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Validation and application of a quantitative real-time PCR assay to detect common wheat adulteration of durum wheat for pasta production

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ABSTRACT

Pasta is the Italian product par excellence and it is now popular worldwide. Pasta of a superior quality is made with pure durum wheat. In Italy, addition of *Triticum aestivum* (common wheat) during manufacturing is not allowed and, without adequate labeling, its presence is considered an adulteration. PCR-related techniques can be employed for the detection of common wheat contaminations. In this work, we demonstrated that a previously published method for the detection of *T. aestivum*, based on the gliadin gene, is inadequate. Moreover, a new molecular method, based on DNA extraction from semolina and real-time PCR determination of *T. aestivum* in *Triticum* spp., was validated. This multiplex real-time PCR, based on the dual-labeled probe strategy, guarantees target detection specificity and sensitivity in a short period of time. Moreover, the molecular analysis of common wheat contamination in commercial wheat and flours is described for the first time.

1. Introduction

Wheat is the most important cereal in diets worldwide, and flour is the primary product. Flour obtained from the *Triticum aestivum* L. species (common wheat) is used for manufacturing bread, biscuits, and other leavened products, while *T. durum* Desf. (durum wheat) is used for semolina and dried pasta production. Pasta is the most traditional Italian product and a mainstay of the Italian diet, with national pasta consumption ca. 1.5 million tonnes and annual per capita consumption of ca. 25.3 kg (International Pasta Organization., 2014). Pasta is consumed in many other countries including the United States, Brazil, Russia, and Germany, and is one of a few worldwide foods (International Pasta Organization, 2014).

Pasta produced exclusively with durum wheat has good cooking properties and stability with incomparable eating quality (Sissons, 2008). Therefore, to guarantee the best product for consumers, a Decree of the President of the Italian Republic states that dried pasta must be produced only with durum wheat and the use of every other cereal is considered fraudulent (DPR n. 187/2001) (Sissons, 2008). Since cross contaminations are frequent during growing, harvesting, and flour milling practices, the current Italian law tolerates a maximum of 3% common wheat in dried pasta. However, for export trade, the same Italian legislative decree allows the production of dried pasta with common wheat flour only if appropriately labeled (DPR n. 187, 2001). However, even in other countries, such as Spain and France, consumers prefer dried pasta made from only durum wheat. Therefore, clear and accurate information about product composition must be given to consumers to enable informed choice (Woolfe & Primrose, 2004). In a previous study, Kelly and Bhave (2007) demonstrated the inaccurate labeling of four commercial Australian pasta samples, finding common wheat was not reported in the ingredient list. In this context, efficient analytical methods for the detection of accidental or intentional contamination with common wheat are essential. Many different methods have been proposed for the qualitative/quantitative determination of common wheat contamination in pasta.

Until a few years ago, most of these analytical techniques (electrophoretic, chromatographic, and immunological assays) were based on the detection of particular proteins, such as albumins, gliadins or friabilin (Bonetti et al., 2004; Barnwell, McCarthy, Lumley, & Griffin, 1994; Stevenson, McCarthy, & Griffin, 1994). Among these, a method based on albumin separation using isoelectric focusing (Resmini, 1969) had become the most commonly used

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in Italy. Since 1980, the Resmini method has been supported by an immunochemical assay based on the albumin fraction (Piazzi & Cantagalli, 1969; Piazzi et al., 1972). However, protein denaturation during manufacturing is a significant limitation of the protein-based methods (Aktan & Khan, 1992). These analytical methods are inappropriate for high quality pasta obtained using very high temperature drying (Lamacchia et al., 2007; Wagner, Morel, Bonicel, & Cuq, 2011). Moreover, proteins can be synthesized in different amounts in plant tissues (Tilley, 2003) and the target protein expression profile could be influenced by environmental factors (Blumenthal, Barlow, & Wrigley, 1993), compromising the quantitative analysis.

To eliminate these drawbacks, a new generation of methods based on DNA analysis to detect common wheat adulteration has been devised (Alary, Serin, Duviau, Jourdrier, & Gautier, 2002; Arlorio et al., 2003;Kelly & Bhave, 2007; Sonnante et al., 2009). These molecular approaches are based on the detection of specific sequences in D-genome DNA, which is the specific target in common wheat and absent in durum wheat (Bryan, Dixon, Gale, & Wiseman, 1998). Techniques based on the DNA analysis have been investigated for several reasons. First of all, DNA can withstand degradation caused by high temperatures and, therefore, fragments of sufficient length and integrity should still be available for amplification (Kelly & Bhave, 2007). Second, PCR amplification is distinguished by high sensitivity and specificity, allowing the analysis of very small amounts of DNA and the detection of low levels of common wheat adulteration (Kelly & Bhave, 2007). Another advantage is that genomic DNA can be extracted from any plant tissue and is not affected by environmental conditions or the developmental stage of the plant (Tilley, 2003). Finally, PCR methods are particularly convenient because of their simple and rapid set up.

Some PCR-based methods have been described recently. Pasqualone, Montemurro, Grinn-Gofron, Sonnante, and Blanco (2007) developed a SYBR Green real-time PCR assay to quantify common wheat adulteration in semolina and bread products. Terzi, Malnati, Barbanera, Stanca, and Faccioli (2003) applied a real-time PCR protocol with the aim of discriminating common and durum wheats through the amplification of gliadin and glutenin genes. The aim of this work was to find an effective method for the quantitative analysis of common wheat adulteration of durum wheat. A pre-existing method developed by Terzi et al. (2003) was evaluated initially. After this preliminary check, a new commercial amplification method for determination of the relative DNA ratio of *T. aestivum* in *Triticum* spp. was validated and used to quantify common wheat adulteration in wheat flours. The analysis of common wheat contamination in commercial raw materials, such as wheat and flours, using a molecular biology assay, is described for the first time.

2. Materials and methods

2.1. Materials and DNA extraction and quantification

Kernels from the durum wheat cultivar 'Claudio' and the common wheat cultivar 'Bolero' were kindly provided by Dr. Antonella Petrini, Research and Experimentation Centre for Plant Improvement (CERMIS, Macerata, Italy). Commercial samples of six different cereals (kamut, spelt, corn, millet, oat, and rice) in five commercial brands, purchased from various Italian food businesses, were used.

Each cereal species was milled separately using a commercial grinder (Kenwood, Havant, United Kingdom). To prepare the common/durum wheat flour mixtures (0.2%, 1%, 3%, 10%, and 15%), common and durum wheat semolina were weighed, mixed, and mechanically homogenized for at least 60 min using the tube rotator EU-plug (VWR International, INC., West Chester, Pennsylvania).

Genomic DNA was extracted using the Grains DNA extraction kit (Diatheva, Fano, Italy) according to the manufacturer's instructions, and the DNA concentration measured using the Nanodrop ND-1000 System (NanoDrop Technologies, Wilmington, Delaware). Genomic units (GU) for *T. turgidum* and *T. aestivum* were calculated assuming that the genomic molecular weights were 12.84 and 17.67 fg, respectively, as previously shown by Eilam, Anikster, Millet, Manisterski, and Feldman (2008).

2.2. Real-time PCR analysis

Real-time amplifications were carried out in a RotorGene Q thermocycler (Qiagen, Hilden, Germany) and in an Applied Biosystems 7500 Instrument (Life Technologies, Carlsbad, California).

Glud and Glia primer and probe sequences, used in the preliminary step of this work, were developed previously by Terzi et al. (2003). Reactions were performed using the Hot-Rescue Real-Time PCR Kit – FP (Diatheva). The final 25 μl reaction volume contained 900 nM forward and reverse primers, 200 nM dual-labelled probes, and 100 ng of DNA template. Real-time amplifications were performed under conditions described by Terzi et al. (2003). Further analyses were performed using the Grain quantitative kit (Diatheva) following manufacturer's instructions. The Grain quantitative kit was specifically designed to determine the relative DNA copy number ratio of *T. aestivum* in *Triticum* spp. by comparing amplification results from a *T. aestivum* specific target with a sequence generally present in all species of *Triticum* genus, used as normalizer. This test is based on the dual-labelled probes real-time PCR assay, where the *Triticum* spp. amplification is detected in the yellow channel (VIC: ex 538 nm – em 554 nm) and *T. aestivum* in the green channel (FAM: ex 495 nm – em 520 nm). The absolute quantification of each target is obtained through two calibration curves, one for each specific target gene. Data were analyzed using the optical system software RotorGene Q v2.1.0 (Qiagen) and the 7500 Software v2.0.6 (Life Technologies), respectively, for the RotorGene Q thermocycler and the Applied Biosystems 7500 Instrument.

2.3. In silico and experimental specificity analysis

The specificity of Glud and Glia primers/probes developed by Terzi et al. (2003) was examined using *in silico* analysis. Sequence alignment studies were performed using the BLAST online program (http://blast.ncbi.nlm.nih.gov), searching in the nucleotide collection (nr/nt) database and using Megablast (optimized for highly similar sequences). DNA (100 ng) from all the cereal species stated above was tested separately with Glud and Glia primers/probes and with the Grain quantitative kit (Diatheva).

2.4. Limit of detection, calibration function and validation of the real-time PCR assay

The limit of detection (LOD) is defined the smallest number of GU which gives a positive amplification result in at least 90% of cases (Omiccioli et al., 2015). To analyze extreme dilutions, three series of 10-fold dilutions of a DNA mixture containing *T. aestivum* in *T. turgidum* (10% ratio) were tested, starting from 3.2×10^4 and going up to 3.2 GU/PCR of *Triticum* spp. Every dilution point was repeated three times in the same amplification run.

To study the calibration function, another mixture of *T. turgidum* and *T. aestivum* DNAs at a ratio of 3%, reflecting the Italian law limit, was prepared and serially (2-fold) diluted in three independent series, on different days and by different operators. *T. turgidum* ranged from 828.6 to 51.7 ng/PCR while *T. aestivum*