QUALITATIVE DETECTION OF GENETICALLY MODIFIED ORGANISMS IN FOODS BY REAL TIME PCR

Dilek Bengü Yaman
Gözde Türköz Fatih Bakırcı

Aybak Natura Food Analyses Laboratory

November 2010
Antalya

What are GMOs?

- GMOs can be defined as organisms in which the genetic material (DNA) has been altered in a way that does not occur naturally by mating or natural recombination, i.e. by being genetically modified (GM) or by recombinant DNA technology.
- How?
  - Inserting a specific gene
  - Switching a gene on or off
  - Removing a specific gene

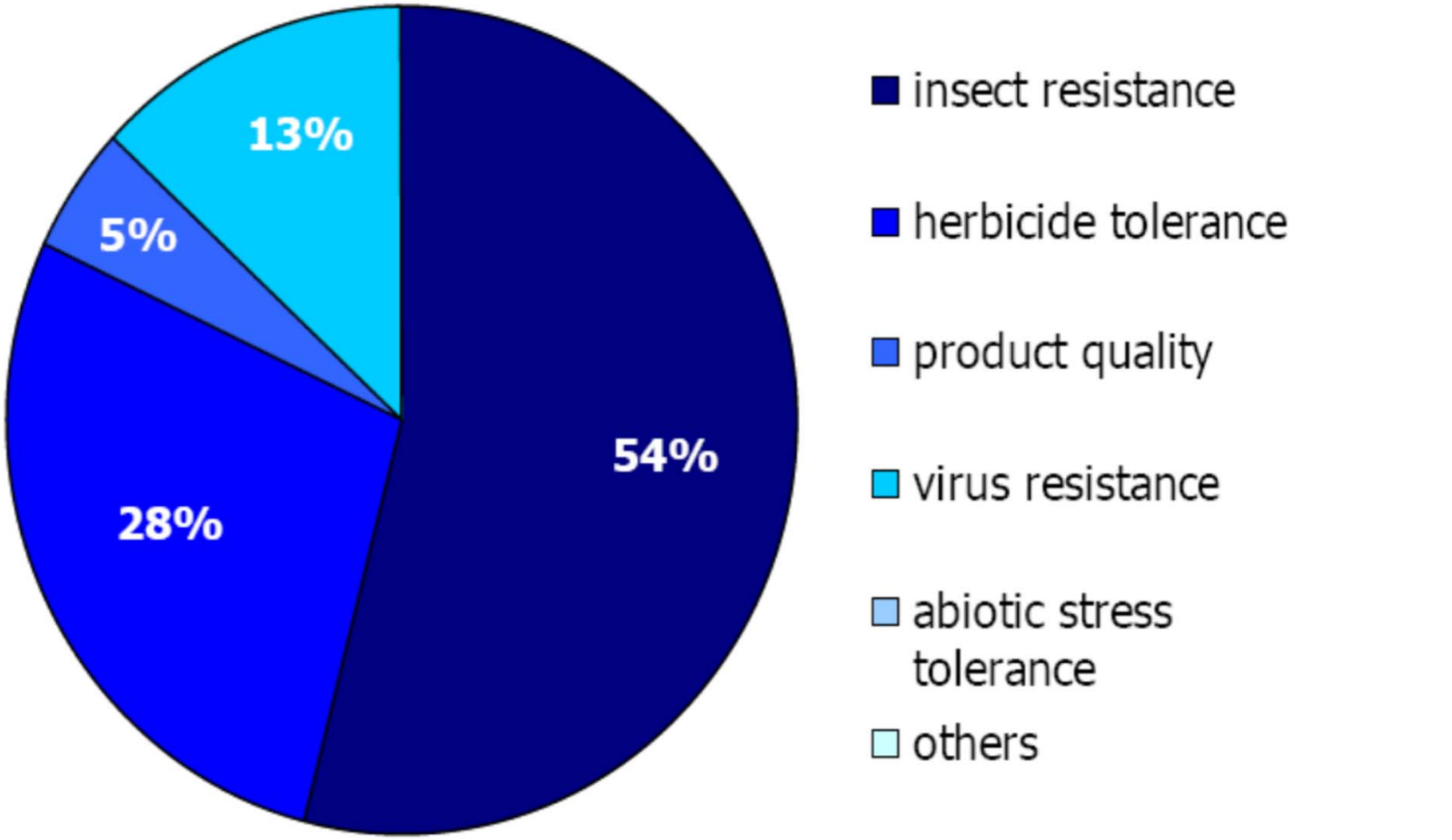
The flavr savr tomato was the first commercially used GMO plant (1994). An introduced antisense RNA gene blocked the expression of a pectin degrading enzyme. It was cultivated and introduced in the USA.

Why GMOs ?

- GM plants have been deliberately developed for a variety of reasons:

✓ to provide longer shelf life,
✓ to provide good quality products
✓ virus resistance,
✓ insect resistance,
✓ herbicide tolerance,
✓ nutritional improvement,
✓ resistance to such a biological stresses,
✓ resistance to against geologic,
✓ climatic challenges,
✓ higher yields

Source: Gachet et all., 1999

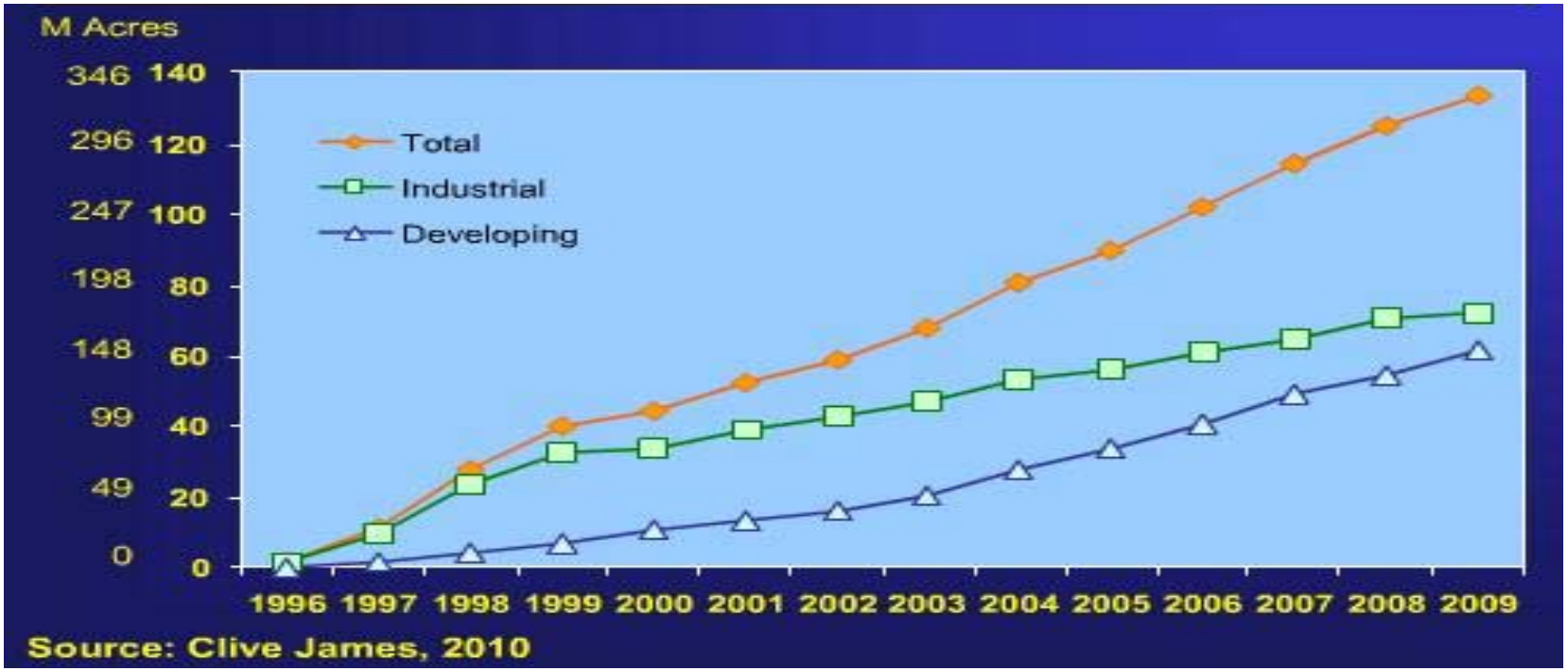


- Attributes of GMO plants commercialized in 2008 (Source: Nature Biotechnology 28, 23-25 (2010))

Global Area of Biotech Crops,1996 to 2009 by Industrial and Developing Countries

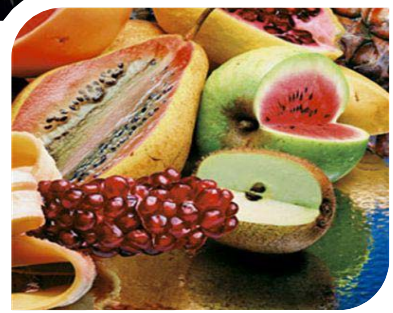
In recent years worldwide production and the use of GMOs in foods is becoming more and more widespread.

In 2009, biotech crop area grew seven percent or by 9 million hectares (22.23 million acres) to reach 134 million hectares (330 million acres).



Possible Risks of GM Foods

- GMO cause risks to human and animal health like toxicity, allergenicity, anti-nutrition effects, and unintended effects.
- Insects might develop resistance to pesticide-producing GM crops
- Herbicide-tolerant crops may cross-pollinate weeds, resulting in "superweeds“
- Edible safety becomes the main focus, when agri-GMOs are applied in producing and processing foods.
- Decrease of the plant species
- Environmental Polution

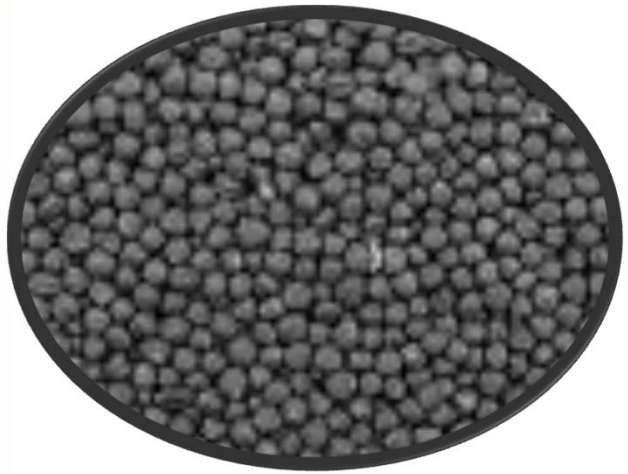


Source: Gachet et all., 1999

August 6, 2010

NEWS

GM crop escapes into the American wild



*Transgenic canola found growing freely in North Dakota.*A genetically modified (GM) crop has been found thriving in the wild for the first time in the United States. Transgenic canola is growing freely in parts of North Dakota, researchers told the Ecological Society of America conference in Pittsburgh, Pennsylvania, today. The scientists behind the discovery say this highlights a lack of proper monitoring and control of GM crops in the United States.

June 7, 2010

NEWS

Genetically modified corn contaminates crops in seven German states

A Greenpeace report says seven German states have had seed supplies contaminated by genetically modified corn. Losses for farmers could be in the millions of euros. Despite a Europe-wide ban, genetically modified corn has contaminated crops in seven German states, according to research from the environmental organization Greenpeace. The seeds were supplied by the firm Pioneer Hi-Bred, based in the town of Buxtehude outside Hamburg, in Lower Saxony.

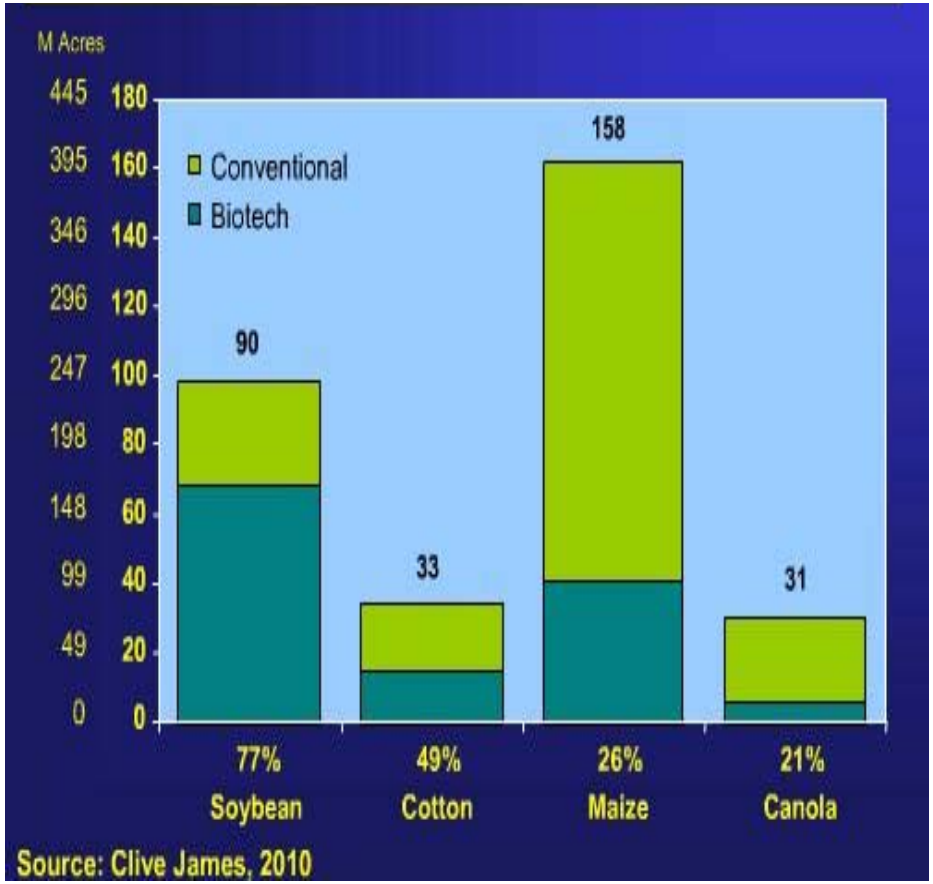
Cultivation areas for GM plants according to country in 2009; field are in millions of hectares

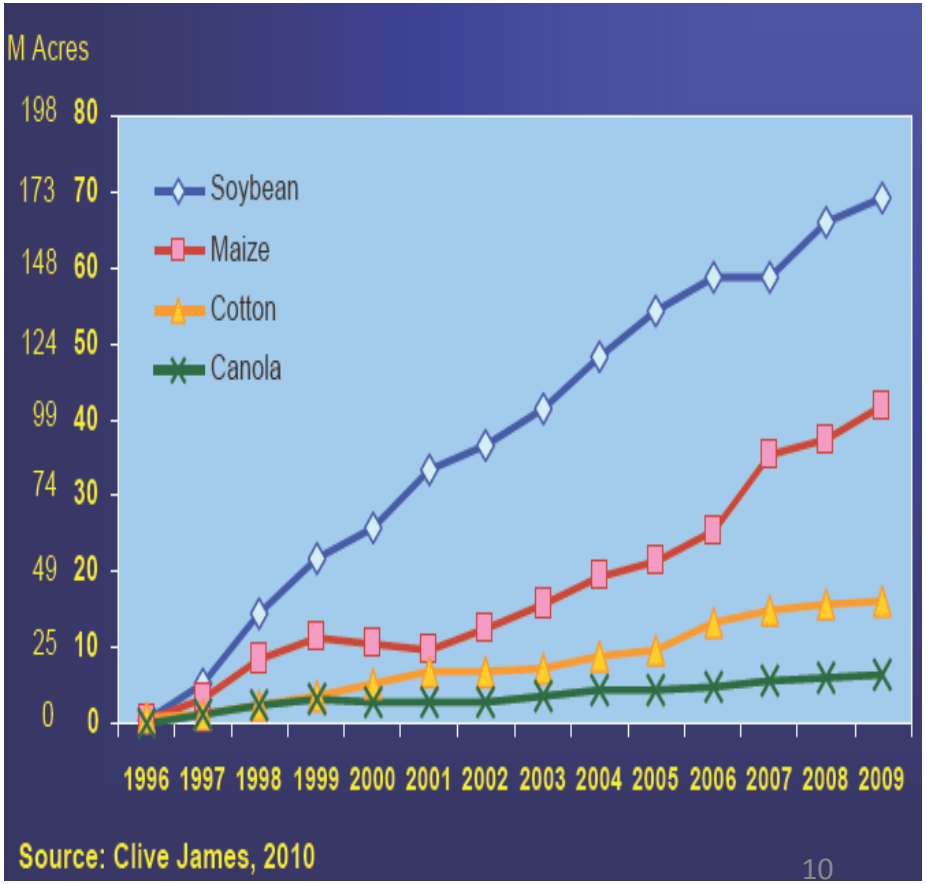
Rank	Countries	Area (Million Hectar)	Transgenic Plants
1	USA	64.0	Soybean, Maize, Cotton, Canola, Papaya, Sugar Beet
2	Brezil	21.4	Soybean, Maize, Cotton
3	Argentina	21.3	Soybean, Maize, Cotton
4	India	8.4	Cotton
5	Canada	8.2	Canola, Maize, Soybean, Sugar Beet
6	China	3.7	Cotton, Tomato, Papaya, Pepper
7	Paraguayan	2.2	Soybean
8	South Africa	2.1	Maize, Soybean, Cotton
9	Uruguay	0.8	Soybean, Maize
10	Bolivia	0.8	Soybean
11	Philippines	0.5	Maize
12	Australia	0.2	Cotton, Canola
13	Burkina Faso	0.1	Cotton
14	Spain	0.1	Maize
15	Mexica	0.1	Cotton, Soybean

Clives James, 2009

Global Adoption Rates (%) for Principal Biotech Crops

The major transgenic foodstuffs are soybean, cotton, maize and canola. In the world, 77% of soy, 49 % of cotton, 26 % of maize and 21 % canola are transgenic products.





Transgenic foodstuffs

- Soybean and Soybean Products
- Maize and Maize Products
- Rice
- Papaya
- Potato
- Tomato and Tomato Products
- Oilseed, Canola, Cotton
- Sausage and some meat products
- Chocolate
- Soup Mixes
- Cola and fruit juices
- Baby infant formula
- Sugar Beet
- Biscuits, Cakes, Cookies and the others products

Regulations about GMO..

Different levels of regulations concerning authorization of such products exist: some countries, including EU Member States, follow a mandatory authorization procedure and a labeling provision for GM food and feed products, while others, like the USA, have only a voluntary labeling procedure.

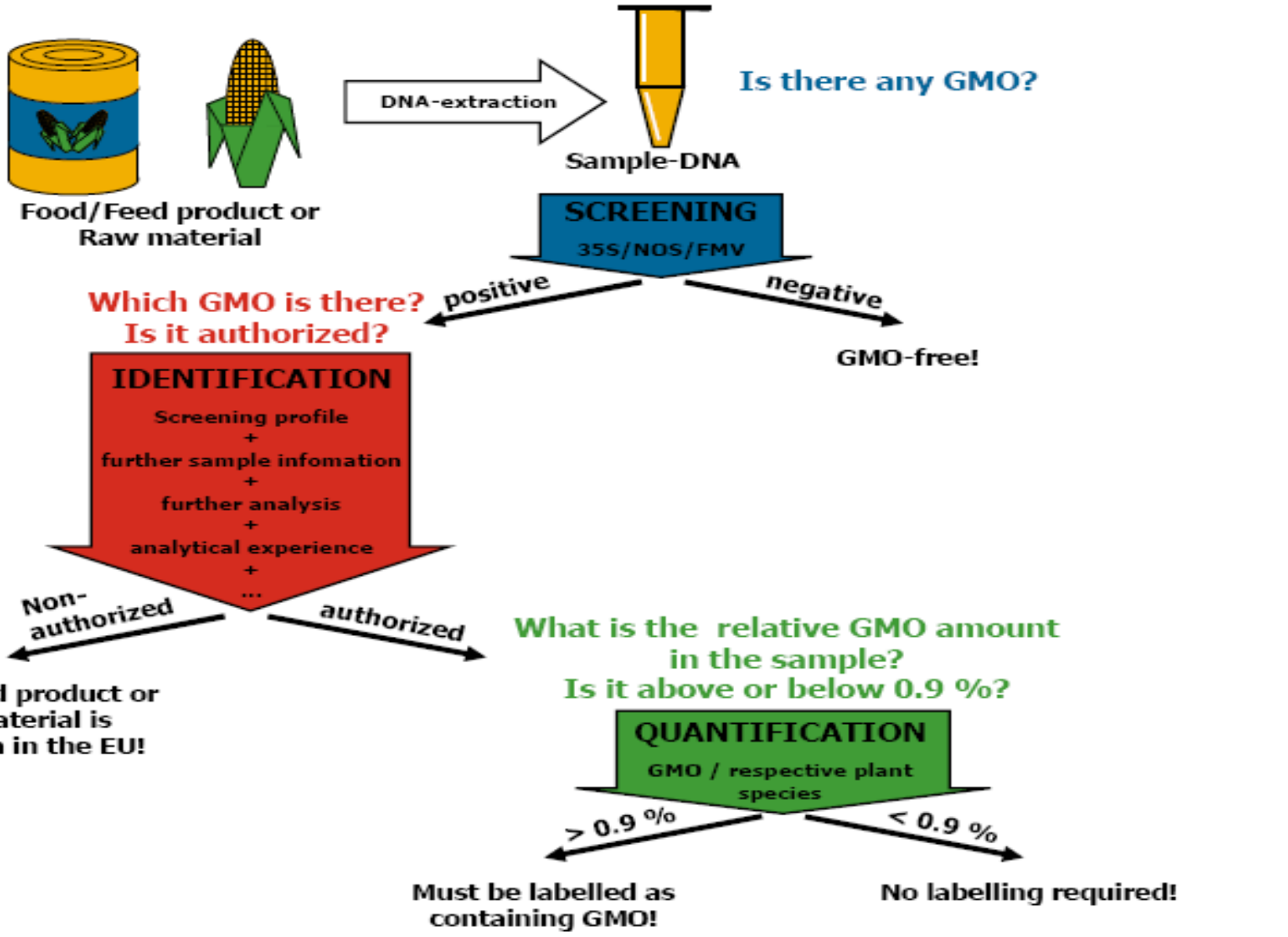
Country	Labeling Mandatory/Voluntary	Labeling Tolerance (%)
Australia	M	1
New Zeland	M	1
Brezil	M	1
Canada	V	5
Chinese	M	0
EU Member States	M	0.9
Japan	M-V	5
Korea	M	3
Rusian	M	1
USA	V	5

Turkish Food Regulation…

- According to Turkish legislation about import, export, control and usage of the genetically modified organisms(GMO) which are aimed to be used as food or lure that was published in 2010;

✓ Cultivation of GMOs are not permitted
✓ GMO products must be labelled
✓ Imports of GM products are not permitted*

* Food that contain soy, maize, tomato, potato and/or rice are controlled before importation to Turkey to detect the foods are GMO or not? If the foods are GMOs, foods are analysed for the identification of the transgenic gene. There is a list of 31 permitted GMO events (all of the plants have also an authorization in EU). If these events are present less than 0.9% of an authorized GMO, the products are imported. If a not authorized GMO is present (zero tolerance), the product are not imported to Turkey.


Food/Feed product or
Raw material
DNA-extraction
Sample-DNA
Is there any GMO?
SCREENING
35S/NOS/FMV
positive
negative
GMO-free!
Which GMO is there?
Is it authorized?
IDENTIFICATION
Screening profile
+
further sample infomation
+
further analysis
+
analytical experience
+
...
Non-authorized
authorized
Food/Feed product or
raw material is
forbidden in the EU!
What is the relative GMO amount
in the sample?
Is it above or below 0.9 %?
QUANTIFICATION
GMO / respective plant species
> 0.9 %
< 0.9 %
Must be labelled as
containing GMO!
No labelling required!

SOY IMPORTATION TO TURKEY (TON)

	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008
ROMANIA	23.839	31.790	11.562	5.462	0	15.600	7.809	29.995	36.021	14.695	0
UKRAIN	402	2.899	2.654	851	5	7.611	33.430	73.969	131.977	141.821	32.345
USA	158.892	258.254	301.328	301.354	541.243	399.328	317.625	555.618	637.161	456.278	**330.304**
CANADA	0	0	0	0	0	0	10.058	0	0	33.803	2.021
BRAZIL	90.761	20.564	53.140	7.087	0	56.745	18.123	188.732	169.638	99.939	**120.165**
PARAGUAY	0	0	0	0	0	13.849	53.620	66.049	8.789	0	64.978
URUGUAY	0	0	0	0	0	0	2.349	32.905	0	38.449	20.787
ARGENTINA	11.301	38.034	17.991	0	71.231	336.991	238.948	207.236	33.260	445.804	**500.583**

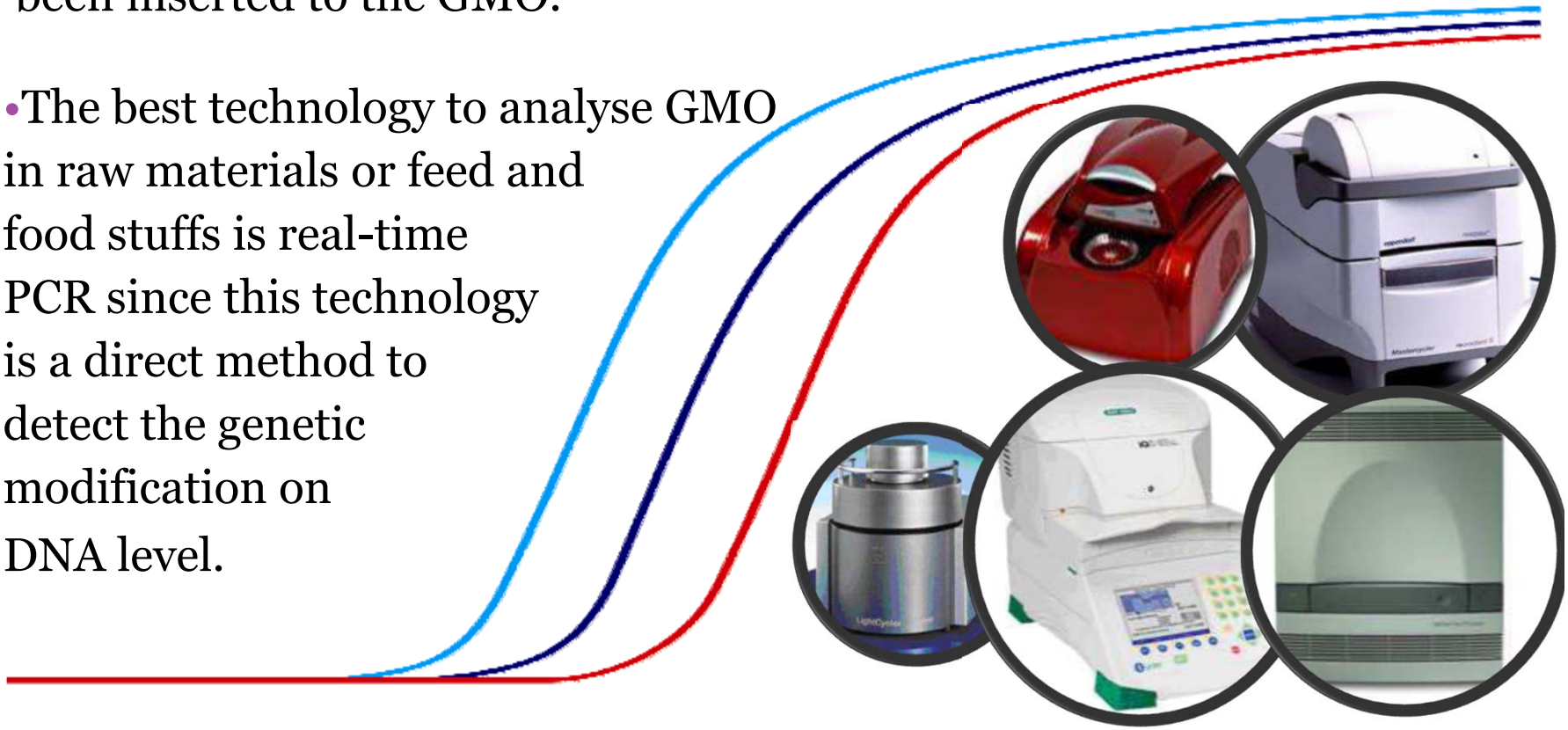
MAIZE IMPORTATION TO TURKEY (TON)

	2001	2002	2003	2004	2005	2006	2007
FRANCE	0	919	21.432	24.105	208	184	384
ITALIA	144	116	25	585	311	389	113
SPAIN	23	459	213	4.517	399	212	83
HUNGARY	5.061	315.861	163.621	45.361	32.043	34	131.064
ROMANIA	1.469	90.800	40.227	27.451	78.214	5.802	97.338
BULGARIA	2.459	10.123	37.508	14.329	6.437	0	93.261
UKRAIN	0	3.911	42.856	47.794	41.755	7.725	268.598
MOLDOVIA	0	10.992	2.407	2.597	7.835	4.820	8.068
CROATIA	130	193	7.809	95	145	11	5.997
SERBIA	0	0	0	0	0	113	19.739
THE REPUBLIC OF SOUTH AFRİCA	1.652	2.639	215	107	65	228	0
USA	460.959	697.429	1.113.684	682.471	15.030	1.661	**366.815**
BRAZIL	62.739	0	31.352	0	0	0	0
CHILE	0	21	69	90.512	10	87	**619.525**
ARGENTINA	2.100	39.524	356.753	197.460	35.013	9.262	**136.149**
AFGHANISTAN	0	994	0	500	0	0	0
AUSTRALIA	0	5.347	14	6	0	0	0

DETECTION OF GMO…

- A detection method of GMOs or derivatives of a GMO can be constructed from detection of molecules (DNAs, RNAs or proteins) that specifically targets for the specific sequences or genes that have been inserted to the GMO.

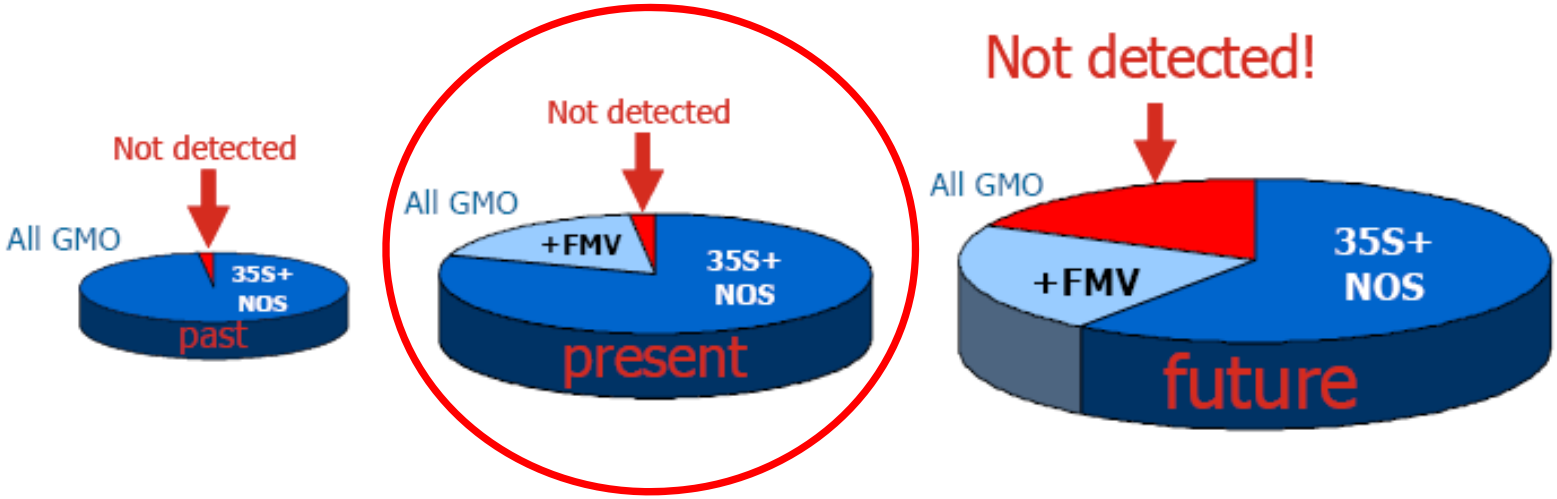
- The best technology to analyse GMO in raw materials or feed and food stuffs is real-time PCR since this technology is a direct method to detect the genetic modification on DNA level.



The objective of this study was to screen;

- ✓ the CaMV 35S promotor (Cauliflower mosaic virus)
- ✓ NOS terminator (nopaline synthase gene)
- ✓34S FMV promotor (Figworth mosaic virus)

in the some kind of foods using Real Time PCR to determine the genetically modified microorganisms.



Materials

- Totally 120 samples were collected from a commercial market in Izmir.

- **These samples are:**

✓ Flour
✓ Chocolate
✓ Soup mix
✓ Cornflakes
✓ Chocolate-flavored hazelnut spread
✓ Wafer-Biscuits
✓ Meat products
✓ Maize products
✓ Tahini halvah
✓ Salad Sauces (soy sauce, tomato sauce, etc.)
✓ Rice
✓ Maize Oil

Methods

- The procedure includes the following steps:

➢ Grinding and homogenization of laboratory samples

➢ Extraction of the analyte

➢ Testing and interpretation

SAMPLE HOMOGENIZATION

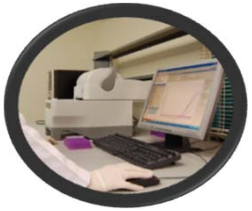
⇩

DNA EXTRACTION

⇩

MASTER MIX PREPARATION

⇩

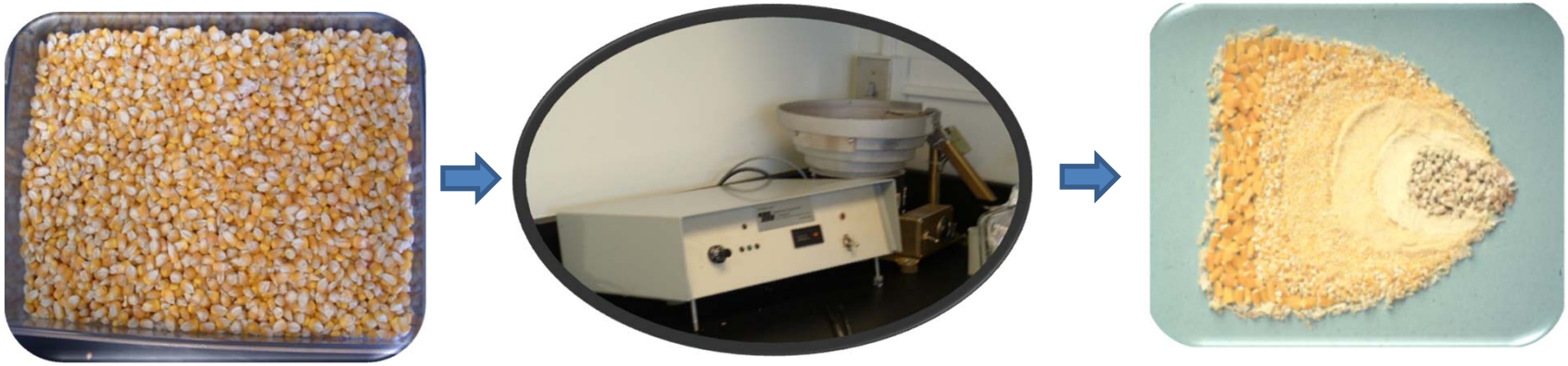


REAL TIME PCR

Screening Method Steps

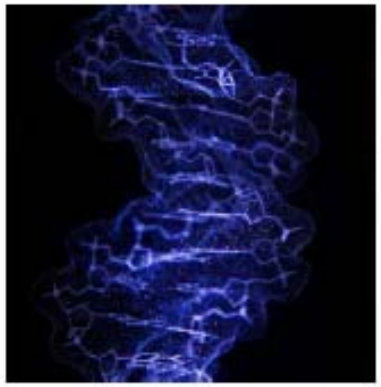
Homogenization

The sample preparation and DNA extraction are very crucial steps in each PCR assay and the basis for accurate and reliable results. Crops (corn, soybean, wheat, etc. and their products) and animal (beef, sausage, etc.) samples must be grinded and homogenized very well with equipment such as blender or grinding mills. DNA is extracted in DNA Extraction Room after the grinding or homogenization.



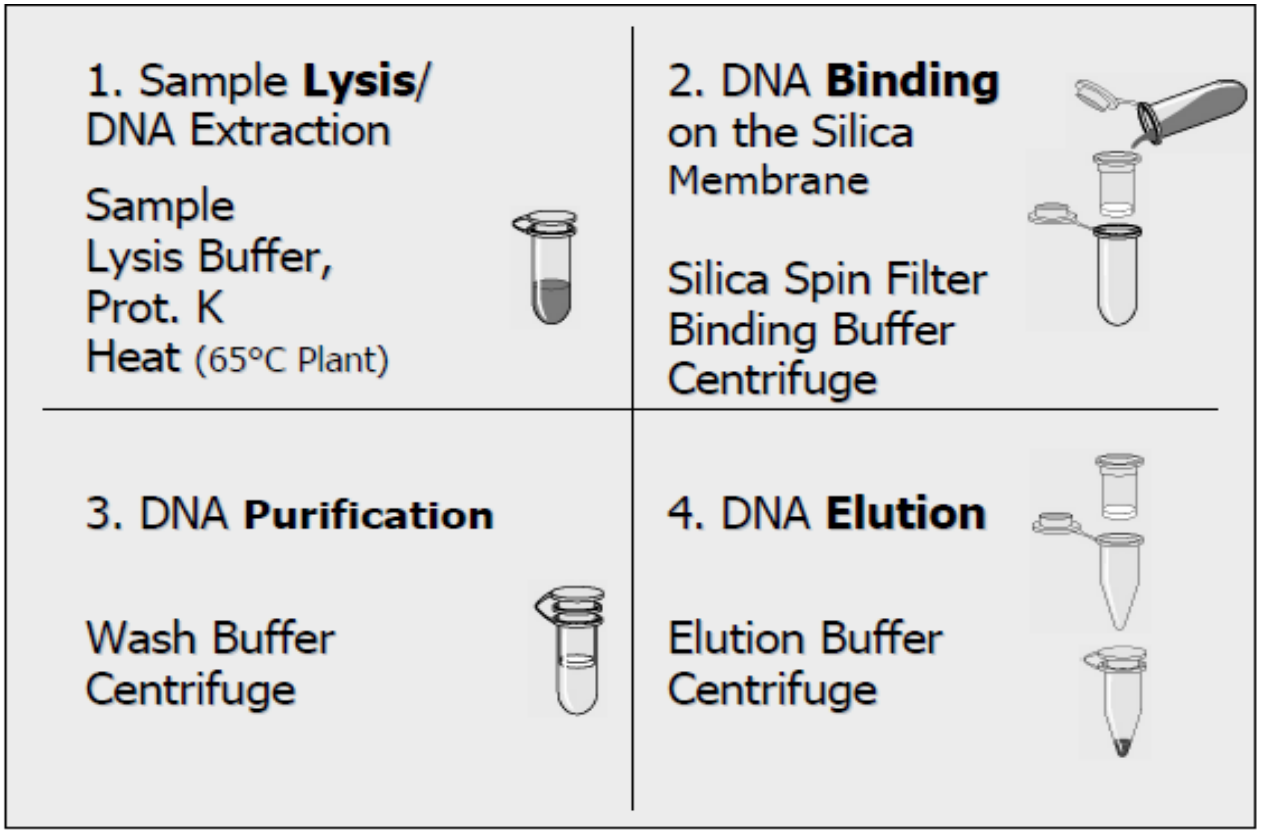
DNA Extraction and Purification Step

DNA-
extraction



The effective **extraction of plant DNA** from raw material and food samples and the **purification from PCR inhibiting substances** is the initial step in succesfull GMO-analysis.

DNA Extraction and Purification Step



R-Biopharm Sure Food kits were used for the extraction of genomic DNA from processed foods. For processed foods Plant X kit was used and for the raw materials Plant kit was used. All DNA extraction was done in duplicates.

DNA Control…

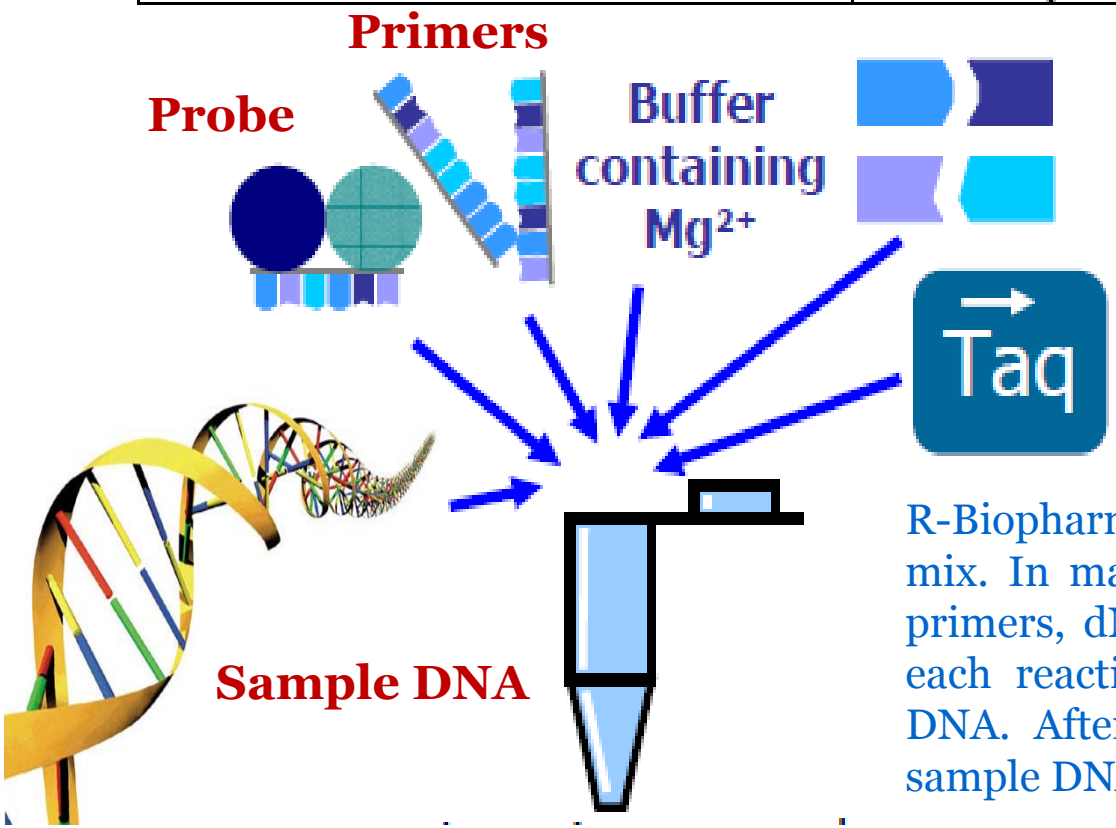
- To exclude false negative GMO screening results through an inefficient plant DNA extraction it is necessary to control the plant DNA concentration. An inefficient DNA extraction will limit the sensitivity of GMO analysis.
- The quality and purity of the extracted DNA were checked by Plant measurements kit ;(Plant Plus) using Real Time PCR for the preventing wrong negative results.

↓

These DNA samples were used for the Real Time PCR analyses.

Master Mix Preparation

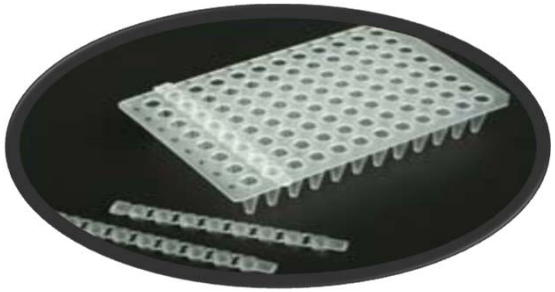
Components for master-mix	Amount per reaction	10 reactions (with 10% excess)
35S Reaction Mix, NOS Reaction Mix, FMV Reaction Mix or Inhibition Control Mix	18.8 µl	206.8 µl
FDE	1.1 µl	12.1 µl
Taq Polymerase	0.1 µl	1.1 µl
Total volume	**20 µl**	**220 µl**

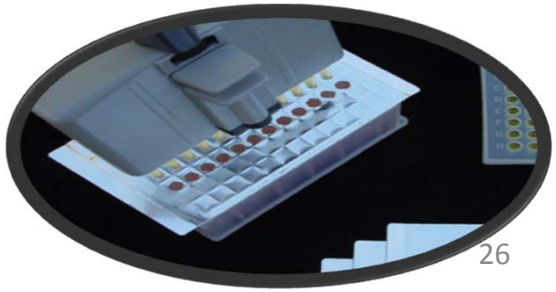


R-Biopharm Sure Food kits were used for master mix. In master mix room, reaction mix (contain primers, dNTP, buffer and etc.) are prepared for each reaction and then mixed with the sample DNA. After the preparation of master mix and sample DNA were processed in **Real Time PCR.**

Real Time PCR Step…

- For each reaction, extaction control, inhibition control, positive control, environmental control and negative control were used to prevent wrong negative and positive results.
- ➢ Extraction control is performing that the DNA extraction procedure except the addition of the test portion.
- ➢ Inhibition Control is reaction mixture that provides the means to monitor PCR inhibition of the assay for the specific sample of the target analyte.
- ➢ Positive Control is known positive sample representative of the sequence or organisms under study.
- ➢ Negative Control is known negative sample not containing the sequence under study.
- ➢ Environmental Control is fof determining that there is no nucleic acid contamination from, the air in the laboratory.





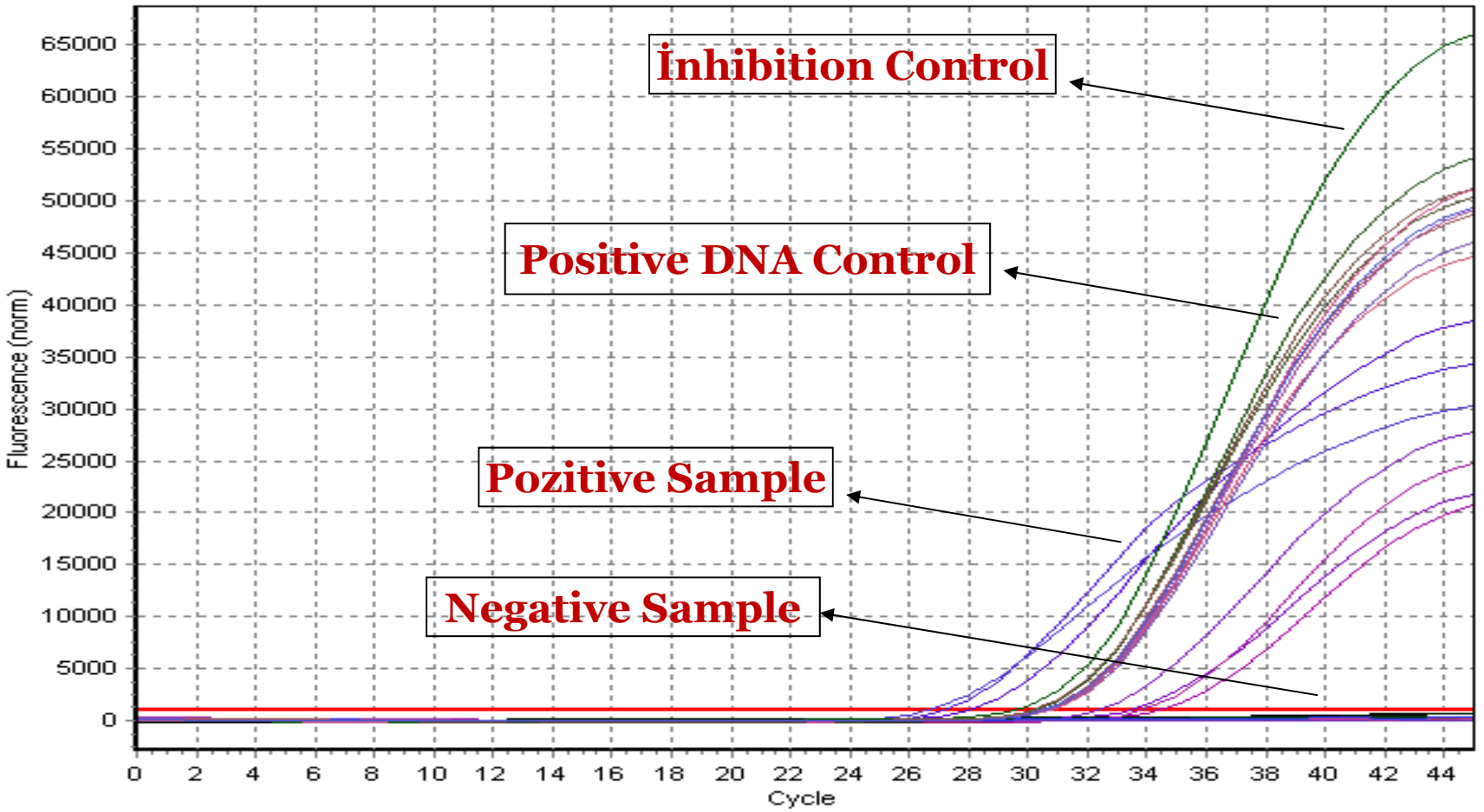
Real Time PCR Set up…

Amplifications were carried out in a Mastercycler ep gradient S termocycler (Eppendorf).

Program	Eppendorf Blockcycler	
Initial Denaturation (HOLD)	5min 95◦C	
Denaturation	15 sec 95◦C	**45 cycles**
Annealing/Extension	30 sec 60◦C	

The real time PCR is used for the amplification and detection of a GMO specific sequence.
This process takes one hour. Totally all analyses including homogenization takes eight hours.
Real Time PCR is a fast and reliable technique to analyze.
The reactions were run in duplicate in a reaction volume of 25 µL, too.

Real Time PCR Results Interpretation



Interpretation of the Real Time PCR Process

Test Sample	Positive Control	Negative Control	Extraction Blank Control	Inhibition Control	Environmental Control	Interpreted result of samples
+	+	-	-	+	-	Positive
-	+	-	-	+	-	Negative
+	+	-	+	+	+	inconclusive*
+	+	-	-	+	+	İnconclusive*
+	+	+	+	+	+	İnconclusive*
-	+	-	-	-	-	İnconclusive*
-	-	-	-	-	-	İnconclusive*
+	-	-	-	+	-	İnconclusive*

*The procedure should be repeated beginning with the extractionstep.

Results and Discussion...

- The importance of the detection of genetically modified organism (GMOs) in food is increasing. The analyses methods should be reliable, sensitive and accurate.

- Real Time PCR is the primary tool for screening, qualitative and quantitative GMO analyses.

- Using the R-Biopharm Plant Extraction kits, high-quality DNA were extracted from the samples except the maize oil.

- For the this screening methods, the detection limit is 5 DNA copies.

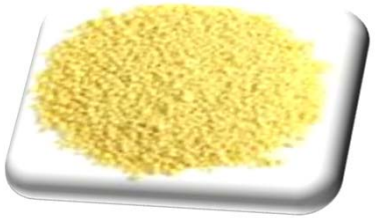
- For the detection metods some control reactions were used and interpretated to prevent wrong negative and positive results according to TS EN ISO 24276 standards.

Results and Discussion...

- In this study, Real Time PCR method was established for a total of 120 samples which specifically amplify the 35 S promoter, NOS terminator and 34 S promoter FMV to screen for the presence of transgenic material.
- No amplification could be observed for all the analysed biscuits, salad sauces and rice samples. These products are not GMOs.
- DNA could not have been isolated from maize oil samples.
- The results showed that 24 out of 120 examined samples were GMO positive.
- These positive samples are: Processed Meat Products, Wafer, Dehydrated Soups, Flour, Maize products, Halvah, Hazelnut spread
- 34S FMV promoter only detected in one halvah, one salami, and one dehydrated soup samples. The others contain 35 S promoter and NOS terminator.

Results of food testing

5 out of 17 wafer samples were positive.



3 out of 7 different kind of flour samples were positive.

3 out of 9 different dehydrated soup samples were positive.

2 out of 37 maize products samples were positive.

Results of food testing

2 out of 5 hazelnut spread samples were positive.

3 out of 9 tahini halvah samples were positive.

6 out of 19 processed meat samples were positive.

Conclusion…

- %20 of the examined samples are GMO positive.
- The results indicate that most of the positive samples contain soy.
- These results demonstrate that the presence of GMOs in the Turkey market in 2009.
- On the basis of our results, GMO screening analyses are requisite all of the imported raw materials and processed foods to provide to be traceable of the food in Turkey market.
- GMOs be traceable throughout the chain from farm to table and provide consumers with information by labeling all food consisting of; containing or produced from GMO.
- GMO testing is needed as a precursor to promote high standards of regulation, tracking developments, acting on new evidence and instituting population health surveillance.

References…

- E. Anklam, F. Gadani, P. Heinze, H. Pijnenburg, G.V. Den Eede, Analytical methods for detection and determination of genetically modified organisms in agricultural crops and plant-derived food products, Eur Food Res Technol 214 (2002) pp 3–26.
- M. Miragliaa,, K.G. Berdalb, C. Breraa, P. Corbisierc, A. Holst-Jensenb, E.J. Kokd, H.J.P. Marvind, H. Schimmelc, J. Rentsche, J.P.P.F. van Ried, J. Zagonf, Detection and traceability of genetically modified organisms in the food production chain, Food and Chemical Toxicology 42 (2004) pp 1157–1180.
- A.K. Deisingh, N. Badrie, Detection approaches for genetically modified organisms in foods, Food Research International 38 (2005) pp 639–649.
- P. Hübner, E. Studer, D. Häfliger, M. Stadler, C. Wolf, M. Looser, Detection of genetically modified organisms in food: critical points for quality assurance, Accred Qual Assur 4 (1999) pp 292–298.
-
- F. E.Ahmed, Detection of genetically modified organisms in foods, TRENDS in Biotechnology 20-5 (2002) pp 215-223.
- K. Taski-Ajdukovic, Z. Nikolic, M. Vujakovic, M. Milosevic, M. Ignjatov, D. Petrovic, Detection of genetically modified organisms in processed meat products on the Serbian food market, Meat Science 81 (2009) pp 230–232.
- E. Gachet, G.G. Martin, F. Vigneau and G. Meyer, Detection of genetically modi®ed organisms (GMOs) by PCR: a brief review of methodologies available, Trends in Food Science & Technology 9 (1999) pp 380-388.
- Turkish Regulation about Genetically Modified Organisms Importation, Production and Exportation Procedure- 26.10.2009-27388.
- www.isaaa.org
- S. Vollenhofer, K. Burg, J. Schmidt, and H. Kroath, Genetically Modified Organisms in Foods Screening and Specific Detection by Polymerase Chain Reaction, J. Agric. Food Chem. 47 (1999) pp 5038-□5043.

Thank You For Listening

AYBAK NATURA

Analiz Lab. Hiz. Tur. İnş. San. Ve Tic.
Adres: Kazım Dirik Mahallesi
Üniversite Caddesi No:114/4
Tel: +090 (232) 4610900
Fax: +090 (232) 4612818
Email: info@naturalab.com.tr;

www.naturalab.com.tr;
www.aybaknatura.com.tr