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Compared Use of HPLC and FZCE for Cluster Analysis of *Triticum* spp and for the Identification of *T. durum* Adulteration

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Wheat quality criteria continually evolve in response to market pressure and consumer preference. Characterization of cereal cultivars for quality and agronomic properties, have widely shown the importance of the protein content to ensure good quality products. The aim of this work is a comparison of reversed-phase high performance liquid chromatography (RP-HPLC) and free zone capillary electrophoresis (FZCE) in the identification of Italian wheat cultivars and detection of durum wheat flour adulteration. Mainly alcohol soluble (gliadins) and water soluble (albumins) proteins were extracted from 14 common wheat cultivars and from 9 durum wheat cultivars. In RP-HPLC chromatograms, wheat albumins and gliadins eluted between 3 and 9 min and between 10 and 42 min, respectively. Even if the chosen chromatographic conditions (reversed phase) did not permit a complete resolution of hydrophilic proteins such as albumins, a good reproducibility was observed for both albumins and gliadins. In FZCE electropherograms, wheat albumins and gliadins migrated between 8 and 14 min and 16−25 min, respectively. A good reproducibility was found for wheat albumins, while the relatively poor reproducibility of gliadin fractions was a consequence of the selected separation conditions aimed to separate in the same run either hydrophilic (albumins) and alcohol-soluble (gliadins) proteins. The principal component analysis (PCA) of HPLC and FZCE data evidenced that both techniques allowed the univocal identification of the great proportion of investigated wheat cultivars. Three peaks were exclusively detected in RP-HPLC chromatograms of common wheat cultivars, while three unique peaks were found in FZCE electropherograms of common wheat cultivars. These peaks were investigated as a basis for detecting and estimating the adulteration of durum wheat flour with flour from common wheat. The direct relationship between the area of the peaks and adulteration level enabled standard curves to be constructed. The standard curves showed that adulteration may be quantified by either RP-HPLC or FZCE.

KEYWORDS: RP-HPLC; capillary electrophoresis; durum wheat adulteration; cultivar identification; principal component analysis

INTRODUCTION

Several studies have been carried out to characterize cereal cultivars for quality and agronomic properties. In particular, the use of biochemical analyses to identify wheat cultivars has been ongoing for many years. The ability to clearly identify a wheat sample before the beginning of a breeding program may save years of no-result efforts. For example, Jones et al. (*1*) discovered that 3% of samples given to breeders from USDA National Germplasm Center were incorrectly marked. On the other hand, cultivar identification represents one of the most important parameters for end-use quality.

Durum wheat has found traditional use for pasta and some specialty breads, particularly in the Mediterranean countries (*2*). In Italy, the pasta is guaranteed by the law, which foresees that the dry industrial pasta has to be exclusively produced with durum wheat flour. In other EU countries and in the Unites States, the common flour is allowed for the preparation of pasta. Since durum wheat is approximately 20% more expensive than common wheat, efficient methods for the detection of the contamination in pasta and bread, made with durum wheat flour, are basic requirements to underline the quality of Italian productions. The official Italian method for the detection and quantification of common wheat adulteration of durum wheat

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semolina and pasta is based on the separation of albumins by polyacrylamide gel electrophoresis (Native-PAGE) or isoelectric focusing (IEF) in thin-layer polyacrylamide gels (*3*, *4*). The albumin analysis on native PAGE permits to identify two protein bands specific of common wheat (not present in durum wheat) and characterized by a lower electrophoretic mobility with respect to the other albumin fractions of both common and durum wheats (*4*). According to Palumbo et al. (*5*), in Italy in the last 10 years, the share of durum wheat used for bread making has increased from 4 to 10% of Italian productions. In addition, several researchers stressed the suitability of durum wheat for making high volume bread and pan bread (*6*, *7*). Wheat quality (both durum and common wheat) criteria continually evolve in response to market pressure and consumer preference. Increasing demand for durum wheat quality attributes for different end-products requires development of more rapid objective means to grade and classify wheat on the basis of processing potential. The protein content continues to play a fundamental role to ensure good- quality products (*8*). Gliadins are all encoded by genes located on chromosomes 1 and 6 of the A, B, and D genomes (*9*). As a consequence, common wheat presenting a hexaploid genome (AABBDD) is characterized by a higher number of gliadin fractions than durum wheat holding a tetraploid genome (AABB) (*10*).

Column chromatography of seed reserve proteins is one of the major analytical techniques used to differentiate wheat cultivars. Reversed-phase high performance liquid chromatography (RP-HPLC) was first used to study the protein content of wheat phenotypes, permitting cultivar identification based on analysis of gliadins (*11*). McCarthy et al. (*10*) used RP-HPLC to study purity of F1 hybrid wheat samples. RP-HPLC has been shown to be a rapid procedure (usually 10−60 min), which can be automated, is sensitive and reproducible, and gives high resolution. HPLC analysis has been shown to be predictive of pasta cooking quality, has potentialities for predicting bread making quality (*12*, *13*), and is a useful tool in breeding and genetic studies (*13*). Identification and registration of bread wheat cultivars is mainly based on morphologic and physiologic characteristics. Even though these descriptors are useful, they are limited in number and may be affected by environmental factors. Molecular markers are a useful complement to morphological and physiological characterization of cultivars because they are plentiful, independent of tissue or environmental effects, and allow cultivar identification early in plant development. Molecular characterization of cultivars is also useful to evaluate potential genetic erosion, defined here as a reduction of genetic diversity in time and to perform DNA-based analyses of food products to determine cultivar identification, product alteration, presence of food-borne pathogens, and detection of ingredients derived from genetically modified sources (*14*−*16*).

The development of high performance capillary electrophoresis (HPCE) has introduced the capability of very fast electrophoretic separations (*17*). HPCE permits the separation of materials based on analytical principles different than those of HPLC or PAGE and therefore provides a complementary analytical approach. In particular, a number of papers have utilized one particular mode of HPCE, referred to as free zone capillary electrophoresis (FZCE), to separate cereal proteins and considerable development has been done with FZCE (*18*). Lookhart and Wrigley (*19*) have first described the potential use and benefit of FZCE for cereal cultivar identification. The technique is useful for selection during wheat breeding and in genetic studies (*20*). Werner et al. (*21*) reported the detection of variation among *Glu-D1* high molecular weight glutenin subunits in several wheat cultivars by FZCE.

Table 1. Investigated Common (C) and Durum (D) Wheat Cultivars and Their Pedigrees

wheat cultivars	pedigree
Bolero (C)	Line 2527/267 × Talent
Brasilia (C)	(Osijeka20 × Libellula) × (Bezostaja × Zladina)
Centauro (C)	Strimpelli × Irnerio
Eureka (C)	(Mironvskaja × M. Hunstman′) × (R.5.1.2 × Courtot)
Francia (C)	Manital × Pandas
Golia (C)	Manital × Orso
Manital (C)	Mendos × Marzotto
Mec (C)	Marzotto × Combine
Mieti (C)	Mec × Vinci
Mol (C)	Manital × (Orso × Loreto)
Nobel (C)	Mec × (Generoso × Mendos)
Pandas (C)	(Orso × Bezostaja) × (Generoso × Marzotto)
Santerno (C)	Nettuno × Orso
Spada (C)	(Trebbo × Kansas) × T. turgidum
Appio (D)	Cappelli × (Gaviota × Yuma)
Cirillo (D)	(Jucci × Polesine) × (Creso × Montanari)
Cosmodur (D)	D881 line (natural hybridization)
Creso (D)	CpB144 × (Yt54-N10B × p263Tc3)
Duilio (D)	Cappelli × (Anhinga × Flamingo)
Grazia (D)	M6800 127 × Valselva
Neodur (D)	(184/7 × Valdur) × Edmore
Vitron (D)	Turchia 77 × ((Jori S × Anhinga) × Flamingo))
Zenit (D)	Valriccardo × Vic

In the present research, RP-HPLC and FZCE techniques were employed to demonstrate the complementary use of these two analytical techniques for the characterization of Italian durum and common wheat cultivars and for the detection of common wheat flour contamination in durum wheat flour.

MATERIALS AND METHODS

Wheat Samples. Seeds of Italian durum wheat cultivars (*Triticum durum* Desf) and common wheat cultivars (*Triticum aestivum* L.) were obtained from authenticated stocks held at the Laboratory of Seed Research and Analysis (LaRAS, University of Bologna, Italy). The fourteen common wheat varieties used were Bolero, Brasilia, Centauro, Eureka, Francia, Golia, Manital, Mec, Mieti, Mol, Nobel, Pandas, Santerno and Spada, while the nine durum wheat cultivars used were Appio, Cirillo, Cosmodur, Creso, Duilio, Grazia, Neodur, Vitron, and Zenit. The pedigrees of the investigated common and durum cultivars are reported in **Table 1**.

HPLC Analysis. Seed samples of each investigated cultivar were milled in a PBI mill to a fine flour. Proteins were extracted from 200 mg of flour in 1.5-mL Eppendorf tubes with 800 μL of the extraction solution (50% 2-propanol (v/v), 4% DTT (w/v), 1% acetic acid (v/v)) for 60 min at 60 °C (*22*). Samples were centrifuged at 13500 rpm for 15 min. The supernatant was collected, filtered through a 0.45-μm GV Millipore filter, held constantly at 4 °C prior the analysis, and directly injected. All samples were extracted in triplicate.

The flours of the common wheat cultivar Santerno and of the durum wheat cultivar Appio were extracted according to the sequential extraction procedure, first described by Osborne (*23*) and modified by Lookhart and Bean (*20*). Briefly, the wheat meal (100 mg) was extracted with deionized water (500 μL). After centrifugation for 5 min at 12000 rpm, the decanted supernatant was collected. The extraction was repeated twice, and all the collected supernatants (albumin fraction) were pooled. The same extraction pattern was repeated for globulin extraction, using the centrifugate of the above procedure, but instead of deionized water, 400 μL of 0.5 N NaCl was employed. The extraction yielded the globulin fraction. The gliadin extraction was carried out with the remaining centrifugate in three steps in a similar way with 400 μL of 70% aqueous ethanol. Finally, the remaining pellet was extracted three times with 50% 2-propanol containing 1% (w/v) of 2-mercaptoethanol (400 μL). The supernatants collected after centrifugation were pooled and saved as glutenin fraction.

Another set of samples was prepared to simulate contamination of durum wheat flours by common wheat flour. As adulteration is usually caused by a mixture of common wheats rather than a single cultivar, the common wheat mixture chosen for use as adulterant was a blend containing in equal part the flour of the fourteen investigated common wheat varieties. The adulterant common wheat blend was added with decreasing percentages (50, 25, 10, and 5%) in the flour of the durum wheat (cv. Appio). The obtained samples were extracted in triplicate as described before.

The RP-HPLC system comprised a Beckman (Palo Alto, Ca) System Gold 126 with two pumps, a Rheodyne 7000 injection valve connected to a Spark Holland (Emmen, The Netherlands) Basic Marathon autosampler, a water circulator, which was used to maintain a constant column temperature and a Beckman Model 168 diode array detector. Separations were performed at 45 °C using a SGE Nucleosil C_{18}, 300-A pore diameter, 5-μm particle size 250- × 4.6-mm reversed phase column (SGE, Canberra, Australia), preceded by a nucleosil-based C_{18} cartridge guard column (SGE, Canberra, Australia). A 20-μL sample volume, roughly corresponding to 250−500 μg protein, was injected, and proteins were eluted at 1.0 mL/min using a gradient formed from solvent A (water containing 0.06% (v/v) trifluoroacetic acid) and solvent B (acetonitrile containing 0.06 (v/v) trifluoroacetic acid). Both solvents were filtered with a 0.22-μm Millipore filter and degassed before use. The ratio of solvents A/B (v/v) was decreased linearly from 65:35 to 40:60 (0−45 min) then to 0:100 (45−55 min) and held there for 10 min to wash the column before returning to initial conditions (55−65 min) to allow column reequilibration before subsequent injections. The column effluent was monitored at 210 nm. The software Beckman GOLD (Version 6.00) was employed for storing, manipulating and comparing chromatograms. The chromatogram of each wheat cultivar represents the average track obtained from nine runs (three extractions analyzed on different days per each accession and three injections analyzed on the same day per each extract).

FZCE Analysis. Wheat seed samples of each investigated cultivar were milled in a Buhler-Miag mill to a fine flour. Proteins were extracted from 500 mg of flour in 6-mL centrifuge tubes containing 4 mL of an ethanol/water solution (30%, v/v). Tubes were vortexed for 1 min and subsequently held at 30 °C for 30 min. Afterward, tubes were placed at 4 °C for 15 min and centrifuged at 2500 rpm for 10 min. Supernatant was transferred in Vectospin tubes and centrifuged at 2500 rpm for 5 min. A 30-μL aliquot of urea solution (60 mM) were added to 300 μL of the clear supernatant and analyzed. All samples were extracted in triplicate.

The sequential extracts of the common wheat cultivar Santerno and of the durum wheat cultivar Appio, obtained as previously described according to the procedure of Osborne (*23*) and modified by Lookhart and Bean (*20*), were also analyzed by FZCE.

The set of samples, obtained as previously described to simulate contamination of durum wheat flours by common wheat flour, was extracted in triplicate by using the same procedure employed for the analysis of wheat cultivars by FZCE as described before.

FZCE analyses were carried out with a Beckman P/ACE 2100 equipped with UV detector, set at 214 nm. Separations were carried out in an uncoated silica-fused capillary 50 cm long (from injection point to detector) and 75 μm i.d. The electrolyte buffer was 16 mM boric acid, 16 mM sodium tetraborate, 33 mM phosphoric acid, 1% (w/v) PEG 400, and 10% (v/v) ethanol. Samples were injected by electromigration, applying 8 kV for 15 s. Separations were obtained at 30 °C by applying a differential of potential of 20 kV. Capillary was washed daily and between runs with 1 M NaOH and separation buffer for 1 and 5 min, respectively. The software Beckman GOLD (Version 6.00) was employed for storing, manipulating and comparing electropherograms. The electropherogram of each wheat cultivar represents the average track obtained from nine runs (three extractions analyzed on different days per each accession and three injections analyzed on the same day per each extract).

Statistical Analysis. The reproducibility of RP-HPLC and FZCE methods was determined by calculating the variation coefficient of retention or migration times of peaks (*24*). For each investigated accession, the reproducibility was calculated on nine different runs (three extractions analyzed on different days per each accession and three injections analyzed on the same day per each extract). Mean reproducibility values for common and durum wheat analyzed by RP-HPLC and FZCE are expressed as the mean variation coefficient of peak retention times ± standard deviation. Chromatograms and electropherograms of wheat varieties were binary coded on the basis of presence or absence of a protein peak. Since peak retention and migration times are unaffected by environmental factors (*25*, *26*), the cultivars were only distinguished on the basis of presence and absence of peaks in the chromatograms and in the electropherograms. The similarity matrix based on simple matching among wheat accessions analyzed by RP-HPLC or CE was determined using the software package NTSYS-pc ver. 2.02 (Exeter Software, NY). Ordination analysis was carried out on the similarity matrixes of wheat accessions analyzed by RP-HPLC and FZCE by principal component analysis (PCA) using the NTSYS-pc ver. 2.02 (Exeter Software, NY). The PCA results were graphically represented by the projection of the first two components.

As concerns the analysis of artificially adulterated samples, total peak area of chromatograms and electropherograms was determined automatically. The adulterant peak areas were calculated through a manual interactive integration process by aligning the cursor at positions corresponding to the valley to valley of the peak. The adulterated peak areas were then corrected, taking into account the total peak areas, which were normalized to a constant value (1×10^7), thereby reducing variation occurring during extraction and injection, using the following formula: normalized peak area = (adulterated peak area × 10^7)/(total peak area) (*27*). Normalized peak area values were subjected to analysis of variance (ANOVA), and confidence intervals ($P < 0.05$) based on the pooled standard deviation for each contamination level were determined.

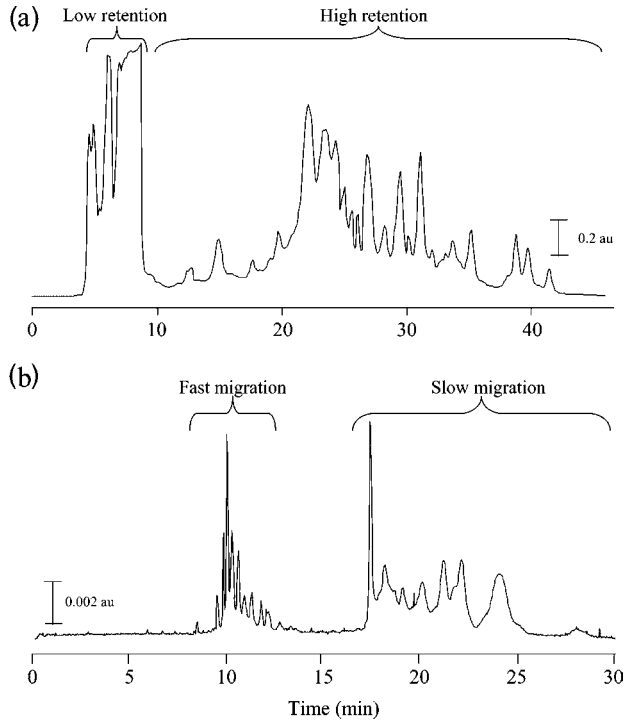

Figure 1. Separation of common wheat proteins (cv. Santerno) by RP-HPLC (**A**) and FZCE (**B**). The RP-HPLC and FZCE separations were carried out according to the procedures outlined in the Materials and Methods section.

RESULTS AND DISCUSSION

RP-HPLC Analysis. The chromatograms obtained by RP-HPLC were divided into two regions: a low retention region (3−9 min) and a high retention region (10−42 min) (**Figure 1a**). The extractant (50% 2-propanol plus DTT) employed for RP-HPLC analysis has been previously used to extract wheat

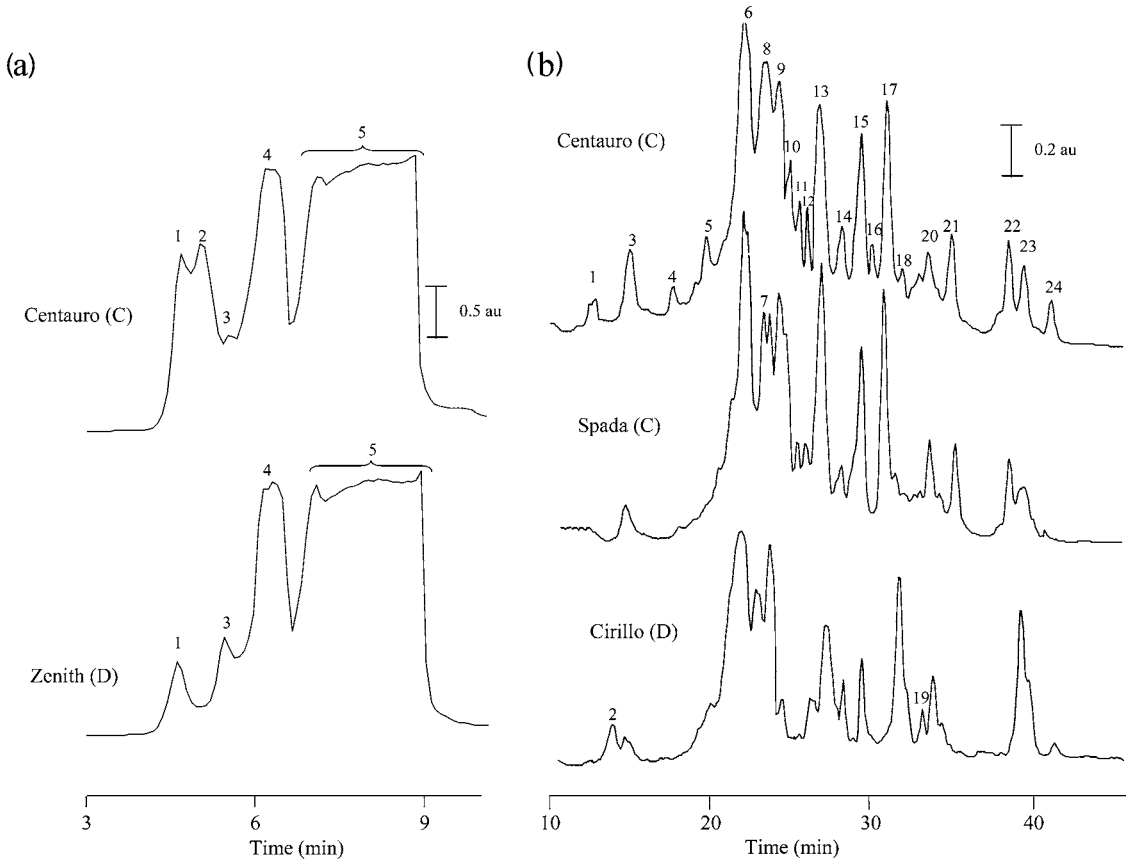

Figure 2. The total number of peaks observed across the analyzed wheat cultivars and detected in the low retention (**A**) and in the high retention (**B**) regions of RP-HPLC chromatograms. Analysis conditions are as given in Materials and Methods.

gliadins (*9*, *22*). DTT improved resolution and increased the amount of protein extraction, but even a considerable proportion of reduced glutenin in addition to gliadins was extracted (*22*). Besides, if a preliminary sequential fractionation is not carried out, some albumins may be present in the extract (*9*). The Osborne sequential extraction procedure confirmed that in addition to gliadins, the wheat extracts contained both albumins and glutenins (data not shown). The peaks of the low retention region (3−9 min) were mainly albumins, while gliadins and glutenins coeluted in the high retention region (10−42 min). Globulins (eluted within 10 min) were present in the analyzed samples in a very low concentration (data not shown).

In the low retention region, the albumin peaks were only partially resolved and coeluted with the solvent front. The chosen separation conditions (reversed phase) did not permit a good resolution of very hydrophilic fractions such as albumins (*28*, *29*). In the albumin elution zone, in all cultivars of common wheat five peaks were detected, while in all durum cultivars, four peaks were observed (**Figure 2a**, **Table 2**). The peak 2a was found only in common wheat cultivars. The mean variation coefficients of the retention times of peaks in this region were 5.7 ± 2.1% and 5.1 ± 1.7% for albumin fractions extracted from common and durum wheat, respectively.

In the high retention region, a total of twenty-four peaks was observed in the analyzed wheat accessions (**Figure 2b**). The mean variation coefficients of the retention times of peaks were 3.1 ± 2.5% and 3.7 ± 1.5% for protein fractions extracted from common and durum wheat, respectively. The mean numbers of peaks were 19.9 ± 1.3 and 15.0 ± 1.1 for common and durum wheat cultivars, respectively (**Table 2**). All common wheat accessions showed three major peaks (11g, 17g, and 22g), not present in durum wheat cultivars (**Table 2**). A similar result is reported by several authors who demonstrated in durum wheat the lacking of 2−3 major peaks usually detected in hexaploid wheat (*29*−*31*).

The principal component analysis (PCA) of distance data calculated from chromatograms of common and durum wheat cultivars is reported in **Figure 3a**. Common wheat cultivars are clearly differentiated from durum wheat cultivars. Durum wheat cultivars are charged on the second factor, while common wheat cultivars are mainly charged on the first factor. As shown by Smith (*32*), multivariate analyses of RP-HPLC data reveal relationships among inbreds that agree with known pedigrees. Thus, biochemical analyses provide fingerprintings that can uniquely identify almost all but exceptionally closely related phenotypes. The PCA analysis revealed relationships among common and durum wheat cultivars in general agreement with those expected from their pedigrees (**Figure 3a**, **Table 1**). The common wheat cultivars were divided in three clusters. The first, second, and third clusters were formed by cultivars characterized by a mean similarity of 78 ± 8%, 67 ± 5% and 60 ± 15%, respectively. In the first cluster, the cultivars Santerno and Golia shared a common parent (cv. Orso), contributing 50% of their genome. In the second cluster, the cultivar Pandas was a parent of the cultivar Francia. In the third cluster, the cultivar Mec contributed 50% of the genome of cultivars Mieti and Nobel. In addition, cultivars Nobel and Manital shared a common parent (cv. Mendos), while cultivar Manital contributed 50% of the genome of cultivar Mol. Finally, cultivars Mec and Manital shared cultivar Marzotto as a common parent. Two different clusters of durum wheat cultivars were found (cluster four and five). The fourth and fifth clusters were made of cultivars characterized by a mean similarity of 81 ± 8% and 81 ± 9%, respectively. In the fifth cluster, the cultivar Cappelli was a

Table 2. Peak Scoring (+ = Presence, – = Absence) in Low and High Retention Regions of RP-HPLC Chromatograms of Common (C) and Durum (D) Wheat Cultivars

	low retention					high retention																						
cultivar	1a	2a	3a	4a	5a	1g	2g	3g	4g	5g	6g	7g	8g	9g	10g	11g	12g	13g	14g	15g	16g	17g	18g	19g	20g	21g	22g	23g	24g
Bolero (C)	+	+	+	+	+	+	-	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Brasilia (C)	+	+	+	+	+	+	-	+	-	-	+	+	+	+	-	+	+	+	+	+	-	+	+	-	+	+	+	+	+
Centauro (C)	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Eureka (C)	+	+	+	+	+	+	-	+	-	-	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
Francia (C)	+	+	+	+	+	+	-	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Golia (C)	+	+	+	+	+	+	-	+	-	-	+	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+
Manital (C)	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mec (C)	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
Mieti (C)	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
Mol (C)	+	+	+	+	+	+	-	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nobel (C)	+	+	+	+	+	+	-	+	-	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+
Pandas (C)	+	+	+	+	+	+	-	+	+	-	+	-	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+
Santerno (C)	+	+	+	+	+	+	-	+	-	-	+	+	+	+	-	+	+	+	+	+	-	+	+	-	+	+	+	+	+
Spada (C)	+	+	+	+	+	+	-	+	-	-	+	+	+	+	-	+	+	+	+	+	-	+	+	-	+	+	+	+	+
Appio (D)	+	-	+	+	+	-	+	+	+	+	+	-	+	+	-	+	+	+	+	-	+	-	+	-	+	-	-	+	+
Cirillo (D)	+	-	+	+	+	-	+	+	-	+	+	+	-	+	+	-	+	+	+	+	-	-	+	+	+	-	-	+	+
Cosmodur (D)	+	-	+	+	+	+	-	+	-	+	+	-	+	+	+	-	+	+	+	+	-	-	+	-	+	+	-	+	-
Creso (D)	+	-	+	+	+	-	+	+	-	+	+	+	-	+	+	-	+	+	+	+	-	-	+	+	+	-	-	+	+
Duilio (D)	+	-	+	+	+	-	-	+	+	+	+	-	+	+	+	-	+	+	+	-	+	-	+	+	+	+	-	+	-
Grazia (D)	+	-	+	+	+	+	-	+	-	+	+	-	+	+	+	-	+	+	+	+	-	-	+	+	+	-	-	+	-
Neodur (D)	+	-	+	+	+	-	+	+	-	+	+	-	+	+	+	-	+	+	+	+	-	-	+	-	+	-	-	+	-
Vitron (D)	+	-	+	+	+	-	-	+	-	-	+	-	+	+	+	-	+	+	+	+	-	-	+	+	+	-	-	+	-
Zenit (D)	+	-	+	+	+	+	-	+	-	+	+	-	+	+	+	-	+	+	+	+	-	-	+	-	+	-	-	+	-

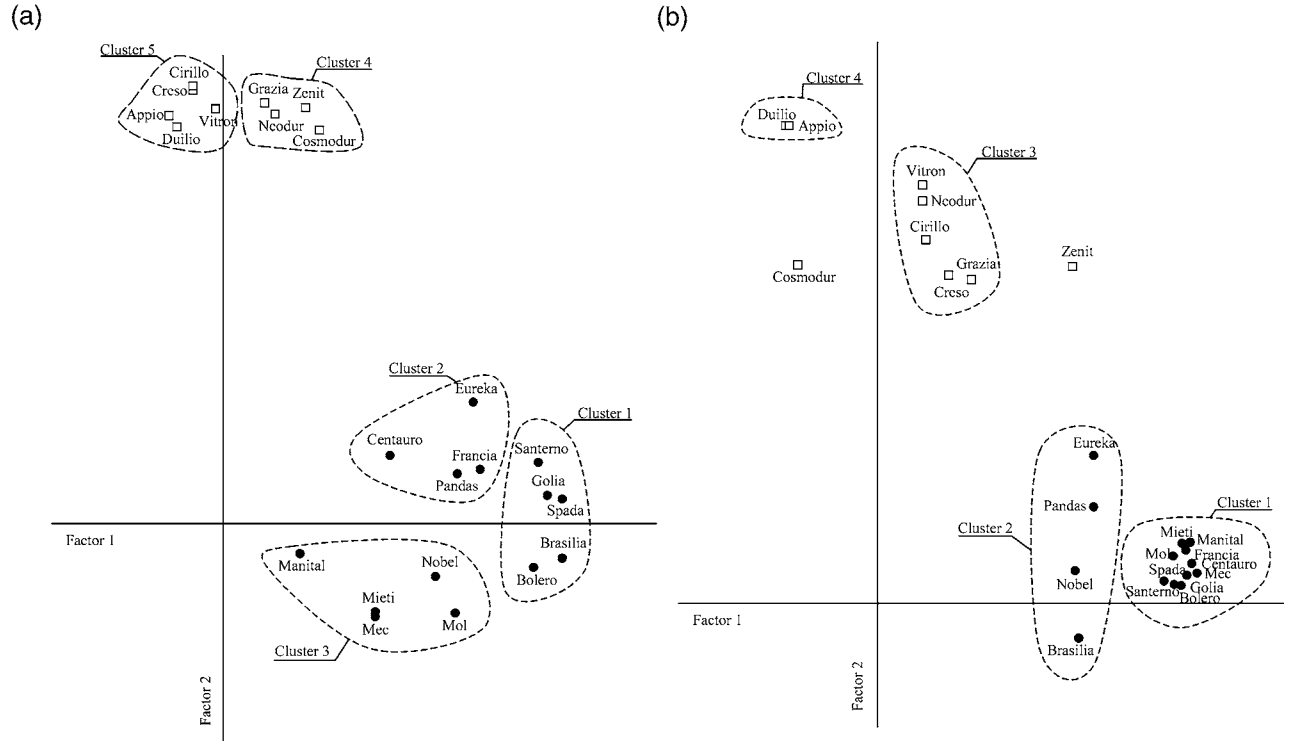

Figure 3. Principal component analysis of investigated common (●) and durum (□) wheat cultivars analyzed by RP-HPLC (**A**) and FZCE (**B**). Variance explained by PCA based on RP-HPLC data is 42.2% for the first factor and 21.1% for the second factor (total variance explained = 63.3%). Variance explained by PCA based on FZCE data is 31.5% for the first factor and 18.7% for the second factor (total variance explained = 50.2%). Dotted lines represent the different clusters outlined by the ordination.

common parent of both Appio and Duilio cultivars, while Duilio and Vitron cultivars shared a common ancestor (cv. Anhinga). Finally, 25% of the genome of the cultivar Cirillo is derived from the genome of cultivar Creso.

FZCE Analysis. The electropherograms obtained by FZCE were divided into two regions: a fast migration region (8−14 min) and a slow migration region (16−25 min) (**Figure 1b**). The extractant (30% ethanol) employed for FZCE analysis has been previously used to extract wheat gliadins (*20*, *33*). If a preliminary sequential fractionation is not carried out, gliadins are co-extracted with albumins (*34*). The Osborne sequential extraction procedure confirmed that in addition to gliadins, the wheat extracts contained albumins (data not shown). The peaks of the fast migration region (8−14 min) were mainly albumins, while gliadins migrated in the slow migration region (16−25 min). Globulins (eluted within 10 min) and glutenins (eluted between 21 and 25 min) were present in the analyzed samples in a very low concentration (data not shown).

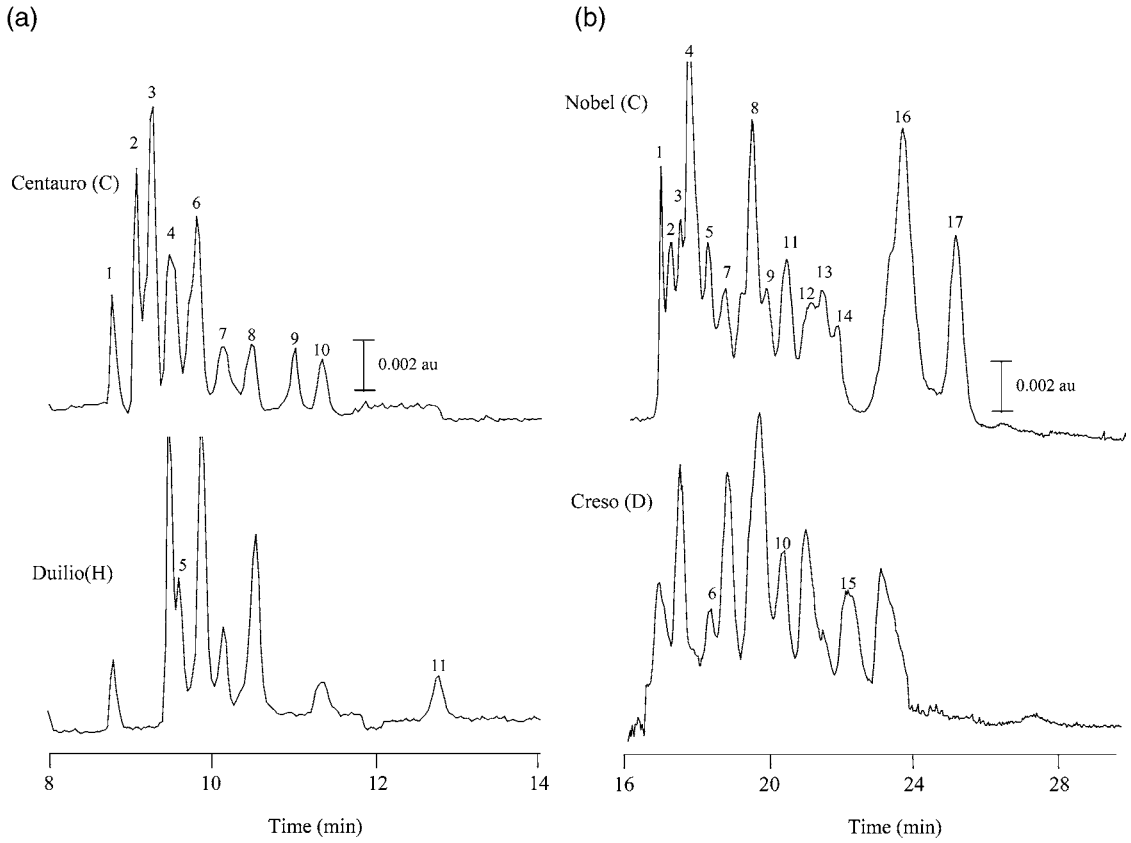

Figure 4. The total number of peaks observed across the analyzed wheat cultivars and detected in the fast migration (**A**) and in the slow migration (**B**) regions of FZCE electropherograms. Analysis conditions are as given in Materials and Methods.

Table 3. Peak Scoring (+ = Presence, – = Absence) in Fast and Slow Migration Regions of FZCE Electropherograms of Common (C) and Durum (D) Wheat Cultivars

cultivar	fast migration											slow migration																
	1a	2a	3a	4a	5a	6a	7a	8a	9a	10a	11a	1g	2g	3g	4g	5g	6g	7g	8g	9g	10g	11g	12g	13g	14g	15g	16g	17g
Bolero (C)	+	+	+	+	-	+	+	+	+	+	-	+	-	+	+	+	-	+	-	+	+	-	+	-	+	+	-	+
Brasilia (C)	+	+	+	+	-	+	+	+	+	+	-	+	-	+	+	+	-	+	+	-	+	+	+	-	+	+	-	+
Centauro (C)	+	+	+	+	-	+	+	+	+	+	-	+	-	+	+	+	-	+	-	+	+	+	+	-	+	+	-	+
Eureka (C)	+	+	+	+	-	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	+	+	-	+	+	+	-	+
Francia (C)	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	-	+	-	+	+	+	-	-	+	+	-	+
Golia (C)	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	-	+	-	+	+	+	+	-	+	+	-	+
Manital (C)	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	-	+	-	-	+	+	+	-	+	+	-	+
Mec (C)	+	+	+	+	-	+	+	+	+	+	-	+	-	+	+	+	+	+	-	+	+	-	-	-	+	+	-	+
Mieti (C)	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	-	-	-	+	+	-	+
Mol (C)	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	-	+	-	+	+	+	-	-	+	+	-	+
Nobel (C)	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+	-	+	+	+	+	-	+	+
Pandas (C)	+	+	+	+	-	+	+	+	+	+	-	+	+	+	-	+	-	+	-	+	+	+	-	-	+	+	+	+
Santerno (C)	+	+	+	+	-	+	+	+	+	+	-	+	-	+	+	+	-	+	-	-	+	-	+	-	+	+	-	+
Spada (C)	+	+	+	+	-	+	+	+	+	+	-	+	-	+	+	+	-	+	-	+	+	+	-	-	+	+	-	+
Appio (D)	+	-	-	+	+	+	+	+	-	+	+	+	+	+	-	-	-	+	-	+	+	-	+	+	+	-	+	-
Cirillo (D)	+	-	-	+	-	-	+	+	-	+	-	+	+	+	-	-	-	+	-	+	-	+	+	+	+	+	-	-
Cosmodur (D)	+	-	-	+	-	-	+	+	-	+	+	+	-	+	+	+	-	-	-	+	+	+	-	+	+	-	+	-
Creso (D)	+	-	-	+	-	-	+	+	-	+	-	+	-	+	-	+	+	+	-	+	+	-	+	-	-	+	+	-
Duilio (D)	+	-	-	+	+	+	+	+	-	+	+	+	+	+	-	-	-	+	-	+	+	-	+	+	+	-	+	-
Grazia (D)	+	-	-	+	-	-	+	+	-	+	-	+	-	+	-	+	-	+	-	-	+	+	-	+	+	-	-	-
Neodur (D)	+	-	-	+	+	+	+	+	-	+	+	+	+	+	-	-	-	+	-	-	+	+	+	-	+	+	-	-
Vitron (D)	+	-	-	+	+	+	+	+	-	+	+	+	+	+	-	-	-	-	-	+	+	-	-	-	+	+	+	+
Zenit	+	-	-	+	-	-	+	+	-	+	-	+	+	+	-	-	-	+	-	+	-	+	-	-	+	+	-	+

In the fast migration region, a total of eleven peaks was observed across all the analyzed wheat accessions (**Figure 4a**). The mean variation coefficients of the migration times of peaks were 3.2 ± 0.8% and 3.5 ± 0.5% for albumin fractions extracted from common and durum wheat, respectively. All the investigated common wheat cultivars were monomorphic and showed the same nine albumin fractions (**Table 3**). The mean number of peaks observed in durum wheat cultivars was 6.4 ± 1.5 (**Table 3**). All common wheat accessions showed three major peaks (2a, 3a, and 9a), absent in durum wheat cultivars. In particular, the lack of peaks 2a and 3a in the electropherograms of durum wheat accessions is in agreement with literature (*34*, *35*). Although in the present study no direct evidence is presented, it could be suggested that the two albumin peaks exclusively detected in the electropherograms of common wheat (peaks 2a and 3a) and characterized by a fast electrophoretic

migration are comparable to the two albumin bands disclosed by the method of Resmini and De Bernardi (*4*).

In the slow migration region, a total of 17 peaks across the analyzed wheat accessions were observed (**Figure 4b**). The mean variation coefficients of the migration times of peaks were 17.5 ± 3.6% and 19.1 ± 2.9% for gliadin fractions extracted from common and durum wheat, respectively. The relatively poor reproducibility is a direct consequence of the selected separation conditions (alkaline electrolyte buffer containing borate and 75-μm i.d. uncoated capillary). First Bietz and Schmalzried (*36*) adopted an alkaline buffer system, consisting of 60 mM borate with 1% SDS and 20% acetonitrile for the separation of wheat gliadins in 57-cm long, 50-μm i.d., uncoated capillary. This system was soon abandoned due to the binding of some components to the negatively charged silanols of inner capillary wall causing a low reproducibility in peak areas and migration times (*36*). At the present, to prevent binding phenomena and improve reproducibility, the FZCE separation of wheat gliadins is usually carried out with acidic buffers (phosphate, lactate, or aspartic acid buffer with a pH ranging between 2.2 and 3.6) in 50 or 20-μm i.d. capillary dynamically coated with linear polymers (hydroxypropylmethylcellulose or hydroxyethylcellulose) (*20*, *21*, *34*, *36*). However, these separation systems did not permit the separation of hydrophilic wheat albumins with a sufficient selectivity and efficiency (data not shown). As a consequence, the FZCE separation condition adopted for the present study was chosen as the best compromise to obtain in the same run an acceptable separation of both wheat albumins and gliadins. In the slow migration region, the mean number of peaks was 11.8 ± 1.3 and 9.4 ± 1.1 for common and durum wheat cultivars, respectively (**Table 3**). As previously observed by RP-HPLC analysis, CE analysis confirmed a statistically higher number of gliadin fractions in hexaploid common wheat than in tetraploid durum wheat. However, no diagnostic peak (exclusively present in common wheat accessions but absent in durum wheat cultivars) was found (**Table 3**). This result suggests that some diagnostic fractions of common wheat probably comigrate with protein fractions shared by both common and durum wheat.

The PCA of distance data calculated from electropherograms of common and durum wheat cultivars is reported in **Figure 3b**. The PCA based on FZCE data was in general agreement with that based on RP-HPLC chromatograms. Common wheat cultivars, mainly charged on the first factor, are clearly differentiated from durum wheat cultivars, mainly charged on the second factor (**Figure 3b**). However, the PCA of FZCE data highlighted different relationships within common and durum wheat accessions with respect to the ordination based on RP-HPLC data (**Figure 3**, **Table 1**). The common wheat cultivars were divided in two clusters. In the first cluster, 10 cultivars characterized by a mean similarity of 78 ± 4% were included. In this cluster, the cultivar Mec contributed for 50% of the genome of cultivar Mieti, while cultivar Manital was a parent of the cultivars Francia, Golia and Mol. In addition, Golia, Mol, and Santerno shared one parent (cv. Orso), while Marzotto was a common parent of both Manital and Mec. The second cluster was formed by four accessions with a mean similarity of 55 ± 14%. In this cluster, the cultivar Generoso was a common ancestor of cultivars Nobel and Pandas, while the cultivar Bezostaja contributed 25% of the genome of the cultivars Brasilia and Pandas. Two clusters were also found for durum wheat accessions (cluster three and four). In the third cluster, five cultivars with a mean similarity of 58 ± 9% were included. In this cluster, except for the cultivar Creso contributing 25% of the genome of the cultivar Cirillo, no other apparent relationship based on pedigree was found. Finally, the fourth cluster was formed by only two cultivars (Appio and Duilio) sharing a common parent (cv. Cappelli).

A comparison of the PCA diagrams based on RP-HPLC and FZCE data evidenced the potentiality of the complementary use of these analytical techniques. In particular, both techniques permitted wheat cultivar clustering, emphasizing different relationships between cultivars on the basis of their pedigrees. In the case of very close pedigrees, protein composition may be so similar that neither RP-HPLC or FZCE can differentiate varieties (*35*, *37*). However, the combined application of RP-HPLC and FZCE can provide very detailed and unique biochemical fingerprints useful for germplasm identification, investigation of pedigree relationships, and checking of pedigree validity.

Detection of Durum Wheat Flour Adulteration by RP-HPLC and FZCE. The differences of RP-HPLC elution profiles and FZCE electropherograms obtained from common and durum wheat cultivars were further investigated as a basis for detecting and estimating the adulteration of durum wheat flour with flour from common wheat varieties.

As concerns RP-HPLC analysis, four potential diagnostic peaks (namely 2a, 11g, 17g, and 22g) were found (**Table 2**). As expected on the basis of the chosen HPLC separation conditions (reversed phase) not ensuring a sufficient resolution of hydrophilic proteins, preliminary trials showed that the albumin fraction (peak 2a) eluting between 4.5 and 5.5 min permitted only the detection of severe adulteration (> 50% w/w) and was inappropriate for the purpose of the present study (data not shown). In contrast, the effect of common wheat adulteration of durum wheat flour on RP-HPLC chromatogram is illustrated in **Figure 5**, parts **a**−**c**. The areas of the peaks 11g (eluting between 25.5 and 26 min), 17g (eluting between 31 and 31.5 min) and 22g (eluting between 33.5 and 34.5 min) increased with the level of adulteration. For peak 11g, the adulteration was visible at 10% (w/w), while for peaks 17g and 22g, the adulteration threshold was 5% (w/w). The direct relationship between the area of the peaks 11g, 17g, and 22g, and adulteration level enabled standard curves to be constructed (**Figure 6a**). The normalized peak area of unknown wheat flours can be compared with these curves in a manner similar to that when a normal standard curve is used, and a quantitative estimate of the level of adulteration can be obtained. **Figure 6a** shows that adulteration may be quantified accurately between 10 and 50% (w/w) by peak 11g, and between 5 and 50% (w/w) by peaks 17g and 22g.

Three potential diagnostic peaks (namely 2a, 3a, and 9a) were found by FZCE analysis (**Table 3**). For peaks 2a (migrating between 9 and 9.25 min) and 3a (migrating between 9.25 and 9.5 min), the adulteration was detectable at 5% (w/w), while for peak 9a (migrating between 10.8 and 11.2 min), the detection threshold of adulteration was 10% (w/w) (**Figure 5**, parts **d** and **e**). The standard curves show that adulteration may be quantified accurately between 5 and 50% by peaks 2a and 3a, and between 10 and 50% by peak 9a (**Figure 6b**).

Any technique intended to be used routinely for the detection of common wheat adulteration of wheat flours requires that the measured component should be independent of wheat cultivar, and should also be determined in an accurate and reproducible manner. The data presented in this study indicate that both the RP-HPLC and the FZCE method are close to satisfying these conditions. The presented RP-HPLC method is basically similar to the other RP-HPLC methods (based on the analysis of gliadin

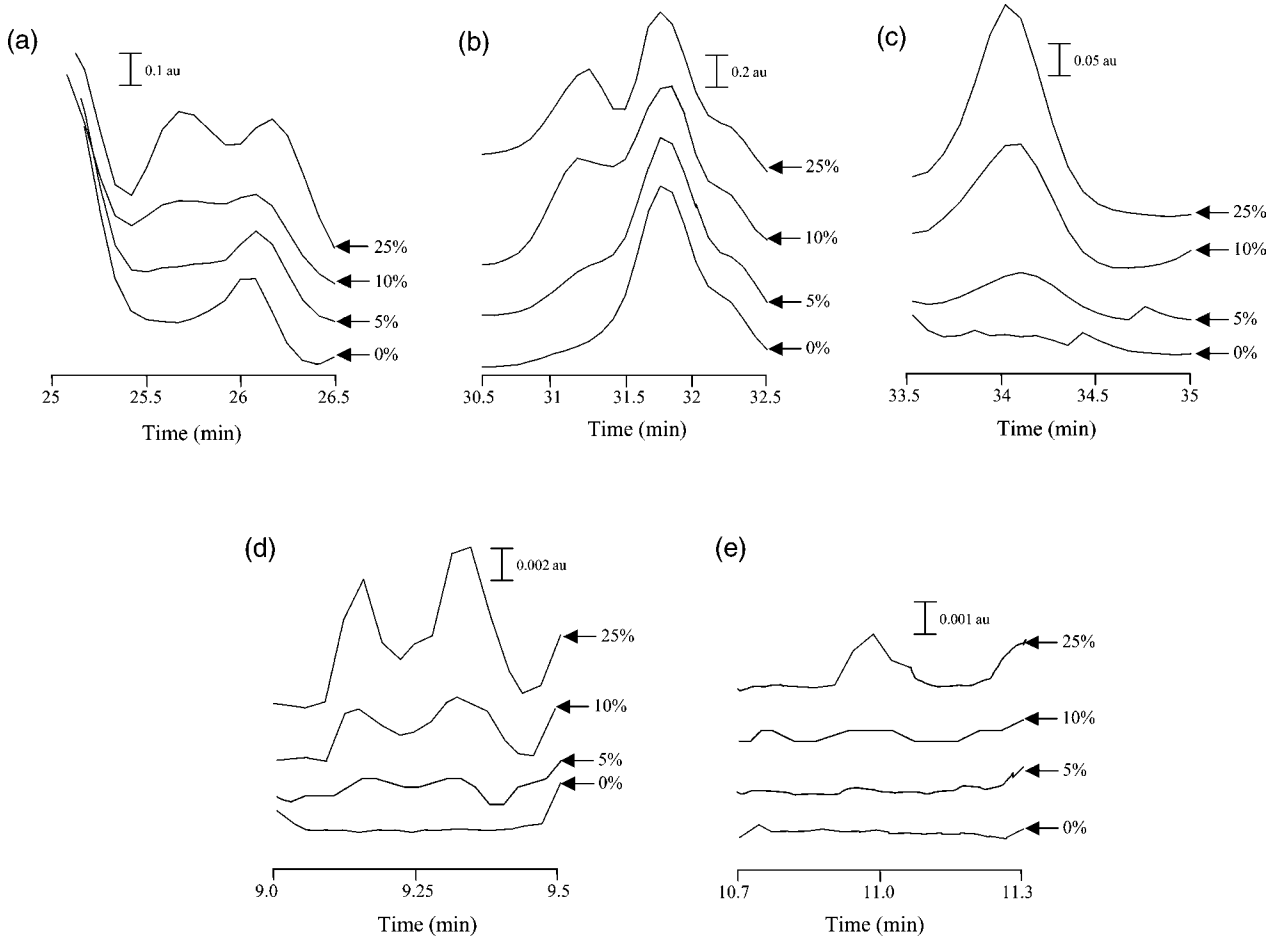

Figure 5. RP-HPLC and FZCE separations illustrating the effect of adulteration of durum wheat flour (cv. Appio) with a common wheat flour mixture. RP-HPLC elution profiles: (**A**) peak 11g eluting between 25.5 and 26 min, (**B**) peak 17g eluting between 31 and 31.5 min, (**C**) peak 22g eluting between 33.5 and 34.5 min. FZCE migration profiles: (**D**) peaks 2a and 3a (migrating between 9 and 9.25 min and between 9.25 and 9.5 min, respectively), (**E**) peak 9a migrating between 10.8 and 11.2 min. The arrows indicate different levels of adulteration (w/w).

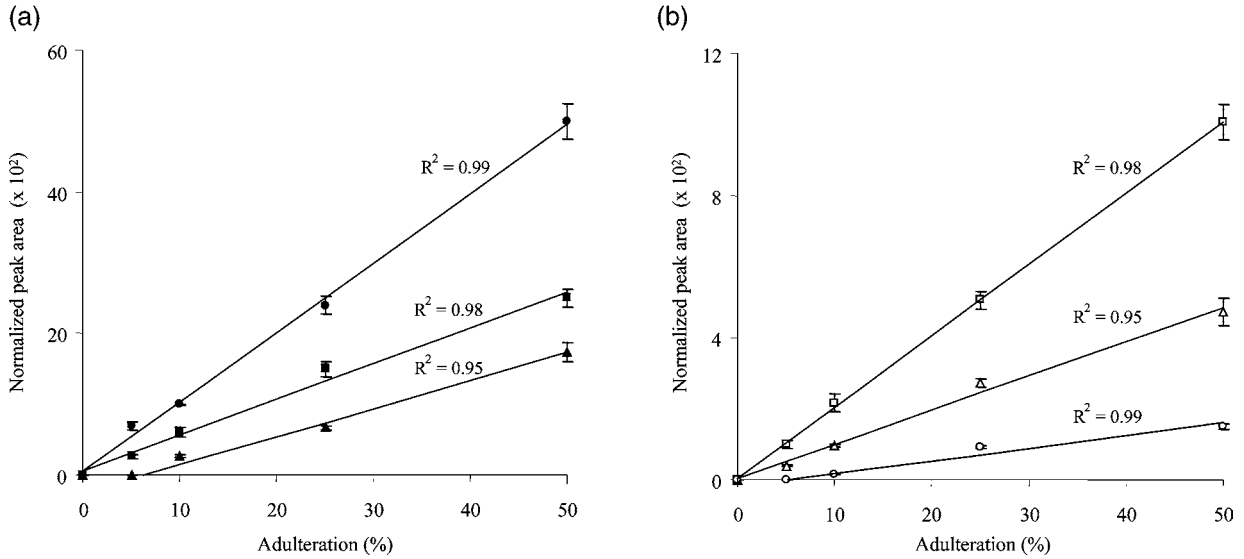

Figure 6. Standard curves showing the relationships between normalized area of RP-HPLC (**A**) and FZCE (**B**) diagnostic peaks and common wheat adulteration level (0–50%, w/w). RP-HPLC peaks: ▲ = 11g; ■ = 17g; 22g = ● FZCE peaks: 2a = △; 3a = □; 9a = ○. In the graphs, the coefficient of determination (R^2) of standard curves are reported. Data points are means from three experiments, and bars represent SD where larger than symbol size.

fractions) proposed in the last years by several authors (*27*, *31*). In contrast, the presented FZCE method is based on analysis of water-extractable wheat proteins and could be considered similar to the official Italian method of Resmini and De Bernardi (*35*), consisting in the separation of wheat albumins on Native-PAGE. With respect to the analysis on Native-PAGE, the FZCE analysis is more flexible to allow a range of adulteration levels to be calculated from standard curves and is less time-consuming than

the FZCE separation of wheat albumins, taking less than 15 min. The short analysis time and the very low sample volume required (few microliters) make the FZCE method competitive also with respect to available RP-HPLC methods (elution time greater than 40 min and sample volume of several microliters). Finally, to our knowledge, the presented FZCE method is the first capillary electrophoresis method proposed for the detection of durum wheat flour adulteration with common wheat flour. In recent years, several new FZCE methods have been introduced for fast and reliable analysis of wheat glutenins and gliadins mainly for the purpose of cultivar identification (*20*, *21*, *34*, *36*). However, in the literature, no report is available on the application of these FZCE separation systems for the detection and estimation of durum wheat adulteration. Further investigations are in progress to verify the use of FZCE separation of wheat albumins also for the quality control of other wheat flour products such as pasta.

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