

# Quantification of Common Wheat Adulteration of Durum Wheat Pasta Using Real-Time Quantitative Polymerase Chain Reaction (PCR)

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ABSTRACT

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Common wheat adulteration of durum wheat pasta was quantified using real-time duplex polymerase chain reaction (PCR). The total DNA content of pasta was determined by amplifying part of a wheat gene encoding a lipid transfer protein, and common wheat DNA was quantified by amplifying part of the puroindoline-b gene. Under the conditions defined by this study, for pasta with a theoretical adulteration of 3%, the experi-

mentally determined mean value was 2.6–3.4%, depending on drying temperature. Pure durum wheat pastas were distinguished from adulterated pastas without ambiguity. This study demonstrates the feasibility of using real-time duplex PCR to quantify common wheat adulteration of pasta dried at high temperature, quantification that was impossible with the French official peroxidase-marker method.

French legislation stipulates (1934) that pasta must be made exclusively from *Triticum durum* semolina and water, although a 1955 regulation authorizes the addition of salt, eggs, gluten, milk, vegetables or vegetable extracts, and spices. Addition of *T. aestivum* (common wheat) is the most common adulteration in industrially made pastas. In Spain and Italy, pasta must also have been made exclusively with durum wheat, whereas in north European countries, pastas made with common and durum wheat are permitted. In France, labeling of such mixed products must clearly indicate their composition and the label “alimentary pasta” may not be used, as this description is reserved for pure durum wheat pasta. However, because of the possibility of accidental contamination occurring during either wheat harvest or storage and transport of grains and semolina, pasta is only officially regarded as impure when the common wheat level exceeds 3%. This legal threshold of 3% is also applicable for export of durum wheat pasta outside the European community as stipulated by the European Commission regulation (1222/94, EC 1994).

Different techniques have been used to determine the level of common wheat adulteration in pasta. These have been based on research concerning sitosterol palmitate (Matveff 1952) or water-soluble proteins specific to common wheat (Resmini 1968; Garcia-Faure et al 1969; Feillet and Kobrehel 1972). Unfortunately, these methods are either not specific enough or not sensitive enough. A great improvement was achieved with the method of Kobrehel and Feillet (1976), based on the detection of the peroxidase-a7D specific to the D genome (Kobrehel and Gautier 1974). However, over the last 15 years, a significant modification in pasta technology has occurred: use of high temperatures in the drying process. Indeed, most industrially made pastas are now dried at high or very high temperatures (70–100°C). This results in protein degradation that makes quantification of the level of adulteration impossible with previous methods. In response to this development, new methods based on the identification of *T. aestivum* specific gliadins (Kobrehel et al 1985; McCarthy et al 1990) or immunodetection of friabilin (Durotest, Rhone Poulenc Diagnostic Ltd.) have been proposed, but these do not meet the standards required for adoption as official methods. Consequently, the Kobrehel and Feillet method, based on the detection of a peroxidase specific to the D genome, is still the official method in France (Journal Officiel 1975). Recently, because DNA exhibits greater thermal resistance than proteins, a polymerase chain reaction (PCR) based method has been developed to detect the simple presence of common wheat in durum wheat

pasta (Bryan et al 1998). A variety of DNA-based methods are available for food authentication and PCR-related techniques appear to be the methods of choice because of their high sensitivity and specificity, and the need for only a small amount of DNA (Lockley and Bardsley 2000). In addition, the development of real-time PCR (Holland et al 1991; Higuchi et al 1993; Heid et al 1996) has raised the further possibility of accurate quantification of food adulteration by similar methods.

The objectives of this work were to demonstrate the feasibility of quantification of common wheat adulteration of durum wheat pasta using real-time PCR. The puroindoline-b gene that is present in *T. aestivum* and absent in *T. durum* (Gautier et al 2000) was used as a target sequence to quantify the common wheat specific DNA. Because of the maximum 3% adulteration tolerance for common wheat content in pasta, this study focused on pastas with a common wheat content of approximately this value.

## MATERIALS AND METHODS

### Materials

Three series of durum wheat (*Triticum durum* Desf.) pastas (spaghetti) containing different percentages of common wheat (*T. aestivum* L.) adulteration (1, 3, 5, 7.5, and 10%, w/w) were prepared by the Centre de Recherches Européen, Céréales, Riz, Pâtes Alimentaires (CRECERPAL, Marseille, France). Before mixing, industrial durum wheat 3SE semolina (250–300 µm) and common wheat flour (100–150 µm) were conditioned to the same water content. Each sample was prepared starting with 5 kg of semolina. The dough (32% H<sub>2</sub>O) was processed into spaghetti using a small-scale pasta press (AFREM, Lyon, France) and extruded at a pressure of 100 bars. The final step was drying for 14 hr at 30°C (low temperature [LT]), 11 hr at 70°C (high temperature [HT]) or 4.5 hr at 85°C (very high temperature [VHT]) (Table I). Spaghetti (1.5 mm dry diameter) was ground in a ball grinder (Dangoumau S.A., Clermont-Ferrand, France) to obtain particle sizes of 100–150 µm. The pulverizing bowl was washed after each use with bleach containing 1.7% active chlorine to eliminate all DNA traces. One reference sample containing 3% common wheat was also prepared by mixing durum wheat semolina with common wheat flour directly in the tube before the extraction of DNA. To get similar size particles, both semolina and flour were ground as described above for spaghetti.

### Peroxidase Extraction and Detection

Peroxidase extraction was performed as described by Kobrehel and Feillet (1976), starting with 0.2 g of ground pasta and 0.8 mL of 0.1M Na acetate buffer. Proteins were separated in 4% (stacking) and 8% (separating) native acrylamide gels (6.5 cm × 10 cm × 1.5 mm) (minigel system, Hoefer SE 250, Amersham Pharmacia Biotech, Orsay, France). A volume of 12 µL of loading buffer (0.4%, w/v, of methyl green and 50%, v/v, glycerol) was added to 25 µL of protein extract. Gels were run for 7 hr at constant voltage (175V)

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in 24 mM Tris, 100 mM glycine buffer (pH 8.5). Peroxidases were stained as described by Kobrehel et al (1972) using catechol and H<sub>2</sub>O<sub>2</sub>.

### DNA Extraction

The pasta genomic DNA was extracted and purified using a blood and cell culture DNA midi kit (Qiagen S.A., Courtaboeuf, France). Lysis buffer (10 mL) (800 mM guanidine HCl; 30 mM Tris HCl, pH 8.0; 30 mM EDTA, pH 8.0; 5% Tween; 0.5% Triton X-100) containing 200 µg of RNase A were added to 1 g of ground pasta, mixed by vortexing, and incubated at 60°C in a water bath for 1 hr. During incubation, the tubes were mixed by inverting every 10 min. Thereafter, 1 mg of proteinase K was added, and the solution was mixed and incubated as described above. The solution was centrifuged at 3,600 × g for 20 min at 20°C using a swinging-bucket rotor, and the supernatant was transferred to a new tube. Equilibration buffer (0.5 vol) (750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol; 0.15% Triton X-100) was added to the supernatant before loading on Qiagen columns previously equilibrated with 4 mL of the same buffer. A flow rate of ≈10–20 drops/min was used; a syringe piston was used as necessary to achieve this rate. The column was washed twice with 7.5 mL of washing buffer (1.0M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol), then the DNA was eluted by 5 × 1 mL of elution buffer (1.25M NaCl; 50 mM Tris HCl, pH 8.5; 15% isopropanol) prewarmed at 50°C. The DNA was then concentrated with the QIAquick PCR purification kit (Qiagen) using a vacuum manifold (QIAvac 24, Qiagen) according to the manufacturer's recommendations. The DNA was eluted by adding 50 µL of 10 mM Tris HCl, pH 8.5, to the QIAquick column, centrifuging for 1 min at 18,000 × g, and storing at –20°C until needed.

### DNA Quantification

DNA quantification was performed with the PicoGreen dsDNA quantitation kit using λDNA as a standard and according to the manufacturer's instructions (Molecular Probes, Interchim, Montluçon, France) (Singer et al 1997). DNA was diluted 400-fold in 1 mL of 1X TE buffer containing the PicoGreen dsDNA quantitation reagent. After 5 min of incubation at 30°C in a water bath, fluorescence emission was measured at 526 nm with a spectrofluorimeter LS50B (Applied Biosystems, Courtaboeuf, France) following excitation at 480 nm.

TABLE I  
Pasta Samples

Common Wheat (% w/w)	Drying Temperature <sup>a</sup>		
	LT 30°C	HT 70°C	VHT 85°C
0	LT0	HT0	VHT0
1	LT1	HT1	VHT1
3	LT3	HT3	VHT3
5	LT5	HT5	VHT5
7.5	LT7.5	HT7.5	VHT7.5
10	LT10	HT10	VHT10

<sup>a</sup> Dried for 14 hr at 30°C (low temperature [LT]), 11 hr at 70°C (high temperature [HT]) or 4.5 hr at 85°C (very high temperature [VHT]).

TABLE II  
Primers and Probes

Gene	Name	Orientation	Sequence (5'→3')	EMBL Accession
Wheat puroindoline-b <sup>a</sup>	pinbF	Forward primer	AGCACTTCTCCCGAACCTCA	X63669
	pinbR	Reverse primer	CAGTCACCTGGCCCAAAA	
	pinb	Forward probe	CTCACAGCCGCCCTTCCACCA	
Wheat lipid transfer protein	ltp490F	Forward primer	TGCGACGGCGTCAAGAA	X69912
	ltp490R	Reverse primer	AGCGCTTTGGCGATCG	
	ltp490	Forward probe	TCCATAACCAGGCGGATCCCA	

<sup>a</sup> Pinb primers and probe were designed using the complementary strand of the puroindoline-b sequence.

### Agarose Gel Electrophoresis

DNA was analyzed on 0.8% agarose gel using 1X TBE buffer (Sambrook et al 1989), stained with ethidium bromide, visualized under UV with the Image Master VDS (Amersham Pharmacia Biotech). The amount of DNA loaded for each sample was 1.5 µg. A 1 kb DNA ladder (Life Technologies, Cergy Pontoise, France) was used as a control for size.

### PCR Parameters

Analyses were performed using the ABI Prism 7700 and the Sequence Detector software (v. 1.6.3, Applied Biosystems). Amplification plots were generated by plotting the average  $\Delta Rn$ , which was calculated for each cycle, versus the predefined total number of cycles. The  $\Delta Rn$  represented the amount of annealed fluorescent probe cleaved during amplification. It was calculated as  $\Delta Rn = (Rn+) - (Rn-)$ , where  $(Rn+)$  is the emission intensity of the reporter divided by the emission intensity of the passive reference during a specific amplification cycle, and  $(Rn-)$  is the emission intensity of the reporter divided by the emission intensity of the passive reference before amplification. The threshold cycle, or Ct value, corresponds to the cycle number at which the  $\Delta Rn$  rises above baseline and is reported as the fractional cycle number reflecting a positive result.

### Sequences of Primers and Probes

Sequences of primers and TaqMan probes listed in Table II were designed using the Primer Express software (Applied Biosystems) and synthesized by Applied Biosystems. Total wheat DNA was amplified using the ltp490F/ltp490R primer pair, which amplifies a 61 bp portion of a lipid transfer protein (ltp) gene (Diercyk et al 1992). Common wheat DNA was amplified using the pinbF/pinbR primer pair, which amplifies a 63 bp portion of a *T. aestivum* puroindoline-b (pinb) gene (Gautier et al 1994). The ltp probe was labeled at its 5'-end with the fluorescent reporter dye VIC (Applied Biosystems) and the pinb probe with the fluorescent reporter dye FAM (6-carboxy-fluorescein). The fluorescent quencher dye, TAMRA (6-carboxy-tetramethyl-rhodamine), was located at the 3'-end of the probes.

### Real-Time Simplex PCR Conditions

Simplex PCR were performed with the TaqMan Universal PCR Master mix (Applied Biosystems) using 5 µL of 160× diluted DNA, corresponding to 0.3125 µL of the DNA extracted from 1 g of pasta. Reactions were performed with the TaqMan PCR reagent

TABLE III  
Research for PCR Inhibitors in Real-Time Duplex PCR<sup>a</sup>

Dilution Factor	Ct pinb PCR	Ct ltp PCR	$\Delta Ct$ Ct pinb – Ct ltp
0	27.65	23.59	4.06
2	28.55	23.03	5.52
4	29.49	23.36	6.13
16	31.09	25.03	6.06
64	33.38	27.03	6.35

<sup>a</sup> DNA extracted from HT3 pasta and dilution factor zero corresponds to 5 µL of DNA (1/10 sample). Concentrations of pinb and ltp490 primers were 400 nM and 135 nM, respectively.

mix in 25  $\mu$ L containing 1X TaqMan buffer A, 5 mM MgCl<sub>2</sub>, 8% glycerol, 200  $\mu$ M of dNTP (dATP, dCTP, dGTP), 400  $\mu$ M dUTP, 0.125 units of Amperase uracil-N-glycosylase (UNG), 0.625 units of AmpliTaq Gold DNA polymerase, 200 nM of pinbF and pinR primers or 200 nM of ltp490F and ltp490R primers, 200 nM of probe, and the passive reference ROX. As a first step, Amperase UNG was activated at 50°C for 2 min then AmpliTaq Gold DNA polymerase was activated at 95°C for 10 min. This was followed by 45 cycles of 15 sec denaturation at 95°C and 60 sec at 60°C for annealing and chain elongation. A negative control of PCR was included in each series of samples.

### Real-Time Duplex PCR Conditions

Duplex PCR were performed using the same conditions as simplex PCR, except for primer concentrations. Indeed, duplex PCR first requires optimization of the concentration of both pairs of primers used simultaneously. Optimized primer concentrations were 400 nM pinb primers/135 nM ltp primers. A negative control of PCR was included in each series of samples.

### Construction of Standard Curves

To construct the standard curves of Ct values, quantification of wheat DNA was performed using the Picogreen reagent as described above. DNA was extracted from the 3% reference sample to make serial dilutions. Total DNA content was 471–7.36 ng, corresponding to 14–0.22 ng of common wheat DNA. The PCR efficiency was calculated as  $[10^{1/\text{slope}} - 1] \times 100$ .

### Determining Common Wheat Content of Durum Wheat Pasta

Using standard curves, Ct values were used to determine the amount of total DNA (durum and common wheat) using the ltp

PCR system, and the amount of common wheat DNA using the pinb PCR system. The common wheat content of pasta was determined as the ratio of common wheat DNA to total wheat DNA.

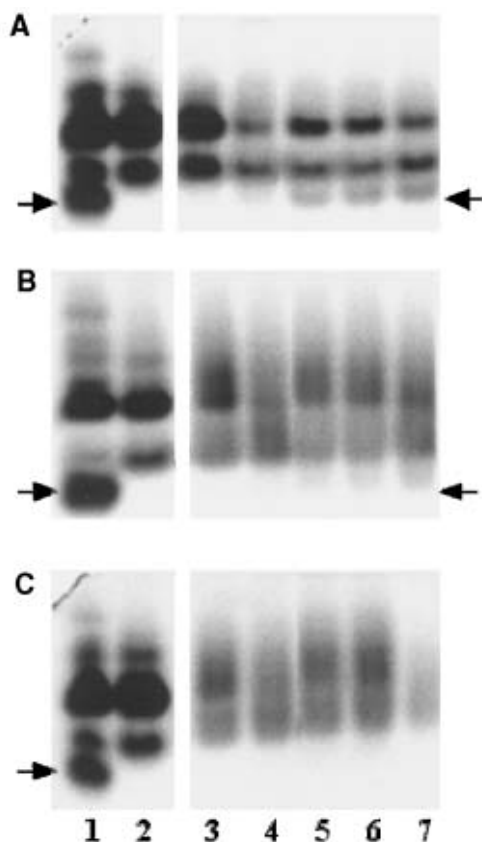
## RESULTS AND DISCUSSION

### Peroxidase Method

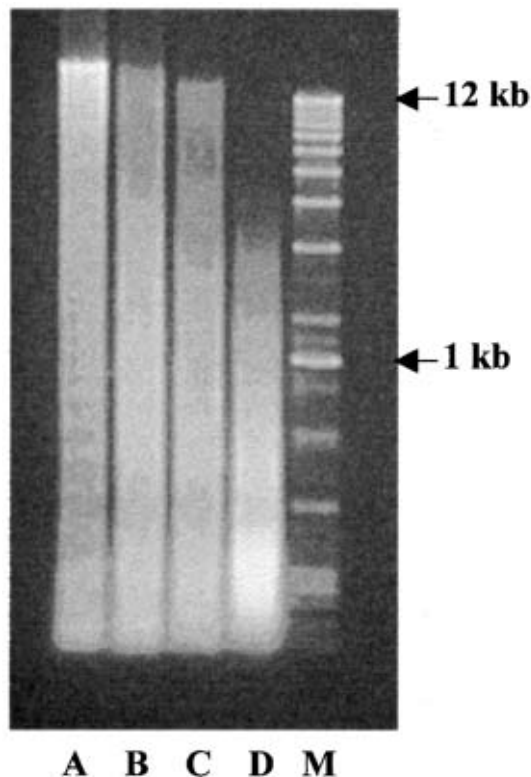
The effect of the drying temperature of pasta on the electrophoretic pattern of peroxidases is illustrated in Fig. 1. When pastas were dried at 30°C, the intensity of peroxidase-a7D, which is specific to *T. aestivum*, increased with the percentage of adulteration (1–10%). When pastas were dried at 70°C, bands were fainter but still visible, although it was difficult to detect adulteration by common wheat at <10%. When pastas were dried at 85°C, the electrophoretic patterns showed that peroxidases were degraded and only smears were visible. The peroxidase-a7D specific of *T. aestivum* was not detected at all.

### Quality of DNA

Before quantification of the common wheat content of pasta, the quality of extracted DNA was analyzed by electrophoresis. The quality of DNA was variable between samples: DNA extracted from VHT pastas were much more highly degraded than those extracted from HT or LT pastas (Fig. 2). To verify that the DNA degradation observed for VHT pastas was not an artifact, DNA extraction was repeated a second time, and identical electrophoretic patterns were observed (data not shown). The DNA extracted from the reference sample was mainly composed of molecules >12 kb, whereas DNA extracted from the VHT pasta was mainly composed of molecules <1 kb. DNA extracted from LT and HT pastas had intermediate patterns. DNA extracted from LT pasta was degraded compared with that extracted from the reference sample, indicating that DNA was degraded during pasta processing (mixing and extrusion), as well as during the drying process.



**Fig. 1.** Effect of drying temperature of pasta on peroxidase electrophoretic pattern for common wheat flour (1), durum wheat semolina (2), common wheat adulteration of 1% (3), 3% (4), 5% (5), 7.5% (6), 10% (7). Pasta dried at low temperature (LT) (A), high temperature (HT) (B), and very high temperature (VHT) (C). Peroxidase-a7D specific to D genome indicated by arrow; 8% native polyacrylamide gels (Tris glycine buffer pH 8.5). Peroxidases stained with catechol and H<sub>2</sub>O<sub>2</sub>.



**Fig. 2.** Quality of genomic DNA extracted from 3% adulterated pasta. DNA (1.5  $\mu$ g) electrophoresed on 0.8% agarose gels in 1 $\times$  TBE buffer and stained with ethidium bromide. A, 3% reference sample; B–D, pasta dried at low (LT3), high (HT3), and very high (VHT3) temperatures. M, 1kb DNA ladder (Life Biotechnologies).

In addition, the yield of DNA extraction was lower for VHT pastas than for LT and HT pastas. On average, taking as reference the amount of DNA extracted from semolina, the yield of DNA extraction was 75% for LT pastas, 60% for HT pastas, and 35% for VHT pastas.

### PCR Inhibitors

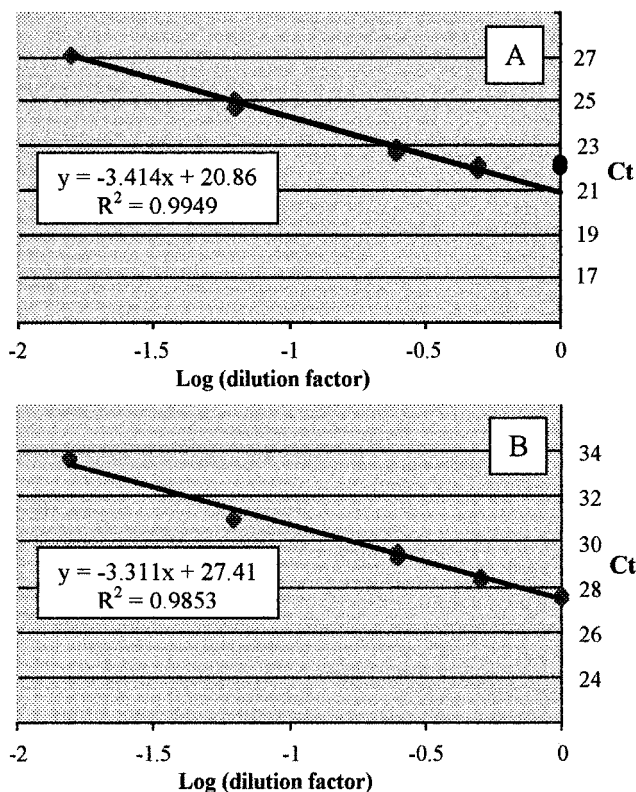
Investigation of *Taq* DNA polymerase inhibitors was undertaken using DNA extracted from the HT3 pasta by serial dilution (0–64×) of DNA extract, the dilution factor zero corresponded to 5 μL of DNA extract. Real-time simplex PCR was performed for both *ltp* and *pinb* PCR (Fig. 3). Theoretically, in the absence of PCR inhibitors within the DNA extract, the *Ct* value increases by one unit for each halving of the number of DNA targets. If the difference between the experimental and theoretical *Ct* values is >0.5, it indicates that the concentration of PCR inhibitors in the volume of DNA extract used for the PCR reaction is too high. Thus, it is necessary to use a lower volume of DNA extract to obtain an experimental *Ct* value identical or very close to the theoretical *Ct* value. For the *ltp* PCR, the undiluted DNA gave an experimental *Ct* value of 22.1, which was higher than the expected theoretical *Ct* value of 20.8, indicating interference by PCR inhibitors (Fig. 3A). Consequently, the *Ct* value for the undiluted DNA was not integrated to calculate the linear regression, showing that such a volume of DNA extract was not suitable for the performance of quantitative PCR. In contrast, for the *pinb* PCR, the undiluted DNA gave a correct experimental *Ct* value, indicating that the *pinb* PCR was less affected by the presence of PCR inhibitors than the *ltp* PCR. In this case, the *Ct* value for the undiluted DNA was integrated in the calculation of the linear regression (Fig. 3B). The linear regression obtained between the *Ct* values and DNA concentrations (expressed on a logarithmic scale) gave calculated values of  $R^2 = 0.9949$  for the *ltp* PCR and  $R^2 = 0.9853$  for the *pinb* PCR. The calculated

PCR efficiencies were 96.3% for the *ltp* PCR and 100% for the *pinb* PCR.

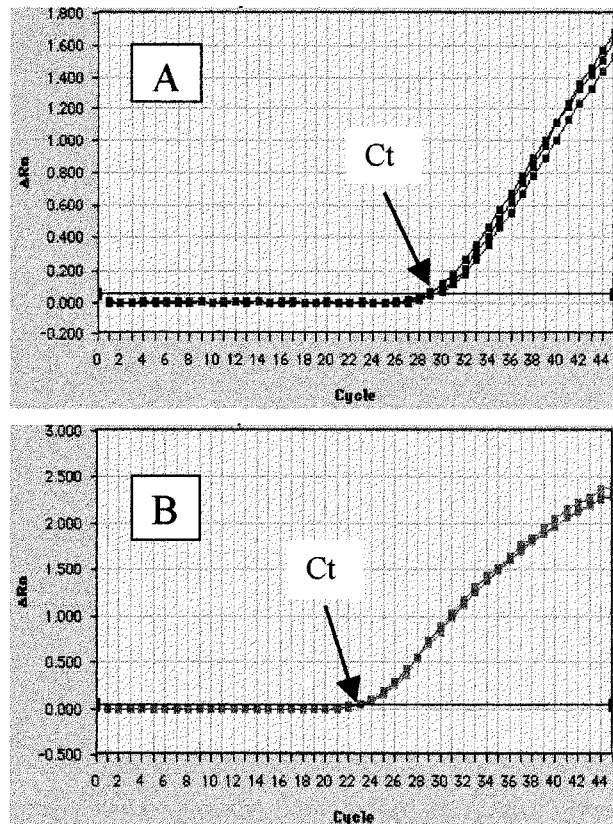
Investigation of PCR inhibitors was conducted using optimized real-time duplex PCR conditions. For pasta containing 3% common wheat, if the number of copies of the *ltp* and *pinb* genes was identical, the theoretical  $\Delta C_t$  between the *pinb* PCR *Ct* and the *ltp* PCR *Ct* is 5.1, because the *Ct* value increases of one unit each time the number of target sequences is divided by two. However if, as is the case, there are two copies of the *ltp* gene versus one copy of the *pinb* gene (unpublished results), the theoretical  $\Delta C_t$  between the *pinb* PCR *Ct* and *ltp* PCR *Ct* is 6.1. For undiluted DNA and 2× diluted DNA, the measured  $\Delta C_t$  values were 4.06 and 5.52, respectively (Table III), indicating the presence of inhibitors at a concentration that interfered with PCR efficiency. In contrast, when the DNA was diluted 4× or more, the measured  $\Delta C_t$  (6.06–6.35) was very close to the theoretical  $\Delta C_t$  (6.1). Because the level of PCR inhibitors may vary from one DNA extract to another, all the real-time duplex PCR were performed with 16× diluted DNA.

### Real-Time Duplex PCR

For this study, DNA was extracted from HT3 pasta with the maximum legal 3% adulteration for accidental contamination by common wheat. Duplex PCR first requires optimization of the concentration of both pairs of primers used simultaneously. To do this, the PCR efficiency was determined for each pair of primers. To avoid competition between the two PCR, the primer concentration for the *ltp* PCR, in which the number of target sequences is much higher than for the *pinb* PCR, must be lower than the primer concentration of the *pinb* PCR. The concentration of *pinb* primers was set to 400 nM and the concentration of *ltp* primers varied at 120–200 nM. In these conditions, the efficiency of *pinb* PCR was 75–150% and the efficiency of *ltp* PCR was 96–166% (Table IV). Within the



**Fig. 3.** Real-time simplex PCR standard curves on HT3 pasta. Total DNA content was 7.36–471 ng and common wheat DNA content was 0.22–14 ng. (A) *ltp* PCR (200 mM *ltp*490F/*ltp*490R primers and 200 mM *ltp*490 probe); (B) *pinb* PCR (200 mM *pinb*F/*pinb*R primers and 200 mM *pinb* probe). Each determination made in triplicate.



**Fig. 4.** Amplification plots of real-time duplex PCR on HT3 pasta. (A) *pinb* PCR (400 mM *pinb*F/*pinb*R primers and 200 mM *pinb* probe); (B) *ltp* PCR (135 mM *ltp*490F/*ltp*490R primers and 200 mM *ltp*490 probe). Each curve corresponds to three replicates.

five pairs of primer concentrations tested, the 400 nM pinb/135 nM ltp primer pair was selected, which gave the same efficiency for both PCR and a value close to the theoretical value of 100%.

The common wheat content of all pastas was determined using the real-time duplex PCR conditions defined above, starting with 16× diluted DNA extract and using standard curves constructed from the 3% reference sample. Amplification plots for the pinb and ltp PCR for HT3 pasta are shown in Fig. 4. For a given sample, the overlap of the three curves indicated good repeatability of the real-time duplex PCR. Theoretical and experimentally determined common wheat adulterations of pastas were in good agreement for all but the LT10 and HT7.5 pastas, which were overestimated (Fig. 5). The discrepancy observed between the theoretical and experimentally determined values was more likely to be due to sampling problems at the pasta-grinding level. For accurate determination using real-time PCR, it is preferable to work with ≈100 copies of the target gene, and in any event, at least 30 copies. Below this value, the probability that repetitions contain relevant DNA copies is insufficiently high. Because all PCR were performed with the same volume of DNA extract, the number of targets for the pinb PCR was, on average, lower for VHT pastas than for others.

For pastas with a theoretical adulteration of 3%, the mean value determined by real-time duplex PCR was 2.6–3.4%, depending on drying temperature. The 100% durum wheat pastas (LT0, HT0, and VHT0) were also analyzed, and the results of real-time duplex PCR (data not shown) indicated that no accidental contamination had occurred during pasta fabrication or DNA extraction. Consequently, there was no ambiguity in the discrimination of pure durum wheat pastas from adulterated pastas.

## CONCLUSIONS

Using the method based on the detection of the peroxidase-a7D specific to the D genome, it was not possible to quantify the adulteration of HT pasta, or to even detect the adulteration of VHT pasta. Thus, new methods for the detection of adulteration of durum wheat pasta by common wheat were required.

Because of the high thermal resistance of DNA, PCR-based methods are more appropriate than protein-based methods, and a PCR-based method was previously developed for simple detection of hexaploid wheat in durum wheat pasta (Bryan et al 1998). This current study proposes the use of real-time PCR to quantify the adulteration of durum wheat pasta by common wheat. Real-time PCR generates small amplicons and therefore amplification of degraded DNA templates, such as those obtained from manufactured pasta products, is much more likely to occur. In addition, amplicons are fully specific to the target sequences because of the use of two template-specific primers and the use of a third oligonucleotide probe which supplies a second level of specificity. Another advantage is that real-time PCR does not require post-PCR sample handling, preventing contamination and resulting in much faster and higher throughput assays. Puroindolines whose genes are located on the 5D chromosome of hexaploid wheat (Sourdille et al 1996) and are present in diploid species and absent in tetraploid species (Gautier et al 2000) were selected as a D-genome specific sequence.

This study focused on real-time duplex PCR because simultaneous quantification of total wheat and common wheat DNA is more accurate, less labor intensive, and also less expensive than real-time simplex PCR. A prerequisite for real-time duplex PCR is the optimization of primer concentrations to verify that both PCR show good efficiency. Because of the 3% maximum adulteration tolerated for common wheat content in pasta, this study focused on pasta with a common wheat content of approximately this value, and standard curves were constructed with a 3% reference sample. In the conditions used in this study, for all pastas (LT, HT, and VHT) with a theoretical adulteration of 3%, the mean value determined by real-time duplex PCR was 3% ± 0.4 at a 95% confi-

dence limit. Pure durum wheat pastas were identified without ambiguity, which is clearly important for commercial application.

To obtain accurate results, real-time PCR requires a minimum of 30 copies of the target sequence to be amplified. Under the conditions defined by this study, for 3% adulterated pastas the number of copies of the pinb sequence ranged from 120 in LT pasta to 35 in VHT pasta. These estimates were obtained using a value of 33 pg of DNA/wheat haploid genome. All duplex real-time PCR were performed with 16× diluted DNA, giving a more limited number of copies of the pinb target sequence for VHT pastas than others because of the lower yield of DNA extraction. Investigation of *Taq* DNA polymerase inhibitors indicated that 4× diluted DNA still gave good PCR efficiency. This means that 8× diluted DNA could be safely used, so doubling the number of the pinb target sequence present and thus improving pinb PCR accuracy. To quantify low-level adulteration by real-time PCR, an equilibrium should be found between two negatively correlated parameters: 1) the amount of DNA template and 2) the amount of PCR inhibitors. Indeed, once all PCR parameters have been optimized, the efficiency of real-time PCR is highly sensitive to the incidence of PCR inhibitors, which are present mainly in DNA extracted from transformed products. Real-time PCR is a convenient technique for the quantification of adulteration in other types of food products. For example, we have used it to determine the GMO content of maize and soybean (Alary et al 2002).

This study demonstrates the feasibility of using real-time duplex PCR to quantify the adulteration of durum wheat pasta by common wheat, and from these results a method can be developed. However, in developing such a method, a number of important factors need to be taken into consideration, including the checking across a wide range of commercial wheats that the copy number of both genes is constant. Sampling and grinding conditions of pasta and the reproducibility of the quantification by duplex real-time PCR in different laboratories will have to be studied further.

TABLE IV  
Real-Time Duplex PCR Efficiency<sup>a</sup>

Primers	Primer Concentration (nM)	PCR Efficiency (%)
pinb/ltp	400/120	150/166
pinb/ltp	400/130	89/125
pinb/ltp	400/135	104/104
pinb/ltp	400/140	92/116
pinb/ltp	400/200	75/96

<sup>a</sup> DNA extracted from HT3 pasta. For each primer concentration, PCR was conducted on 5 μL of 4, 16, and 64× diluted DNA. PCR efficiency calculated as  $[10^{1/\text{slope}} - 1] \times 100$ .

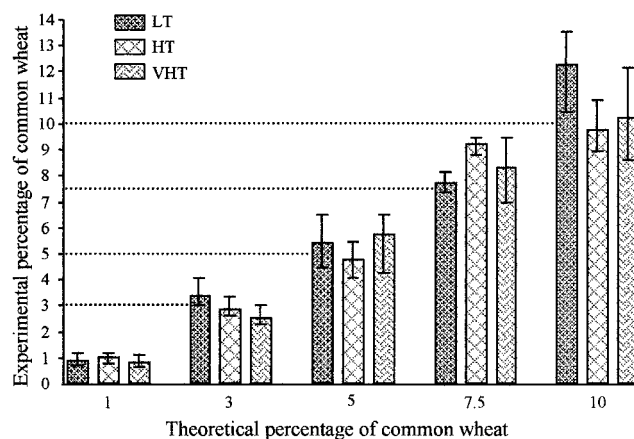


Fig. 5. Real-time duplex PCR determination of common wheat adulteration (1–10%) of pastas dried at low, high, and very high temperatures (LT, HT, and VHT). Data correspond to mean value of triplicate analysis. Vertical bars indicate minimum and maximum values; horizontal dotted lines show theoretical percentage adulteration of pasta.

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