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DNA Microsatellite Region for a Reliable Quantification of Soft Wheat Adulteration in Durum Wheat-Based Foodstuffs by Real-Time PCR

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Italian industrial pasta and durum wheat typical breads must be prepared using exclusively durum wheat semolina. Previously, a microsatellite sequence specific of the wheat D-genome had been chosen for traceability of soft wheat in semolina and bread samples, using qualitative and quantitative Sybr green-based real-time experiments. In this work, we describe an improved method based on the same soft wheat genomic region by means of a quantitative real-time PCR using a dual-labeled probe. Standard curves based on dilutions of 100% soft wheat flour, pasta, or bread were constructed. Durum wheat semolina, pasta, and bread samples were prepared with increasing amounts of soft wheat to verify the accuracy of the method. Results show that reliable quantifications were obtained especially for the samples containing a lower amount of soft wheat DNA, fulfilling the need to verify labeling of pasta and typical durum wheat breads.

KEYWORDS: DNA microsatellite region; soft wheat adulteration; real-time PCR; semolina; pasta; bread

INTRODUCTION

Pasta is traditionally an Italian product made of durum wheat (*Triticum turgidum* L. Thell. subsp. *turgidum* convar. *durum* Desf. MK.), and currently Italian rules prohibit the manufacture of pasta containing soft wheat (*Triticum aestivum* L. Thell. subsp. *vulgare* Vill. MK.) for domestic market to fulfill consumers' quality expectative in terms of high tenacity and consistency. Only a maximum of 3% *T. aestivum* can be tolerated to account for cross contamination during the agricultural process (*1*). However, the same Italian law allows import−export of pasta totally or partially prepared using *T. aestivum*, which in this case requiring a clear indication on the label.

Moreover, some breads from Southern Italy, awarded with the Protected Designation of Origin (PDO) or Protected Geographical Indication (PGI) marks at European level (*2*), have to be prepared exclusively employing durum wheat semolina to maintain their typical properties and to gain the quality marks. This is the case of "Pane di Altamura" and "Pane di Matera", awarded with PDO and PGI marks, respectively (*3*, *4*), as well as "Pagnotta del Dittaino", whose application for PGI is currently under consideration by EU Commission (*5*).

Consequently, a strong interest toward the detection of soft wheat has stimulated the development of numerous analytical methods, generally aimed at searching for specific protein fractions, the first set up as early as at the end of 1960s (*6*). More recently, a new generation of methods that employ DNA screening for sequences localized in the D-genome, characteristic for soft wheat, has become available (*7*−*12*).

In a previous paper (*11*), the analysis of a DNA microsatellite specific for D-genome has been applied to set up a method for the detection of soft wheat in semolina and durum wheat bread. Microsatellites are sequences of repeated DNA that can be analyzed by means of a single PCR reaction, providing short-sized amplicons (*13*). The small size of generated amplicons (around 200 base pairs) is crucial if analyzing food samples that have undergone, during their production process, high-temperature and/or strong mechanical treatments, such as in the case of bread and pasta. In fact, the quantity and quality of DNA recovered from processed food is generally low, and using small DNA regions for amplification can bypass the high level of DNA degradation. Pasqualone and colleagues (*11*) used Sybr green real-time PCR to quantify soft wheat adulteration in semolina and bread preparations. However, while detection by Sybr green is relatively simple and straightforward, it indiscriminately binds all double-stranded DNA products. Therefore, nonspecific amplification will result in an increased fluorescent signal, and problems in specificity can prevail when target DNA is highly degraded. Consequently, a reliable quantification of *T. aestivum* was only obtained for semolina samples as compared to bread.

Fluorescent oligonucleotide probes enable real-time monitoring of the PCR assay and ensure that increases in fluorescence result only from the accumulation of the desired product. One popular probe strategy is the assay based on dual-labeled probes,

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which exploits the 5′ to 3′ exonuclease activity of *Taq* DNA polymerase to cleave a labeled hybridization probe during the extension phase of PCR (*14*).

The aim of this work has been to improve the specificity of the method previously set up, by means of real-time experiments based on the use of a dual-labeled probe, with the purpose of accurately quantifying *T. aestivum* contaminations in semolina, bread, and pasta products.

MATERIALS AND METHODS

Sample Preparation. Kernels from one durum wheat cultivar (Appulo), and one soft wheat cultivar (Pandas), grown at the experimental field of Genetics and Breeding Section, DIBCA Dept., Bari University (Bari, Italy), were separately milled by a Buhler MLU 202 mill. From Appulo semolina and Pandas flour experimental mixtures were prepared in the ratios 60:40, 80:20, 90:10, 95:5, 97.5:2.5, and 98.75:1.25. An additional sample with a 3% content of soft wheat was also prepared because this level represents the limit for indication of soft wheat presence in pasta label according to Italian law (*1*). These mixtures, as well as pure Pandas flour and pure Appulo semolina (100%), were used to produce pasta samples (spaghetti shaped) by adding 32% tap water, kneading, shaping, and cutting with a manual pasta-machine SP 150 (Imperia, S. Ambrogio di Torino, Italy) equipped with STS spaghetti-maker (Imperia, S. Ambrogio di Torino, Italy). Pasta was then dried at high temperature (maximum value reached 80 °C) by means of an industrial plant belonging to a local pasta factory (Riscossa, Corato, Italy). The same mixtures, as well as pure Pandas flour and pure Appulo semolina, were also used to produce bread at a local bakery (Digesù, Bari, Italy). At this purpose 500 g of flour/semolina, 20 g of baker's yeast, 12 g of sodium chloride, and about 600 mL of water, were mechanically kneaded for 15 min. After manual portioning and shaping, the dough was left to rise at 28−30 °C for 1 h, then manually kneaded for few minutes, shaped, and again left to rise for 1 h. Baking was carried out at 250 °C for 45 min. A sample of commercial bread and pasta was also collected. Prior to DNA extraction, 10 g of each type of pasta and 10 g of lyophilized crumb of each kind of bread were powdered in a mortar.

DNA Extraction. Various DNA extraction methods were tested on commercial samples of bread and pasta to assess the best method to use with the experimental samples. Dellaporta (*15*) and Doyle and Doyle (*16*) extraction methods and the commercial kits NucleoSpin Plant (Macherey-Nagel, Düren, Germany) and NucleoSpin Food (Macherey-Nagel) were considered. The kits were used to extract DNA according to the manufacturer's instructions with some modifications. An amount of 50 mg and 200 mg of lyophilized bread crumb or pasta was used for NucleoSpin Plant or NucleoSpin Food (Macherey-Nagel), respectively. For NucleoSpin Plant (Macherey-Nagel), with the aim of increasing DNA yield at the end of the extraction procedure DNA was eluted twice by using 50 μL of CE buffer (instead of a single time with 100 μL buffer), then the eluted solution was incubated at 70 °C for 3 min, centrifuged at 11.000 × g throughout a mini-chromatographic column for 1 min, and then the clear solution containing DNA was recovered. The two aliquots of this solution were then joined together. For NucleoSpin Food (Macherey-Nagel), the same procedure was carried out, but 100 μL of CE buffer instead of 50 μL was used. Moreover, for both procedures the time of lysis of the cell structures was prolonged from 30 min to 12 h. For NucleoSpin Food (Macherey-Nagel), 200 mg of each sample was used instead of 100 mg, and the volumes of the lysis solution and k proteinase were increased from 550 to 775 μL, and from 10 to 15 μL, respectively. Dellaporta protocol (*15*) was applied on 50 mg of ground lyophilized bread crumb or pasta with the only modification of prolonging the time of isopropanol DNA precipitation from 30 min to 12 h to increase the amount of precipitate. The CTAB-method described by Doyle and Doyle (*16*) was used to extract DNA from each sample with the following modifications: 200 mg of each sample was used instead of 100 mg; 900 μL instead of 700 μL of the extraction buffer was added to each sample; the first and the second centrifugations were extended to 25 min to better separate DNA from proteins and cell debris; DNA was incubated at 37 °C for 1−2 h to achieve a complete resuspension. The preferred protocol was the Doyle and Doyle method (*16*), which was used for the extraction of all the samples of this study, that is, experimental semolina, flour, pasta, and bread.

Quali-Quantitative Evaluation of Extracted DNA. The concentration of 1 μL of DNA extracted from each sample (durum and soft semolina/flour, pasta, and bread) was measured by means of a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA), using 1× TE buffer as a reference. The quantity of some random DNA samples was checked on 1% agarose gel by comparison with 500 ng/μL λ-DNA (New England Biolabs, Ipswich, MA) as a concentration reference. Their quality was also checked on the same gels by comparison with molecular weight standard 100-base pair (Bio-Rad Laboratories, Hercules, CA), to evaluate the extent of DNA fragmentation.

DNA Sequencing, Primer, and Probe Design. Primers specific for the microsatellite region GDM111 (*17*) were used to amplify the expected fragment of about 200 bp in *T. aestivum* cv. Pandas. A final volume of 25 μL contained 30 ng DNA, 1× PCR buffer, 0.25 mM dNTP, 2.5 μM of each primer, 1U recombinant *Taq* DNA polimerase (Invitrogen, Carlsbad, CA). The PCR conditions were as follows: 95 °C for 5 min, 35 cycles of 95 °C for 1 min, 58 °C for 1 min, 72 °C for 2 min, and a final extension at 72 °C for 10 min. The amplified fragment was visualized on a 1% agarose gel, purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and subsequently cloned using a Gene JET PCR Cloning Kit (Fermentas, Burlington, Canada). The cloned fragment was sequenced by means of an automated DNA sequencer CEQ 8800 (Beckman Coulter, Fullerton, CA).

On the obtained DNA sequence, forward and reverse primers and a dual-labeled probe were designed using Primer Quest program (http://www.idtdna.com/Scitools/Applications/Primerquest/), excluding the microsatellite repeat motif, and setting the optimal conditions for real-time PCR: probe Tm 7−10 °C higher than the primer Tm, primer Tm 55−60 °C, primer length 18−22 bp, probe length 27−32 bp, GC content 35−50%, a product length comprised between 80 and 150 bp. The probe was labeled with 6-carboxy-fluorescein (FAM) on the 5′-end, and the fluorescent quencher dye 6-carboxytetramethylrhodamine (TAMRA) was attached to the 3′-end.

Real-Time Amplification. Real-time amplifications were carried out in a RotorGene 6000 (Corbett, Mortlake, NSW, Australia). The reaction mixtures were prepared using a CAS-1200 liquid handling system (Corbett, Mortlake, NSW, Australia), and each one contained 12.5 μL of Brilliant Q-PCR Master Mix II (Stratagene, Santa Clara, CA), 600 nM each forward and reverse primers, 300 nM probe, and 200 ng of DNA target in a final volume of 25 μL. Real-time PCR amplifications were performed under the following conditions: 95 °C for 10 min, 40 cycles at 95 °C for 30 s and 60 °C for 1 min. All samples were prepared in triplicates and each set of analysis was repeated three times.

Construction of Standard Curves. To construct the standard curve for each sample set (flour, pasta, and bread), three sets were prepared using the following dilutions: 1.25, 2.5, 5, 10, 20, 40% soft wheat DNA starting from 100% soft wheat flour, pasta, or bread, so as to progressively increase by a factor of 2 the concentration of the standard samples. In each real-time experiment, the samples of semolina, pasta, or bread mixtures as described above were amplified together with the corresponding standard curve samples. All standards were amplified in triplicates.

The data obtained from the amplification runs were analyzed using the absolute quantitative method in the RotorGene 6000 software. Calculated concentrations were obtained from a simple linear regression model, with the log concentrations (x) as the known values and the Ct values (y) as the experimental ones. Deviation from the expected concentration was calculated as the difference between the percentage of soft wheat contained in the standard sample and the percentage of soft wheat in the corresponding experimental sample.

RESULTS AND DISCUSSION

The use of the real-time PCR technique to identify, quantify, or trace the DNA present in food and feed is an established practice in many laboratories. Within these fields, real-time PCR analysis was initially developed for detecting and quantifying exogenous DNA of bacterial or viral origin, and, more recently, for genetically modified organism (GMO) detection (*18*). However, the advances in plant genomics and marker discovery have provided researchers with a lot of useful DNA markers able to specifically detect *taxa* or fingerprint varieties (*19*). These tools can be now

used not only to verify the presence of nondesirable components in processed food, but also to quantify them through real-time PCR with the aim to protect the food industry from loss of income due to fraud and the ensure the consumer a certified quality product.

Comparison of DNA Extraction Methods. In this context, the capability to efficiently extract DNA from the various plant tissues, as well as from processed food, such as pasta or bread, is a basic prerequisite for the overall research (*20*, *21*). Moreover, DNA extraction techniques must be adapted to the real-time PCR assay. Thus, meticulous work was necessary to set up a suitable protocol that could increase DNA quantity and purity, and improve PCR efficiency. The quality of DNA extractable from food is often quite low: DNA is scarce, degraded, and can be contaminated by inhibitors of DNA polymerase. Hence, prior to performing real-time experiments, the efficiency of different extraction methods was evaluated in terms of DNA extraction yield, quality, and purity. At this purpose, commercial samples of bread and pasta were used. The laboratory protocols of Dellaporta (*15*), Doyle and Doyle (*16*), and the commercial kits NucleoSpin Plant (Macherey-Nagel) and NucleoSpin Food (Macherey-Nagel) were compared. Their procedures were modified to better adapt to food samples.

Table 1 reports the extraction yield and DNA concentration obtained by applying different extraction methods to pasta and bread samples. NucleoSpin Plant kit (Macherey-Nagel) allowed to extract 5 μg of DNA from a 50 mg sample at a concentration of 50 ng/μL. This kit is marketed for giving 10−30 μg of DNA from 100 mg leaf tissue; hence the obtained yield for processed food was coherent with the expected. Besides, this kit is set up for 100 mg plant tissue, while only 50 mg of lyophilized bread crumb or pasta were used due to their high water absorption to obtain a sufficiently fluid suspension. Similar results were observed in other studies by comparing the yield of various kits in extracting DNA from olive oils (*22*).

NucleoSpin Food kit (Macherey-Nagel), marketed specifically for the extraction of DNA from food matrices, gave high yields, leading to 55 μg of DNA from 200 mg sample at the very high concentration of 550 ng/μL on average. The manufacturer's leaflet indicates that this kit had been tested over various cereal-based foodstuffs, such as biscuits, corn-flakes, and bread, but not with pasta. The esteemed yield was in the range 0.10−10 μg DNA per mg sample, hence the obtained result, 0.27 μg/mg, was as expected. No significant differences were observed between the amount of DNA extracted from pasta or bread.

DNA extracted with the Dellaporta method (*15*) gave a yield of 25 μg DNA/g sample. This was an acceptable value for processed food, considering that the expected yield for leaf tissue should be 50−100 μg DNA/g sample (*15*). Doyle and Doyle method (*16*) was able to furnish the highest yield from pasta or bread, that is, an average value of 300 μg DNA/g sample, with a very high concentration (600 ng/μL); consequently, this procedure was used to extract all the samples of the study. This method was particularly suitable also because of its minor costs compared to the commercial kits. No significant differences were observed regarding the quality of the extracted DNA by comparing the above cited methods.

Real-Time Assays and Standard Curves. The aim of this research was to establish quantification methods and protocols for real-time PCR for the detection of soft wheat contamination in durum wheat foodstuffs. In a previous study, a microsatellite region mapping on wheat D-genome was chosen after testing among several other markers as a target region for quantifying the presence of soft wheat in durum wheat semolina and bread preparations. This microsatellite region proved to amplify on various wheat varieties and was therefore used in both qualitative PCR and real-time Sybr green assays (*11*). However, due to high degradation of DNA, especially in bread, a high level of primer-dimer formation was observed. Nevertheless, it was possible to quantify contaminations from soft wheat in semolina, but not, for instance, in bread. On the basis of the sequence obtained using the primer pairs GDM111 (*17*), a new couple of primers and a dual-labeled probe were designed in order to obtain a short fragment. The use of a dual-labeled probe assures a higher specificity compared to an aspecific dye, such as Sybr green. In fact, Sybr green is an intercalating dye, and for this reason all amplified double strand DNA is detected during amplification cycles, including possible primer−dimers.

Table 1. Extraction Yield and DNA Concentration Obtained by Applying Different DNA Extraction Methods to Pasta and Bread Samples

	method			
parameter	Dellaporta et al.	Doyle and Doyle	NucleoSpin Plant	NucleoSpin Food
extraction yield (μg DNA/g sample)	25	300	100	275
DNA concentration (ng/μL)	25	600	50	550

Another crucial aspect of real-time PCR quantification concerns the assembly of a standard calibration curve that needs to be built with reference DNA. To construct our standard curves, DNA from flour, pasta, and bread entirely prepared with soft wheat were diluted, ranging from 1.25 to 40% soft wheat concentration. The standard curves based on these dilutions showed a linear relationship between log input DNA and threshold of cycle (Ct) values. The PCR reaction efficiency, calculated from the instrument software, on the basis of the standard curve slope, ranged from 97 to 99%. In these conditions, accurate quantifications can be obtained. The square regression coefficients (R^2) ranged between 0.9824 to 0.9936 for the different assays. The good linearity between DNA quantities and fluorescence values (Ct) confirms that the assays are well suited for quantitative measurements (**Figures 1−3**).

To verify reproducibility of the Ct measurements, the DNA dilutions and the experiments were performed in triplicate. Real-time assays gave average Ct values varying from 27.65 to 33.30 for flour, 30.23 to 35.74 for pasta, and 28.45 to 33.92 for bread. In all the experiments, standard deviation (SD) values were in the range of 0.03−0.13, indicating that the quantitative conditions reported in this paper are quite stable and reliable.

Quantification of Semolina, Pasta, and Bread Samples. For the three foods considered in this study, namely flour/semolina, pasta, and bread, samples were prepared using 100% soft wheat (our positive control), 100% durum wheat (our negative control), and mixtures based on durum wheat with varying percentages of soft wheat (1.25, 2.5, 3, 5, 10, 20, or 40%). The samples with a known content of soft wheat were used to evaluate the accuracy and precision of the real-time PCR methods in this study.

Real-time PCR amplification of all samples was carried out in triplicate, and quantification was verified on the basis of the standard curve constructed separately for flour, pasta, or bread (**Figure 4**). The deviation of the experimental values from the expected ones was comprised between 0.04 and 1.50 in semolina samples, 0.02 and 4.65 for pasta samples, and 0.02 and 2.05 for bread samples (**Table 2**).

It is interesting to note that if we consider the lower soft wheat concentrations (from 1.25 to 3%) the experimental error was very low even when DNA derived from pasta or bread, and therefore it was extremely degraded. A lower accuracy of the quantification

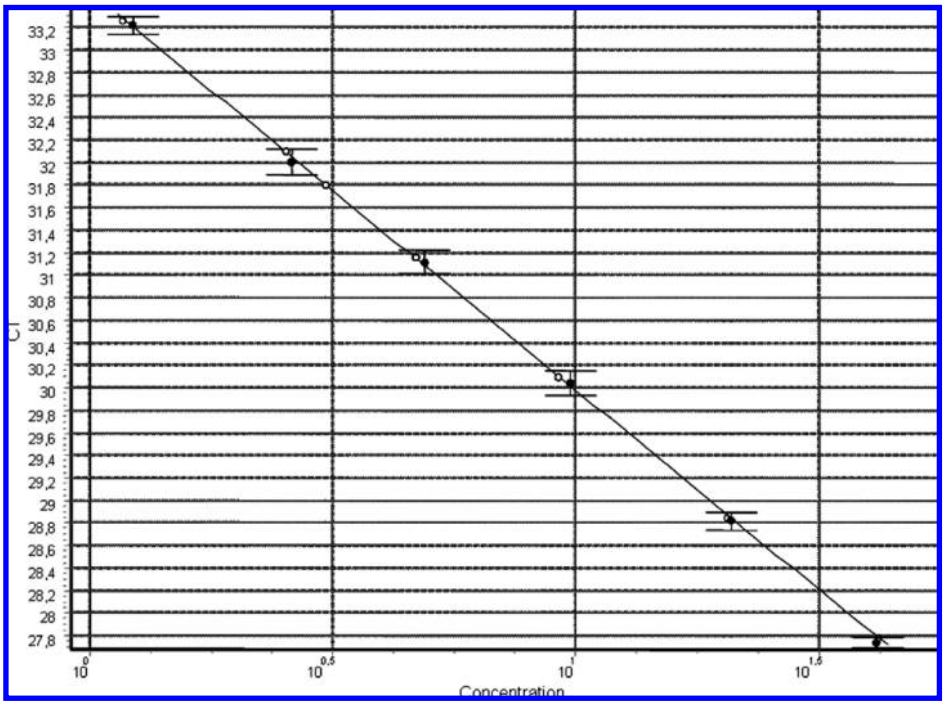

Figure 1. Real-time PCR standard curves and positioning of samples of semolina. Black dots indicate the standard curve dilutions; white circles indicate the experimental samples obtained from the mixtures as indicated in **Table 2**.

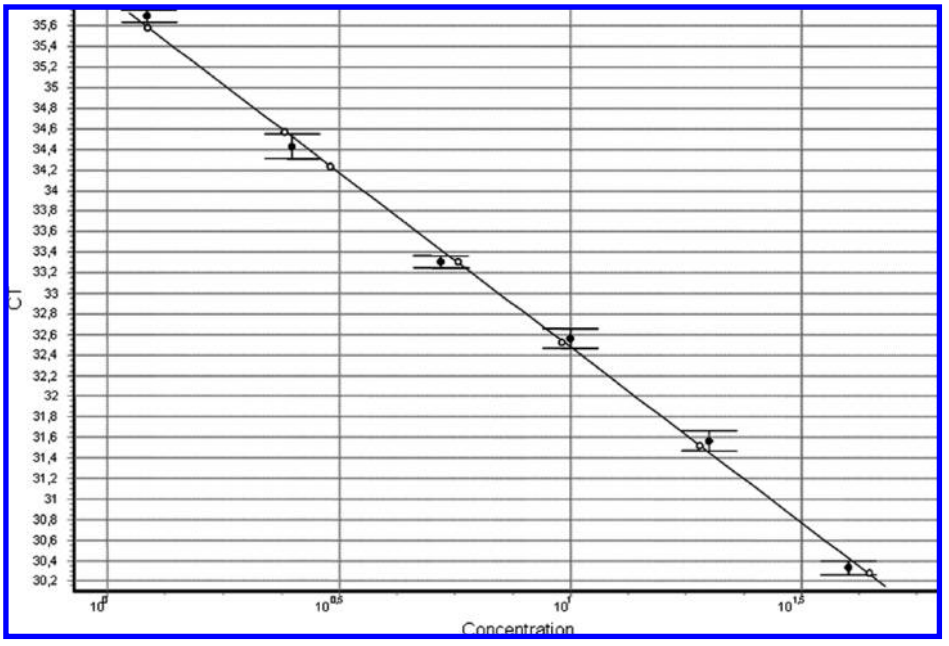

Figure 2. Real-time PCR standard curves and positioning of samples of pasta. Black dots indicate the standard curve dilutions; white circles indicate the experimental samples obtained from the mixtures as indicated in **Table 2**.

was observed in the preparations containing higher amounts of soft wheat, but this may be expected since it is difficult to get precise quantifications when the DNA template is present in high quantity. However, for our purpose it is important to get accurate results on lower concentrations. In fact, for instance Italian laws forbid the use of soft wheat in pasta, considering the threshold of 3% as deriving from accidental contamination (*1*), whereas no soft wheat presence is allowed in PDO or PGI durum wheat typical breads. It is therefore crucial to detect the precise amount of occasional soft wheat contamination in these products, so to be able to possibly discover intentional addition.

With the aim of detecting adulteration of durum wheat pasta, Alary et al. (*8*) used a method based on real-time PCR amplification of a puroindoline-b gene selected as a D-genome specific sequence. However, their study only referred to a theoretical adulteration of 3%, and only one pasta sample was used. Like in our study, Alary et al. (*8*) also evidenced real-time PCR as a more appropriate method as compared to protein and qualitative PCR analyses thanks to the use of very small amplicons, which are highly specific to the target sequence, and to the lack of post-PCR sample handling. In the study by Terzi et al. (*10*), a real-time based analysis was performed, using a gliadine sequence, to identify

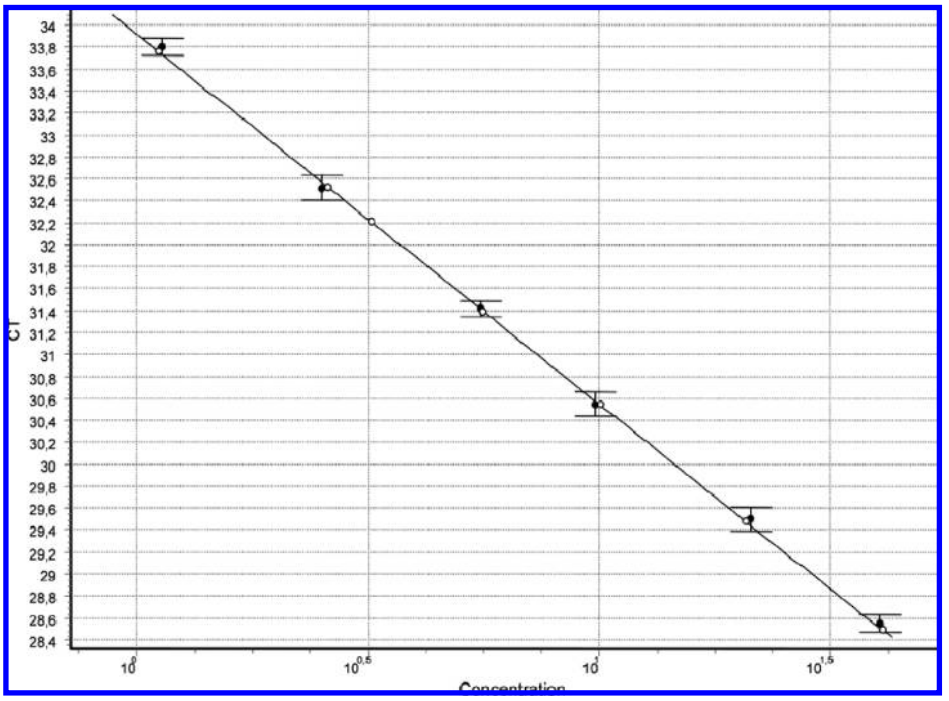

Figure 3. Real-time PCR standard curves and positioning of samples of bread. Black dots indicate the standard curve dilutions; white circles indicate the experimental samples obtained from the mixtures as indicated in **Table 2**.

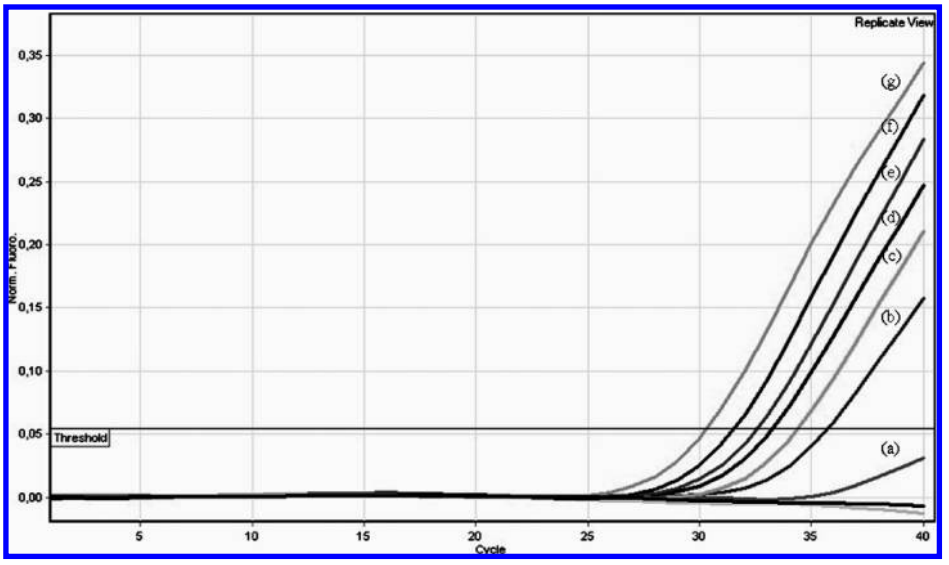

Figure 4. Amplification plot of real-time PCR on pasta DNA standard dilutions at (b) 1.25, (c) 2.5, (d) 5, (e) 10, (f) 20, and (g) 40% soft wheat. Negative control (100% durum wheat pasta) is indicated with (a).

Table 2. Quantification of Soft Wheat in Semolina, Pasta, or Bread Samples (Mean of Three Values)[a]

	soft wheat concentration (%)						
	1.25	2.5	3.0	5.0	10.0	20.0	40.0
semolina	1.21 (−0.04)	2.41 (−0.09)	3.05 (+0.05)	4.88 (−0.12)	9.52 (−0.48)	19.87 (−0.13)	41.50 (+1.50)
pasta	1.27 (+0.02)	2.38 (−0.12)	2.98 (−0.02)	5.62 (+0.62)	9.44 (−0.56)	18.84 (−1.16)	44.65 (+4.65)
bread	1.23 (−0.02)	2.54 (+0.04)	3.23 (+0.23)	5.57 (+0.57)	10.23 (+0.23)	18.93 (−1.07)	42.05 (+2.05)

[a] The upper line refers to the concentration of the prepared soft wheat samples. In brackets, the experimental deviation from the expected value is reported.

adulterations in durum wheat semolina and spaghetti. They were able to detect up to a limit of 1% soft wheat. Differently from others, our study, besides semolina and pasta, also considers bread, the DNA of which is extremely degraded. Moreover, we also confirmed the reliability of our results by quantifying ad hoc prepared samples, in comparison to the standard dilutions.

In conclusion, the obtained results indicate that the above-reported analytical method is a reliable and effective system to

detect soft wheat in durum-wheat based foodstuffs. The design of a dual-labeled probe combined with an appropriate primer pair allowed to get a more precise quantification of soft wheat contamination in durum wheat-based bread and pasta. Particularly accurate at low levels of contamination, it fulfils the need to verify labeling of pasta and typical durum wheat breads, possibly warranting better quality for consumers.

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