



Analytical Methods

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



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ARTICLE INFO

Article history:

Received 1 April 2015

Received in revised form 4 December 2015

Accepted 11 May 2016

Available online 12 May 2016

Keywords:

Real-time PCR

Gluten-containing cereals

Wheat

Celiac disease

Wheat allergy

Agglutinin isolectin

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.

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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 (Martín-Fernández, Costa, Oliveira, Lopez-Ruiz, & Mafra, 2015; Martín-Fernández 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 – ATTCTTGGAAGAGTGGAGTTGG) was used to amplify a fragment of 125 bp by real-time PCR (Martín-Fernández 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 – CTGCCATCAACTTTCATGGTA, 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).

2.4. Sequencing

In order to verify the identity of the amplified fragments, a second set of primers (Tri18-FS/Tri18-RS) was specifically designed to produce a longer fragment for wheat and related species, covering the target region amplified by the primers (Tri18-F/Tri18-R). PCR products were purified with Jetquick PCR purification kit (Genomed, Löhne, Germany) to remove any possible interfering components and sent to a specialised research facility (STABVIDA, Lisbon, Portugal) for sequencing. Each target fragment was sequenced twice, performing the direct sequencing of both strands in opposite directions, which allowed the production of two complementary sequences of high quality.

2.5. End-point PCR

PCR amplifications were carried out in 25 µL of total reaction volume containing 2 µL of DNA extract (200 ng), 67 mM of Tris-HCl (pH 8.8), 16 mM of (NH₄)₂SO₄, 0.01% of Tween 20, 200 µM of each dNTP, 1.0 U of SuperHot Taq DNA Polymerase (Genaxxon Bioscience, Ulm, Germany), 3.0 mM of MgCl₂ and 120 nM of each primer (Tri18-F/Tri18-R). For sequencing, the PCR components were the same, but using the set of primers Tri18-FS/Tri18-RS. The reactions were performed in a MJ Mini thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA), using the following program: initial denaturation at 95 °C for 5 min; 40 cycles at 95 °C for 30 s, 63 °C for 30 s or 60 °C for 45 s and 72 °C for 30 s or 1 min, respectively for primers Tri18-F/Tri18-R or Tri18-FS/Tri18-RS; and a final extension at 72 °C for 5 min. PCR amplifications targeting the universal region (18S rRNA) were performed as described by Costa et al. (2013).

The amplified fragments were analysed by electrophoresis in a 1.5% agarose gel containing 1× Gel Red (Biotium, CA, USA) for staining and carried out in 1× SGTB buffer (GRISP, Porto, Portugal) for 20 min at 200 V. The agarose gel was visualised under a UV light tray Gel Doc[™] EZ System (BioRad Laboratories, Hercules, CA, USA) and a digital image was obtained with Image Lab software version 5.1 (Bio-Rad Laboratories, Hercules, CA, USA).

2.6. Real-time PCR assays and HRM analysis

Real-time PCR assays were performed in 20 µL of total reaction volume containing 2 µL of DNA extract (200 ng), 1× of SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA, USA) and 200 nM of each primer Tri18-F/Tri18-R. The reactions were performed in a fluorometric thermal cycler CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA), following the program: 95 °C for 5 min, 50 cycles at 95 °C for 15 s and 63 °C for 45 s, with the fluorescence signal acquisition at the end of each cycle. Data were collected and processed using the software Bio-Rad CFX Manager 3.1 (Bio-Rad Laboratories, Hercules, CA, USA).

For HRM analysis, PCR products were denatured at 95 °C for 1 min and then annealed at 63 °C for 5 min, in order to allow the correct annealing of the DNA double strands. The melt curve ranged from 63 °C to 95 °C with temperature increments of 0.2 °C every 10 s and with acquisition of fluorescence data at the end of each melting temperature. The collected data were further processed using the Precision Melt Analysis Software 1.2 (Bio-Rad Laboratories, Hercules, CA, USA) to generate melting curves as a function of temperature and difference curves for easier visualisation of the clusters. Melting curve shape sensitivity determines the stringency used to classify melting curves into different clusters, while temperature of melting (T_m) difference threshold is a parameter that determines the lowest amount of T_m difference between samples. In both cases, cluster detection parameters were

set to high sensitivity and threshold yields aiming at providing more heterozygote clusters. Therefore, melting curve shape sensitivity was adjusted to a percentage value of 70% and T_m difference threshold was set as 0.20. Samples were analysed in four replicates in two independent assays.

3. Results and discussion

Tri a 18 is a wheat germ agglutinin that was firstly identified as an allergen for bakers by Sutton, Skeritt, Baldo, and Wrigley (1984). This small disulphide-rich lectin protein has a homodimeric structure (Breiteneder & Radauer, 2004) and appears in multiple molecular forms due to the polyploidy nature of wheat. In hexaploid wheat (*T. aestivum*), agglutinin presents three isolectins, namely A, B and D, which are encoded by homologous genes, presenting 95–97% and 91–94% sequence identity at the amino acid (Wright & Raikhel, 1989) and at nucleotide levels (Raikhel & Wilkins, 1987; Smith & Raikhel, 1989), respectively. Based on a previous study concerning the selection of new DNA markers for gluten detection (Martín-Fernández et al., 2015), the Tri a 18 gene was selected for the development of a novel HRM approach for the discrimination of gluten-containing cereals.

3.1. End-point PCR

Prior to the target gene specific amplification, to eliminate any false negative results, all DNA extracts were evaluated for their amplifiability by end-point PCR targeting a conserved nuclear 18S rRNA gene, as a universal eukaryotic marker sequence (Costa et al., 2013). All samples amplified positively the expected fragment of 113 bp, confirming that the DNA extracts had the adequate quality, yield and purity for PCR amplification. The specificity of the primers targeting Tri a 18 encoding sequence was previously assayed using a total of 28 plant species, from which only wheat, barley, rye and oat amplified the expected fragment (Martín-Fernández et al., 2015). Therefore, there was no cross-reactivity with other plant species rather than gluten-containing cereals (barley, wheat, rye and oat).

3.2. Conventional melting analysis

Real-time PCR assays using EvaGreen as fluorescence dye were carried out with different wheat species and varieties along with other gluten-containing cereals (rye, barley and oat) targeting the Tri a 18 encoding gene. Confirming the end-point PCR results, wheat, rye, barley and oat were all amplified, but with different cycle threshold (C_t) values. For the same amount of template DNA (200 ng), wheat species and varieties (*Triticum* spp.) amplified at approximately 26.15 ± 0.50 cycles, being followed by rye, barley and oat with differences of 2.1, 7.3 and 8.0 cycles, respectively (Table 1, Fig. 1A). From the amplification results, *Triticum* spp. products seem to present higher similarity with rye, but more distantly related to barley and oat. By conventional melting analysis, the fragments from the five cereal species presented two distinct melting profiles at ~87.80 °C for *Triticum* spp. and oat and at ~89 °C for barley and rye (Fig. 1B, Table 1). As a result, from the melting profiles, fragments from wheat/oat and from barley/rye seemed to have close composition and/or size, disabling the unequivocal identification of each cereal.

3.3. HRM analysis

HRM analysis is reported as a fast, simple and cost-effective approach for scanning and genotyping similar DNA sequences, which allows their identification based on small variations in size

Table 1

Conventional melting and high resolution melting (HRM) analysis applied to wheat (*Triticum* spp.) and other gluten-containing cereals (rye, barley and oat).

Gluten-containing cereal	Ct $\pm$ SD ^a	Tm $\pm$ SD ^b (°C)	Cluster ^c	Level of confidence $\pm$ SD ^d (%)
<i>Triticum aestivum</i> commercial sample	25.77 $\pm$ 0.10	87.80 $\pm$ 0.00	1	99.7 $\pm$ 0.1
<i>Triticum aestivum</i> variety "Antonius"	26.01 $\pm$ 0.04	87.80 $\pm$ 0.00	1	99.6 $\pm$ 0.4
<i>Triticum aestivum</i> variety "Xerxes"	25.56 $\pm$ 0.36	87.80 $\pm$ 0.00	1	99.4 $\pm$ 0.8
<i>Triticum aestivum</i> variety "Capo"	25.99 $\pm$ 0.07	87.80 $\pm$ 0.00	1	99.7 $\pm$ 0.2
<i>Triticum aestivum</i> variety "Energo"	25.96 $\pm$ 0.14	87.80 $\pm$ 0.00	1	99.9 $\pm$ 0.1
<i>Triticum aestivum</i> variety "Arnold"	26.07 $\pm$ 0.03	87.80 $\pm$ 0.00	1	99.7 $\pm$ 0.1
<i>Triticum aestivum</i> variety "Lukullus"	26.25 $\pm$ 0.10	87.80 $\pm$ 0.00	1	99.6 $\pm$ 0.3
<i>Triticum aestivum</i> variety "Donnato"	26.17 $\pm$ 0.08	87.80 $\pm$ 0.00	1	99.7 $\pm$ 0.3
<i>Triticum aestivum</i> subsp. <i>spelta</i>	26.24 $\pm$ 0.04	87.80 $\pm$ 0.00	1	98.2 $\pm$ 0.1
<i>Triticum turgidum</i> subsp. <i>durum</i>	26.72 $\pm$ 0.19	87.80 $\pm$ 0.00	1	99.1 $\pm$ 0.3
<i>Avena sativa</i> (Oat)	34.11 $\pm$ 0.38	87.80 $\pm$ 0.00	2	99.4 $\pm$ 0.4
<i>Secale cereale</i> (Rye)	28.25 $\pm$ 0.08	88.80 $\pm$ 0.00	3	99.5 $\pm$ 0.2
<i>Hordeum vulgare</i> (Barley)	33.41 $\pm$ 0.16	89.15 $\pm$ 0.10	4	99.0 $\pm$ 0.8

^a Mean cycle threshold (Ct) values $\pm$ standard deviation (SD) of ($n = 8$) replicates in two independent real-time PCR runs.

^b Mean melting temperature (Tm) values $\pm$ SD.

^c Cluster 1 is considered as the reference.

^d Mean level of confidence values $\pm$ SD.

and nucleotide composition. Therefore, it is a very useful tool to discriminate sequences from phylogenetically related species, such as the gluten-containing cereals. As a result, HRM analysis was applied aiming at differentiating the four cereal taxa, for which conventional melting analysis was not able to do.

Fig. 1C shows the application of HRM analysis to discriminate wheat, rye, barley and oat. Data shows that all wheat species/varieties (*T. durum*, *T. aestivum* and *T. spelt*) were included in the reference cluster (cluster 1) with high level of confidence (Table 1), confirming the similarity among the size and composition of *Triticum* spp. sequences. Oat amplicons were included in cluster 2, with high level of confidence ($>99.4 \pm 0.4\%$) (Fig. 1C, Table 1), being now differentiated from wheat species/varieties. Rye was included in a distinct group (cluster 3) with a level of confidence above 99.5%. Barley was also differentiated from wheat and the other gluten-containing cereals, being identified as cluster 4 with $99.0 \pm 0.8\%$ of confidence (Fig. 1C, Table 1). Accordingly, HRM analysis targeting the Tri a 18 encoding sequence was able to differentiate wheat varieties/species from other closely related species (oat, rye and barley), which is in good agreement with other reports (Costa et al., 2012a; Madesis et al., 2013).

Despite the fact that oat is not clearly related to celiac or wheat allergy, its consumption is commonly not recommended due to the possibility of cross-reactivity with allergenic proteins from wheat (Tatham & Shewry, 2008). However, close genetic proximity can be attributed to all these cereals (wheat, rye, barley and oat), which

is herein highlighted by real-time PCR results, suggesting potential cross-reactivity phenomena among gluten-containing cereals. These species produced fragments with similar size and melting temperatures, mostly due to their phylogenetic proximity and the conserved nature of the targeted sequence, a wheat germ agglutinin (Chapot, Peumans, & Strisberg, 1986). With the application of HRM analysis, it was possible to distinguish small variations among these related species, contributing to discriminate wheat from other gluten-containing cereals.

3.4. Sequencing results

The results presented by HRM analysis were further investigated and confirmed by PCR-sequencing. For this purpose, sequencing primers (Tri18-FS/Tri18-RS) producing 649 bp fragments were specifically used, covering the target DNA sequence of 125 bp flanked by Tri18-F/Tri18-R primers. Although oat DNA could be amplified for the target fragment of 125 bp, only minor product amount was obtained with Tri18-FS/Tri18-RS primers, thus precluding its sequencing. This fact might indicate that oat is probably the species more distantly related with wheat.

Sequencing results from wheat, rye and barley were aligned and compared with an available consensus sequence from the Genbank database of NCBI (M25536.1). Since each analysed cereal was amplified twice, with direct sequencing of both strands in opposite directions, a total of $n = 4$ strands were obtained. All sequencing results evidenced high resolution electropherograms, being consistent among replicates of each target. Considering that the platform often does not allow perfect resolution for the reading of the first ~ 50 bp at the 5'-end of each sequence, data of Fig. 2 refers to sequencing of 532 bp regions with double coverage. Marked differences could be observed among wheat, rye and barley sequences, as well as with the sequence from the Genbank database. Within the region of interest (125 bp) used for HRM analysis, some differences could also be observed among the same sequences. In relation to the available Genbank sequence, a total of 5 nucleotide differences were evidenced for the all the tested species, namely in positions 121, 160, and 181, where sequenced products present residues of cytosine (C) instead of thymine (T) and in positions 236–237 guanine (G) and cytosine (C) are present instead of cytosine (C) and adenine (A), respectively. These consistent nucleotide differences could be attributed to potential sequencing mistakes on the available Genbank sequence or to the genetic variability of multiploid species, such as wheat (Eilam, Anikster, Millet, Manisterski, & Feldman, 2008; Wright & Raikhel, 1989).

Besides the 5 nucleotide differences with the consensus sequence that were common to all species, two and five additional mismatches were identified in rye and barley sequences, respectively. Regarding the alignment of rye with wheat, the two additional differences were located in positions 166 and 195, with two C residues instead of T and G, respectively (Fig. 2). These two nucleotide differences could be responsible for the shift in melting temperature verified between rye and wheat, as well as for the 2.1 differences in Ct of values of rye amplification (Table 1). The 5 additional nucleotide differences present in barley were located in positions 124 and 166, corresponding to C $\rightarrow$ T, in position 172 (T $\rightarrow$ C), in position 215 (G $\rightarrow$ A) and in position 233 (G $\rightarrow$ C). Since two of the additional differences were located in the region of primer annealing, in both forward and reverse, this might be responsible for the much delayed amplification of barley (7.3 cycles) in relation to wheat (Table 1). Oat also presented a delayed amplification (8.0 cycles), suggesting that a high number of nucleotide differences with wheat sequences. However, since oat sequencing was not possible, the number of nucleotide mismatches, as well as their respective location within oat sequence could not be confirmed.

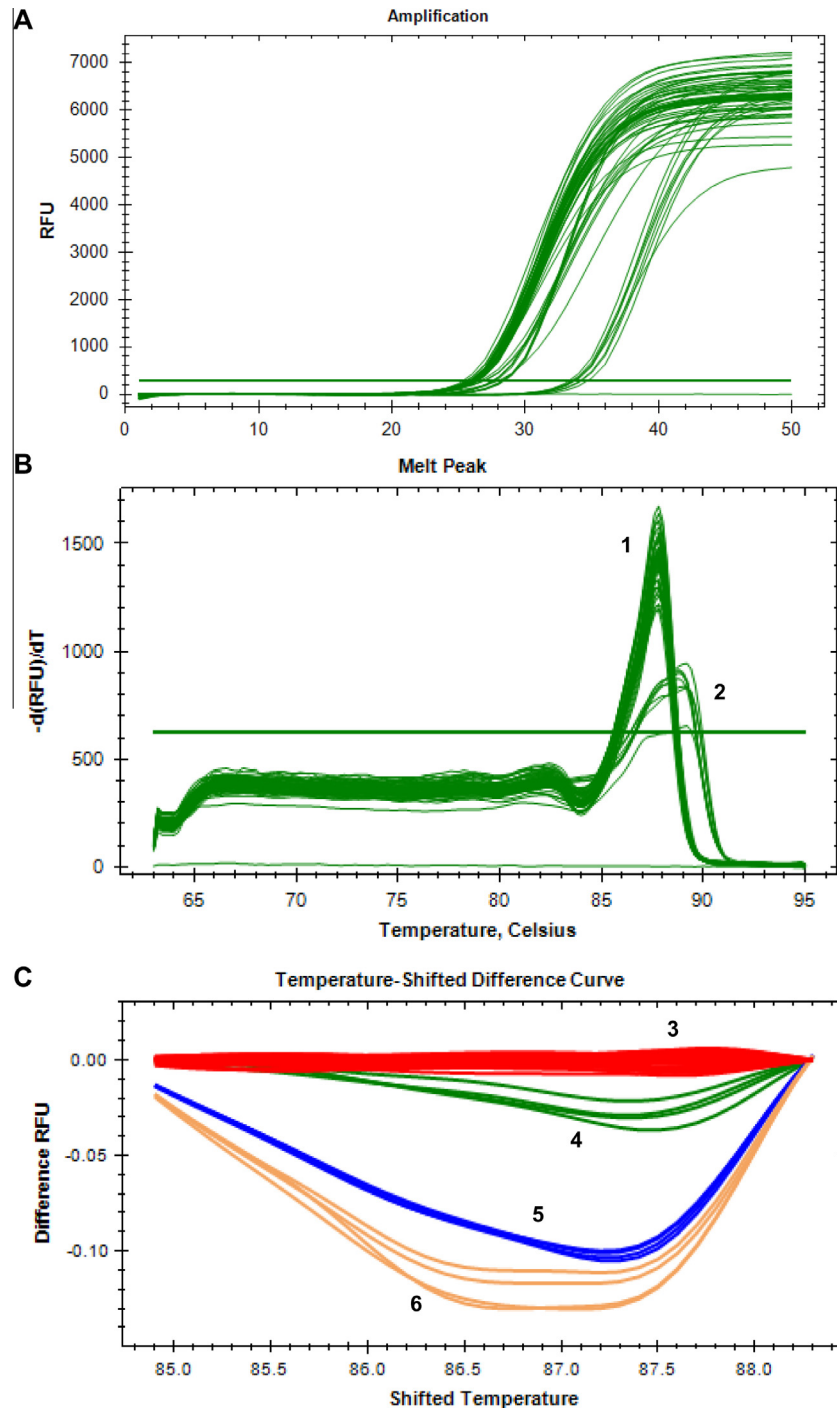


Fig. 1. HRM analysis of real-time PCR products with EvaGreen® dye targeting the Tri a 18 gene and applied to different *Triticum* spp. and other gluten-containing cereals; (A) amplification curves; (B) conventional melting curves and (C) difference curves from HRM analysis. Legend: 1 – wheat varieties (Antonius, Xerxes, Capo, Energo, Arnold, Lukullus, Donnato) and species (*T. aestivum* subsp. *spelta* and *T. turgidum* subsp. *durum*); 2 – rye, barley; 3 – wheat varieties (Antonius, Xerxes, Capo, Energo, Arnold, Lukullus, Donnato) and species (*T. aestivum* subsp. *spelta* and *T. turgidum* subsp. *durum*); 4 – oat; 5 – rye, 6 – barley.

According to studies on immunological and biochemical properties, similar lectins and wheat agglutinin isolectin can be found in rye and barley (Peumans, Stinissen, & Carlier, 1982). Barley and wheat B-isolectin share 95% of sequence identity at protein level. Also, cDNA clones encoding barley lectin are homologous to the wheat agglutinin (Lerner & Raikhel, 1989). Despite the small nucleotide differences between wheat and other cereals from the Poaceae family (rye and barley), this can justify the exclusion of rye, barley and oat, with high level of confidence (>99.0%), from the same cluster of wheat by HRM analysis. Therefore, sequencing

enabled to confirm and reinforce the results of the HRM analysis. These findings are in good agreement with previous reports. In the work described by Costa et al. (2012a), the proposed HRM system allowed the discrimination of almond (*Prunus dulcis*) from other *Prunus* species, such as apricot (*Prunus armeniaca*) and peach (*Prunus persica*) based on two nucleotide differences in Pru du 5 allergen encoding gene. Likewise, Madesis et al. (2013) used an universal chloroplast *trnL* region (barcode) to amplify polymorphic products in eleven nut species, being further discriminated by HRM analysis.

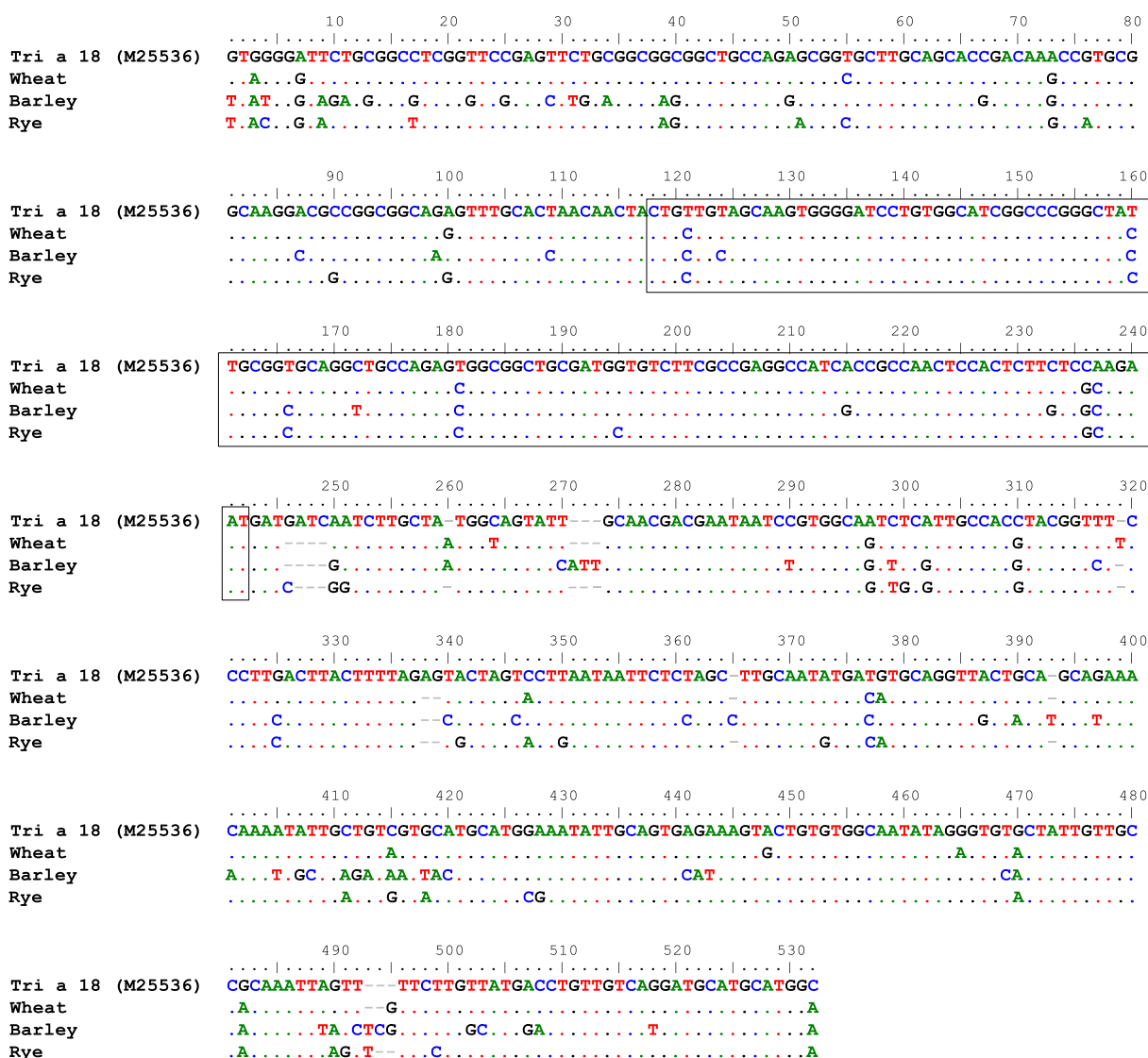


Fig. 2. Sequence analysis of wheat, barley and rye, evidencing the differences in DNA alignments. The region inside the boxes refers to the amplified sequences with primers (Tri18-F/Tri18-R).

3.5. Development of a quantitative real-time PCR assay

The real-time PCR system targeting the Tri a 18 encoding gene was further exploited to develop a quantitative model with the use of reference mixtures with known amounts of wheat flour in rice. The performance parameters of real-time PCR assays were considered in good agreement with the criteria defined for this type of assays (Bustin et al., 2009; ENGL, 2015). Amplification curves presented similar shape and the melting curve analysis enables confirming the identity of wheat fragments (Fig. 3A and B). Parameters of correlation coefficient ($R^2 > 0.98$), PCR efficiency (90–110%) and slope (−3.6 to −3.1) were obtained in all real-time PCR assays (Fig. 3C), which were in good agreement with the defined criteria of acceptance. Limits of detection (LOD) and quantification (LOQ) were also determined for the proposed real-time PCR system, considering the lowest level of the target analyte with at least 95% of positive replicates as the LOD (Bustin et al., 2009; ENGL, 2015). So, the obtained relative LOD was 20 mg/kg (0.002%), considering the total number of replicates ($n = 8$). Since the lowest level of positive amplification was within the linear range of the calibration curve, the LOQ identical to the LOD (Fig. 3). To obtain the absolute sensitivity of the assay, a wheat

DNA extract was used until the 10,000-fold dilution factor, suggesting an absolute LOD of 20 pg of wheat DNA (Table 2), which corresponds to 1.1 DNA copies assuming the size of wheat genome of 17.33 pg (Royal Botanic Gardens, <http://data.kew.org/cvalues/CvalServlet?querytype=1>).

Therefore, the proposed real-time PCR system reached the adequate sensitivity as set by legislation (Regulation (EU) No. 41/2009) to protect celiac individuals and it was found to be in the same order of magnitude (Martín-Fernández et al., 2015) or even better than other works (Piknova, Brezna, & Kuchta, 2008; Sonnante et al., 2009). Gluten in wheat flour corresponds to about 9% of the protein fraction (Zeltner, Glomb, & Maede, 2009), indicating a sensitivity of 1.8 mg/kg of gluten in rice flour.

3.6. Analysis of processed foods

The application of the proposed real-time PCR system to commercial foods enabled to detect wheat in 6 out of 12 tested samples (Table 3). From the selected food samples that cover different cookies, breads and wafers, 6 were labelled as gluten-free and the other 6 included wheat as ingredient. The later samples were 5-fold diluted in order to amplify within the dynamic range of

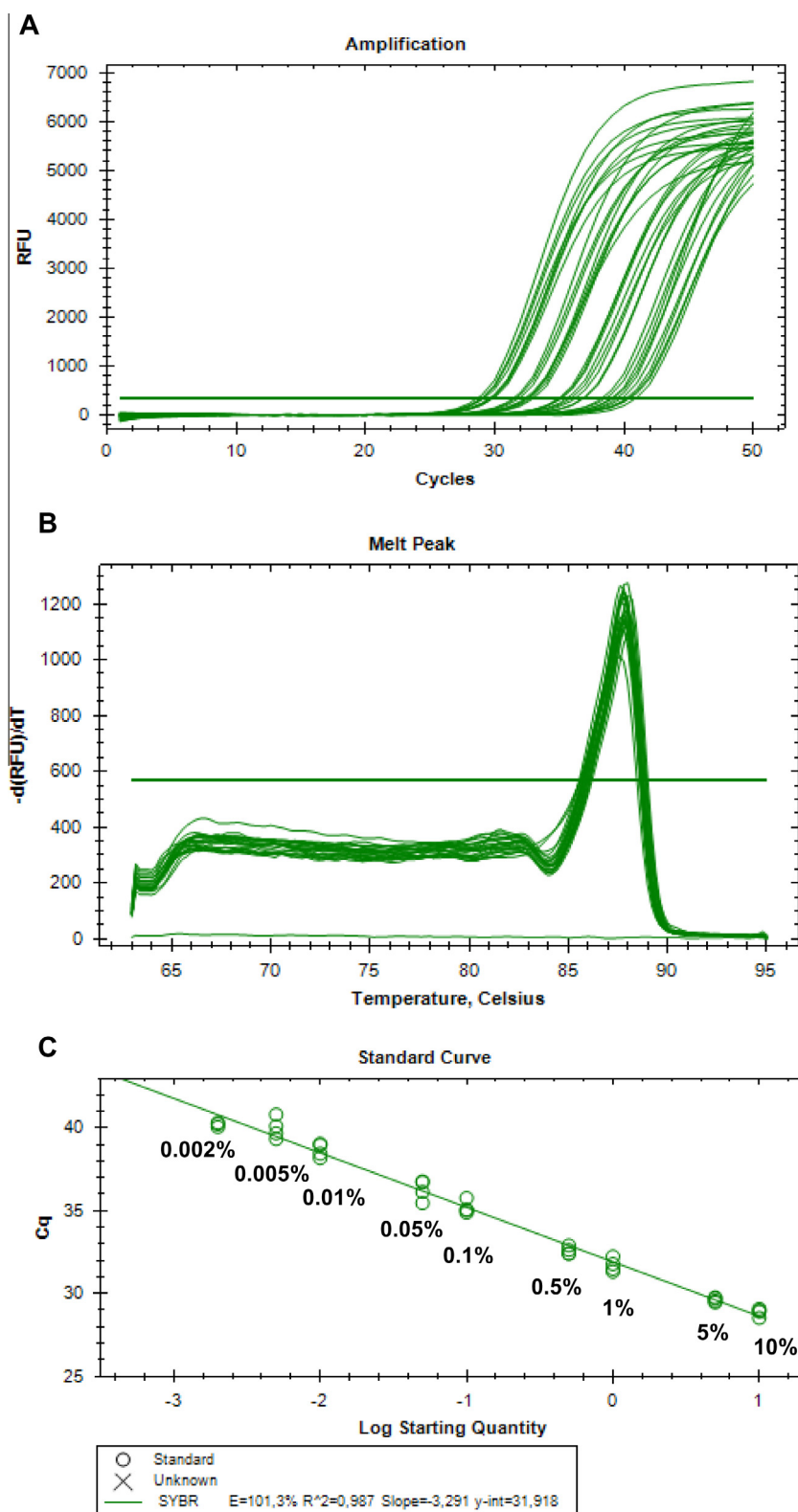


Fig. 3. Real-time PCR targeting Tri a 18 allergen encoding sequence in model mixtures of known amounts of wheat (10–0.0001%) in rice flour. (A) Amplification curves; (B) conventional melting curves and (C) calibration curve.

the calibration curve. In these cases, wheat content was determined considering the applied dilution factor, which allowed estimating a relative amount of wheat ranging from 11% to 49% in cookies and wafers (Table 3). Concerning the gluten-free foods,

three of them did not amplify, while the others presented very few positive replicates at very late C_t values, being considered below the LOD of 20 mg/kg and therefore in good agreement with gluten/wheat label information.

Table 2

Results of quantitative data of the real-time PCR assay targeting Tri a 18 encoding sequence of wheat.

Spiked level (mg/kg)	Ct $\pm$ SD ^a	Absolute quantity (pg)	Ct $\pm$ SD ^a	DNA copies ^d
10	NC ^b	–	–	–
20	40.89 $\pm$ 1.06 (8/8) ^c	–	–	–
50	39.98 $\pm$ 0.74 (8/8)	–	–	–
100	38.48 $\pm$ 0.52 (8/8)	–	–	–
500	36.55 $\pm$ 0.57 (8/8)	2	nd	–
1000	35.51 $\pm$ 0.51 (8/8)	20	38.10 $\pm$ 0.67 (8/8)	1.15
5000	32.71 $\pm$ 0.22 (8/8)	200	34.41 $\pm$ 0.32 (8/8)	11.54
10,000	31.70 $\pm$ 0.28 (8/8)	2000	30.73 $\pm$ 0.20 (8/8)	115.4
50,000	29.78 $\pm$ 0.19 (8/8)	20,000	27.43 $\pm$ 0.08 (8/8)	1154
100,000	29.00 $\pm$ 0.23 (8/8)	200,000	25.51 $\pm$ 0.11 (8/8)	11,540

^a Mean cycle threshold (Ct) values $\pm$ standard deviation (SD).

^b NC, not considered (positive amplification but different melting peaks).

^c (Positive replicates/total replicates) of independent real-time PCR runs.

^d Number of wheat haploid genome copies (17.33 pg).

Table 3

Results of the application of the real-time PCR assay targeting Tri a 18 encoding sequence to different commercial foodstuffs.

Commercial food	Labelling	Ct $\pm$ SD ^a	Estimated amount $\pm$ SD ^c (%)
Bread	Gluten-free	47.03 $\pm$ 4.15 (3/8) ^b	<LOD
Cereal bread #1	Gluten-free	45.20 $\pm$ 3.56 (4/8)	<LOD
Cereal bread #2	Gluten-free	NA (0/8)	–
Chocolate cookies	Gluten-free	NA (0/8)	–
Apricot cookies	Wheat as ingredient	30.07 $\pm$ 0.18 (8/8)	3.96 $\pm$ 0.48
Lupine and lemon cookies	Wheat as ingredient	30.49 $\pm$ 0.17 (8/8)	2.96 $\pm$ 0.31 (14.8) ^d
Cookies #1	Wheat as ingredient	29.19 $\pm$ 0.17 (8/8)	7.30 $\pm$ 0.46 (36.5)
Cookies #2	Gluten-free	NA (0/8)	–
Cookies #3	Gluten-free	46.57 $\pm$ 0.71 (2/8)	<LOD
Wafers	Wheat as ingredient	28.76 $\pm$ 0.19 (8/8)	9.80 $\pm$ 0.66 (49.0)
Chocolate wafers	Wheat as ingredient	30.91 $\pm$ 0.24 (8/8)	2.21 $\pm$ 0.24 (11.1)
Cream wafers	Wheat as ingredient	30.06 $\pm$ 0.18 (8/8)	3.99 $\pm$ 0.41 (20.0)

^a Mean cycle threshold (Ct) values $\pm$ standard deviation (SD) (n = 8) from two independent real-time PCR assays.

^b (Positive replicates/total of replicates).

^c Mean relative amount of wheat $\pm$ SD.

^d Values between brackets correspond to samples that were previously 5-fold diluted before real-time PCR amplification.

4. Conclusions

Herein, we report the development and application of a HRM method for wheat detection/discrimination from other phylogenetically related cereals, namely barley, rye and oat based on the DNA encoding the wheat germ agglutinin isolectin A (Tri a 18) allergen. The specific amplification was successfully obtained for gluten-containing cereals by real-time PCR targeting the Tri a 18 gene, providing products with similar melting temperatures owing to their close relationship. By means of HRM analysis we were able to discriminate *Triticum* spp. from the other cereal species, proposing an innovative and fast method for gluten-containing cereal detection. Additionally, a cost-effective quantitative real-time PCR system was successfully developed and applied to processed food analysis. The proposed method enabled relative and absolute sensitivities of 20 mg/kg and 2 pg (1.1 wheat DNA copies), respectively, which allow verifying labelling compliance of gluten-free foodstuffs. To the best of our knowledge, this is the first time that HRM analysis was applied as a tool for simple, fast and high-throughput identification of gluten-containing cereals in foods.

Acknowledgments

This work was supported by FCT/MEC through national funds and co-financed by FEDER, under the Partnership Agreement PT2020 with grant no. UID/QUI/50006/2013 – POCI/01/0145/FEDER/007265, the bilateral project Acções Integradas Luso-Espanholas/2012 between University of Porto (E-38/12) and University of Oviedo (Ministerio de Ciencia e Innovación, PRI-AIBPT-2011-0769). B. Martín-Fernández and J. Costa are grateful to Complutense University PhD grant (BE45/10) and to FCT post-doctoral grant SFRH/BPD/102404/2014, respectively. The authors are grateful to Imprint Analytics (Neutal, Austria) for the kind supply of seed samples of some wheat varieties.

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