 (Roundup Ready-RRS). Analytical methods based on PCR were used to detect DNA sequences specific to GTS 40-3-2 line (RRS) [36,37].

The identification of the DNA specific to the GTS 40-3-2 line (qualitative detection method) involves a double-checking system: PCR specific to *Glycine max.* L soybean and PCR specific to the genetically modified construct [38,39].

The classical PCR method allows for the selective amplification of specific segments of DNA that occur at reduced frequency in a complex mixture with other DNA sequences, whereas the real time PCR method allows for the determination of the amount of genetically modified DNA [40].

With regard to GMOs, Romania has adopted the European legislation represented by directives and regulations on GMOs.

In the present study, nine samples, consisting of food products (three samples), soybean-based feed products (one sample) and soy beans (five samples), coming from Romanian supply companies interested in the processing/marketing of these products, were investigated. The products on which sampling was based were not labeled "GMO free", and they were tested so as to check compliance with the regulations in force, which is of paramount importance for the credibility of the food biosecurity system.

Samples, which come from different sources, were harvested at the request of various food and feed companies to verify the existence of GMOs for labeling, according to a national strategic program approved by the National Sanitary Veterinary and Food Safety Authority.

Also, the samples where the DNA specific to GTS 40-3-2 (Roundup Ready)/DNA GM was identified, and DNA quantification was performed in duplicate (4 replicates/1 sample) were examined.

Each PCR cycle was noted by the fluorescence emission, which was measured directly by thermocycler. The obtained results were interpreted using the dedicated software, then visualized as amplification curves, and a Ct (the cycle at which the fluorescence significantly increased) was determined for each reaction.

The RRS soybean identification is performed by detecting a DNA fragment, including a 35S promoter sequence derived from CaMV (Cauliflower Mosaic Virus) and the chloroplast transit peptide (CTP4) sequence derived from *Petunia hybrida*.

The amplification and detection of a lectin gene fragment (specific to soybean) allows for the control of the DNA integrity used in the reaction, while being the reference for relative quantification. The quantification method using the Light Cycler tool complies with the basic principles of real-time PCR detection and quantification by using external standards. The absolute concentrations of the

DNA specific to RRS and species-specific DNA are determined by generating two calibration curves, one calibration curve for each of the detection systems (absolute quantification method). The generation of each calibration curve consists of measuring four calibration points (using the calibration DNA solutions in the kit). The result at the end of each sample analysis is the ratio between Roundup Ready soybean DNA/total soybean DNA (as percentage).

Calculation of the result: multiplying the RRS soybean value by 100 and dividing that number by the reference gene value (RRSsoybean DNA/total soybean DNA ratio calculation).

3. Results

Three samples of food (931, 932, 447), five soybean samples (342, 343, 344, 345, 346) and one soybean meal (71) were analysed using the classical PCR and real-time PCR methods.

DNA-GMO was undetectable in two samples—food products (931, 932); detectable below the 0.9% quantification limit in four samples—a sample of food (447) and three samples of soy beans (342, 343, 345), and detectable beyond the limit of 0.9% in two samples—soy beans (344, 446) and soybean meal (71) (Table 1).

Table 1. Detection of DNA-GMO in the examined samples.

GMO Content	Sample Type (Matrix)			
	Processed Food	Soybean Protein Concentrate	Feed Products	Soybean Grains
Undetectable	2	-	-	-
Detectable, below 0.9%	-	1	-	3
Detectable, above 0.9%	-	-	1	2

The results obtained through real time PCR quantification, as presented in Table 2, reveal that DNA-RRS was detectable in a relative amount of 0.27% in soy bean (sample 344) and 9.36% in soybean grains (sample 446), and in soybean meal (sample 446) amounting to 50.98%.

Table 2. Examined samples, their origin and results of real time polymerase chain reaction (PCR) quantification.

Sample	Type	Origin	%
931	Chocolate cream	local product	Undetectable
932	Chocolate cream	local product	Undetectable
447	Soybean protein concentrate	local product	Detectable, below the quantification limit of 0.08%
342	Soy beans	local product	Detectable, below the quantification limit of 0.08%
343	Soy beans	local product	Detectable, below the quantification limit of 0.08%
344	Soy beans	local product	0.27 ± 0.03
345	Soy beans	local product	Detectable, below the quantification limit of 0.08%
446	Soybean grains	local product	9.36 ± 1.02
71	Soybean meal	local product	50.98 ± 5.57

The results presented in Table 2 showed that, out of the total of nine examined samples, DNA-GMO above the limit of 0.9% was present in two samples of soybean (344, 446) and in the soybean meal (71).

Figures A1–A6 (see the Appendix A) display the results generated by the software associated with the Light Cycler, for each sample where the maximum limit of 0.9% of DNA-GMO was exceeded. The diagrams and results are presented consecutively for both the lectin (soybean) gene and DNA-RRS (genetic construct) sequence. Absolute concentrations of specific DNA and DNA-RRS were determined by generating two calibration curves, one curve for each of the detection systems. The generation of each calibration curve was the result of measuring four calibration points in the calibration DNA solutions in the kit. The result of the analysis of each sample is indicated as the percentage of the

Roundup Ready DNA/total soybean DNA ratio, which shows that in three of the examined samples (344, 446 and 71) DNA-RRS ranges above the quantification limit of 0.9%.

4. Discussion and Conclusions

Research on genetic engineering of plants has a particular theoretical significance, facilitating the knowledge of how genes of these organisms are acting, the effects of phytohormones on plant development, genes inactivation mechanisms, and so on. [41]. Also, by applying molecular biology techniques, useful information can be obtained on the plant genomes particularities used in amelioration, the localization of genes of interest, the degree of kinship between different species, and so on [42,43].

The control of weeds and plant diseases through genetic engineering technology allows the rapid introduction into crops of resistance mechanisms that mimic natural processes. Genetic engineering technologies should lead to sustainability [44], and food system sustainability is a widespread concern going beyond the potential of plant crops or any related philosophy. From the perspective of private farmers, varietal diversity may play an increased role in productivity and risk-reducing effects. It has been hypothesized that transgenic crops can also increase productivity and reduce the risk of production, so it could replace the diversity of varieties. However, a transgenic technology is not only a new variety because the same genes encoding certain traits can be inserted into varieties that are well suited to different soil and climate conditions [45]. Agro-biodiversity can be preserved if several transgenic varieties with the same traits and adapted to certain conditions are developed.

There is a large gap between the rapid acceptance of GM crops for the exploitation by farmers in many countries and on the global food and feed markets, acceptance being often limited by consumers in terms of perceiving risks or benefits [46].

Recently, it seems that the GMO-related problems refer mostly to the consumers' sovereignty rather than to human and/or environmental safety [47].

A rigorous analysis of GMOs requires their assessment both from the point of view of the influences on food security and the potential effects on sustainable development. Another important aspect is that of assessing the long-term costs of new environmental technologies. In general, the benefits of production increase are diminished by consumer mistrust.

In accordance with the legislation regarding the activities with biotech products, in order to market a GMO, the manufacturer must first prove that the product is safe, reliable and meets all the requirements laid down by the European legislation in force. At the experimental stage, the plants obtained by genetic modification are examined with respect to their safety on human, animal and environmental health.

Generally, new methods and concepts are needed to probe into the safety of the genetic techniques used in developing GM crops for allay the concern of the general public towards this modern genetic technology [14].

The regulation (EC) No 1830/2003 regarding the traceability and labeling of GMOs and the traceability of food products and feed obtained from genetically modified organisms requires operators to label it as "This product contains GMOs" or "This product contains GMOs (and the name of the genetically modified organism or organisms)" [10]. Labeling provides information for the consumer and the user of the product in order to enable them to make informed choices [48].The research findings served the food manufacturers for the accurate labeling of products, in accordance with the EU regulations, resulting in the labelling of the products in which GMO is above 0.9%. Also, the detection of GMO above the limit of 0.9% in three of the nine samples in our study leads to the conclusion that, although in Romania crops of genetically modified bean have been banned since 2007, the presence of GMO, especially in beans and feed products, is due to imports of soybean and soy beans from major producing countries. The monitoring of numerous samples through appropriate testing techniques carried out within this research shows that there is no complete control over GM crops, and in some cases importers do not ensure their proper labeling.

It is the case of the Portuguese maize bread, similar research showed that in more than 40% of the samples the limit of 0.9% GMO was exceeded, with no labeling in this respect. This type of bread corresponds to the GM maize, which is not cultivated in the EU, but it enters the chain through international trade [49].

Such studies are likely to enable the market differentiation of the products containing GMOs, so as to raise the consumers' awareness. Securing traceability will lead to the verification of the origin of the food products, heavily impacting on both the end consumers and policy makers.

Labeling in a reliable and transparent manner is a must and it provides useful information for implementing traceability in the agri-food industry by reducing the risk exposure of consumers/food business operators. Based on the adequate functioning of the traceability system, operators in the agricultural, food and feed sector must be able to identify at any time who has supplied the raw materials for production, processing and marketing. These issues are of major importance in the case of contamination of products (food/feed) in order to identify, isolate and correct the problem quickly and efficiently, which otherwise may endanger the quality and safety of products.

In Romania, the current monitoring of GM foods is coordinated by the Genetically Modified Organisms Inspection (IOMG), a body authorized to carry out official controls to ensure the traceability of GM plants from the farmer to the storehouse. IOMG County Departments ensure the monitoring of the application of GMO legislation [50].

In the context of food policies and consumers' choice, it has been found that European consumers consider not only the price but also the quality when buying food. In Romania, in addition to the economic factors (price and income), non-economic factors (habits, quality, freshness, taste, family preferences) can have an impact on the consumer's choice [51]. Over recent years, Romanians have made substantial changes in their diets by becoming selective for the food they consume [52]. Some studies confirm that the Romanians' perception of organic food is different, as 33% of a survey respondents think that organic food is "fashionable" and 18% see it as an exaggeration [53].

Failure to implement specific legislation on the labeling of products containing GMO actually violates the consumer's right to choose products that are not genetically modified, simply by failing to identify those products on the market.

Certainly, our investigation may be subject to further comments and refinements, yet, we think that it provides well-grounded scientific data aiming primarily at raising consumers' awareness in this respect. More complex research and triangulation of data pertaining to agriculture, food security and environmental protection will improve outcomes with reference to food sustainability.

Author Contributions: Catalin Aurelian Rosculete and Elena Teleanu are responsible for taking samples and analyzing them. Elena Rosculete collected and interpreted the data and wrote the manuscript. Elena Bonciu critically revised the manuscript. All authors made important contributions to the manuscript and approved the final version.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix

Figures A1–A6 samples with exceeded admissible DNA-GMO limit.

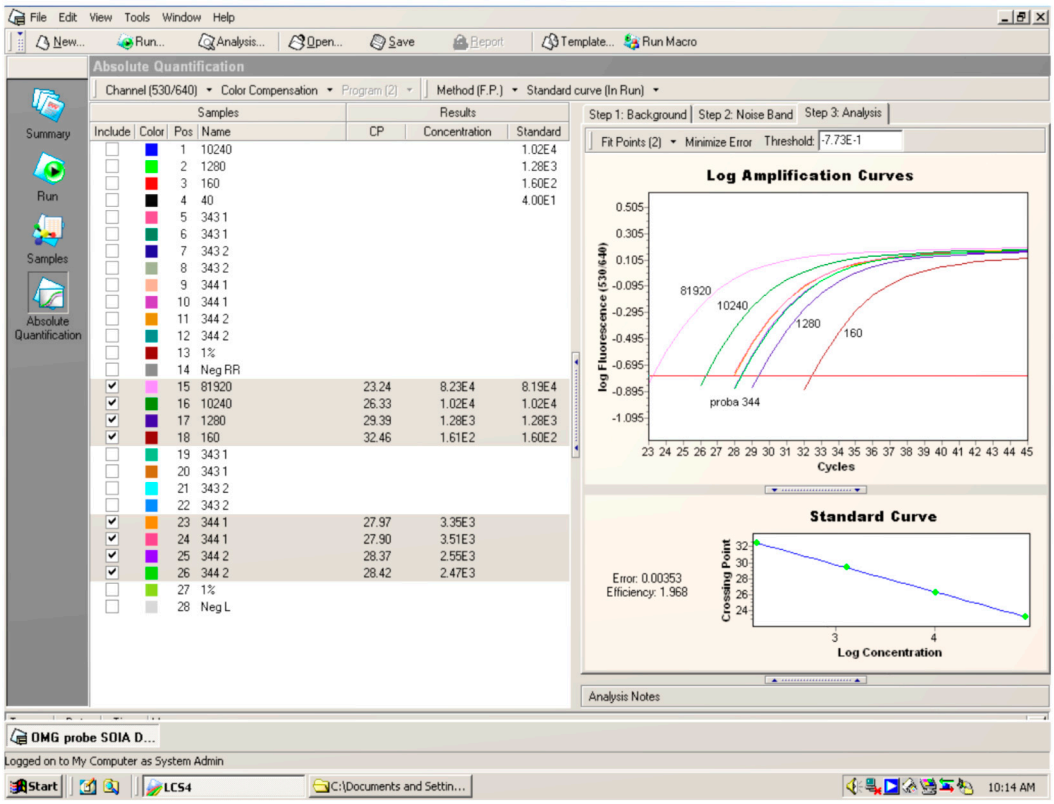

Figure A1. Soy beans (sample 344L). Source: Authors' computation.

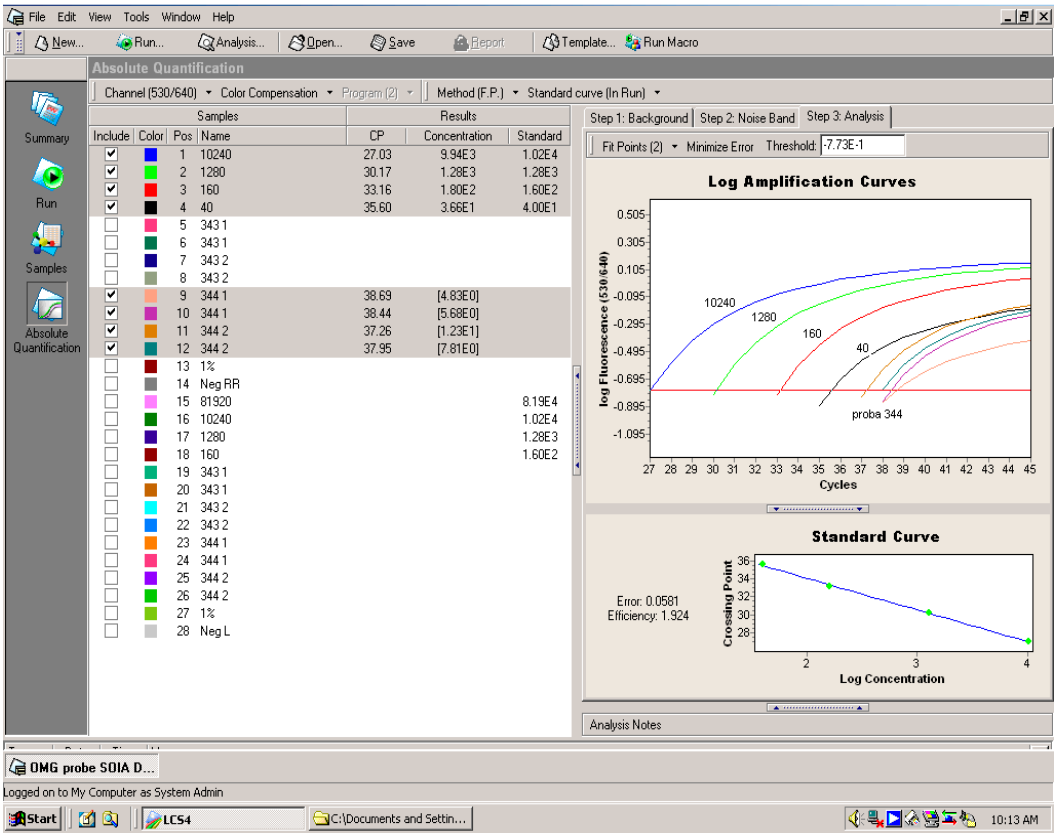

Figure A2. Soy beans (sample 344RR). Source: Authors' computation.

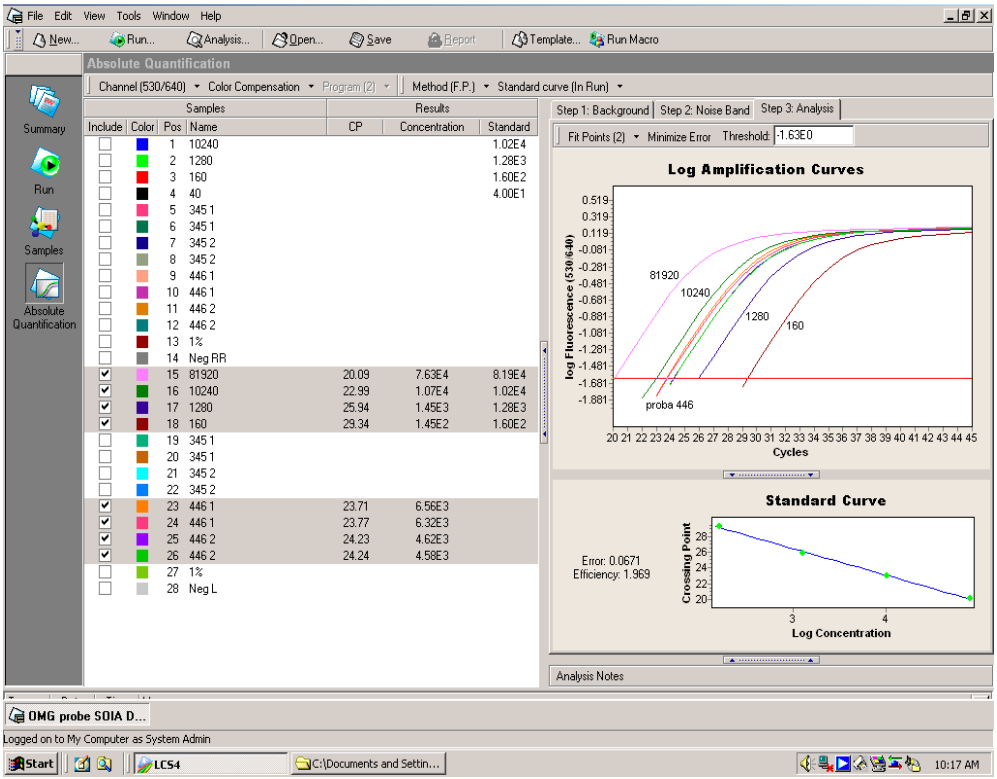

Figure A3. Soy beans (sample 446L). Source: Authors' computation.

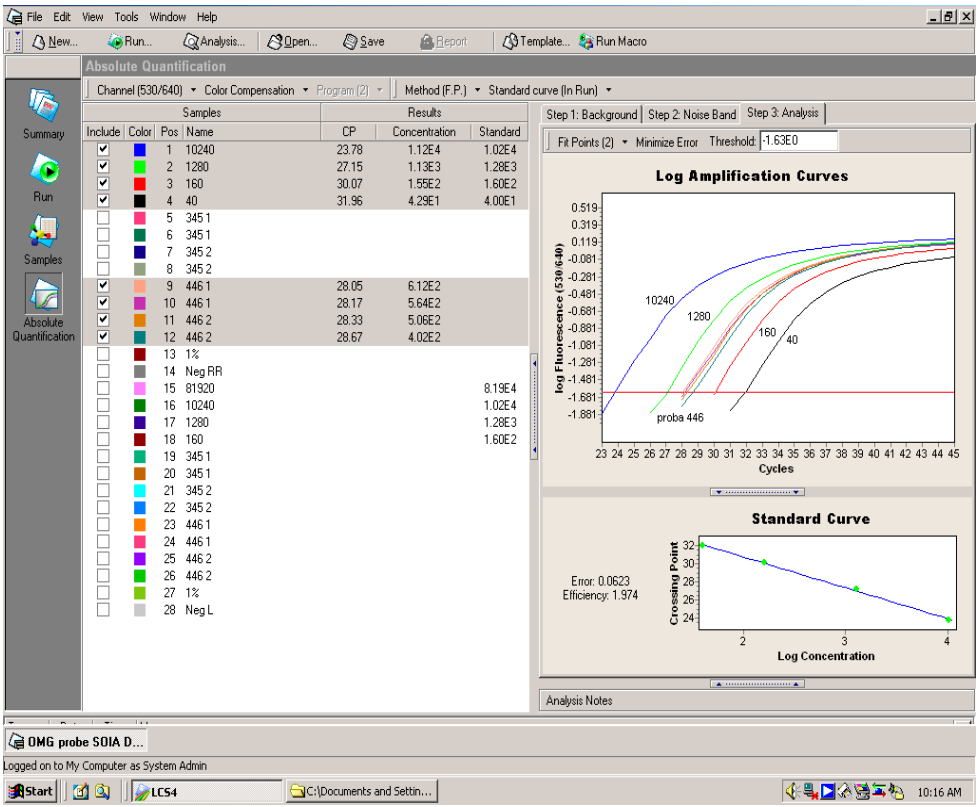

Figure A4. Soy beans (sample 446RR). Source: Authors' computation.

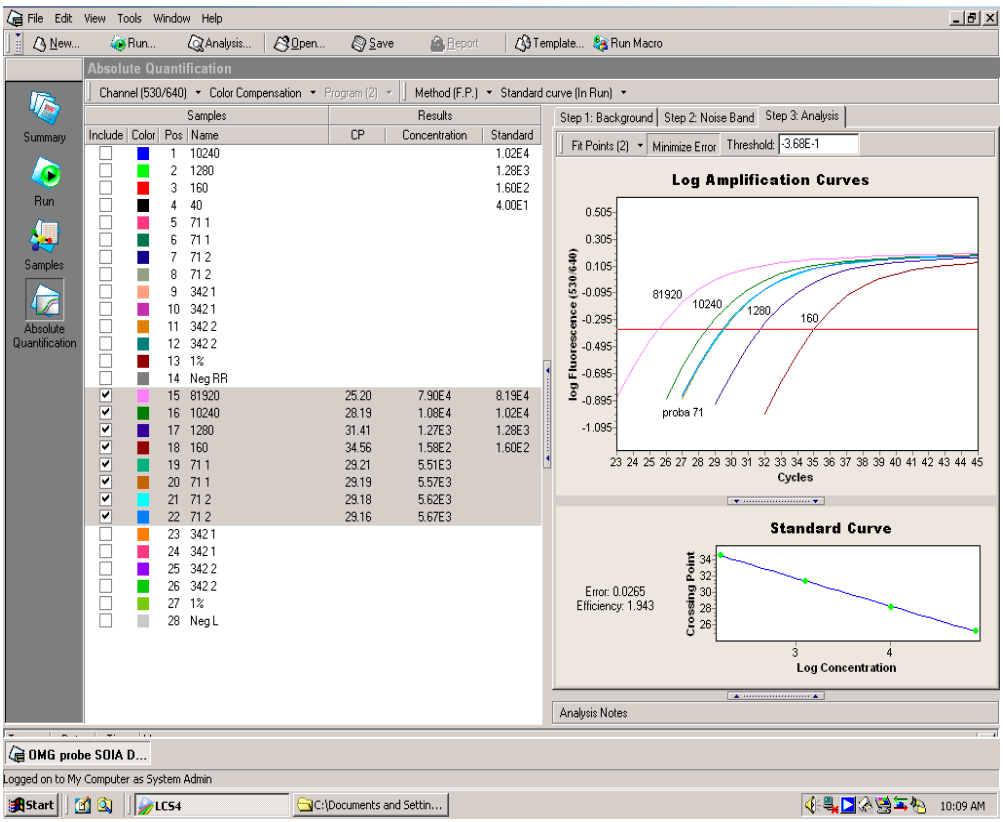

Figure A5. Soy bean meal (sample 71L). Source: Authors' computation.

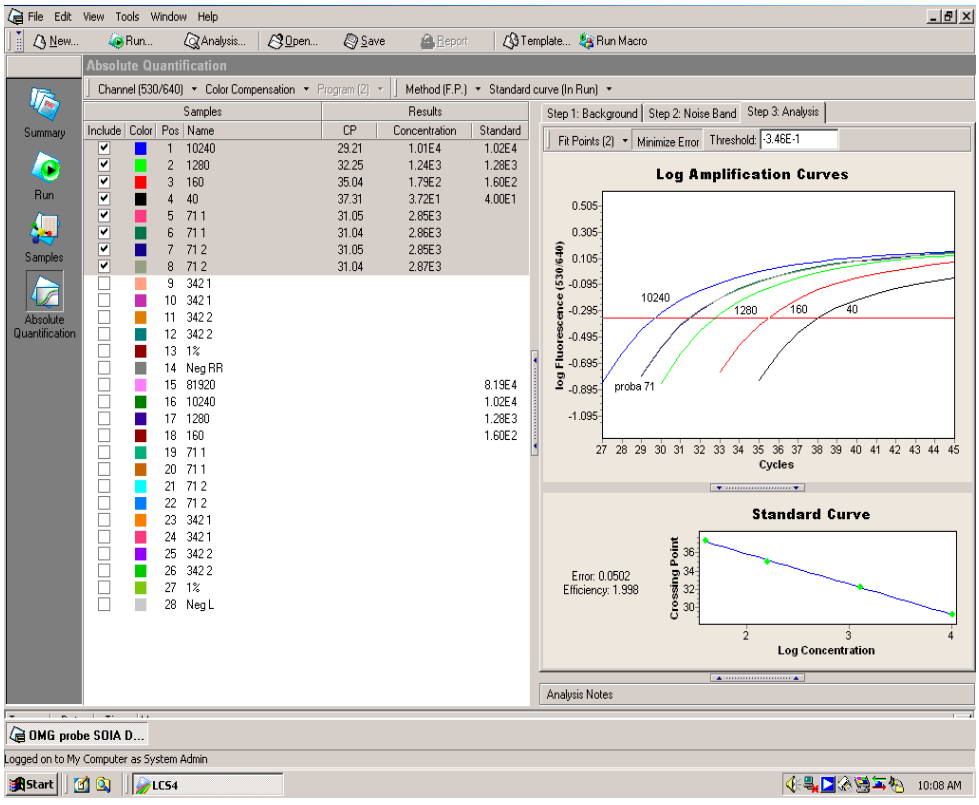

Figure A6. Soybean meal (sample 71RR). Source: Authors' computation.

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