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Analytical Methods

High resolution melting analysis as a new approach to discriminate gluten-containing cereals

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ABSTRACT

With this work, it is intended to propose a novel approach based on high resolution melting (HRM) analysis to detect wheat and discriminate it from other gluten-containing cereals. The method consisted of a real-time PCR assay targeting the gene encoding for the germ agglutinin isolectin A protein (Tri a 18 allergen), using the fluorescent Evagreen dye combined with HRM analysis. The results enabled wheat differentiation from other phylogenetically related cereals, namely barley, rye and oat with high level of confidence. Additionally, a quantitative real-time PCR approach was proposed, allowing detecting and quantifying wheat down to 20 mg/kg in rice flour and 20 pg of wheat DNA (~1.1 DNA copies). Its application was successfully achieved in the analysis of processed foods to verify labelling compliance, being considered as a cost-effective tool for the specific detection of cereals in gluten-free foods.

1. Introduction

Food allergy is defined as an adverse immunological response in sensitised individuals that occurs reproducibly upon exposure to a given food(s) (Boyce et al., 2010). Among foods classified as allergenic, wheat (*Triticum* spp.) and other gluten-containing cereals are part of one of the eight groups responsible for triggering almost 90% of the reported food allergic reactions. Allergies related to the consumption of wheat (or other gluten-containing cereals) are mainly classified as IgE-mediated (baker's asthma and wheat-dependent exercise-induced anaphylaxis) or as non-IgE mediated (celiac disease), depending on the activation mechanism (Tatham & Shewry, 2008). In IgE-mediated allergy, wheat allergens are the main cause for most of clinical symptoms, while in celiac disease the immunological inflammatory response is triggered by the gluten fraction (glutenins and gliadins) that is common to several cereals: wheat (*Triticum aestivum*), rye (*Secale cereale*), barley (*Hordeum vulgare*), kamut (*Triticum turgidum* subsp. *turanicum*), spelt (*Triticum aestivum* subsp. *spelta*), and, in some cases, oat (*Avena sativa*) (Denham & Hill, 2013; Haraszi, Chassaigne, Maquet, & Ulberth, 2011; Tatham & Shewry, 2008).

To protect the health of patients with celiac disease or wheat allergy, the avoidance of gluten-containing cereals is required. However, their total elimination from most diets is extremely difficult. In the specific case of celiac disease, the concept of "gluten-free", referring to a level of gluten that is harmless when ingested indefinitely, is currently widely accepted (Hischenhuber et al., 2006). Presently, food products containing a maximum of 20 mg/kg of gluten are labelled as "gluten-free" (Regulation (EU) No. 41/2009), since this limit is considered a safe clinical threshold level for most celiac individuals. Nevertheless, the definition of an upper safe limit is still not universally accepted in food allergy since the sensitivity of celiac/wheat-allergic patients to gluten varies on an individual basis (Hischenhuber et al., 2006). Therefore, the referred threshold level (20 mg/kg of gluten) represents a reference value for both celiac and wheat-allergic individuals, contributing to a better management of their personal allergies.

In spite of the recent legislation that establishes the limits of 20 mg/kg or 100 mg/kg for "gluten-free" or "very low gluten" products, respectively, no specific analytical methodology has yet been recommended for gluten monitoring. Due to the high stability of DNA molecules, methods relying on their analysis have attained an increasing role in food safety control. In the specific case of

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allergen analysis, DNA-based techniques have been pointed out as highly reliable, sensitive and specific tools for the detection, identification and quantification of gluten-containing cereals in foods. Presently, methods based on polymerase chain reaction (PCR) are widely used for gluten analysis, being available either commercially (e.g. SureFood® Allergen Quant Gluten, R-Biopharm AG, Darmstadt, Germany) or reported in the literature (Martin-Fernandez, Costa, Oliveira, Lopez-Ruiz, & Mafra, 2015; Martin-Fernandez et al., 2014; Mujico, Lombardia, Mena, Méndez, & Albar, 2011; Pinto et al., 2014).

With the recent advances on high resolution instrumentation and with the development of specialised and more efficient fluorescent DNA-binding dyes, the novel approach of high-resolution melting (HRM) analysis has been recently applied to food analysis. HRM measures the rate of dissociation of double stranded to single stranded DNA through small increments of temperature (0.01–0.2 °C/s) (Druml & Cichna-Markl, 2014; Reed, Kent, & Wittwer, 2007), generating DNA melt curve profiles that are both specific and sensitive for species discrimination based on minor nucleotide differences. In theory, a single substitution of a nucleotide (e.g. adenine by guanine) is sufficient to discriminate two fragments of similar size and composition (Reed et al., 2007). With respect to food analysis, HRM has been applied to identify/differentiate varieties and closely related species (e.g. Ganopoulos, Argiriou, & Tsaftaris, 2011; Montemurro, Miazzi, Fanelli, Sabetta, & di Rienzo, 2015), genotyping and serotyping pathogenic microorganisms (e.g. Sanzani, Montemurro, Di Rienzo, Solfrizzo, & Ippolito, 2013), screening genetically modified organisms and detecting food allergens (see reviews of Druml & Cichna-Markl, 2014; Madesis, Ganopoulos, Sakaridis, Argiriou, & Tsaftaris, 2014). In the specific cases of food allergen analysis, reports in the literature describe the successful discrimination of almond allergens from other related species namely peach, nectarine and apricot (Costa, Mafra, & Oliveira, 2012a) and hazelnut from other nuts (Madesis, Ganopoulos, Bosmali, & Tsaftaris, 2013) by HRM analysis. Following the referred achievements, this work intends to propose, to the best of our knowledge, a first attempt of using HRM analysis to differentiate and detect gluten-containing cereals targeting the gene encoding the germ agglutinin isolectin A protein, known as Tri a 18 allergen. Additionally, it was also aimed to propose a rapid and cost-effective method for the detection and relative quantification of wheat in complex foods consumed by celiac and allergic individuals.

2. Materials and methods

2.1. Sample preparation

Flours of different plants from the Poaceae family (wheat, rye, barley, oat, maize, soybean and rice) as well as other 21 plant species, namely peach, nectarine, apricot, pine nut, brazil nut, pecan nut, hazelnut, macadamia nut, chestnut, cashew, walnut, almond, raspberry, blueberry, strawberry, lupine, cherry, plum, sunflower, potato and cassava were obtained at local markets. Different wheat varieties (Donnato, Antonius, Xerxes, Energo, Arnold, Capo and Lukullus) were provided by Imprint Analytics (Austria) and other wheat species (*Triticum aestivum* subsp. *spelta* and *Triticum turgidum* subsp. *durum*) were obtained at local markets. In the absence of certified or testing reference standards for wheat, binary model mixtures containing: 1 mg/kg, 5 mg/kg, 10 mg/kg, 20 mg/kg, 50 mg/kg, 100 mg/kg, 500 mg/kg, 1000 mg/kg and 5000 mg/kg, 10,000 mg/kg, 50,000 mg/kg, 100,000 mg/kg and 500,000 mg/kg of wheat flour in rice material were prepared. The first sample containing 500,000 mg/kg (50%) of wheat was prepared by adding 50 g of wheat flour to 50 g of rice flour. All the other model mixtures were prepared by successive additions of rice flour until the spiked level of 1 mg/kg (0.0001%) in the equivalent proportion to a final weight of 100 g in a non-target plant matrix (rice flour). All the mixtures were thoroughly homogenised separately in a laboratory knife mill Grindomix GM200 (Retsch, Haan, Germany), using different containers and material, previously treated with a DNA decontamination solution (DNA-ExitusPlus™, AppliChem, Darmstadt, Germany). To avoid accidental cross-contaminations among samples, cereals and reference mixtures were prepared on different days. After preparation, all samples and reference mixtures were immediately stored at −20 °C until further DNA extraction.

2.2. DNA extraction

DNA was extracted from 200 mg of each sample or model mixture using the commercial NucleoSpin Food kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions with minor alterations, as previously described (Costa, Mafra, Kuchta, & Oliveira, 2012b). Yield and purity of extracts were assessed by agarose gel electrophoresis and by UV spectrophotometric DNA quantification on a SynergyHT multi-mode microplate reader (BioTek Instruments, Inc., Vermont, USA), using a Take3 micro-volume plate accessory. DNA content was determined using the nucleic acid quantification protocol with sample type defined for double-strand DNA in the Gen5 data analysis software version 2.01 (BioTek Instruments, Inc., Vermont, USA).

2.3. Target gene selection and oligonucleotide primers

In the official list of allergens of WHO/IUIS (World Health Organization/International Union of Immunological Societies) there are 21 wheat allergenic proteins, from which 9 are classified as food allergens: Tri a 12, Tri a 14, Tri a 18, Tri a 19, Tri a 20, Tri a 25, Tri a 26, Tri a 36 and Tri a 37. As a potential DNA marker for the detection of wheat and other gluten-containing cereals, the nucleotide sequence encoding the allergenic protein agglutinin isolectin A (Tri a 18) was retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/). This allergen belongs to the pathogenesis-related (PR)-3 family of proteins or class I chitinases, which is an important family of allergenic proteins (Breiteneder & Radauer, 2004). Targeting the referred allergen a set of primers (Tri18-F – CTGTTGTAGCAAGTGGGGATCCT, Tri18-R – ATTCTTGGAGAAGAGTGGAGTTGG) was used to amplify a fragment of 125 bp by real-time PCR (Martín-Fernandez et al., 2015). For sequencing analysis other primers were specifically designed (Tri18-FS – TACGGGTACTGCGGCTTCGG, Tri 18-RS – CCATGCATGCATCCTGACAACAG) to amplify a larger fragment (649 bp). (GenBank accession No. M25536.1), using the software Primer-BLAST designing tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The basic local alignment search tool BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was also used to identify regions of local similarity between the chosen nucleotide and homologue sequences of different species. *In silico* analysis estimated the statistical significance of the matches and confirmed the specificity of the designed primers with 100% and 93% of identity for wheat species (*T. aestivum* and *T. durum*) and 91% identity for barley (*Hordeum vulgare*) considering 100% of query cover. As a positive amplification control for end-point PCR and real-time PCR assays, universal primers (18SRG-F – CTGCCCTATCAACTTTCGATGGTA, 18SRG-R – TTGGATGTGGTAGCCGTTTCTCA) targeting a conserved DNA region (nuclear 18S rRNA gene) were also used (Costa, Oliveira, & Mafra, 2013). Primers were synthesised by Eurofins MWG Operon (Ebersberg, Germany).