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Simple and Rapid Method for the Differentiation of *Lens culinaris* Medik. from False Lentil Species

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The practice of using both common vetch (*Vicia sativa* L.) and single-flower vetch (*Vicia articulata* Hornem.) seeds as food or feed is encouraged by the very high resemblance of their seeds with those of small-seeded lentil cultivars. Among the *Vicieae*, antinutritional and toxic factors are particularly important, because many species, containing high levels of these compounds, are not safe. A simple and fast capillary electrophoresis (CE) method was proposed for the differentiation of lentil cultivars from false lentil species (i.e., single-flour vetch and common vetch). Proteins were extracted from defatted milled seeds with an alcoholic/saline solution. Extracts were separated in an uncoated fused-silica capillary with iminodiacetic acid (IDA) isolectric buffer containing 0.05% hydroxypropylmethylcellulose (HPMC) and 20% acetonitrile. The presented method is useful also for the detection of contamination of whole or split seeds of lentil by vetch species. With respect to alternative techniques, such as DNA-based markers or thin-layer chromatography (TLC), CE has the advantages of being less expensive, faster, and fully automated.

KEYWORDS: Capillary electrophoresis; seed storage proteins; species differentiation; *Vicia articulata* Hornem; *Vicia sativa* L.

INTRODUCTION

Monogastric animals, included humans, are the major global end-users for grain legume products. Among the grain legumes, lentil (*Lens culinaris* Medik.) is unique because it can be grown satisfactorily in marginal environments in which other pulses cannot be cultivated (*1*). Moreover, lentil seeds are characterized by very low levels of antinutritional compounds (*2*). Almost the half of the world's area of lentil cultivation is located in southwestern Asia, where it is urgent to increase the production of both food and animal feed (*3*).

Over the past decades the less domesticated grain legumes, such as *Vicia* and *Lathyrus* spp., have come under considerable interest in the search for grain and forage legume alternatives for temperate agro-ecosystems (*4*−*6*). Vetches are multipurpose crops, allowing for either fodder conservation or immediate cash returns through hay or grain production, while at the same time providing a green manure option. Among the two hundred species belonging to the genus *Vicia*, only a few species are candidates for further domestication toward grain legume status (*7*). Common vetch (*V. sativa* L.) and single-flower vetch (*V. articulata* Hornem.) have attracted attention because of their wide adaptation, which includes extremes of winter cold and very dry conditions, as well as for the high protein content of their seeds (*8*−*9*). The custom to use both these species as food or animal feed is attributable to the very high resemblance of their seeds (shape, size, pattern, and colour of coat) with the small-seeded lentil cultivars. For example, it is known that Chilean rural populations use both lentil and common vetch seeds for food as well as for animal feed (*8*). Similarly, the habit of eating single-flower vetch seeds has been observed in some Mediterranean countries (*10*−*11*). The frequent confusion between lentil and single-flower vetch is confirmed by the name used by local people to designate this species. It is locally known as "algarroba or lenteja de Aragon" (lentil from Aragona) in the Iberian peninsula (*12*), while it is named "lenticchia nera" (black lentil) in Italy (*11*).

Among the *Vicieae*, antinutritional and toxic factors are likely to be particularly important, because many species, containing high levels of these compounds, are not safe (*13*−*14*). The cultivar Blanchefleur, a common vetch originally introduced to Australia as a hay, forage, and green manure crop, is an exemplary case. In a very short time, the high similarity of decorticated and split seeds with those of red lentil cultivars produced the habit of selling the cv Blanchefleur as a cheap replacement for lentils in ignorance of the presence of γ-glutamyl-β-cyanoalanine and the favism toxin in common vetch seeds (*14*−*15*). As a consequence, several cases of poisoning occurred (*16*). The size of the problem caused the ban on importation of the cv. Blanchefleur in India and Egypt where split lentil is an important source of dietary protein.

The development of reliable and accurate methods can assist both farmers and consumers in the detection of contamination of lentil cultivars by seeds of *Vicia* spp. or in the identification of batches of seeds that are incorrectly marked. This is a basic requirement to prevent accidental intoxication. Methods based

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Table 1. Seed Morphological Traits of Studied Lentil Cultivars and *Vicia* Accessions

cultivar name or accession code	seed morphological traits	1000 seeds wt (g)
Blue lentil	yellow cotyledon-type with dark green and speckled coat	31.1
Eston	pale yellow colored seed with green coat	37.7
French dark speckled	yellow cotyledon-type with dark brown and speckled coat	28.5
Pardina	yellow cotyledon-type with brown and speckled coat	41.7
Regular	yellow cotyledon-type with green coat	48.9
ILL 7202	orange cotyledon-type with brown coat	29.6
ILL 7535	orange cotyledon-type with brown and speckled coat	29.0
PI 602370	yellow cotyledon-type with brown and speckled coat	57.6
MG 103292	orange cotyledon-type with dark brown coat	42.4

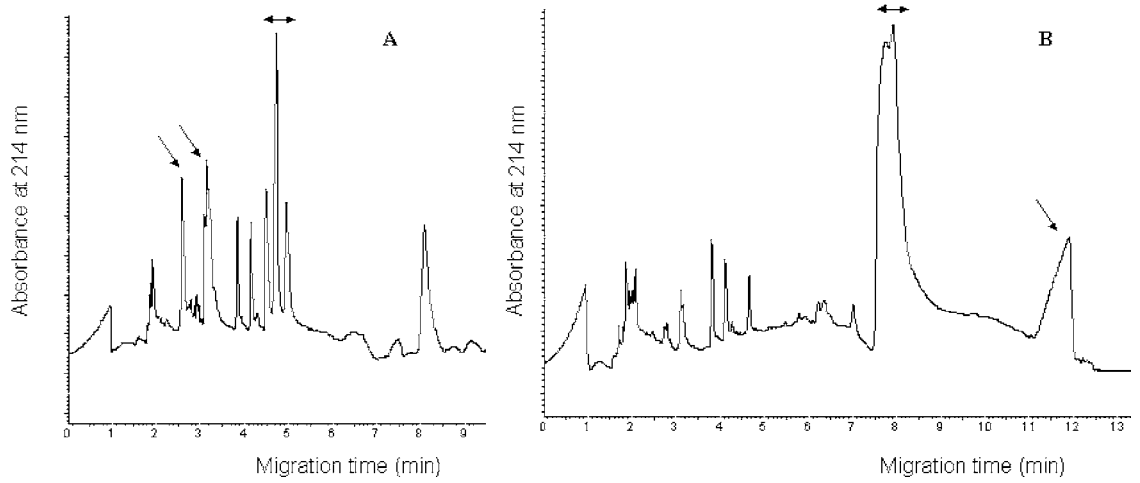

Figure 1. Capillary electrophoretic patterns of the alcoholic/saline extract from (**A**) single-flower vetch (PI 602370) and (**B**) common vetch (MG 103292). The arrows indicate the species-specific peaks of each species. The separations were performed in 50 mM IDA, 0.05% HPMC, 20% ACN at 35 °C and 25 kV.

on morphological characters of plants or on DNA-based analyses are expensive and they demand a long time. Conversely, biochemical analyses are cheap and require a relatively short time of analysis. Electrophoresis of seed storage proteins on polyacrilammide gel (SDS−PAGE) has been ongoing for many years. It has been used to successfully discriminated between several species as well as cultivars belonging to the same species. Several studies on the electrophoresis of lentil seed storage proteins are available in the literature (*17*−*19*). Recently, the development of capillary electrophoresis (CE) has introduced the capability of very fast and fully automated electrophoretic separation of seed storage proteins. A number of papers have evidenced the potentialities of CE for the discrimination of legume varieties (*20*) or legume species (*21*−*23*).

In the present research, capillary zone electrophoresis (CZE) was employed to discriminate lentil cultivars from *V. sativa* and *V. articulata* populations. The utilization of this technique to detect contamination of lentil cultivars by seeds belonging to these species was also investigated.

MATERIALS AND METHODS

Five small-seeded lentil commercial cultivars (Blue, Eston, French dark speckled, Regular, and Pardina, also named Spanish brown) and two improved lines (ILL 7202 and ILL 7535) selected at International Center for Agricultural Research in Dry Areas (ICARDA, Aleppo, Syria) were analyzed in this study. For each cultivar, three batches of seeds were examined to test the batch-to-batch reproducibility of the CE profile. Moreover, one single-flower vetch(*V. articulata* Hornem.) accession (PI 602370) from the USDA (USA) and one common vetch (*V. sativa* L.) accession (MG 103292) from the Istituto di Genetica Vegetale-CNR (Bari, Italy) were analyzed. Seed morphological traits of the studied samples are summarized in **Table 1**.

Different levels of lentil contamination were simulated by mixing milled seeds of lentil with the meal of the single-flower vetch or the common vetch. The obtained mixtures were processed as described for cultivars.

Protein Extraction. Dehulled seeds, 8−10 individuals for each sample, were manually milled to a fine meal. The protein extraction for CE analysis was carried out according to Piergiovanni and Taranto (*23*). A pre-extraction was done to remove fat, pigments, etc. by mixing the meal with acetone (1/10, w/v) at room temperature. The pre-extraction was repeated twice for 20 min. The suspension was vortexed periodically, centrifuged at 6800 *g* for 10 min, and air-dried. The defatted meal was suspended (1/8, w/v) in 70% (v/v) ethanol/0.5 M NaCl buffer for 2 h at room temperature with brief vortexing every 20 min. The suspension was centrifuged at 6800 *g* for 10 min, and the supernatant was used for CE and analyzed on the day of extraction.

Capillary Zone Electrophoresis. Beckman P/ACE model MDQ equipment (Beckman Coulter, USA) was used to separate the protein extracts. Separations were achieved as previously described (*23*) using 30 cm long (22 cm to detector), 50 μm i.d., uncoated fused-silica capillaries. The extracts were pressure injected, and the separated proteins were detected by UV absorbance at 214 nm. An isoelectric buffer based on imino diacetic acid (IDA) and containing 0.05% hydroxypropylmethyl cellulose (HPMC) and 20% acetonitrile (ACN) was used. The capillary was cleaned between consecutive runs with the procedure of Olivieri et al. (*24*). Beckman Karat 32 software was used for the acquisition and elaboration of the electropherograms.

Diagnostic peaks were first identified on the basis of migration time (MT). The reproducibility of the MT was evaluated by repeated injections ($n = 4$) of the same extract. The area of the diagnostic peaks was normalized according to the method of Bonetti et al. (*25*) before the evaluation of the linearity of the response against the percentage of lentil contamination.

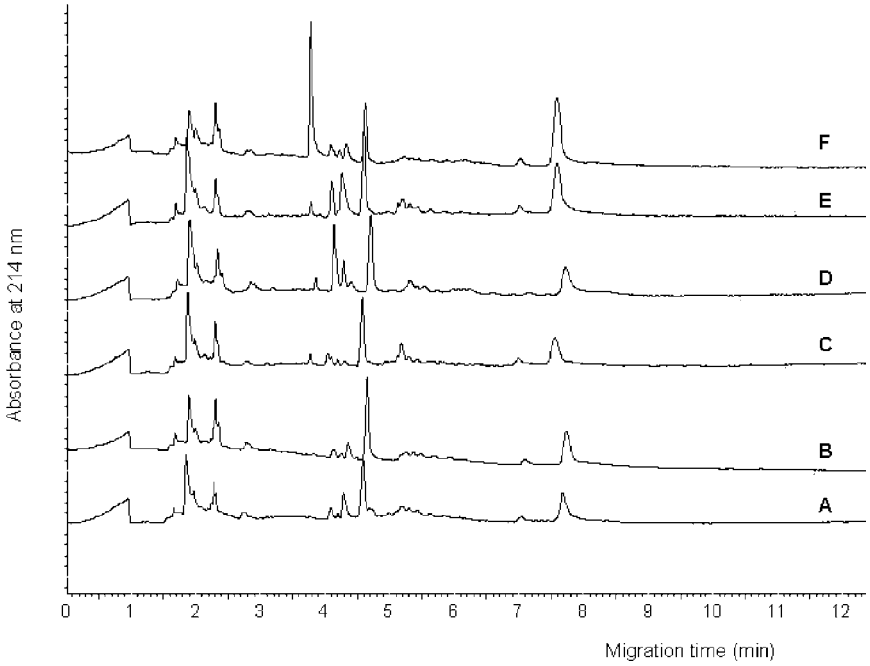

Figure 2. Electrophoretic patterns of lentil cultivars: (**A**) Eston; (**B**) Regular; (**C**) Blue lentil; (**D**) French dark speckled; (**E**) ILL 7535; (**F**) ILL 7202. The separation conditions are the same as those in **Figure 1**.

RESULTS AND DISCUSSION

A recent study showed that several *Vicia* species can be discriminated by submitting the proteins soluble in an alcoholic/saline solution to CZE analysis (*23*). The comparison of the electropherograms of single-flower vetch (**Figure 1A**) and common vetch (**Figure 1B**) clearly shows that different electrophoretic patterns are associated to these species. The same study provided evidence that one or more species-specific peak(s) can be associated to each *Vicia* species (see arrows, **Figure 1**) and that only minor differences can be observed among the profiles of different accessions belonging to the same species (*23*). These results suggest that an efficient and fast method based on CZE could be established to differentiate lentil, common vetch, and single-flower vetch.

The analysis of seven lentil samples showed that the seed proteins extracted with an alcoholic/saline solution are separated by CZE in a variable number of peaks embracing a wide range of mobility (**Figure 2**). As expected, the analysis, for each cultivar, of different batches of seeds did not show differences among the electropherograms. Pardina cultivar was the only exception, showing significant differences in the height of three peaks (see arrow, **Figure 3**) among the tested batches of seeds.

Though the proteins detected in the profiles of the tested lentils are not completely resolved under the chosen separation conditions, they can be easily arranged into three main groups. The first migration region (1.6−2.4 min) is comprised of the faster fractions, proteins with intermediate mobility were eluted between 3.8 and 4.6 min, while the slower fractions migrated at more than 7.0 min (**Figure 2**). At the opposite of *Triticeae* cultivars, but similarly to *Vicia* spp., the discrimination of lentil cultivars using the described procedure appears to be of little feasibility, because the differences detectable by comparing the cultivar profiles are mainly related to the peak heights rather than to the presence/absence of some fractions. For example, the blue lentil (**Figure 2**, trace **C**) was characterized by a very low expression of almost all the proteins with intermediate and low mobility. The lack of the peak migrating at 3.8 min was observed for Eston and regular cultivars (**Figure 2**, traces **A** and **B**, respectively). At the opposite, this peak was very pronounced in two batches of Pardina (**Figure 3**, traces **B** and **C**) as well as in ILL 7202 (**Figure 2**, trace **E**). Since it is well established that the content of seed storage proteins may be affected by environmental conditions and fertilizer factors (*26*−*28*), the differentiation of cultivars based mainly on quantitative differences of some protein fractions has a limited usefulness.

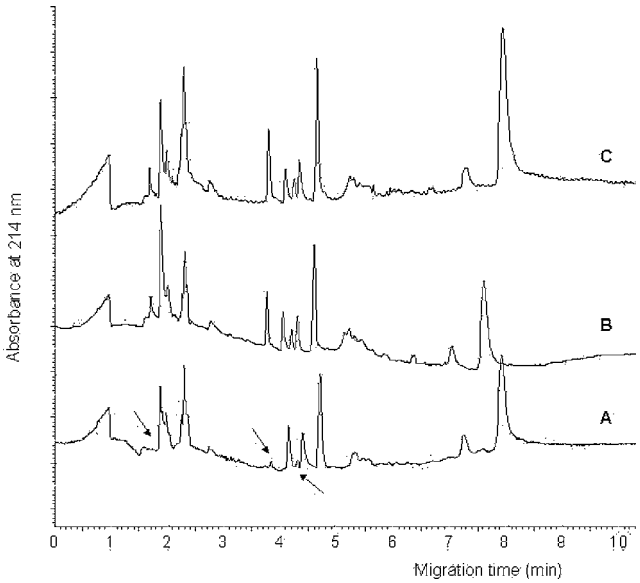

Figure 3. Electrophoretic patterns of three batches of Pardina cv. The separation conditions are the same as those in **Figure 1**.

On the other hand, if the high number of peaks shared by lentil cultivars is a restriction in achieving their differentiation, it could be an undoubted advantage to discriminate lentil, common vetch, and single-flower vetch seeds by using CZE.

Actually, by comparing the electropherograms relative to the studied samples, species-specific peaks can be easily identified. For example, the protein fractions migrating between 2.5 and 3.5 min were detected only in the single-flower vetch profile (**Figure 1A**), and they were absent in all lentil cultivars (**Figure**

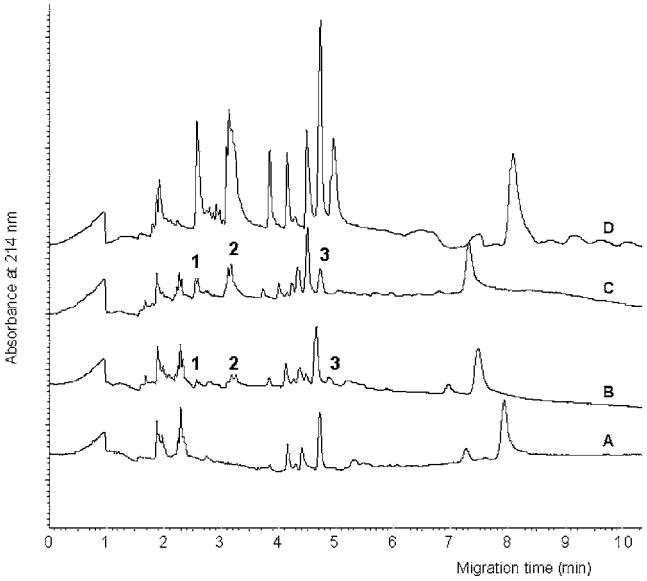

Figure 4. Electrophoretic patterns of lentil, single-flower vetch, and their mixtures: (**A**) Pardina; (**B**) Pardina/single-flower vetch mixture (9/1 w/w); (**C**) Pardina/ single-flower vetch mixture (4/1 w/w); (**D**) single-flower vetch (PI 602370). The progressive number indicates the diagnostic peaks. The separation conditions are the same as those in **Figure 1**.

2). Similarly, the protein fractions with high migration time (more than 8 min), observed in the common vetch electropherogram (**Figure 1B**), were not observed in the lentil ones (**Figure 2**). Consequently, these peaks can be considered diagnostic because they uniquely identify both whole as well as split seeds belonging to lentil, common vetch, and single-flower vetch.

The advantages of CZE as compared to both DNA-based methods and thin-layer chromatography (TLC) of alcoholic extracts (30% EtOH) of seeds (*29*) are undoubted: (1) as compared to molecular markers, CZE is less expensive and faster, requiring less than 4 h to complete the analysis; (2) as compared to TLC, CZE uses very low amounts of organic solvents; (3) it has a high reproducibility and is completely automated.

In addition to the confusion between lentil and *Vicia* spp., alien seeds are sometimes present in lentil batches. This contamination, very often due to the common vetch, is a trouble generally associated with local lentil populations. In general, the local populations survive in marginal geographical areas where farmers traditionally cultivate them (*30*). The harvest is mainly used for selfconsumption, and only small quantities are sold in local markets. The conditions of cultivation and utilization summarized before do not entail particular care toward the genetic homogeneity of local populations. Sometimes farmers deliberately grow populations that are mixtures of genotypes or subspecies (*31*). Moreover, lentil local populations are frequently cultivated in areas close to those where forage crops grow, and for this reason, accidental contamination could occur. From a nutritional point of view, the significance of lentil contamination is strictly related to its level, but it is certainly less dangerous than the misclassification. However, this study has showed that alien seeds introduce in the diet of end-users some protein fractions not present in lentil seeds.

The existence of species-specific peaks associated to the tested *Vicia* species suggests that CZE could be adequate to detect lentil contamination. The electropherograms of cultivar Pardina, single-flower vetch (accession PI 602370), and two mixtures of their meals are shown in **Figure 4**. The extracts of lentil/single-flower vetch mixtures (**Figure 4**, traces **B** and **C**) can be easily identified due to the presence of additional protein fractions. In fact, in the contrast with Pardina (**Figure 4**, trace **A**), single-flower vetch (**Figure 4**, trace **D**) and both mixtures (**Figure 4**, traces **B** and **C**) showed the peaks 1, 2, and 3. These three peaks are diagnostic for the detection of single-flower vetch contamination because no one of tested lentil cultivars (**Figure 2**) showed protein fractions having comparable migration times. Similarly, it is possible detect lentil contamination by common vetch seeds (**Figure 5**). The two late migrating proteins (**Figure 5**, peaks **1** and **2**) were diagnostic, being observed in the electropherograms of both common vetch and lentil/common vetch mixture (**Figure 5**, traces **C** and **B**, respectively) but never in lentil ones (**Figure 5**, trace **A** and **Figure 2**). As known, the reproducibility of migration time of

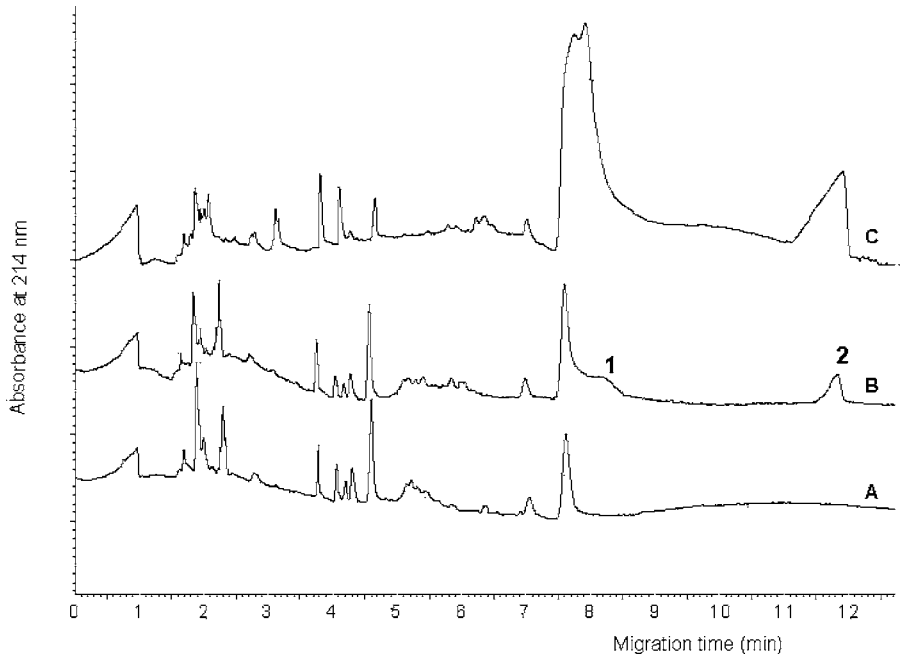

Figure 5. Electrophoretic patterns of lentil, common vetch, and their mixtures: (**A**) Pardina; (**B**) Pardina/common vetch mixture (9/1 w/w); (**C**) common vetch (MG 103292). The progressive number indicates the diagnostic peaks. The separation conditions are the same as those in **Figure 1**.

CE peaks decreased with increasing migration times. In this study, the standard error of migration time associated with the diagnostic peaks ranged from 0.019 (**Figure 4**, peak **1**) to 1.352 (**Figure 5**, peak **2**). However, the separation of protein fractions obtained under the described condition of analysis makes certain the identification of all the diagnostic peaks.

The effectiveness of this methodology is enhanced by the excellent linearity of the area of diagnostic peaks vs the percentage of contamination over the tested range (5−25 wt %) of single-flower vetch or common vetch contamination. The lowest correlation ($r = 0.9739$) was observed for peak 1 relative to single-flower vetch contamination.

CONCLUSIONS

Any technique intended to be used routinely for the detection of lentil misclassification as well as lentil contamination requires that the used parameters should be independent of cultivar and determined in an accurate and reproducible manner. The data discussed in this study indicate that the analysis by CZE of proteins extracted with an alcoholic/saline solution can satisfy these conditions. With respect to alternative techniques, CZE has the advantages of being less expensive, faster, and fully automated. Finally, the presented method can be used for the detection of both misclassification and contamination of whole or split seeds of lentil.

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