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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

Running Title: A reliable assay for the quantification of common wheat adulteration

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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.

KEYWORDS

common wheat adulteration; real-time PCR; semolina; durum wheat; pasta

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 (Sisson, 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) (Sisson, 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, 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 in Italy. Since 1980, the Resmini method has been supported by an immunochemical assay based on the albumin fraction (Piazzi & Cantagalli, 1969; Piazzi, Riparbelli, Sordi, Cantagalli, Pocchiari & Silano, 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, 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 factrors (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, Coïsson, Cereti, Travaglia, Capasso & Martelli, 2003;Kelly & Bhave, 2007; Sonnante, Montemurro, Morgese, Sabetta, Blanco & Pasqualone, 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 avaialble 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, Schiavano, Ceppetelli, Amagliani, Magnani & Brandi, 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 x 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* from 33.9 to 2.1 ng/PCR. Each dilution was amplified in triplicate using the Grain quantitative kit, and the calibration function was calculated by linear regression analysis of threshold cycles (Ct) measured for each amplification vs. the $\log_2$ copy number for each standard dilution for the two fluorescence acquisition channels. The software calculated automatically the correlation coefficient (R^2), slope and efficiency of the two standard curves. Data were compared with those obtained from the amplification of standard DNA provided in the commercial kit, containing both *T. turgidum* and *T. aestivum* DNAs, diluted, according to manufacturer's instructions, and analyzed as described above.

Quantitative analyses were performed on binary mixtures of DNA extracted from common wheat flour cv. Bolero and durum wheat semolina cv. Claudio in the ratios 0.2%, 1%, 3%, 10%, and 15%. DNA amplification was performed as previously described.

2.5. Limit of detection and validation of the whole method

For LOD determination of the entire method (DNA extraction and amplification test in multiplex real-time PCR), two distinct (0.15%) wheat mixtures containing common wheat flour in durum wheat semolina were prepared. For the two-wheat mixture, an aliquot of 200 mg was used for DNA extraction and each sample was amplified in 10 replicates, for a total of 20 samples.

Validation of the method was carried out by testing flour mixtures in the ratios 0.15%, 0.2%, 1%, 3%, 10%, and 15%. Three separate extractions for each flour mixture were performed and each DNA extract was amplified twice.

2.6. Sample analysis

Fifteen Italian wheat samples, four wheat samples from EU countries, 29 non-European (non-EU) wheat samples, and 33 wheat flour samples were analyzed. Genomic DNA was extracted using the Grains DNA extraction kit (Diatheva) according to the manufacturer's instructions, and common wheat contamination ratios were evaluated by multiplex real-time PCR using the Grain quantitative kit (Diatheva). Results from samples containing an common wheat contamination less than 2% were expressed as "< 2%", without an exact value.

2.7. Statistical analysis

Statistical analyses, such as means, atandard deviation (SD) and relative SD (RSD %), were undertaken using GraphPad Prism 5.0 (GraphPad Software, Inc. California). The distribution of sample values are represented in a scatter plot (vertical) created using GraphPad Prism 5.0 software.

3. Results and Discussion

3.1. Glud and Glia primers/probes specificity

In the first phase of the present work, Glud and Glia primers/probes, designed by Terzi et al. (2003) from the U86029.1 and AF234648.1 GenBank sequences, were tested for specificity with DNA from certified durum wheat cv. Claudio and common wheat cv. Bolero. The low-molecular-weight glutenin gene was selected as a specific target for plants belonging to the *Triticum* genus, while the gliadin gene was chosen to selectively target *T. aestivum* genotypes, allowing the establishment of a qualitative real-time PCR for the detection of common wheat in durum wheat. In the present work, the Glud specific amplification product was obtained for both durum wheat cv. Claudio and common wheat cv. Bolero (Table 1), reconfirming that low-molecular-weight glutenin is a *Triticum* genus-specific target. However, Glia primers/probe also gave positive results for both genotypes (Table 1), in contrast with Terzi et al. (2003) who demonstrated previously the capacity of these primers/probe to discriminate 26 common and 28 durum wheat cultivars, including Claudio. In support of our results, *in silico* analyses were performed using the BLAST online program. Glia primers have 100% identity with sequences in *T. durum* partial GAG56B gene for gamma-gliadin of cultivar Wascana (AJ389704.1), Rugby (AJ389703.1), Fortore (AJ389702.1), DT433 (AJ389701.1), and Astrodur (AJ389699.1). The Glia probe has 100% identity with *T. durum* partial GAG56B gene for gamma-gliadin of cultivar Wascana and Rugby, and a 96% identity for cultivar Fortore, DT433, and Astrodur. The gamma-gliadins are deemed to be the most ancient of the gliadins and low molecular weight glutenins (Shewry & Tatham, 1990). It has been already shown in Genbank that gamma-gliadin sequences belong primarily to tetraploid *T. durum* and hexaploid *T. aestivum* (Goryunova et al., 2012), in accordance with experimental data.

The results reported by Terzi et al. (2003) could be ascribed to the use of different real-time PCR instrumentation and amplification reagents, affecting assay sensitivity. To obtain more information about the species specificity of selected targets, the same real-time PCR amplification was carried out using commercial samples of kamut, spelt, corn, millet, oat and rice (Table 1). All cereals belonging to the *Triticum* genus (spelt, *T. spelta* and kamut, *T. turgidum* ssp. *turanicum*) tested positive for both glud and glia. Spelt DNA amplification agreed with previous outcomes (Terzi et al., 2003), while kamut DNA, which should be gliadin negative, was not tested previously (Martel et al., 2004). Millet, belonging to *Panicum* genus, also produced a gliadin-specific amplification product. Thus, our results confirmed the genus specificity of glud and the lack of *T. aestivum* specificity of glia. The gliadin sequence, AF234648.1, is not a common wheat specific target, so the combination of glud and glia targets, previously proposed for durum and common wheat discrimination, respectively, cannot be used reliably for quantification of common wheat contamination in durum semolina. This new knowledge could be essential for diagnostic labs that use this method to detect the adulteration with common wheat in durum wheat for pasta production. Therefore, the potential for using a commercial Grain quantitative kit (Diatheva) was assessed and its performances were evaluated.

3.2 Performance study of the Grain quantitative kit

Validation experiments were carried out to establish whether the chosen commercial kit could be a specific and sensitive amplification assay.

3.2.1 Specificity

During wheat harvesting and grinding, grain contamination by other cereals is very common. Therefore, the specificity of a system should be guaranteed for both: (a) *Triticum* genus, to avoid erroneous quantification of non *Triticum* cereals, which leads to the underestimation of common wheat; (b) *T. aestivum*, to prevent the over-estimation of the amount, with the risk of finding values that erroneously exceed the legal Italian limit. This mistake could cause enormous economic damage to flour and pasta manufacturing companies.

The specificity of the amplification kit assay was assessed on a panel of target and non-target commercial cereals from different species. The results shown in Table 1 demonstrate the selectivity of this assay for *Triticum* genus and *T. aestivum* species. The *Triticum* genus target was reliably detected in all species from *Triticum* genus, such as durum and common wheat cultivars, spelt, and kamut (Table 1). Negative results were obtained for the other cereals belonging to different genera.

The *T. aestivum* primers/probe provided in the kit showed positive amplification results for the Bolero cultivar. Neither *T. durum* Claudio, nor all the other cereal species used for the specificity tests, including millet, gave any amplification products with the *T. aestivum*-specific oligonucleotide set. These results highlighted the greater specificity of the commercial system compared to the Terzi et al. (2003) protocol, confirming its suitability for the quantitative assessment of common wheat presence in durum wheat flour.

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

To establish the threshold for common and total wheat DNA by means of the dual-labelled probes PCR assay, mixtures of DNA extracted from durum wheat flour cv. Claudio and common wheat flour cv. Bolero in a 9:1 ratio were subjected to amplification. LOD for both *T. aestivum* and *Triticum* spp. corresponded to three GU/PCR, detected in 100% cases.

Therefore, the amplification assay was very sensitive, allowing up to 3 GU of both targets to be detected in DNA mixtures.

The proportion of common wheat in durum wheat should be calculated after absolute quantification of the specific target DNA sequences using two separate standard curves. To obtain quantification data with high specificity, sensitivity and reproducibility, the reliability and validity of the standard curve and standard materials used should be assessed (Pfaffl, 2004; Reischl & Kochanowski, 1995; Bustin, 2000; Pfaffl & Hageleit, 2001; Reiter, Kirchner, Müller, Holzhauer, Mann, & Pfaffl, 2011).

Table 2 summarizes the means and standard deviation of values obtained from a standard curve study conducted by amplifying three series of two-fold diluted *T. turgidum* and *T. aestivum* DNA mixtures. A set of standard curves was constructed using standard DNA provided in the commercial kit while another set used wheat DNA that was extracted in laboratory. Efficiency and slope for *Triticum* spp. and *T. aestivum* standard curves were acceptable, allowing accurate quantifications to be obtained (Table 2). The two targets investigated, irrespective of the standard DNA mixture used, generated satisfactory R^2 median values (Table 2). Specifically, the R^2 means for the *T. aestivum* standard curves were less than the R^2 means of *Triticum* spp. standard curves. This small difference could be because of the high quantity DNA from the *Triticum* targets, which was to the disadvantage of the common wheat standard curve linearity. However, R^2 values were similar to those obtained by another research group quantifying common wheat adulteration using real-time PCR (Sonnante et al., 2009). To verify the reproducibility of Ct measurements and the resulting assay stability, DNA two-fold dilutions were performed in triplicate. In all three experiments, for the two sets of standard curves, standard deviation (SD) values were in the range 0.03 – 0.17 for the *Triticum* spp. standard curve and 0.05 – 0.28 for the *T. aestivum* curve. Although closer ranges of SD values were found in another study (Sonnante et al.,

2009), standard deviations were satisfactory, indicating that the quantitative commercial kit used in this study is stable and reliable. The lower and upper limits of quantification (LLOQ and ULOQ) were the lowest and highest standard curve points that could still be used for quantification. Therefore, the LLOQ of the assay was 4 x 10^3 and 1.2 x 10^2 GU, while the ULOQ was 6.4 x 10^4 and 1.92 x 10^3 GU for *Triticum* spp. and *T. aestivum*, respectively. This real-time PCR assay was performed to obtain a ratio of common wheat in a specific matrix. To quantify the threshold of for common wheat in foods, using this amplification assay, a mixture of common and durum wheat semolina were subjected to amplification. The relative standard deviation (RSD) values ranged from 14.16 to 19.53 %, proportionally to the decrease in common wheat flour contamination, revealing good precision of the amplification assay (Table 3). Different from other methods, which give only data regarding compliance of the analyzed sample with the Italian legal limit, the commercial system evaluated in this study, because of its very wide quantification range (0.2% - 15%), might give specific information about the degree of common wheat adulteration. The experimental deviation from the expected ratio was very low for common wheat concentrations from 0.2 to 10% while lower quantification accuracy was observed in the mixture containing 15% common wheat (Table 3). These results agree with previous reports of difficulties in obtaining exact quantification values for samples with high DNA quantities (Sonnante et al., 2009), although the %RSD value at 15% was not the highest (Table 3). However, considering that the Italian legal threshold of common wheat in durum wheat is 3%, for our purpose, accurate results were more important for the lower concentration range than for the higher one.

3.2.3 *Limit of detection and validation of the whole method*

Significant differences between the amplification results from lab-prepared DNA mixtures compared with DNA extracted from the food samples were observed. This discrepancy was due to the extraction method in a complex matrix (Jankiewicz, Broll, & Zagon, 1999). Results determined using DNA mixtures allow only a "theoretical" threshold to be obtained, while the "real" minimum detectable level can be determined starting with food samples (Jankiewicz et al., 1999). Therefore, wheat flour mixtures were also analyzed to assess sensitivity. However, that there is a lack of specific guidelines for the validation of biomolecular methods for quantification of common wheat contamination should be taken into account.

For LOD determination, 0.15% wheat mixtures were analyzed. The two targets, *Triticum* spp. and *T. aestivum*, were amplified correctly and revealed in all samples (100%). Therefore, the LOD for this method was 0.15%, lower than the limits of sensitivity identified using previous methods (Arlorio et al., 2003; Casazza, Morcia, Ponzoni, Gavazzi, Benedettelli, & Breviario, 2012) and considerably lower than the limit permitted by Italian law. The entire method, including DNA extraction and amplification, therefore, allows the detection of very small amounts of common wheat in durum wheat.

Other wheat flour mixtures (0.2%, 1%, 3%, 10%, and 15%) were analyzed to validate the whole method. Table 3 shows that the experimental deviations from the expected ratio of the flour mixture (FM) are higher than the experimental deviations of the respective DNA mixture (DM) amplification results. Moreover, considering the experimental deviation from the expected value, quantification accuracy was lower for flour mixtures containing more than 10% of common wheat, even if the RSD% values were inversely proportional to the ratio of common wheat contamination and, in particular, the 0.2% ratio was associated with a very high RSD% (Table 3). Nevertheless, this quantification method is more sensitive than previous ones that are able to detect up to 1% common wheat (Sonnante et al., 2009).

Adulteration with concentrations below 1% is not economically advantageous and unlikely to be perpetrated, so this quantification method meets the needs of the market. In conclusion, the validation results indicate that this new method is reliable and effective for detection and quantification of common wheat flour in durum wheat semolina.

3.3 *Sample analysis*

A serious concern for consumers and food authorities is ensuring the authenticity of foods. Correct and detailed labeling of food composition has become a crucial element in the global market (Ibrahim, Al-Hmoud, Al-Rousan, & Hayek, 2011). In this context, the production of pastas from mixtures of durum and common wheats, without adequate labelling, is usually considered adulteration. Kelly and Bhave (2007) identified previously mislabeling of durum pastas, and Ibrahim et al. (2011) showed that 65.4% of presumed wheat durum pasta products sold in Jordan were adulterated with common wheat. Thus, the analysis of pasta products, and related basic ingredients (e.g. flour), is essential for the production of high quality pasta worldwide, not just in Italy.

In line with Italian law, all of the Italian and EU wheat samples analyzed had a common wheat contamination less than 2% (Fig. 1). Therefore, these wheats could be used for pasta sold in Italy or on the global market. On the contrary, 41.4% of the non-EU wheat samples analyzed contained more than 3%, with the highest value being 7.90% (Fig. 1). Flour samples ranged from < 2% to 7% of *Triticum aestivum* in *Triticum* spp. and 27.3% of semolina samples are illegal on the Italian market (Fig. 1). Until now, investigations have analyzed pasta samples, as the final product in the manufacturing chain (Kelly & Bhave, 2007; Ibrahim et al., 2011; Casazza et al., 2012). This is the first study in which commercial raw materials, such as wheat and flour, have been analyzed using a molecular assay to detect the percentage of *T. aestivum* present. Raw product examination allows the classification of wheat and

flours, converging in their commercial use, for appropriate and legal trade and reducing unreliable manufacturers.

4 Conclusion

In the first phase of this work, we demonstrated that the combination of glud and glia targets should not be used for reliable quantification of common wheat in durum semolina. Therefore, the study focused on evaluation of the "Grain quantitative kit" commercial method. This assay, a multiplex real-time PCR based on the dual-labeled probe strategy, guarantees specific and sensitive target detection, and quantification in a short period of time. Moreover, the limit of sensitivity of this method (0.15%), less than the Italian legal limit (3%), would allow easy detection of common wheat in durum wheat for pasta production. This commercial system could impede fraudulent pasta manufacturing and make labeling on pasta packages more accurate. Lastly, for the first time, commercial raw materials were analyzed using a molecular assay and, in line with Italian law, no Italian and EU wheat samples showed *T. aestivum* adulteration above 3%.

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