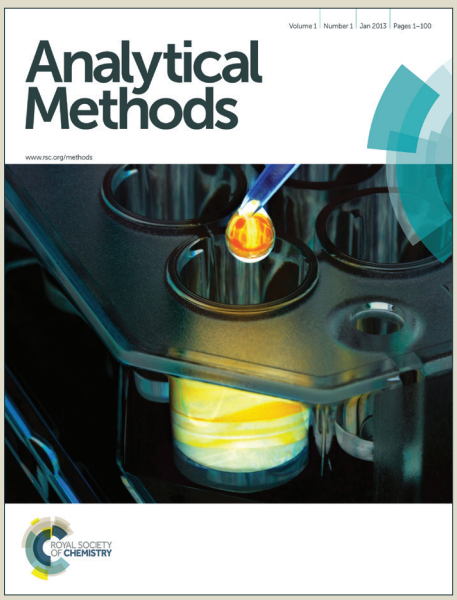
Analytical Methods

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods

SOY DETECTION IN CANNED TUNA BY PCR AND CAPILLARY ELECTROPHORESIS

Roberto Rodríguez-Ramírez[1,2], Belinda Vallejo-Cordoba[1], Miguel A. Mazorra-Manzano[1], & Aarón F. González-Córdova[1]*

1.- Laboratorio de Calidad, Autenticidad y Trazabilidad de los Alimentos.
Coordinación de Tecnología de Alimentos de Origen Animal (CTAOA)
Centro de Investigación en Alimentación y Desarrollo, A.C. (CIAD)
Carretera a La Victoria Km 0.6, Apartado 1735
Hermosillo, Sonora, 83304. Mexico.
2.- Laboratorio de Biotecnología y Trazabilidad Molecular de los Alimentos.
Departamento de Biotecnología y Ciencias Alimentarias.
Instituto Tecnológico de Sonora (ITSON).
5 de Febrero y Chihuahua, Edificio 600 Campus Centro, Cd. Obregón, Sonora 85000. Mexico
E-mail: roberto.rodriguez@itson.edu.mx,vallejo@ciad.mx, mazorra@ciad.mx, aaronglz@ciad.mx

Corresponding author:
Phone: +52 (662) 289-2400 Local 303
Fax: +52 (662) 280-0421
E-mail address: aaronglz@ciad.mx

Manuscript submitted to the Editor of *Analytical Methods*.
October 2014

Analytical Methods Accepted Manuscript

ABSTRACT

Tuna is a commercially important fish species that accounts for a significant proportion of the global fish market. The annual canned-tuna consumption in Mexico reaches 1.6 kg per capita. Consequently, tuna is more than likely to be fraudulently substituted with lower-priced fish species or mixed with soy products. Recently, interest has focused on DNA analysis instead of on protein-based assays. DNA is more thermo-stable than protein and it can be used to analyze processed products such as canned fish. Polymerase chain reaction (PCR)-based methods are frequently used for soy detection in different heat-processed foods. Usually, amplified DNA fragments are separated by conventional electrophoretic methods. As a result, the present study aimed to develop a capillary gel electrophoresis (CGE) method, using laser-induced fluorescence (LIF), to detect soy DNA in canned tuna. The conditions for DNA extraction and PCR were optimized. DNA extraction was carried out using the GENCLEAN® commercial kit protocol with modifications. The PCR products of the constituent gene Le1 (118 bp) were analyzed for the detection of soy in canned tuna. For the *Thunnus albacares* detection, the 350 bp from Cytb gene fragment was used. Results showed that DNA extraction was accurate for different soy percentages since concentration ranged from 1-70 ng/μL (R^2 = 0.99). Additionally, the selected primer for either tuna or soy was shown to be specific by gel electrophoresis, nevertheless some band smearing was shown for canned tuna. On the other hand, the characteristic tuna fragment (350 bp) and soy fragment (118 bp) were unequivocally identified by CGE using the Low DNA mass ladder and ΦX174 RF DNA/*Hae* III standards, respectively. The presence of soy in commercial canned tuna as revealed with these results, is an adulteration and a consumer fraud. Thus, the PCR-CGE method presented is a suitable technique for the semi-quantitative detection of soy in canned tuna. However, further studies are required in order to quantify soy in canned tuna by using quantitative competitive PCR followed by CGE.

Key words: Food authentication, food deoxyribonucleic acid, capillary electrophoresis, soy detection, canned tuna.

1. INTRODUCTION

Food adulteration has been performed over time for different purposes, including cost reduction, increased performance for industrial production, as well as to cover up process malpractices[1]. Despite the existence of several more reasons for food adulteration practice, most of them are equally aimed to generate economic benefits for industrial workers who are engaged in unfair competition towards both the productive sector and the global trade, where such actions represent fraud against consumers[2]. Soy utilization in food is neither new nor expected to decline in usage at any time in the near future. Nevertheless, soy additive types and the amounts used should be regulated in accordance with the standards set by the law[3], as well as in

Analytical Methods Accepted Manuscript

Europe[4-5]. Such restrictions were created to set authenticity parameters, with the goal of alerting individuals sensitive to allergies derived from the intake of soy[6]. Moreover, the use of transgenic soy in several foods has caused controversy among consumers[7-9].

Studies aiming to detect soy adulteration have been documented for the meat industry[3, 10-12]. Most of these food adulterations are performed in order to replace animal proteins with vegetable proteins, resulting a decrease in cost. Another purpose is to utilize either substitutes or meat-type products based on soy because these can expand the shape of a meat product, adopting a meat appearance[13-14]. Consequently, since a high quality product is expected to be consumed, these actions represent fraud against the potential consumer[15-16].

A new law that prohibits the use of plant proteins in meat products came into effect in 1995 in Germany; ever since, their use is meant to be stated in the labeling of foods, turning into a new ability to protect consumers from both fraud and food allergies[17]. Even though soy utilization, either as an additive or as an adulterant, has been rather common for both the meat industry and dairy products[18], marine products have not been exempted from such practice[19]. Some canned marine species in Mexico are mostly prepared from fresh species, including canned tuna[20]. Therefore, the canned tuna industry has engaged in excessive use of soy products (textured), whose function is to rehydrate and expand the fish during the canning process.

The Mexican law NOM-084-SCFI-1994[21] is responsible for the regulation of soy addition, stating a food that contains it shall have it declared so on the list of ingredients, due to the fact that it is associated with being a promoter of allergic reactions. Among the additives permitted for canned fish, in accordance with the NOM-028-SSA1-1993[22] standard regulation, soy is not listed as such; hence, the presence of soy in canned fish without being declared on the label must be regarded as an adulteration.

Most effort appears to have gone into the development of methods to detect and determine soy-based ingredients. Among them, electrophoresis based on proteins has been used to detect and estimate the amount of soy protein in different foods[23]. To circumvent these problems, DNA replace protein due to its stability at high temperatures and consequently a variety of DNA-based techniques were developed. While most methods used conventional gel electrophoresis for the separation of polymerase chain reaction (PCR) products, PCR-based capillary electrophoresis (CE) was the most used for fish and seafood species identification. PCR followed by CE take advantage of the high specificity and sensitivity of the former and the high resolving power and automation of the later[24]. Furthermore, capillary gel electrophoresis (CGE) separation of PCR products was demonstrated to be a powerful analytical technique for the detection of genetically modified organisms (GMOs) [25-27]; food-borne pathogens[28-29] and species identification[30]. Thus, the determination of soy in canned tuna by PCR-CGE appeared to be a good alternative. The present study aimed to develop a CGE method with laser induced fluorescence (LIF) detection in order to detect the presence of soy in canned tuna by means of the analysis of PCR products.

Analytical Methods Accepted Manuscript

2. MATERIALS AND METHODS

2.1. Sampling

Commercial canned tuna samples were obtained from the local market and from different states of Mexico (Guaymas, Sonora; Mexicali, Baja California; Mazatlan, Sinaloa). Fresh tuna *(Thunnus albacares)* samples were provided by the Universidad Autónoma de Baja California Sur (UABCS, La Paz, Baja California, Mexico). Textured soy samples were provided by Nutrimientos y Complementos Alimenticios S.A. de C.V. (NUTRICASA, Mexico, Distrito Federal).

2.2. Preparation of simulated canned tuna with added soy

Tuna mixtures with 1, 5, 10, 25 and 100% (w/w) of textured soy were assessed for the detection of added soy at different levels. A total of 120 g were taken from each of the samples once the mixtures were ready, then 20 mL of water were added followed by a thermal process, simulating tuna canning conditions[19]. Such conditions consisted of submitting the tuna-soy mixtures to a temperature of 120 °C for 20 minutes at 1.1 kg/cm^2 (units of pressure); the samples were then stored at room temperature until use.

2.3. Genomic DNA extraction

Samples DNA extraction was performed based upon the GENCLEAN® (Qbiogene, Pasadena, CA, USA) commercial kit protocol with a few modifications, where a total of 600 µL of lysis mixture (0.1 M EDTA, 0.1 M NaCl, 1% SDS in 0.45 M Tris, pH 8) were added to a 100 mg sample, previously homogenized using an Ultra-Turrax apparatus (IKA®, Staufen, GR). Next, samples were centrifuged at 9279 g for 5 minutes. A total of 100 µL were taken from the supernatant, which were subsequently added to 300 µL of an affinity matrix (Gene Clean Spin Glassmilk) in a new micro tube. Samples were then left to rest for 5 minutes at room temperature and occasionally mixed by inversion.

Next, samples were centrifuged at 9279 g, the supernatant was decanted and the micro tube was washed with 150 µL of washing solution (50% ethanol/physiological saline solution as the solvent), which were centrifuged again at 9279 g at 20 °C; this step was repeated three times. After the third wash, the residual liquid was evaporated for 10 minutes in a Centri Vap (Labconco, CA, USA), and then the pellet (DNA) was resuspended in 50 µL of sterile water (PCR-grade), which was centrifuged at 3921 g for 2 minutes. The supernatant (water) was collected and placed into a new tube for storage (-20 °C).

The DNA in simulated and commercial canned tuna samples, was obtained using the procedure described above, differing only by the fact that the samples were also incubated for 1 h at 60 °C with 20 µL of proteinase K (20 mg/mL). After lysis solution homogenization, the volume of the affinity matrix (Gene Clean Spin Glassmilk) was 400 µL. The DNA concentration in samples was evaluated by spectrophotometry using a Cary BIO 50 spectrophotometer (VARIAN, Palo Alto, CA) at wavelength of A_{260nm} and sample purity was assessed by the $A_{260/280\ nm}$ absorbance ratio. The DNA from samples was quantified using a Synergy MxF

Analytical Methods Accepted Manuscript

Fluorescence Microplate Reader (Bioteck, USA) and the commercial Broad Range Quant-iT™ DNA Assay Kit (Invitrogen, USA) according to the manufacturer's instructions.

2.4. Primer sequences

The primer sequences used for soy detection were set by establishing the constitutive lectin gene (Le1) (amplicon 118 bp) [31] and tuna identification was carried out through the primer sequences of the mitochondrial cytochrome b gene (Cytb) (amplicon 350 bp) reported by Quinteiro *et al.* [32] The different sets of primers were synthesized by Invitrogen (CA, USA); both the sequence and GenBank access number are shown in Table 1.

2.5. PCR DNA amplification

The DNA amplification by PCR for soy constitutive gene and mitochondrial cytochrome b gene for tuna was performed using puReTaq ready-To-Go PCR commercial kit reactions, in accordance with the supplier's specifications, where each reaction had 50 ng of DNA template (1 µL) and 0.5 mM of each primer added to it. The amplification cycles were performed in an Eppendorf Thermocycler™ (San Diego, CA, USA) and programmed in accordance with the parameters presented in Table 2.

Negative controls were included for all PCR reactions in order to confirm the amplification specificity and to discard possible contaminated samples; in other words, no template PCR (H_2O) and PCR with non-specific DNA. A total of 1.5 to 2.5 µL was taken from the PCR sampling products afterwards, to be deposited in vials for their subsequent analysis by capillary electrophoresis.

2.6. Tuna and soy PCR products detection by gel electrophoresis

PCR product determination by gel electrophoresis was performed on precast gels (E-Gel Agarose®) at 1.2% and 2% agarose, in a Power Base chamber (Invitrogen, CA, USA); separation conditions were set by applying 15 kV for 15 minutes, in accordance with the protocol established by the supplier (Invitrogen, CA, USA). The marker used was Low DNA mass ladder and 1 Kb DNA extension ladder. A Kodak camera DC265 (Kodak, USA) was used to store images which were digitized by means of Adobe PhotoDeluxe (Adobe Systems Inc.) software.

2.7. Tuna and soy PCR products detection by Capillary gel electrophoresis (CGE)

Analysis was carried out using PACE-MDQ capillary electrophoresis equipment (Beckman Instruments, Fullerton, CA, USA). Separation was performed on a non-coated capillary (Beckman Coulter) with a total length of 38 cm, an effective length of 28 cm and an internal diameter of 75 µm. The separation buffer was 2-hydroxyethylcellulose (HEC) at 3%, which contained 20mM Tris-HCl, 2.5 µM YOPRO®, 10mM H_3PO_4, and EDTA 2 mM, at pH 7.3. Capillary conditioning was carried out with 0.1 N HCl for 10 minutes before the sample run; the capillary fill sequence among introduced sample was 1% PVA for 2 minutes and separation buffer for 4 minutes, all applied at 30 psi. The temperature and separation constant current were 40 °C at 72 µA, respectively, with inverse polarity.

Analytical Methods Accepted Manuscript

The introduced sample volume was 1.5 to 5 µL for 25 seconds at 0.5 psi; the standard ΦX174 RF DNA/*Hae* III was introduced at a concentration of 250 µg/mL under the same time and pressure conditions as the samples. Detection by laser induced fluorescence (LIF) was performed with excitation at 488 nm and emission at 520 nm.

3. RESULTS AND DISCUSSION

3.1. Genomic DNA extraction and isolation

The quality of the DNA obtained by the 260/280 absorbance ratio for the soy was 1.88-1.92. The concentration obtained for soy was revealed at in the range of 32.5-178.25 ng/µL, thus showing that the method was suitable for DNA extraction in soy, and allowed the amplification of constitutive soy genes.

Using the commercial extraction protocol for the proper analysis of simulated canned tuna with added soy did not make it possible to distinguish DNA bands in the samples and, in some cases, little smearing were revealed; perhaps such behavior was partially due to the thermal treatment that the samples were submitted to, to its high protein content and/or to possible sample contaminants[32-33]. Consequently, it was not possible to either quantify or determine the DNA purity by UV analysis methods. As quantification errors were encountered by UV analysis, and since more precise quantification of the DNA was required, it was decided to quantify DNA by fluorescence, with the goal of more accurately determining the amounts of PCR products and ensuring successful amplification.

The DNA concentration ranges found for the different percentages of added soy ranged from 1 to 70 ng/µL; the standard curve obtained for the determinations provided R^2 = 0.99, i.e. the fluorescence determination was more accurate than the UV method. This could be due to the fact that either the intercalant or the fluorophore directly binds to double stranded DNA molecules, emitting a signal at a certain wavelength, providing more accurate DNA quantification in the sample as a consequence[34]. Is it important to emphasize that knowing DNA concentration in canned food samples was required for running PCR reactions, and this determination was not intended for soy quantitation.

3.2 PCR products detection by gel electrophoresis

Tuna PCR products by gel electrophoresis are shown in Figure 1A (raw) and B (simulated canned), 350 bp from Cytb gene fragments were obtained, consistent with those reported by Bartlett and Davison[35]. Thus, the selected primers in the study were remarkably specific for the tuna analysis. The lack of a band for soy DNA in Figure 1 showed that the primers did not promote unspecific reactions with soy DNA. It is noteworthy that the bands found in the agarose gel for the simulated canned tuna (Figure 1B) were weaker than those from raw tuna (Figure 1A); this could be due to the factors mentioned above with regards to degradation and to the smearing that is obtained with gel electrophoresis of canned food samples[32].

Analytical Methods Accepted Manuscript

Soy PCR products by gel electrophoresis are shown in Figure 2A. The lectin gene detection (Le1) was observed as amplification fragments with a size of 118 bp sizes in soy; such fragments are consistent with those reported by the Official Collection of Test Methods and by Querci *et al* (2006). Lectin is a minor protein found in soy; nevertheless, this gene has been adopted in most conventional methods to detect both conventional and transgenic soy[36-37]. Le1 gene primer specificity was verified by having them tested with raw tuna samples (Figure 2B), as well as with simulated canned tuna with added soy. The sample of raw tuna showed negative amplification with the soy gene primers, whereas the blends containing soy had the fragment of interest amplified without non-specific reactions. Soy was detected in eight from 30 commercial canned tuna samples.

3.3. PCR products detection by CGE

Characteristic electrophoresis patterns were obtained for the ΦX174 RF DNA/*Hae* III, Low DNA Mass Ladder standard, as well as for the PCR products from soy, simulated and commercial canned tuna, as well as their respective amplification genes used in the study hereby. A signal of 20 relative fluorescence units (RFU) was observed (Figure 3) in the electrophoretic patterns obtained for the molecular weight markers, which was considered acceptable in terms of sensitivity for the analytical method, according to García-Cañas *et al* (2004).

It is noteworthy that signals up to 100 RFU for molecular markers were obtained in some experiments performed under different YOPRO® concentrations, but due to poor peak resolution and reproducibility in the migration time, it was decided to focus on signals ranging from 20 to 30 RFU. Table 3 shows migration times for the ΦX174 RF DNA/*Hae* III and Low DNA Mass Ladder molecular weight standards, where the total run times of 12.97 and 13.42 minutes respectively were observed. The method was reproducible since the standard deviation was lower than 0.13 and the variation coefficient was lower than 1.1 for migration times. The prediction equation for the base pairs (molecular weight) was obtained by plotting the reciprocal of the migration time against the base pair logarithm obtained by means of the following equation: $y = -21.042x + 4.6302$,with $R^2 = 0.9772$ ($n = 5$). High reproducibility was also observed for the low DNA mass ladder marker with regards to migration times, where standard deviation was lower than 0.1 and the variation coefficient was lower than 0.9; the prediction equation was $y = -24.05x + 4.9334$ with $R^2 = 0.99$ ($n = 5$). Thus, this method was useful for detecting and unequivocally identifying the fragments of interest 118 and 350 bp.

The characteristic tuna fragment (350 bp) was identified at 10.3 minutes by using the low DNA mass ladder standard. Similarly, this fragment was clearly detected in simulated canned tuna. On the other hand, smeared bands were obtained by gel electrophoresis (Figure 1B), which could result in the misinterpretation of the data. Detection by capillary electrophoresis was more reliable than gel electrophoresis, as the peak was clearly defined and the PCR product (350 bp) migration times was consistent when compared with the standard.

Analytical Methods Accepted Manuscript

A typical CGE electropherogram showing soy PCR product at 8.0 minutes of migration time is depicted in Figure 4A. The characteristic soy fragment (118 bp) was identifying by using ΦX174 RF DNA/*Hae* III standard. Figure 4B depicts the negative control showing the absence of peaks at 8.0 minutes.

Figure 5 shows a typical CGE electropherogram for simulated canned tuna with different soy percentages. The characteristic soy fragment (118 bp) peak increased it size proportionally to the soy percentage. Thus, the develop method was usefully for soy detection over a wide substitution range.

The electrophoretic pattern of a commercial canned tuna sample showing the presence of soy is depicted in Figure 6. Similarly, seven more samples were positive for soy by this method. Therefore, the presence of soy as revealed with these results, which if not declared, represents an adulteration and consumer fraud. These findings agree with and reaffirm the report made by the Mexican Consumer Protection Agency (PROFECO) in 2005, based on the presence of soy in commercial canned tuna.

4. CONCLUSIONS

The use of soy addition as an adulterant in commercial canned tuna became evident in this study; such actions may end up harming the consumer. The method developed in the present study may be helpful for regulatory agencies in Mexico, since there is a lack of effective analytical tools to help maintain regulatory control of this sort of product. However, further studies are required in order to quantify soy in canned tuna using quantitative competitive PCR (QC-PCR) followed by CGE.

5. ACKNOWLEDGEMENTS

The technical assistance of Maria del Carmen Estrada Montoya and Hector Arvayo-Enríquez were gratefully acknowledged.

6. REFERENCES

[1] C. Cordella, I. Moussa, A.C. Martel, N. Sbirrazzuoli, L.L. Cuvelier, *J. Agric. Food Chem.,* 2002, 50, 1751-1764.

[2] J.M. Dennis, *The Analyst,* 1998, 123, 151R-156R.

[3] J. Belloque, M.C. García, M. Torre, M.L. Marina, *Crit. Rev. Food Sci. Nutr.,* 2002, 42, 507-532

[4] Directive 2000/13/EC. *Official J. European Community*, L109:29–42.

[5] Directive 2003/89/EC. *Official J. European Community,* L308: 15–18.

[6] R.E. Poms, C.L. Klein, E. Anklam, *Food Additives and Contaminants*, 2004, 21, 1-31.

[7] L-T. Lau, R.A. Collins, S-H. Yiu, J. Xing, A.C.H. Yu, *Food Control*, 2004, 15, 471-478.

[8] T. Abdullah, S. Radu, Z. Hassan, J.K. Hashim, *Food Chem.,* 2006, 98, 575-579.

[9] O. Arun-Ozgen, F. Yılmaz, K. Muratoğlu, *Food Control*, 2013, 32, 524-531.

Analytical Methods Accepted Manuscript

[10] A.F. González-Córdova, A.M. Calderón de la Barca, M. Cota, B. Vallejo-Cordoba, *Food Sci. Technol. Int.,* 1998, 4, 257-262.

[11] F. Castro, M.C. García, R. Rodríguez, J. Rodríguez, M.L. Marina, *Food Chem.,* 2007, 100, 468-476.

[12] M. Espiñeira, B. Herrero, J.M. Vieites, F.J. Santaclara, *Food Additives & Contaminants: Part A,* 2010, 27, 426-432.

[13] J. Endres, (2001). Champaign, IL: AOCS Press (Chapter 6).

[14] S.J. Koppelman, C.M.M. Lakemond, R. Vlooswijk, S.L. Hefle, *J. AOAC Int.,* 2004, 87, 1398-1407.

[15] I.D. Lumley, Food Authentification, Blackie Academic & Professional-Chapman & Hall, London, 1996, pp. 108-139.

[16] A. Macedo-Silva, M. Shimokomaki, A.J. Vaz, Y.Y. Yamamoto, A. Tenuta, *J. Food Composition and Analysis*, 2001, 14, 469–478.

[17] O. Mellenthin, R. Galensa, *J. Agric. Food Chem.*, 1999, 47, 594-602.

[18] J. López-Tapia, M.R. García-Risco, M.A. Manso, R. López-Fandiño, *Journal of Chromatography A*, 1999, 836, 153-160.

[19] PROFECO. El laboratorio PROFECO reporta. Atún enlatado. *Revista del Consumidor*, 2005, 337, 34-41.

[20] ICEX. Dirección de Análisis y Estudios Económicos, 2002. Captura de túnidos (atún y Similares) http://www.icex.es.

[21] Mexican Ministry of Commerce and Industry. Regulations: NOM-084-SCFI-1994, Diario Oficial de la Federación: México, Marzo 29, 1994.

[22] Mexican Ministry of Health. Regulations: NOM-028-SSA1-1993. Diario Oficial de la Federación: México, Abril 14, 1994.

[23] B. Vallejo-Cordoba, A. F. González-Córdova, M. A. Mazorra-Manzano, R. Rodríguez-Ramírez, *J. Sep. Sci.,* 2005, 28, 826-836.

[24] R. Rodríguez-Ramírez, A. F. González-Córdoba, B. Vallejo-Cordoba, *Anal. Chim. Acta,* 2011, 685, 120-126.

[25] V. García-Cañas, R. González, A. Cifuentes, *J. Agric. Food Chem.,* 2002, 50, 1016-1021.

[26] V. García-Cañas, R. González, A. Cifuentes, *J. Agric. Food Chem.,* 2002, 50, 4497-4502.

[27] V. García-Cañas, A. Cifuentes, R. González, *Anal. Chem*., 2004, 76, 2306-2313.

[28] B. Alarcón, V. García-Cañas, A. Cifuentes, R. González, R. Aznar, *J. Agric. Food Chem.,* 2004, 52, 7180-7186.

[29] G.W. Shin, H.S. Hwang, B. Chung, G.Y. Jung, *Electrophoresis*, 2010, 31, 2137-2153.

[30] J.F. Hernández-Chávez, A.F. González-Córdova, R. Rodríguez-Ramírez, B, Vallejo-Cordoba. *Anal. Chim. Acta,* 2011, 708, 149-154.

Analytical Methods Accepted Manuscript

[31] Official collection of test methods in accordance with article 35 lmbg, classification no. L 23.01.22-1. (1998). German Federal Food Act- Food Analysis, Beuth, Berlin Köln. March 1998.

[32] J. Quinteiro, C.G. Sotelo, H. Rebién, I. Medina, R.I. Perez-Martin, M. Rey-Mendez, I.M. Mackie, *J. Agric. Food Chem.,* 1998, 46, 1662-1669.

[33] T. Bauer, P. Weller, W.P. Hammes, C. Hertel, *European Food Research and Technology*, 2003, 217, 338-343.

[34] K.M. Ririe, R.P. Rasmussen, C.T. Wittwer, *Anal. Biochem.,* 1997, 245, 154-160.

[35] S.E. Bartlett, W.S. Davidson, *Can. J. Fish Aquatic Sci.,* 1991, 48, 309-317.

[36] R. Greiner, U. Konietzny, A.L.C.H. Villavincencio, *Food Control*, 2005, 16, 753-759.

[37] M. Querci, M. Maretti, M. Mazzara, In: Qualitative Detection of MON810 Maize, Bt-176 Maize and Roundup Ready® Soy by PCR, in. The Analysis of Food Samples for the Presence of Genetically Modified Organisms. Querci M., Jermini M., Van den Eede G., editors. European Communities; Luxemburg: 2006. Session 9.

[38] V. García-Cañas, R. González, A. Cifuentes, *TrAC Trends Anal. Chem*., 2004, 23, 637-643.

Analytical Methods Accepted Manuscript

Figure Captions

Figure 1. Agarose gel electrophoresis at 2% of tuna (*Thunnus albacares*) and soy PCR products. **A**) Tuna raw muscle. Lane 1: soy DNA, 2: Tuna and 3: Low DNA mass ladder marker. **B)** Thermally processed tuna muscle: Lane 5: Low DNA mass ladder marker; 6-8 thermally processed and 9: soy DNA. **C)** Le1 gene amplification (118 bp). Lane 1: MW marker: Sigma 100 bp. Lanes: 2, 3 and 5: soy DNA; Lane 4: negative control (without DNA).

Figure 2. Le1 gene primer specificity: agarose gel electrophoresis at 1.2% showing PCR primer product Le1 in raw tuna muscle. Lane 1: Low DNA mass ladder marker; 2 and 5: positive control (soy DNA), 3: raw tuna muscle (primers Le1); 4: negative control (without DNA).

Figure 3. Typical electropherograms of molecular markers using HEC at 3%. A) Standard Hae III (72-1353 bp), B) Standard Low Mass (100-2000 bp). Buffer: 20mM Tris-HCl, 2.5 µM YOPRO®, 10mM H_3PO_4, 2 mM EDTA at pH 7.3.Introduced sample: 25 seconds. Current: 72 µA. Temperature: 40°C. Detection: LIF 488nm (excitation), 520nm (emission) Capillary: uncoated, 75 µL DI, 38 cm total length, 28 cm effective length. Polarity: inverse.

Figure 4. Le1 gene amplification shown by typical electropherograms. **A)** Soy (Le1 primers). **B)** negative control soy: water (Le1 primers). Separation conditions were to those used in Figure 6, dNTPs = deoxyribonucleoside triphosphates (variable scale by printing effect).

Figure 5. Typical electropherograms showing Le1 gene amplification in water tuna-soy mixtures thermally processed with different addition percentages. Separation conditions were to those used in Figure 6, dNTPs = deoxyribonucleoside triphosphates.

Figure 6. Typical electropherograms showing Le1 gene amplification in a commercial tuna sample. Separation conditions were to those used in Figure 6, dNTPs = deoxyribonucleoside triphosphates.

Analytical Methods Accepted Manuscript

Table 1. Primers used to detect soy in tuna (*Thunnus albacares*) products.

Primers	Sequences	Gen	GenBank access number
GMO3 (Forward)	5'-GCCCTCTACTCCACCCCCATCC-3'	Lectin (Le1)	K00821
GMO3 (Reverse)	5'-GCCCATCTGCAAGCCTTTTTGTG-3'	Lectin (Le1)	K00821
CytBL (Forward)	5'-CCATCCAACATCTCAGCATGATGAAA-3'	Cytochrome b mt	AB101291
CytBH (Reverse)	5'-CCCCTCAGAATGATATTTGTCCTCA-3'	Cytochrome b mt	AB101291

Official Collection of Test Methods, 1998 and Quinteiro *et al.*, 1998.

Analytical Methods Accepted Manuscript

Table 2. PCR amplification conditions

Parameter	Gene: Gen lectin (Le1)		Gene: Cytochrome b mt	
Initial denaturation	95 °C	3 min		
Denaturation	95 °C	30 sec	92 °C	45 sec
Annealing	63 °C	30 sec	50 °C	60 sec
Extension	72 °C	30 sec	72 °C	60 sec
Number of cycles	40		30	
Final extension	72 °C	3 min		

Analytical Methods Accepted Manuscript

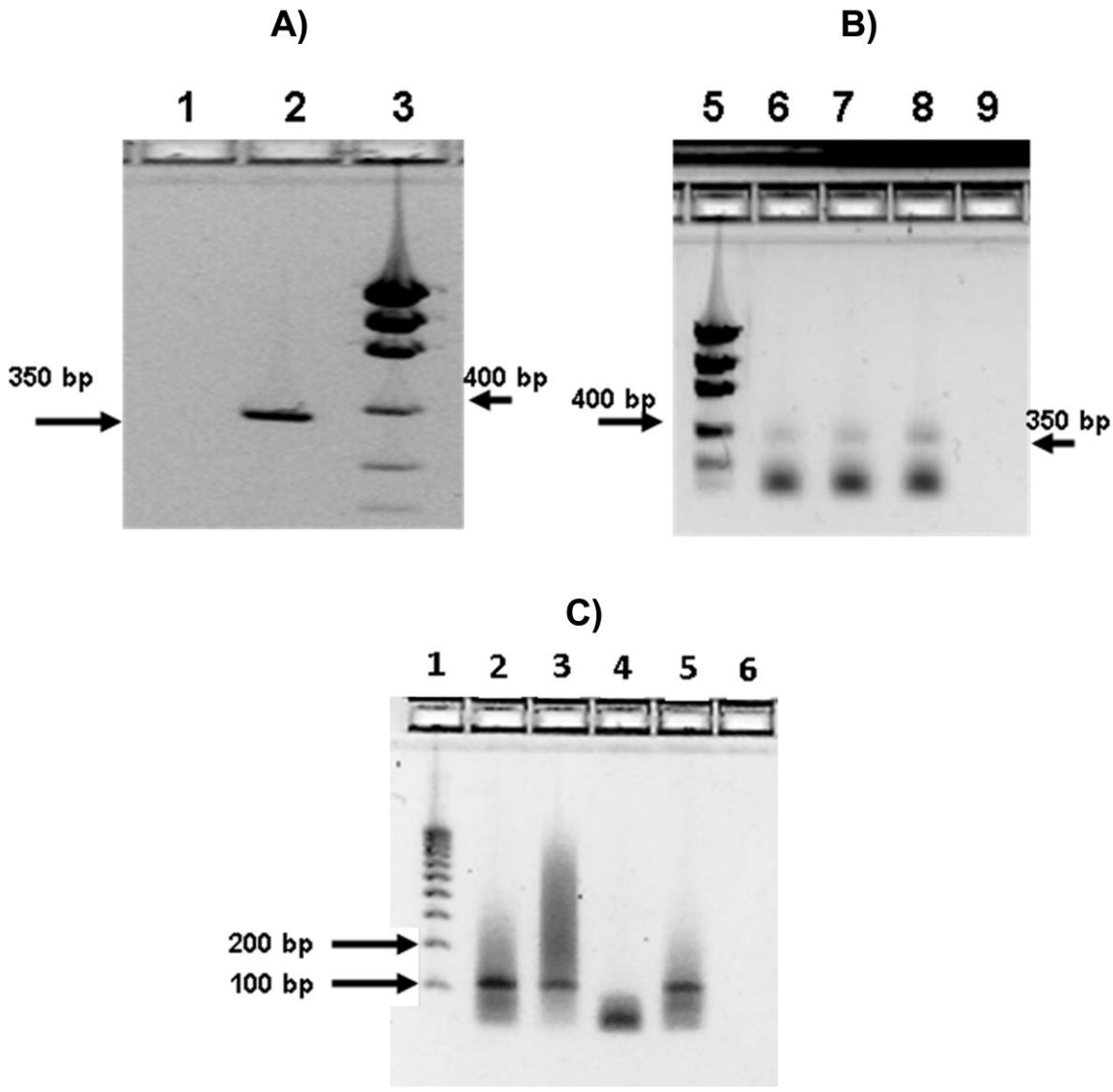
Table 3. Migration times and reproducibility of the molecular weight markers used (n = 5)

ΦX174 RF DNA/*Hae* III											
bp	72*	118	194	234	271	281	310	603	872	1078	1353
Mt	7.22	8.01	9.00	9.39	9.81	10.06	10.20	11.80	12.44	12.71	12.97
SD	0.03	0.06	0.07	0.07	0.05	0.044	0.11	0.10	0.11	0.10	0.12
CV	0.54	0.86	0.088	0.78	0.60	0.44	1.10	0.86	0.95	0.85	0.94
Low DNA mass ladder (100-2000 bp)											
bp	100	200	400	800	1200	2000					
Mt	7.76	9.20	11.03	12.49	12.98	13.42					
SD	0.03	0.06	0.07	0.07	0.05	0.044					
CV	0.54	0.86	0.088	0.78	0.60	0.44					

bp= base pair, Mt = migration time (minutes) SD = standard deviation, CV = coefficient of variation

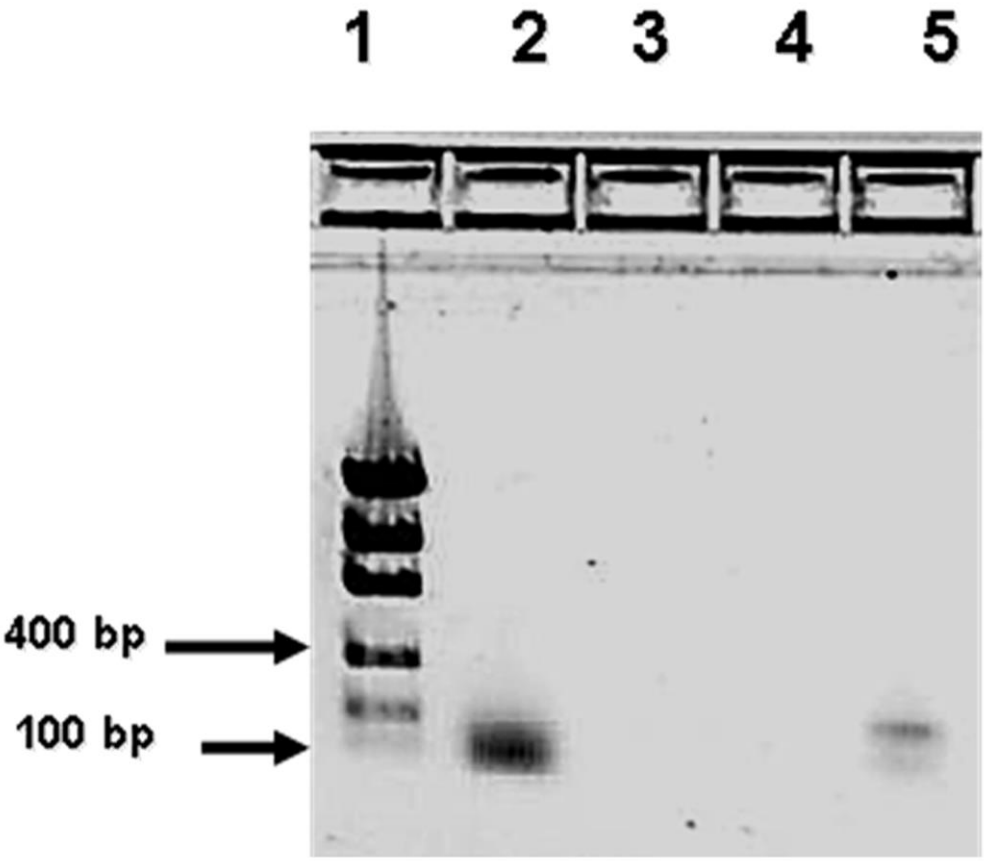
Analytical Methods Accepted Manuscript

Figure 1



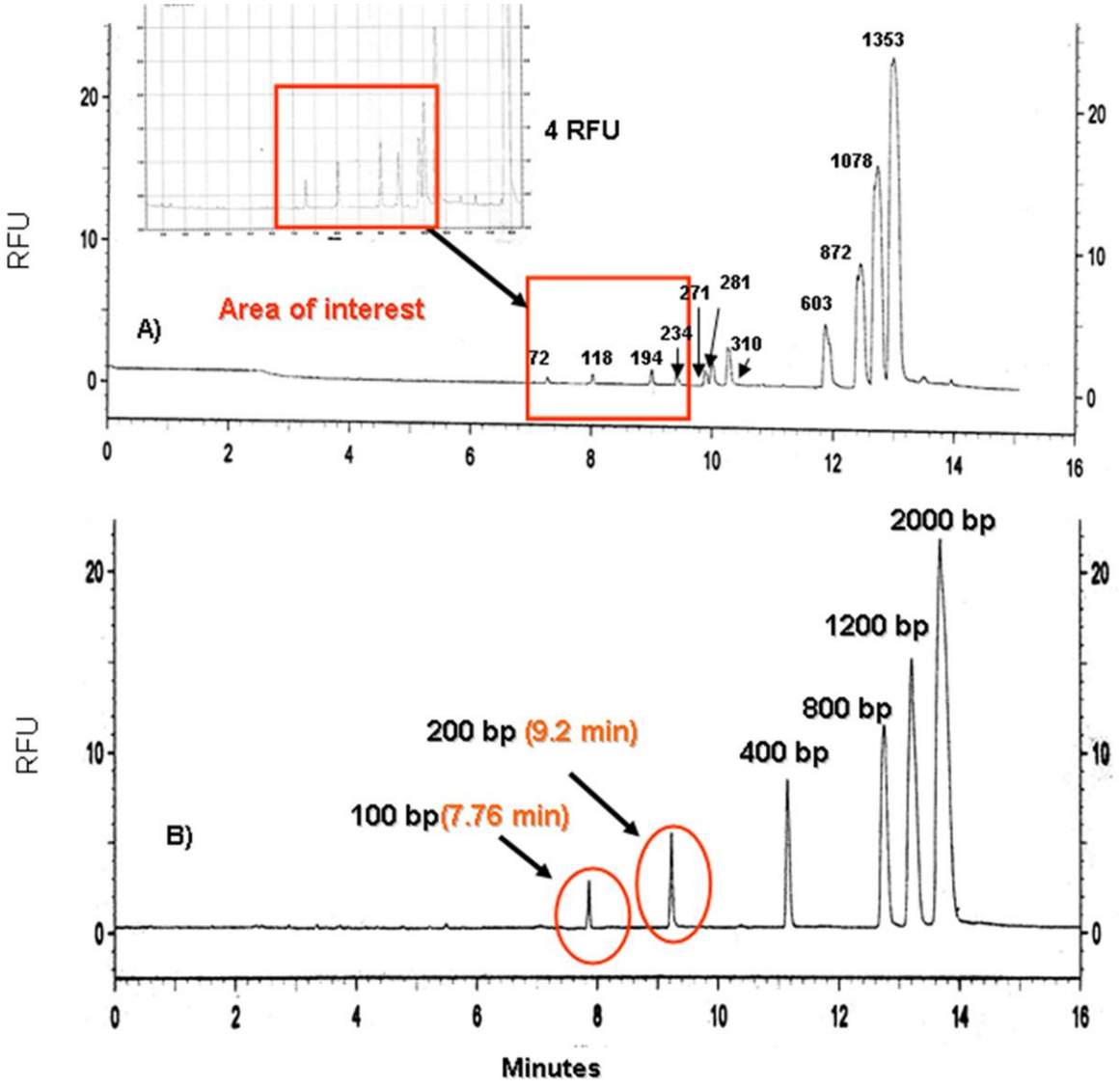
Analytical Methods Accepted Manuscript

Figure 2



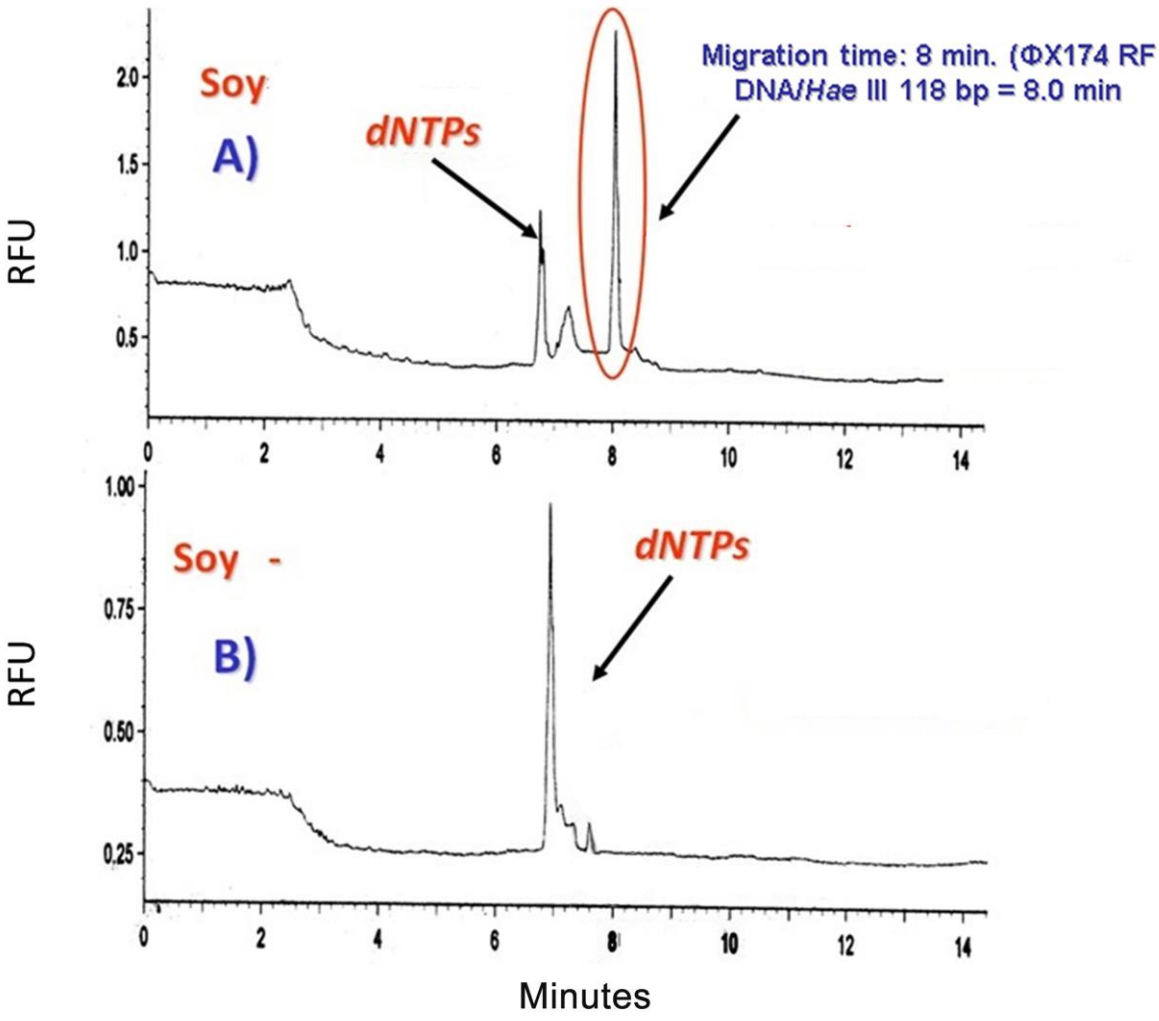
Analytical Methods Accepted Manuscript

Figure 3



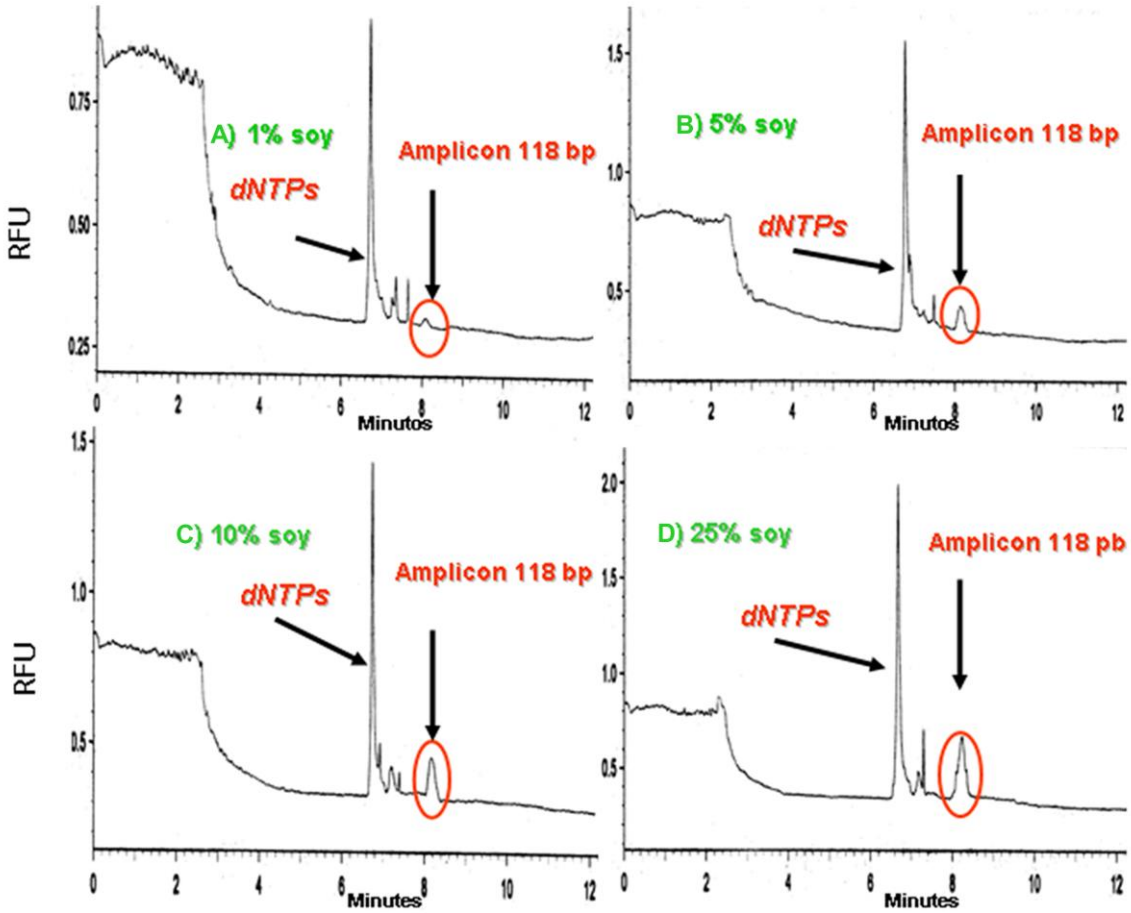
Analytical Methods Accepted Manuscript

Figure 4



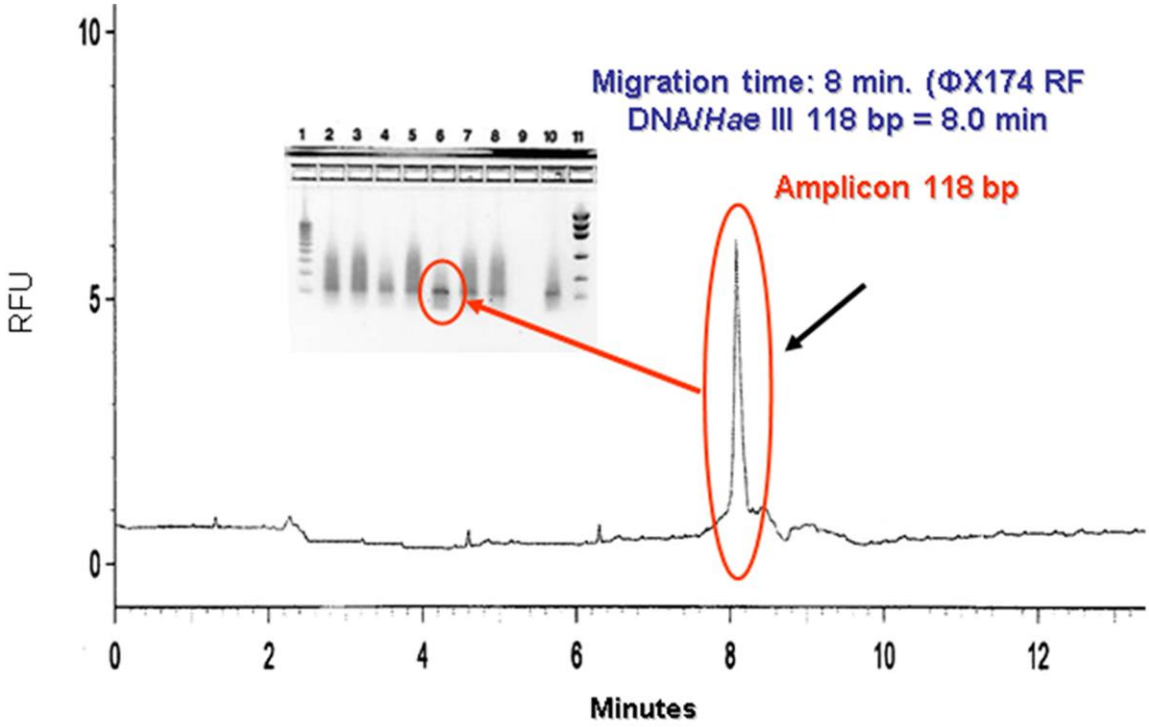
Analytical Methods Accepted Manuscript

Figure 5



Analytical Methods Accepted Manuscript

Figure 6



Analytical Methods Accepted Manuscript