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# THE EFFECT OF FOOD-PROCESSING CONDITIONS ON DETECTION OF THE *IAP* GENE OF *LISTERIA MONOCYTOGENES* PERFORMED USING POLYMERASE CHAIN REACTION TECHNIQUE (PCR)

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## ABSTRACT

The effect of physicochemical factors: temperature (20°C, 60°C, 100°C, 121.1°C), pH (2-9), inorganic and organic components, i.e. sodium chloride (5%, 10%, 15%, 20%), carbohydrates: glucose and starch (10%, 20%, 40%), proteins: casein (2.5%, 5%, 10%) and their combinations (e.g. prolonged incubation time at low pH) on *in vitro* detection of specific fragment of *L. monocytogenes iap* gene (453 bp) carried out using polymerase chain reaction (PCR) were examined. The possibility of detection of the *iap* gene during apple and tomato processing as well as in their processed products was also tested. Results showed that two factors hindering detection of the *iap* gene were casein at all concentrations and processing applied to apples (initial pH 2.5-3.0) and tomatoes (initial pH 4.0-4.5) as well as prolonged incubation in low pH at 60°C. As the *iap* gene and modified genes incorporated in foods are biochemically and structurally identical at the molecular level, results obtained in the studies may reflect the effect of food-processing conditions on detection of genetically modified food.

**Key words:** *Listeria monocytogenes,* PCR, *iap* gene, food processing, genetically modified foods (GMFs), genetically modified organisms (GMOs)

## INTRODUCTION

Presently genetically modified foods (GMFs) created using tools provided by genetic engineering is more frequently available on the market. It includes e.g. delayed ripening tomatoes, bananas or strawberries, nutritionally modified soybeans, rape, potatoes or wheat as well as herbicide resistant maize, apples or bananas. Not to mention nutraceuticals (including pre- and probiotic foods), e.g. vitamin-enhanced rice, which may be also genetically engineered. Food is also constantly modified to satisfy extravagant consumer requirements and preferences based mainly on attributes such as appearance, taste and freshness, e.g. sweeter strawberries, crisper celery or pipless grapes [9, 11, 17].

The first genetically modified fruit available on the market was FlavrSavr tomato purposed to soften more slowly. In 1997 eleven GMO crops were on sale mainly in the United States but also in Europe, including corn, soy bean, tomatoes, potatoes, chicory, squash, melons, papayas, rape seed, cotton and tobacco. They were modified to increase herbicide tolerance, virus and insect resistance, to obtain slower ripening and male sterility and exclusively in case of rape seed to change the oil composition and rise the level of lauric acid [7]. Presently, there are over 30 different fruits, nuts, vegetables and staples under development [19].

The European Commission has issued Directives, Council Decisions and regulations concerning the placing on the market and the mandatory labeling of food containing GMOs [3]. It is important for ethical, economic and legal reasons. Polish producers and importers usually do not inform consumers if particular food products are prepared using genetically modified ingredients or supplements, although the article 47 introduced in the act from 22$^{nd}$ of June 2001 in Polish Library of Acts clearly orders indication of modified components in traded products. Consumers unaware of that fact may buy food containing transgenic components or supplements (e.g. chocolate, soybean products, cooking oils) or inadvertently feed animals with modified fodder [17]. As products usually are processed in harsh conditions (cooking, frying, pasteurizing, autoclaving, lyophilizing etc.) their processing may affect further detection of modified genes.

Testing of raw materials and foods containing GM components or supplements may be conducted by testing for the presence of incorporated DNA or detecting expressed novel proteins [2]. The presence of modified DNA sequences may be easily detected by application of widely known polymerase chain reaction (PCR) with primers aimed specifically at 'novel' fragments. PCR was efficiently applied for detection of genetically engineered corn in flour, meal and processed food. The reaction was e.g. designed to detect the presence of *cry9C* gene which encodes Cry9C protein, a natural toxin of potential insecticidal activity produced by *Bacillus thuringiensis* [16] or 35S promoter and NOS terminator genes in samples from soy bean and maize flour collected in 13 countries participating in this screening project [3].

PCR is not only adapted to support identification of GM components but also to detect fraud substitutions added to final products. It was used to identify meat species e.g. sheep and goat meats [5, 13] to identify bovine milk in high-quality Italian cheeses intended to contain only buffalo milk [4] or different mollusk species [6].

The aim of the studies was to evaluate if physicochemical factors and processing procedures may influence detection of a selected gene using PCR technique. As modified and non-modified genes are biochemically and structurally identical at the molecular level, results obtained in the studies may reflect the effect of food-processing conditions on detection of genetically modified organisms (GMOs) present in food.

## MATERIALS AND METHODS

Suspensions of *L. monocytogenes* strain ATCC 1577 ($10^8$ CFU/mL) were subjected to following physicochemical factors or factor combinations: temperature (20°C/12 h, 60°C/12 h, 100°C/1 h cooking, 121.1°C/15 min autoclaving; each sample tested at pH 5, 7 and 9), pH (2-9 at 4°C, 20°C and 60°C for 24 h, respectively), inorganic components sodium chloride (5%, 10%, 15%, 20% at pH 7 at 20°C/24 h), organic components including carbohydrates and proteins glucose (10%, 20%, 40%), starch (10%, 20%, 40%) and casein (2.5%, 5%, 10%) tested at pH 7 at 20°C/ 24 h and low pH during prolonged incubation (pH 2-4 at 60°C/20 days). Suspensions in a deionized, sterile water were prepared using a Carl Zeiss spectrophotometer. Each experiment included a blank sample 'a positive control' (a $10^8$- CFU/mL bacterial suspension at pH 7 incubated at ambient temperature for fixed time with no reagents added) to avoid false-negative results caused by mistakes made during DNA extraction and PCR identification. Practical simulations of apple and tomato processing with samples containing *L. monocytogenes* ($10^8$ CFU/mL) were also performed. Table 1 presents a detailed protocol of their processing and sampling.

**Table 1. Conditions applied during apple and tomato processing**

| APPLES + *L. monocytogenes* (pH ~ 2.5 -3.0) | | TOMATOES + *L. monocytogenes* (pH ~ 4.0 - 4.5) |
|---|---|---|
| | COOKING | |
| 100°C/ 30 min* | | 100°C/ 30 min* |
| 100°C/ 60 min* | | 100°C/ 60 min* |
| 100°C/ 90 min* | | 100°C/ 90 min* |
| | STERILIZATION | |
| 121.1°C/ 15 min* | | 121.1°C/ 15 * |

*sample collecting

**DNA extraction, oligonucleotides and PCR assay**

DNA extraction and the *iap gene* detection using PCR was carried out as described previously [14].

## RESULTS AND DISCUSSION

To extract DNA for GMO analyses two methods are commonly used: the CTAB (cethyl-trimethyl-ammonium-bromide) method – the basis for an official German method and the Wizard-extraction method – the official Swiss method [14]. In our studies the Wizard-based method was applied. Obtained results are presented in Table 2.

**Table 2. Factor combination applied in the studies and results obtained**

| Factors | Presence of the expected PCR product (453 bp) |
|---|---|
| Temperature<br>20°C/12 h, 60°C/12 h, 100°C/1h, *121.1°C/15 min<br>(at pH 5, 7, 9) | + |
| pH 2-9 each tested<br>at 4°C/24 h, 20°C/24 h, 60°C/24 h, respectively | + |
| PH, 2 incubated at 60°C for 20 days<br>3-4 incubated at 60°C for 20 days | -<br>+ |
| Sodium chloride<br>5%, 10%, 15%<br>(at pH 7 at 20°C/24 h) | + |
| Glucose<br>10%, 20%, 40%<br>(at pH 7 at 20°C/24 h) | + |
| Starch<br>10%, 20%, 40%<br>(at pH 7 at 20°C/24 h) | + |
| Casein<br>2.5%, 5%, 10%<br>(at pH 7 at 20°C/24 h) | - |
| Fruit and vegetable processing<br>after 30 min, 60 min, 90 min at 100°C<br>followed by 15 min at 121.1°C | - |

* autoclaving

The *iap* gene was detected in incubated, cooked or autoclaved suspensions subjected to pH 5, 7 and 9 at all tested temperatures (20°C/12 h, 60°C/12 h, 100°C/1 h, 121.1°C/15 min). The mechanism of DNA destruction by heat is based on its depurination or deamination. At temperatures above 100°C a significant strand scission and irreversible loss of secondary structure occurs [8]. Temperature conditions applied in our experiments did not destroy DNA template as it was still detectable by PCR, even in case of autoclaving when the temperature effect was supported by pressure action. It confirmed results presented by Masters [12] and Herman [8]. Herman [8] also proved that DNA ability to serve as PCR template was lost only then at 124°C.

Our results showed that detectability of the selected gene was not reduced depending on pH treatment. The *iap* gene was detected in samples incubated at wide range of pH (2-9) at refrigeration temperature as well as in suspensions incubated at 20°C and 60°C for 24 h.

Surprisingly, DNA template was not destroyed during a 20-day incubation at pH 3 and 4 at 60°C. After 20 days detection of the *iap* gene was efficiently inhibited only at pH 2. Although, according to Lindahl [10] exposure to acid causes depurination of DNA, our results may confirm other suggestion made by Herman [8] that pH influence is limited by DNA protection due to cell wall structures. At pH 2 the protective structures seem to be inactivated or destroyed. It is worth mentioning that purified DNA is also acid-resistant to some degree. Prince and Andrus [18] demonstrated that its 5-minute incubation in 6.7% HCl did not affect PCR amplification. Detectability of DNA template after prolonged incubation at low pH suggests that after initial cell lysis and preliminary DNA destruction, the enzymes responsible for DNA degradation (endogenous nucleases) are destroyed quicker than DNA itself and its further breakdown was avoided [8]. Niederhauser et al. [15] indicated that prolonged storage of *L. monocytogenes* strains (over 7 days) at refrigeration or freezing temperatures caused false-negative results of their detection due to cell lysis and DNA destruction. Our results revealed that incubation at 4°C for 24 h did not affect detectability even if supported by a low pH.

Sodium chloride, glucose and starch in applied concentrations did not inhibit detection of the selected gene. Casein at all tested concentration as well as fruit and vegetable processing effectively hindered detection of the gene.

PCR inhibition caused by casein was probably due to DNA structure. As a polynucleotide it may interact with other polymers (like phosphoprotein) forming complex compounds and abolish PCR detection. Limited solubility of casein in water solutions is an additional drawback which reduces application of standard DNA extraction kits based on a column purification as applied in our studies. It was proved by examination of purified DNA by agarose gel electrophoresis which indicated varying degrees of DNA degradation.

Industrial food processing masks and/or destroys DNA template, therefore it reduces possibility to detect gene presence in processed cooked or autoclaved products like jams, marmalades or pastes. Individual components tested have no such drastic influence so the inhibitory effect seems to be reinforced by synergistic interactions among them. For instance, Agersborg et al. [1] indicated that too high concentrations of DNA may inhibit PCR. Our results suggest that processed food matrices are too complex chemically to enable uncomplicated detection of selected genes. It indicates that there is a growing need for sampling protocols precisely aimed at challenges linked to detecting genetically modified components and/or supplements in processed food.

## CONCLUSIONS

1. Simulation of particular food-processing conditions (including the effect of physiochemical factors like temperature, pH, addition of sodium chloride, glucose, starch or factor combinations) enabled to detect the expected fragment of a model gene.
2. Addition of casein, prolonged incubation in low pH as well as experiments on processed fruit and vegetables revealed that amplification of the model gene is inhibited and the results of trials are false-negative.
3. Synergistic interactions within complex food matrices may reduce or inhibit detectability of genes in processed food products hindering their identification via molecular biology methods like PCR.
4. As the gene chosen for our studies is biochemically and structurally identical at the molecular level to genes incorporated into food products (genetically modified foods), it is highly probable that also a detection of genetically engineered food components or/and supplements may cause serious drawbacks if analyzed by means of molecular biology methods.

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