Food Chemistry 127 (2011) 834–841

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Development of a real-time PCR method for the simultaneous detection of soya and lupin mitochondrial DNA as markers for the presence of allergens in processed food

Antonio M. Gomez Galan [a], Marcel Brohée [a], Eugénia de Andrade Silva [a], Arjon J. van Hengel [b], Hubert Chassaigne [c,*]

[a] European Commission, Joint Research Centre, Institute for Reference Materials and Measurements, Retieseweg 111, B-2400 Geel, Belgium
[b] European Commission, Research Directorate General, Rue du Champ de Mars 21, B-1050 Brussels, Belgium
[c] European Commission, Joint Research Centre, Institute for Health and Consumer Protection, Via Enrico Fermi 2749, I-21027 Ispra, Italy

ARTICLE INFO

Article history:
Received 13 August 2010
Received in revised form 7 December 2010
Accepted 1 January 2011
Available online 9 January 2011

Keywords:
Food allergens
Peanut
DNA isolation
Mitochondrial DNA
Real-time PCR
Duplex method

ABSTRACT

Lupin and soya are members of the *Leguminosae* family which are recognised as some of the richest source of vegetable proteins. Lupin- and soya-containing products are available on the EU market and could cause severe adverse reactions in allergic individuals, even if consumed at low concentrations. In this context the development of methods for reliable detection of these allergens in food products is a useful tool for the surveillance of established legislation on food labelling within the EU. This work described the development of a duplex real-time PCR method allowing the simultaneous detection of traces of lupin and soya in processed food based on a specific TaqMan® probe designed on a mitochondrial tRNA-MET gene. A set of primers and probes was designed for the amplification of a 168 and 175 bp fragment of lupin and soya mitochondrial DNA, respectively. The performance of the method was established using lupin and soya flours and cookies baked from lupin- and soya-containing dough (different concentrations and baking times). The PCR platform yielded consistent and repeatable results. The specificity of the system was tested with DNA from 28 plant species. The sensitivity of the method was suitable to detect allergenic ingredients in the low mg per kg range. Both lupin and soya at a level of 2.5 mg per kg food matrix could be detected in cookies baked at 180 °C for 10 min. The method was successfully applied to bakery (e.g. bread) and vegetarian (e.g. non-meat sausages) food products that contain or may contain soya and/or lupin as ingredient or contaminant (according to the declaration on the product label).

1. Introduction

In industrialised countries, food allergies represent a health problem of considerable and increasing relevance. Food allergies affect up to 2% of the adult population and up to 8% of children (Hughes & Mills, 2001). Allergens are either deliberately used as ingredients or are present as contaminants in food products. To date, the only effective method to prevent adverse reactions to food allergens is strict allergen avoidance, requiring correct and integral declaration of products and the availability of products not inadvertently contaminated with allergens. The problem of the detection of declared or hidden allergens in food is a major concern for both the food industry and consumers. The most significant cause of uncontrolled exposure to food allergens is incorrect labelling or contamination of a product that the consumer has considered safe.

Among food allergies, lupin and soya allergies are becoming an important public health concern because it affects an increasing number of children and adults in Europe (Hieta, Hasan, Mäkinen-Kiljunen, & Lammintausta, 2009; Lindvik, Holden, Løvik, Cvancarova, & Halvorsen, 2008; Reis et al., 2007; Shaw, Roberts, Grimshaw, White, & Hourihane, 2008) and the ingestion of even minute amounts of lupin or soya, often found as declared ingredients or contaminants in processed foods, may trigger allergic reactions including digestive disorders, respiratory (rhinitis and asthma) and skin reactions (urticaria and atopic dermatitis) (Poms, Anklam, & Kuhn, 2004; Łoza & Lampart-Szczapa, 2008). The estimated prevalence of soya allergy ranges from 0.5% in the general population to 3–6% in children (Article 6, 2000). The number of allergic reactions to lupin and soya in the general EU population is unknown (Ballmer-Weber et al., 2007), although a growing incidence of legume allergy is observed because of its increasing consumption

* Corresponding author. Tel.: +39 033278 9849; fax: +39 033278 9303.
E-mail address: hubert.chassaigne@ec.europa.eu (H. Chassaigne).

0308-8146/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2011.01.019

(Holden, Fæste, & Egaas, 2005; Peeters, Nordlee, Hefle, Bruijnzeel-Koomen, & Knulst, 2005).

Therefore, EU legislation has been issued to inform the consumer about the content of food products by means of a mandatory labelling of food ingredients. The presence of the most important potential allergens needs to be declared on the label of food products whenever they are used as ingredients as required by Directive 2000/13/EC (2000), which was amended by (Directive 2003/89/EC (2003). Lupin and soya are recognised as food allergens by the European Food Safety Authority (EFSA) and belong to the EU food allergen list. While soya was included in the EU foodstuffs allergen list from its origin in 2000, mandatory declaration of lupin was first required by Directive 2006/142/EC, (2006) and was maintained in the recent Directive 2007/68/EC, (2007).

Thus, as far as allergenic contaminants are concerned, there are no legal concentration limits to date. There are only indications that there are levels of allergens (thresholds) below which an allergen poses only a small risk of causing harm to an allergic consumer (Yanez, Ivnovic, Owen, & Ballester, 1983). Due to the variation in individual responses it is not possible to say what constitutes a safe level. To date no threshold level for lupin and soya bean has been established. A study of the severity of soya allergy and the threshold level of soyabean protein inducing allergy symptoms established threshold doses ranging from 10 mg to 50 g of soya for subjective symptoms and from 454 mg to 50 g for objective symptoms (Ballmer-Weber et al., 2007). Regarding lupin, a recent study of the Allergy Vigilance Network (Gayraud et al., 2009) determined the reactivity threshold to lupin to be low, between 625 and 965 mg, which may correspond to amounts found in market products (few mg of soya or lupin per kg final product).

Soya (*Glycine max*) and lupin (*Lupinus*) are recognised as valuable sources of vegetable proteins. They are used by the food industry for the production of bakery products (e.g. bread and cookies), pastry, pasta, sauces, and vegetarian products (e.g. non-meat sausages). The main reasons for possible hidden allergens in food are contamination from previously or simultaneously produced products in the same factory. Contamination of food by either lupin or soya may take place at the stage of the production of the final product. Since lupin and soya are used by the food industry in the same class of products the simultaneously detection of soya and lupin ingredients in a single assay is of great interest.

Reliable methods must be put in place for the specific and sensitive detection of potential allergens in food to ensure compliance with food-labelling regulations and to provide consumer protection (van Hengel, 2007). Because allergens are specific proteins within a food, they are the primary analytes to be targeted.

Immunochemical methods, which exploit the specificity and affinity interactions of antibodies with proteins, such as enzyme-linked immunosorbent assays (ELISAs) have therefore been favoured in allergen detection (Kerbach et al., 2009). An alternative approach which detects the presence of the allergenic food species uses sensitive and specific DNA-based methods like real-time polymerase chain reaction (real-time PCR). The development of methods based on the detection of DNA sequences encoding for allergens or a comparable protein is becoming increasingly popular. The effect on DNA during food processing steps (such as heat treatment) is relatively minor compared to the effect on the expressed protein (Bauer, Weller, Hammes, & Hertel, 2003). Real-time PCR technique has been successfully used to detect genomic DNA from members of the legume family, lupin (Demmel, Hupfer, Hampe, Busch, & Engel, 2008; Scarafoni, Ronchi, & Duranti, 2009), soya (Abdullah, Radu, Hassan, & Khair Hashim, 2006; Gryson, Messens, & Dewettinck, 2008; Pedersen et al., 2008), pea (Brežná, Hudecová, & Kuchta, 2006) and peanut (Hird, Lloyd, Goodier, Brown, & Reece, 2003; Scaravelli, Brohee, Marchelli, & van Hengel, 2008) in food matrices. Limits of detection could be established for some allergens, including soya and lupin, and were in the range of 10–50 mg allergen per kg of food for soya (Köppel et al., 2010) and of 1000 mg allergen per kg of food for lupin (Scarafoni et al., 2009).

Duplex or multiplex PCR methods can be a valuable tool to indicate the presence of allergens if multi-target screening of several allergenic foods is required, even if many allergen combinations are unlikely to be present in certain food products. Previous studies using a duplex real-time PCR approach have been successfully used to simultaneously detect sesame and hazelnut in food (Schöringhumer, Redl, & Cichna-Markl, 2009). More recently two tetraplex real-time PCR assays have shown positive identification of hazelnut, peanut, celery, soya, sesame, milk, almonds and egg at levels of 10–100 mg allergen per kg of food (Köppel et al., 2010). To our knowledge, no simultaneous method for soya and lupin detection in food has been developed. In addition, the detection limits of individual methods are not sufficient for the detection of potentially hazardous lupin and soya residues of undeclared allergens in food. As members of the legume family, lupin and soya have been shown to have allergenic cross-reactivity (Guarneri, Guarneri, & Benvenga, 2005; Moneret-Vautrin et al., 1999; Peeters et al., 2007), therefore the development of a novel, sensitive and specific real-time PCR for the simultaneously detection of lupin and soya in processed foods is of valuable interest to adequately protect lupin or soya allergic patients.

In our previous work we used genomic DNA for the specific detection of lupin traces in a model cookie matrix and in food products currently available on the market (Gomez Galan, Brohee, Scaravelli, van Hengel, & Chassaigne, 2010). The α-conglutin method allowed the detection of 10 mg lupin flour per kg food matrix in cookies baked at 180 °C for 10 min. In this work the selection of mitochondrial DNA (mtDNA) over genomic DNA as the target for the detection of lupin and soya is advantageous because its presence in multiple copies per cell increases the probability of achieving a positive result, even in the case of samples undergoing partial DNA fragmentation due to severe processing conditions (Verkaar, Nijman, Boutaga, & Lenstra, 2002). Large phylogenetic variability of mtDNA compared with nuclear sequences, which undergo a less rapid evolution, is likely to facilitate authenticity studies (Parties et al., 2000).

We report here the development of a duplex real-time PCR method for the simultaneous detection of soya and lupin specific mtDNA sequences in a heat treated model food matrix and in commercial food products. In this study, TaqMan® primers and probes were designed for amplification and detection of the target DNA. Two real-time PCR assays targeting DNA sequences coding for mitochondrial gene for initiator tRNA-MET in the case of lupin, and a sequence almost identical of soya, obtained via inverse PCR, have been developed in-house. Cookies containing both soya and lupin were prepared as a heat treated model matrix material, based on a recipe already used in our previous work (Gomez Galan et al., 2010). The objective of this work was to develop a method with detection limits for lupin and soya in the low mg ingredient per kg range of food product.

2. Materials and methods

2.1. Lupin and soya flours

Raw lupin flour from *Lupinus angustifolius* species (FRALU-NT) was kindly provided by Frank & Miedendorp (Twello, The Netherlands). Soya bean samples were obtained from the Reference Materials Unit of the Institute of Reference Materials and Measurements (IRMM, Geel, Belgium).