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Varietal identification of Thai hot chilli using seed protein analysis by ultrathin layer isoelectric focusing technique

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Abstract

This research aimed to determine the protein extraction solvent and pH of gel acrylamide that are suitable for conducting varietal verification of Thai hot chilli by ultrathin layer isoelectric focusing. Four extraction solvents, EX 3 (urea + ethylene glycol), phosphate buffer, water and HEPES, and two gel pH ranges, 3-10 and 5-8/2-11, forming eight treatment combinations were compared. Ten chilli cultivars, *Capsicum annuum* 'Hua reur yon', 'Mun', 'Gaw', 'Pama' and 'Yod son lek' and *C. frutescens* 'Mong', 'She deuy kai', 'Pop puang', 'Som ka rieang' and 'Kee noo suan', were tested. Protein extraction with EX 3 in combination with gel pH 3-10 gave the best varietal verification of chilli varieties in this study. In pairwise comparisons of the 10 chilli varieties, this treatment combination was able to verify all the 45 pairs with 100% efficacy.

Keywords: *Capsicum annuum*, *Capsicum frutescens*, extraction solvent, gel pH, Thai hot-chilli

Introduction

Hot chilli seed production is a significant business for Thailand, producing approximately 35 MT of high quality chilli seeds, sufficient to meet the annual requirement for planting, over 55,753 Ha of chilli per year, the largest area among vegetables in Thailand. Moreover, each year, Thailand exports 57.55 MT of chilli seeds and the export quantity and value are rising (Thai Seed Trade Association, 2018). Hence, the quality of chilli seeds produced is crucial for the success of chilli seed production in Thailand. Genetic purity is one of the most important aspects of seed quality, especially for the production of F1 hybrid

seeds. Therefore, genetic purity testing is essential in the chilli seed production process, especially for Thailand where a large number of chilli cultivars are produced annually from two major *Capsicum* species, *C. annuum* and *C. frutescens* (Kraikruan *et al*., 2008).

A number of varietal verification methods to determine the genetic purity of seed lots with different effectiveness have been reported. The grow-out test, using the morphological characteristics of the seedlings to verify the varieties, is commonly used by seed companies to determine the genetic purity of seed lots. The seeds are planted in the fields and the morphological characteristics of seedlings are observed and compared. It is relatively easy and requires no complicated equipment to perform the test. However, the major draw-backs of this grow-out test are that it is laborious and space- and time-consuming (Arus, 1983; McDonald, 1998). Moreover, the results can be inconsistent since the characteristics of plants can vary with environmental conditions. The molecular method, using DNA fingerprints to identify the varieties of seed lots, is the most accurate and precise technique, since the DNA fingerprint is unique to each variety. However, this technique is rather expensive since experienced personnel and sophisticated equipment are needed. Therefore, the use of DNA fingerprinting to verify varieties may not be appropriate for large-scale and routine commercial seed testing (Thongket and Chanpreme, 2006). Isozyme analysis based on differences in protein forms of the same enzyme to verify varieties is relative cheap compared with DNA fingerprinting, but has many limitations including the specificity of plant tissues and analytical environment sensitivity (Cooke, 1995; Dou *et al.*, 2012). Ultra-thin layer isoelectric electrophoresis focusing (UTLIEF), a seed protein separation technique, has also been proposed as an effective method to verify varieties among seed lots. Seed proteins are more stable and do not change with environment (Van den Berg, 1990, 1991). The analytical process is relatively simple, cheap and fast. It uses a thin (0.15 mm) polyacrylamide gel to separate different groups of seed storage proteins at a particular pH gradient. Results can be obtained within four hours of applying the seed protein samples into the electrophoresis chamber (Van den Berg, 1990; Onwimol *et al.*, 2010; Yan, 2013). Hence, it should be more appropriate and more cost effective to use this UTLIEF technique as the routine genetic purity test in commercial seed production. ISTA accepted this technique as the standard technique for varietal identification of maize and sunflower in 2012 (ISTA, 2016). The success of UTLIEF for variety identification had been reported for a number of crops including cucumber (Onwimol *et al.*, 2010; Tu *et al.*, 2012), tomato (Wang *et al.*, 2000), rice (Jiamtae *et al.*, 2017), maize (Manum, 2008) and pumpkin (Yan, 2013). However, the successive use of UTLIEF for varietal identification of hot chilli cultivars has not been reported yet.

One of the keys to the success of using UTLIEF to identify crop varieties is to use the appropriate seed protein extracting solution that can dissolve the protein group containing desirable polymorphic marker proteins. Water was reported as the effective protein extracting solution for cucumber (Onwimol *et al.*, 2010) and maize (Manum, 2008); phosphate buffer for mung bean (Nantareeyawatn, 2006) and rice (Jiamtae *et al.*, 2017); and NaCl solution for tomato (Wang *et al.*, 2000). There is no report regarding the appropriate seed protein extracting solution for varietal identification of hot chilli. The selection of appropriate pH gradient on polyacrylamide gel is also critical to

the success of protein separation. A number of reports revealed that the appropriate pH range on polyacrylamide gel for varietal identification varies with crop species. The pH range of 4-8 gave the best result in separation of seed proteins for varietal identification of tomato (Wang *et al.*, 2000) while the pH range of 5-8 was successfully used for separating hybrid-rice protein (Zhao *et al.*, 2003) and the hybriditiy test of cucuumber seeds by UTLIEF was made possible using gel pH range of 2-11 (Onwimol *et al.*, 2014). However, the appropriate pH range of hot-chilli seed protein separation for varietal identification has not been reported.

The objective of this study was therefore, to investigate the effects of different seed protein extracting buffers and pH gradient ranges on the efficacy of the varietal identification of 10 Thai hot-chilli cultivars from two species (*C. annuum* and *C. frutescens*) using the UTLIEF technique.

Materials and methods

The experiment was conducted at the Centre for Agricultural Technology, Augustenberg, Germany between August and November 2015.

Plant materials

The seeds of hot-chilli were brought from the germplasm bank of the Tropical Vegetable Research Centre, Department of Horticulture, Kasetsart University, Nakhon Pathom, Thailand. They consisted of 10 cultivars from two species: *Capsicum annuum* 'Hua reur yon' (HRY), 'Mun' (MN), 'Gaw' (GW), 'Pama' (PM) and 'Yod son lek' (YSL), and *C. frutescens* 'Mong' (MG), 'She deuy kai' (SDK), 'Pop puang' (PP), 'Som ka rieang' (SKR) and 'Kee noo suan' (KNS).

Seed and seed storage protein extracting solvent preparation

Four seed protein extracting solutions were compared: distilled water, EX 3 (32.34 g 3M and urea, 120 mL ethylene glycol, 1 L distilled water), phosphate buffer (0.194 g K_2HPO_4, 0.528 g KH_2PO_4, 0.38 g ethylene diamine tetra acetic acid, 1 g 1,4-dithiothreitol and 25 mL glycerine, 975 mL distilled water) and HEPES buffer (0.5 g 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid, 0.02 g 1,4-dithiothreitol, 1 g glycerine, 5 µL 20% triton x-100, 25 mL distilled water). Bulked hot chilli seeds of each variety were crushed and 300 mg of crushed seeds put in 1.5 mL tubes containing 800 µL of each buffer. They were vortexed and put in a freezer at -20°C over night. Next, each seed sample was thawed, vortexed and centrifuged at 13,000 rpm for 20 minutes. The supernatants were used as seed protein extracts for running the UTLIEF .

UTLIEF gel and pH gradient preparation

The UTLIEF gels were prepared according to the flap technique, described in the International Rules for Seed Testing (ISTA, 2007). The gel solution for 10 gels was prepared at a time by mixing 50 mL acrylamide (6.8% T, 2.5% C) with 1.60 g taurine, 50 µL N N N'N'-tetramethylenediamine (TEMED) and 350 µL of 20% (w/v) ammonium

peroxydisulphate. Two gel pH gradients of pH 5-8/2-11 and pH 3-10 were compared in this study. Each pH gradient was prepared by adding 4.4 mL of either pH 5-8/2-11 or pH 3-10 ampholytes (Sepalytes TM) to the gel polymerisation solution. A few drops of water were applied onto a glass plate before a polyester support film was placed on top. An aliquot of 6.5 mL gel polymerisation solution was dropped onto the film. The cover glass plate, secured with two pieces of adhesive tape (0.15 mm thick) on both the long sides of the plate, as the spacer, was placed on top to facilitate the uniform spreading and thickness of the gel solution over the film. Next, gel polymerisation was allowed for 35 minutes at room temperature.

Ultrathin-layer isoelectric focusing

The UTLIEF was performed on an IEF-SYSTEM horizontal electrophoresis chamber (biostep® DESAGA Technology) that was connected to a cooling bath (HARVEST SC972®) to cool the gel surface down to 7-8°C. An application strip was placed on top of the gel surface about 15 mm away from the anode strip. Then, for each sample, 25 µL of seed protein extract was applied into each well of the application strip. The chamber was connected to a power supply (Consort E833) providing a current of 200-3,000V for three hours and 10 minutes of focusing time. The gels were then fixed in 12% (W/V) trichloroacetic acid (TCA) for 20 minutes, stained with Coomassie Brilliant Blue solution (0.15 g Coomassie R250 (SERVA), 0.45 g Coomassie G250 (SERVA), 110 mL 99% acetic acid, 180 mL 95% ethanol, adjusting volume to 1 L with water) for 50 minutes and destained in destaining solution (300 mL 95% ethanol, 50 mL 99% acetic acid adjusting volume to 1 L with water) for five minutes. After rinsing with water, the gels were air-dried overnight at room temperature and covered with transparent adhesive film before the interpretation of results was performed.

Interpretation electrophoretogram and efficacy of the buffer solution and pH gradient combinations

The gel electrophoretograms as affected by the protein extracting solution and pH range were evaluated to determine the presence or absence of polymorphic protein bands to differentiate, through pairwise comparisons, the 10 hot chilli cultivars. The effectiveness of each treatment combination was calculated as (N - Nnd)/N, where N is the total number of pairs (45) and Nnd is the non-different pair (Jiamtae *et al.*, 2017).

Results

Efficacy of EX 3 seed protein extracting solution in combination with gel pH of 3-10.

The EX 3 seed protein extracting solution in combination with pH 3-10 gel provided 13 polymorphic protein bands across 10 hot chilli cultivars (figure 1). This treatment combination was able to verify all the 45 pairs in pairwise comparisons among the 10 Thai hot chilli cultivars, i.e. gave 100% efficacy (table 1). It was also noticeable that a relatively high number of polymorphic protein bands was found when comparing the electrophoretogram of seed proteins between cultivars from different species than between

cultivars within the same species. The highest polymorphic protein bands of 11 was found when comparing *C. annuum* YSL with *C. frutescens* SKR.

Efficacy of EX 3 seed protein extracting solution in combination with gel pH of 5-8/2-11.
The Ex 3 buffer in combination with pH gradient 5-8/2-11 provided five polymorphic protein bands (figure 2). This treatment combination was able to differentiate 34 pairs in pairwise comparisons among 10 hot chilli cultivars, i.e. gave 76% efficacy (table 2). There were three polymorphic protein bands (No. 1, 3 and 4) that only existed in all cultivars of *C. annuum* but were absent in all cultivars of *C. frutescens.* Thus, all 25 pairs in pairwise comparisons among five cultivars from *C. annuum* and five cultivars from *C. frutescens* were differentiated from each other, while no pairs in pairwise comparison among five *C. annuum* cultivars were differentiated from each other (figure 2). Polymorphic protein band No. 4 existed in SDK and SKR cultivars and No. 5 existed in SKR and KNS cultivars while No. 2 existed in all five cultivars of *C. frutescens*. As the result of this polymorphic protein pattern, nine pairs in pairwise comparisons among five cultivars of *C. frutescens* were differentiated, with the exception being the comparison of MG and PP (table 2).

Efficacy of phosphate buffer as seed protein extracting solution in combination with gel pH range of 3-10.
Phosphate buffer solution in combination with pH gradient in the range of 3-10 gel provided four polymorphic protein bands across the 10 cultivars (figure 3). This treatment combination was able to differentiate 36 pairs in pairwise comparisons, i.e. gave 80% efficacy (table 3). Polymorphic protein band No. 2 existed in all cultivars of *C. anuumm* but was absent from the *C. frutescens* cultivars (figure 3). Thus, this polymorphic protein band pattern could differentiate all 25 pairs in pairwise comparisons between five hot chilli cultivars of *C. annuum* and five hot chilli cultivars of *C. frutescens* (table 3). Polymorphic protein band No. 3 was missing in *C. annuum* YSL (figure 3). Thus, differentiation of YSL from the other four cultivars of *C. annuum* was possible (table 3). Polymorphic protein band No. 1 was missing in SKR and KNS cultivars of *C. frutescens* and could therefore differentiate these two cultivars from the other cultivars of *C. frutescens* and the presence of polymorphic protein band No. 4 in KNS but missing in SKR cultivar made it possible to differentiate these two cultivars from each other (figure 3; table 3).

Efficacy of phosphate buffer as seed protein extracting solution in combination with gel pH range of 5-8/2-11.
The phosphate buffer solution in combination gel pH range 5-8/2-11 provided five polymorphic protein bands (figure 4). This treatment combination was able to verify 41 pairs in pairwise comparisons among 10 hot chilli cultivars, i.e. gave 91% efficacy (table 4). The presence of polymorphic protein band No. 3 in all five cultivars of *C. annuum* and absence from all five cultivars of *C. frutescens* (figure 4) made it possible to differentiate 25 pairs in the pairwise comparisons (table 4). The presence of polymorphic protein band No. 2 in PM differentiated it from the other four cultivars of *C. annuum* and the presence of the polymorphic protein band No. 4 in YSL (figure 4)

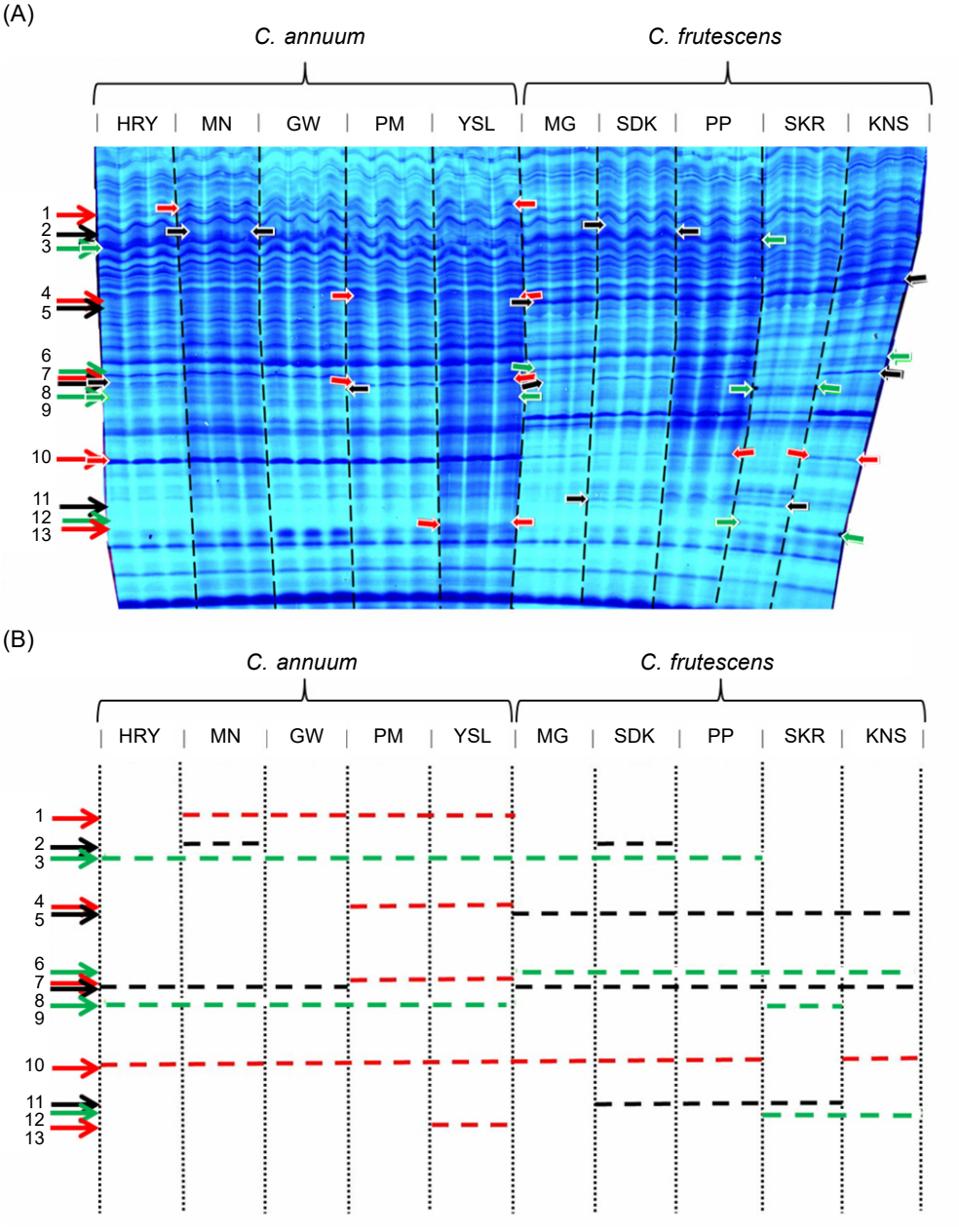

Figure 1. Electrophoretogram (**A**) and diagram (**B**) with 13 polymorphic protein bands (PPB) derived from performing UTLIEF of seed proteins of 10 Thai hot-chili cultivars of *Capsicum annuum* and *C. frutescens* using EX 3 solution as extraction solution and gel pH gradient of 3-10. (numbers 1-13 indicate the position of each PPB).

Table 1. Number of polymorphic bands from pairwise comparisons of seed proteins of 10 Thai hot-chilli cultivars from *Capsicum annuum* and *C. frutescens* by UTLIEF using EX 3 solution as extraction solution and gel pH gradient 3-10.

Species / Cultivars*		*C. annuum*					*C. frutescens*				
		HRY	MN	GW	PM	YSL	MG	SDK	PP	SKR	KNS
C. annuum	HRY	0									
	MN	2	0								
	GW	1	1	0							
	PM	4	4	3	0						
	YSL	5	5	4	1	0					
C. frutescens	MG	3	5	4	7	8	0				
	SDK	5	5	6	9	10	2	0			
	PP	4	6	5	8	9	1	1	0		
	SKR	6	8	7	10	11	5	5	4	0	
	KNS	5	7	6	9	10	2	4	3	3	0

* HRY = ‘Hua reur yon’; MN = ‘Mun’; GW = ‘Gaw’; PM = ‘Pama’; YSL = ‘Yod son lek’; MG = ‘Mong’; SDK = ‘She deuy kai’; PP = ‘Pop puang’; SKR = ‘Som ka rieang’; and KNS = ‘Kee noo suan’.

allowed the differentiation of YSL from the other cultivars of *C. annuum* (table 4). The presence and absence of polymorphic protein bands No. 1 and 5 in the cultivars of *C. frutescens* differentiated nine pairs in pairwise comparisons, the exception being that of MG and PP (figure 4; table 4).

Efficacy of HEPES buffer as seed protein extracting solution in combination with gel pH of 3-10.

The HEPES buffer in combination with gel pH 3-10 provided three polymorphic protein bands (figure 5). This treatment combination was able to differentiate 25 pairs in pairwise comparisons, i.e. had 56% efficacy (table 5). The presence of polymorphic protein bands No. 1 and 2 in all five cultivars of *C. annuum* and No. 3 in all five cultivars of *C. frutescens* gave the successful differentiation of 25 pairs in pairwise comparisons (figure 5; table 5). However, differentiation of cultivars within the same species was not possible.

Efficacy of HEPES buffer as seed protein extracting solution in combination with gel pH of 5-8/2-11.

HEPES buffer as seed protein extracting solution in combination with gel pH of 5-8/2-11 provided six polymorphic protein bands (figure 6). This treatment combination was

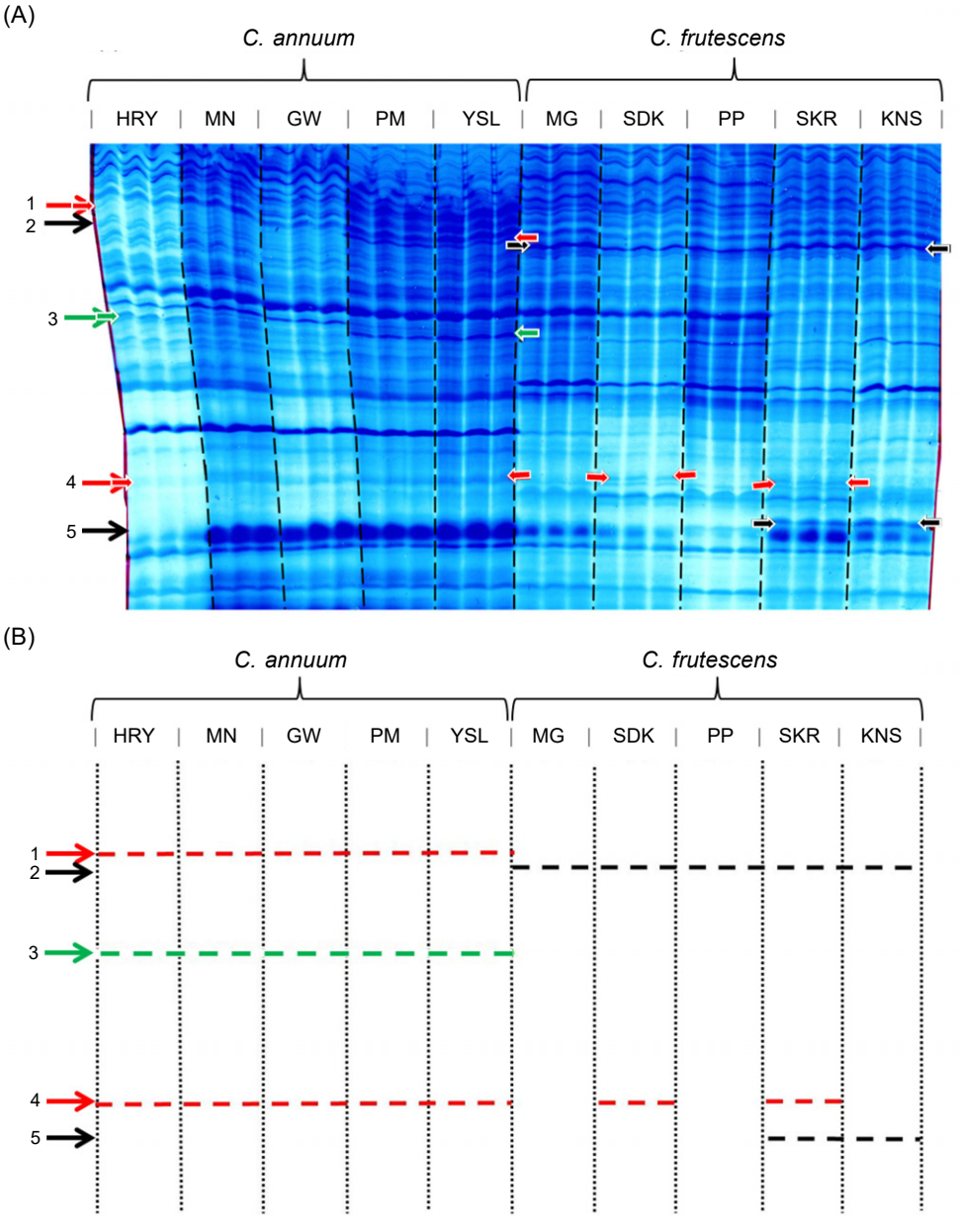

Figure 2. Electrophoretogram (**A**) and diagram (**B**) with five polymorphic protein bands (PPB) derived from performing UTLIEF of seed protein of 10 Thai hot-chili cultivars of *Capsicum annuum* and *C. frutescens* using EX 3 solution as extraction solution and gel pH gradient 5-8/2-11 (numbers 1-5 indicate the position of each PPB).

Table 2. Number of polymorphic bands from pairwise comparisons of seed proteins of 10 Thai hot-chilli cultivars from *Capsicum annuum* and *C. frutescens* by UTLIEF using EX 3 solution as extraction solution and gel pH gradient 5-8/2-11.

Species / Cultivars		*C. annuum*					*C. frutescens*				
		HRY	MN	GW	PM	YSL	MG	SDK	PP	SKR	KNS
C. annuum	HRY	0									
	MN	0	0								
	GW	0	0	0							
	PM	0	0	0	0						
	YSL	0	0	0	0	0					
C. frutescens	MG	4	4	4	4	4	0				
	SDK	3	3	3	3	3	1	0			
	PP	4	4	4	4	4	0	1	0		
	SKR	4	4	4	4	4	2	1	2	0	
	KNS	5	5	5	5	5	1	2	1	1	0

* HRY = ‘Hua reur yon’; MN = ‘Mun’; GW = ‘Gaw’; PM = ‘Pama’; YSL = ‘Yod son lek’; MG = ‘Mong’; SDK = ‘She deuy kai’; PP = ‘Pop puang’; SKR = ‘Som ka rieang’; and KNS = ‘Kee noo suan’.

able to verify 29 pairs in pairwise comparisons, i.e. had 64% efficacy (table 6). The presence of polymorphic protein bands No. 1, 2 and 4 in all five cultivars of *C. annuum* made possible the differentiation of hot chilli cultivars of *C. annuum* and *C. frutescens* but differentiation among cultivars within *C. annuum* was not possible (figure 6; table 6). The presence of polymorphic protein bands No. 5 and 6 in SKR cultivar allowed the successful differentiation of five pairs in pairwise comparisons among the *C. frutescens* cultivars.

Efficacy of water as seed protein extracting solution in combination with gel pH of 3-10. Using water as the protein extract solution in combination with pH gradient 3-10 provided seven polymorphic protein bands (figure 7). This treatment combination was able to verify 40 pairs in pairwise comparisons, i.e. gave 89% efficacy (table 7). Polymorphic protein band No.1 found in all five cultivars of *C. annuum* and No. 3 found in all five cultivars of *C. frutescens* made possible the differentiation of 25 pairs in pairwise comparisons. The presence and absence of polymorphic protein bands No. 2, 4 and 5 in the *C. annuum* cultivars made possible the verification of eight pairs in pairwise comparisons. The presence and absence of polymorphic protein bands No. 6 and 7 made possible the verification of seven pairs in pairwise comparisons among the *C. frutescens* cultivars.

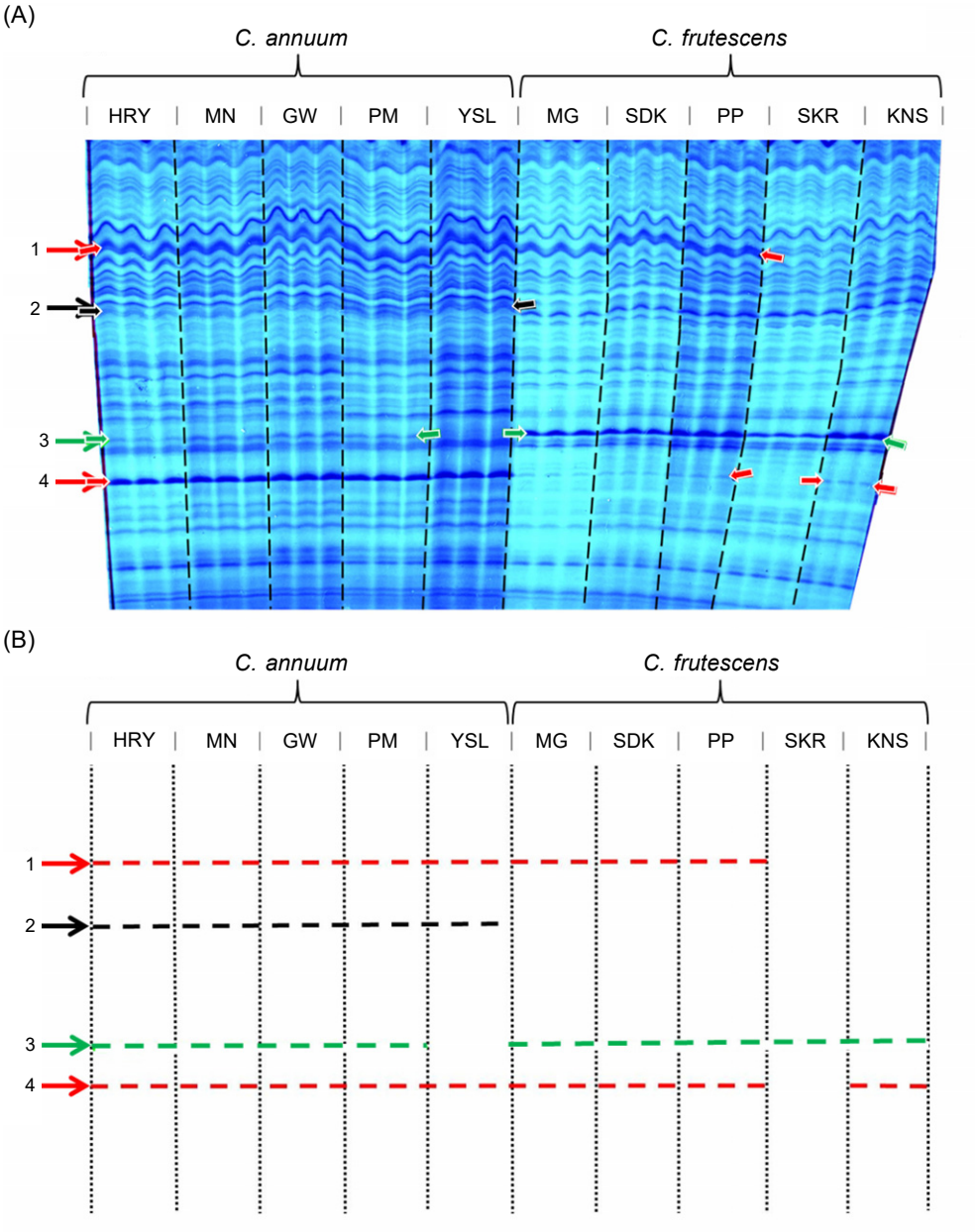

Figure 3. Electrophoretogram (**A**) and diagram (**B**) with four polymorphic protein bands (PPB) derived from performing UTLIEF of seed protein of 10 Thai hot-chili cultivars of *Capsicum annuum* and *C. frutescens* using PO_4 solution as extraction solution and gel pH gradient of 3-10 (numbers 1-4 indicate the position of each PPB).

Table 3. Number of polymorphic bands from pairwise comparisons of seed proteins of 10 Thai hot-chillli cultivars from *Capsicum annuum* and *C. frutescens* by UTLIEF using PO_4 solution as extraction solution and gel pH gradient 3-10.

Species / Cultivars		*C. annuum*					*C. frutescens*				
		HRY	MN	GW	PM	YSL	MG	SDK	PP	SKR	KNS
C. annuum	HRY	0									
	MN	0	0								
	GW	0	0	0							
	PM	0	0	0	0						
	YSL	1	1	1	1	0					
C. frutescens	MG	1	1	1	1	2	0				
	SDK	1	1	1	1	2	0	0			
	PP	1	1	1	1	2	0	0	0		
	SKR	3	3	3	3	4	2	2	2	0	
	KNS	2	2	2	2	3	1	1	1	1	0

* HRY = ‘Hua reur yon’; MN = ‘Mun’; GW = ‘Gaw’; PM = ‘Pama’; YSL = ‘Yod son lek’; MG = ‘Mong’; SDK = ‘She deuy kai’; PP = ‘Pop puang’; SKR = ‘Som ka rieang’; and KNS = ‘Kee noo suan’.

Efficacy of water as seed protein extracting solution in combination with gel pH of 5-8/2-11.

Using water as protein extracting solution with gel pH gradient of 5-8/2-11 gel provided four polymorphic protein bands (figure 8). This treatment combination was able to differentiate 31 pairs in pairwise comparisons, i.e. gave 69% efficacy (table 8). The presence of polymorphic protein bands No. 1, 2, 3 and 4 in all five cultivars of *C. annuum* provided the verification of 25 pairs in pairwise comparisons among five cultivars of *C. annum* and five of *C. frutescens* and also verification of six pairs in pairwise comparison among five cultivars of *C. annuum* (figure 8; table 8). Lastly, the absence of polymorphic protein band in all five hot chilli cultivars of *C. frutescens* made the verification among five hot chilli cultivars in *C. frutescence* not possible.

Discussion

The results of this study revealed that all treatment combinations (four protein extracting solutions and two gel pH ranges) were able to provide essential polymorphic protein bands required for differentiating cultivars of Thai hot chilli, but their efficacy varied. The EX3 solution in combination with gel pH range 3-10 was the most effective method

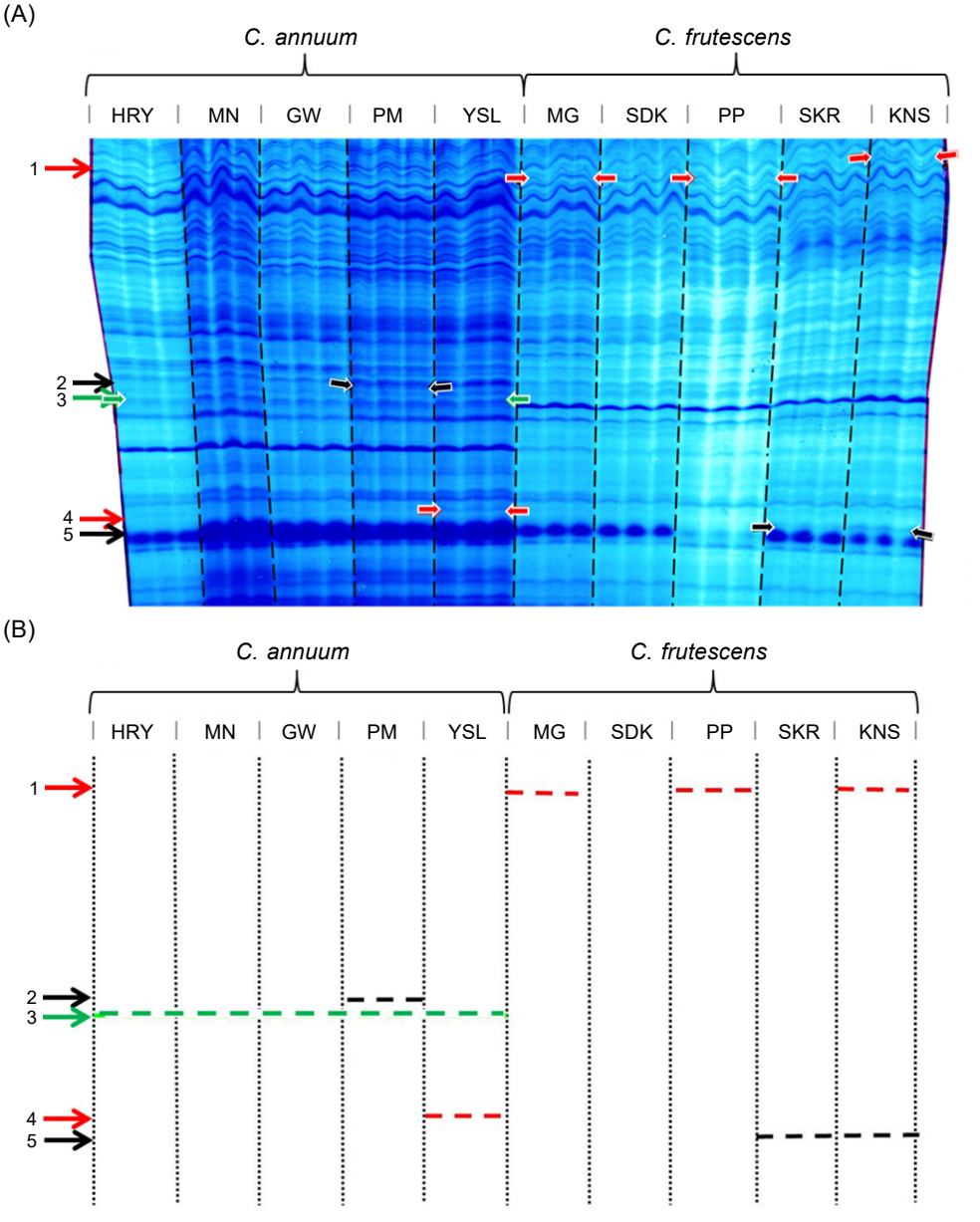

Figure 4. Electrophoretogram (**A**) and diagram (**B**) with five polymorphic protein bands (PPB) derived from performing UTLIEF of seed protein of 10 Thai hot-chili cultivars of *Capsicum annuum* and *C. frutescens* using PO_4 solution as extraction solution and gel pH gradient of 5-8/2-11 (numbers 1-5 indicate the position of each PPB).

Table 4. Number of polymorphic bands from pairwise comparisons of seed proteins of 10 Thai hot-chilli cultivars from *Capsicum. annuum* and *C. frutescens* by U TLIEF using PO_4 solution as extraction solution and gel pH gradient of 5-8/2-11.

Species / Cultivars		*C. annuum*					*C. frutescens*				
		HRY	MN	GW	PM	YSL	MG	SDK	PP	SKR	KNS
C. annuum	HRY	0									
	MN	0	0								
	GW	0	0	0							
	PM	1	1	1	0						
	YSL	1	1	1	2	0					
C. frutescens	MG	2	2	2	3	3	0				
	SDK	1	1	1	2	2	1	0			
	PP	2	2	2	3	3	0	1	0		
	SKR	2	2	2	3	3	2	1	2	0	
	KNS	3	3	3	4	4	1	2	1	1	0

* HRY = ‘Hua reur yon’; MN = ‘Mun’; GW = ‘Gaw’; PM = ‘Pama’; YSL = ‘Yod son lek’; MG = ‘Mong’; SDK = ‘She deuy kai’; PP = ‘Pop puang’; SKR = ‘Som ka rieang’; and KNS = ‘Kee noo suan’.

for verifying these 10 Thai hot-chilli cultivars. This treatment combination provided the highest number of polymorphic protein bands (13) and was able to verify all the 45 pairs in pairwise comparisons among 10 Thai hot chilli cultivars tested in this study, equal to 100% efficacy (table 9). Beside the EX3 solution, PO_4 buffer in combination with gel pH 2-11/5-8 and deionised water in combination with gel pH 3-10 were the second and third most effective combinations. They could verify 41 and 40 pairs in pairwise comparisons with efficacy of 91 and 89%, respectively. Regardless to the similar total efficacy, PO_4 buffer gave higher efficacy than water in verifying cultivars within *C. frutescence* (table 4) while water gave higher efficacy than PO_4 buffer in verifying cultivars within *C. annuum* (table 7). Water is cheap, locally available, non-toxic and it gives no harmful waste to the environment. Hence, in terms of cost and environmental safety, water should be one of the alternative protein extracting solutions to be tested for varietal verification by UTLIEF for hot chilli, especially for newly released cultivars of *C. annumm*.

The major type of protein in the seeds of Solanaceae species is globulin (Vladova *et al.*, 2004) which is dissolvable in diluted salt solutions such as phosphate and HEPES buffer solutions. However, in this study we found that the most polymorphic protein bands occurred when the EX3 solution was used as the protein extracting solution. EX3 solution consists of amide and alcohol compounds in which prolamins proteins can be dissolved. Hence, this study revealed that the majority of polymorphic proteins for

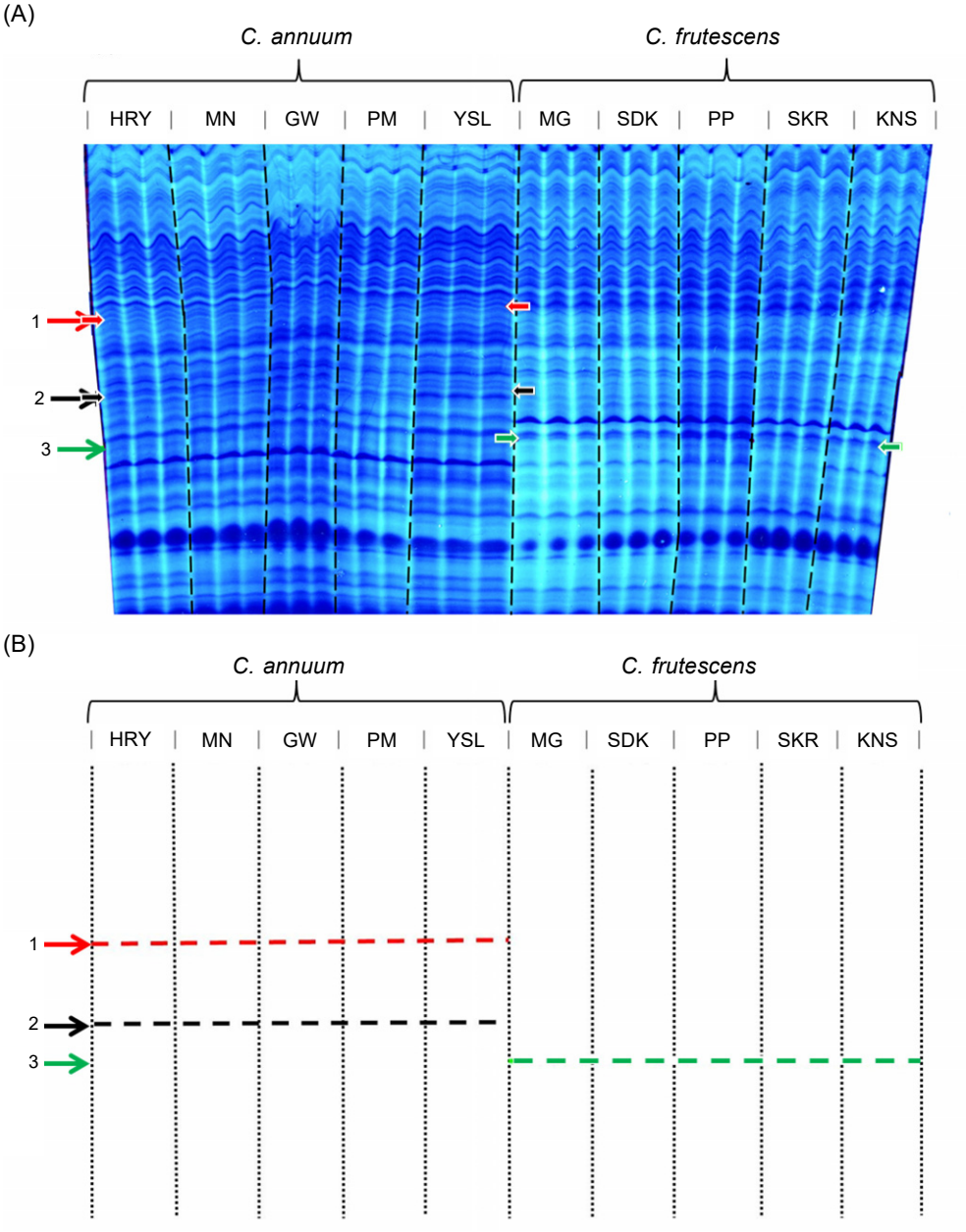

Figure 5. Electrophoretogram (**A**) and diagram (**B**) with three polymorphic protein bands (PPB) derived from performing UTLIEF of seed protein of 10 Thai hot-chili cultivars of *Capsicum annuum* and *C. frutescens* using HEPES solution as extraction solution and gel pH gradient of 3-10 (numbers 1-3 indicate the position of each PPB).

Table 5. Number of polymorphic bands from pairwise comparisons of seed proteins of 10 Thai hot-chilli cultivars from *Capsicum annuum* and *C. frutescens* by UTLIEF using HEPES solution as extraction solution and gel pH gradient 3-10.

Species / Cultivars		*C.annuum*					*C. frutescens*				
		HRY	MN	GW	PM	YSL	MG	SDK	PP	SKR	KNS
C. annuum	HRY	0									
	MN	0	0								
	GW	0	0	0							
	PM	0	0	0	0						
	YSL	0	0	0	0	0					
C. frutescens	MG	3	3	3	3	3	0				
	SDK	3	3	3	3	3	0	0			
	PP	3	3	3	3	3	0	0	0		
	SKR	3	3	3	3	3	0	0	0	0	
	KNS	3	3	3	3	3	0	0	0	0	0

* HRY = ‘Hua reur yon’; MN = ‘Mun’; GW = ‘Gaw’; PM = ‘Pama’; YSL = ‘Yod son lek’; MG = ‘Mong’; SDK = ‘She deuy kai’; PP = ‘Pop puang’; SKR = ‘Som ka rieang’; and KNS = ‘Kee noo suan’.

varietal verification by UTLIEF for hot chilli were prolamins, the minority protein type in hot chilli seeds. This result was in line with the results of previous research which found that more polymorphic proteins for cucumber varietal identification were albumins which was the minor protein type rather than globulins, the major protein (Onwimol *et al*., 2010). Therefore, the most polymorphic proteins which are essential for varietal verification by UTLIEF may be found in the minor protein types of seed storage proteins. It is important to try all the possible protein extracting solutions in order to cover all seed storage protein types so as to increase the possibility to find the one that can provide the highest polymorphic protein bands and maximise varietal verification efficacy.

The results in this study also revealed the crucial role of gel pH gradient on the efficacy of varietal verification of Thai hot chilli cultivars. As shown in table 9, the varietal verification efficacy of EX3 solution was reduced from 100 to 76% and that of water decreased from 93 to 69%, when the gel pH range changed from 3-10 to 5-8/2-11 and in contrast the efficacy of phosphate and HEPES buffer solution increase from 80 and 56 to 90 and 64%, respectively, when the gel pH range changed from 3-10 to 5-8/2-11 (table 9). The pH range of the gel can affect the distance in the gel between two adjacent pH values. Therefore, the appropriate gel pH range ensures sufficient space between pH values and hence that proteins with different isoelectric points stop in different places for easy gel interpretation. In this study, it was found that when EX3

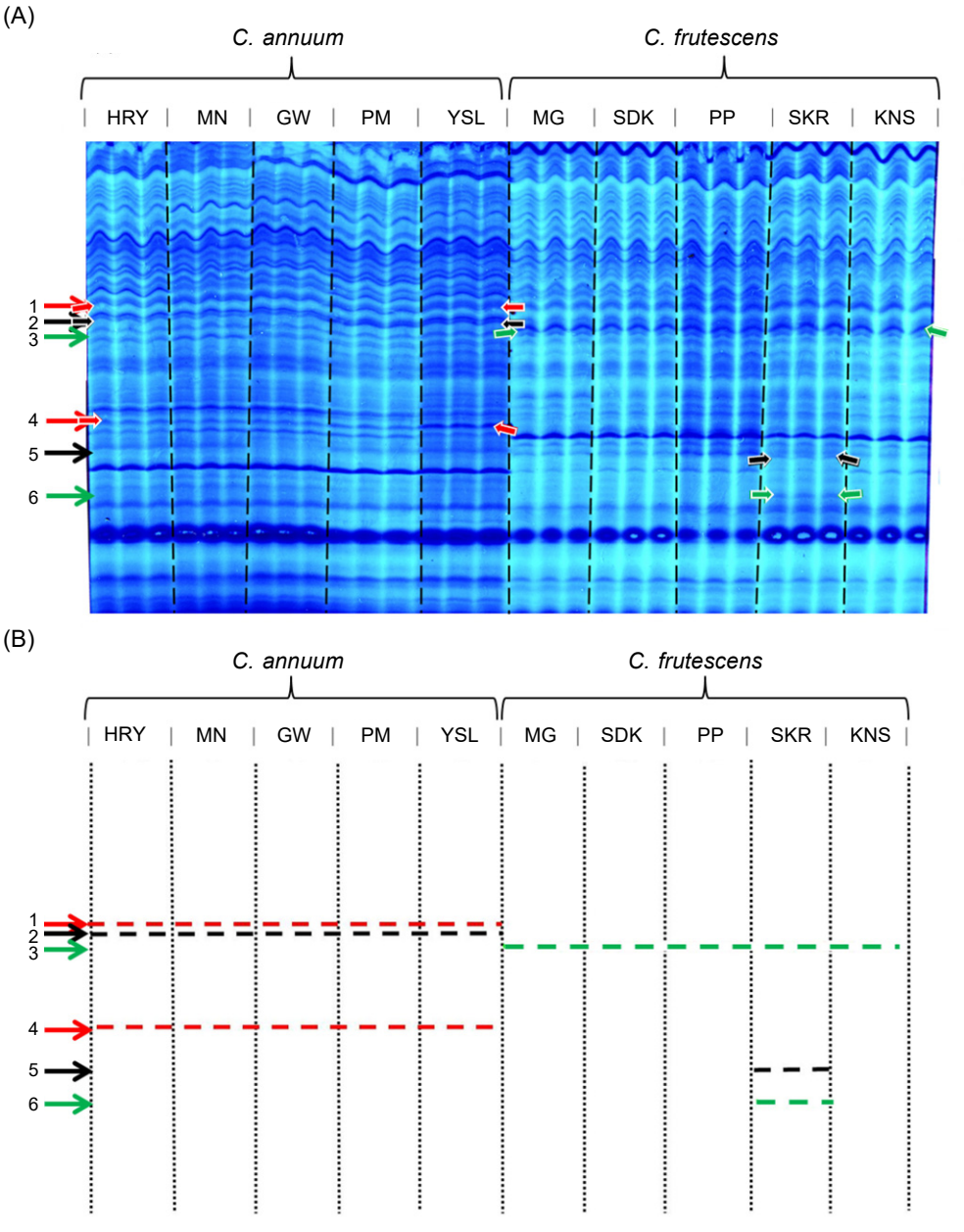

Figure 6. Electrophoretogram (**A**) and diagram (**B**) with six marker protein bands (PPB) derived from performing UTLIEF of seed protein of Thai hot-chili cultivars of *Capsicum annuum* and *C. frutescens* using HEPES solution as extraction solution and gel pH gradient of 5-8/2-11 (numbers 1-6 indicate the position of each PPB).

Table 6. Number of polymorphic bands from pairwise comparisons of seed proteins of 10 Thai hot-chilli cultivars from *Capsicum annuum* and *C. frutescens* by UTLIEF using HEPES solution as extraction solution and gel pH gradient 5-8/2-11.

Species / Cultivars		*C. annuum*					*C. frutescens*				
		HRY	MN	GW	PM	YSL	MG	SDK	PP	SKR	KNS
C. annuum	HRY	0									
	MN	0	0								
	GW	0	0	0							
	PM	0	0	0	0						
	YSL	0	0	0	0	0					
C. frutescens	MG	4	4	4	4	4	0				
	SDK	4	4	4	4	4	0	0			
	PP	4	4	4	4	4	0	0	0		
	SKR	6	6	6	6	6	2	2	2	0	
	KNS	4	4	4	4	4	0	0	0	2	0

* HRY = ‘Hua reur yon’; MN = ‘Mun’; GW = ‘Gaw’; PM = ‘Pama’; YSL = ‘Yod son lek’; MG = ‘Mong’; SDK = ‘She deuy kai’; PP = ‘Pop puang’; SKR = ‘Som ka rieang’; and KNS = ‘Kee noo suan’.

solution and water were used as protein extracting solution, the gel pH range of 3-10 was more appropriate than pH range of 5-8/2-11 since it allowed a higher number of polymorphic protein bands to be visible. This indicates that this 3-10 pH range provided more space between two pH values with respect to the presence of polymorphic protein bands than pH 5-8/2-11. However, when phosphate buffer and HEPES solution were used, the gel pH of 5-8/2/11 gave better performance. Thus, this results suggest that not only is it necessary to use a suitable protein extracting solution, but appropriate gel pH gradient should be taken in to consideration to achieve the highest efficacy in varietal verification.

Amongst the 10 Thai hot chilli cultivars tested in this study, five were *C. annuum* and five were *C. frutescens*. It was clearly found in this study that the UTLIEF technique could effectively differentiate chilli cultivars between these two species. All combinations of protein extracting solution and gel pH ranges could effectively differentiate between the two species. This result revealed that the difference in seed proteins between two species is greater than the difference within the same species for all types of seed proteins. Nevertheless, the greatest number of polymorphic protein bands occurred using EX3 solution as the protein extracting solution in combination with gel pH range of 3-10 (table 1). This result suggests that the greatest difference in seed proteins between these two species is in prolamins.

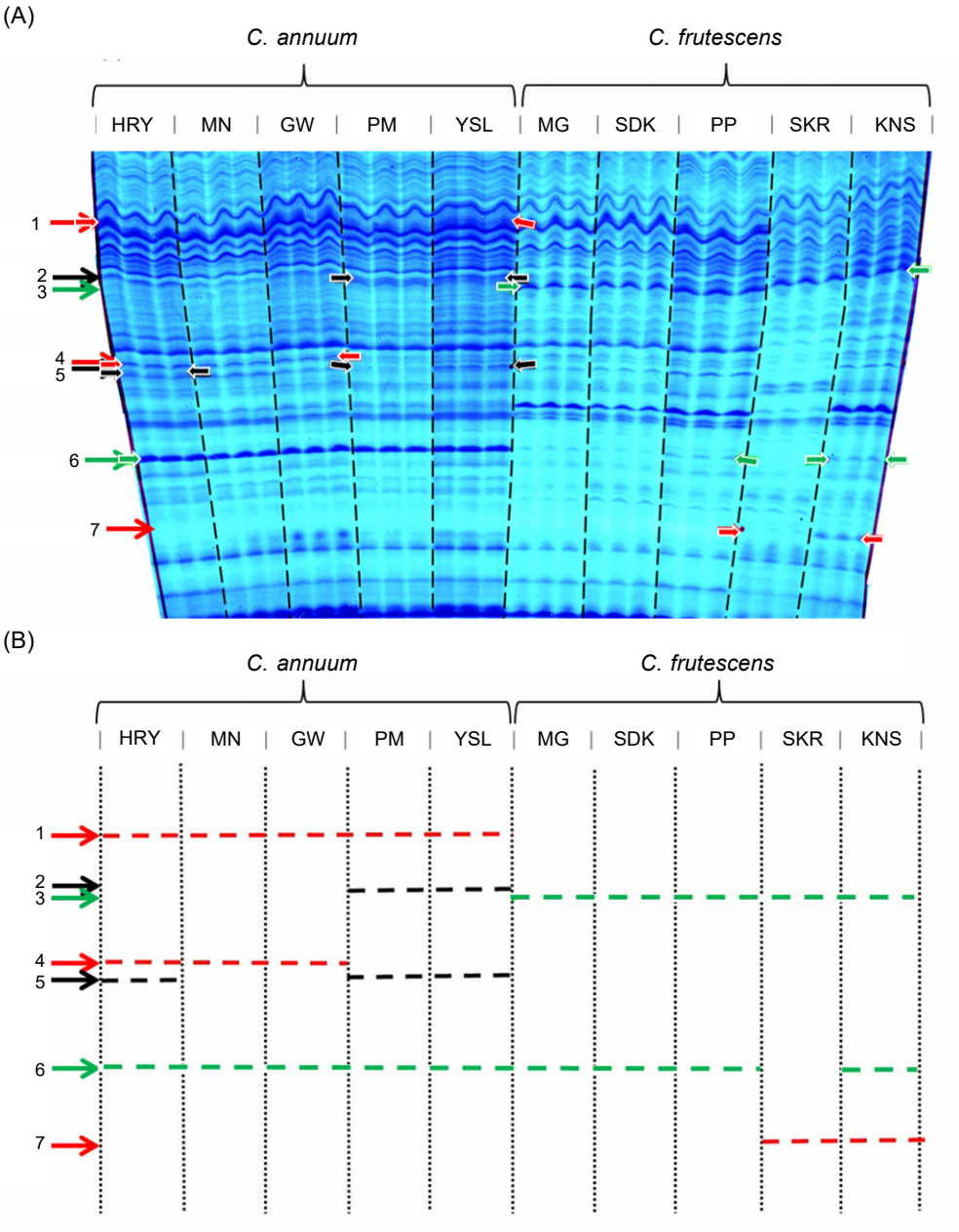

Figure 7. Electrophoretogram (**A**) and diagram (**B**) seven polymorphic protein bands (PPB) derived from performing UTLIEF of seed protein of 10 Thai hot-chili cultivars of *Capsicum annuum* and *C. frutescens* using H_2O solution as extraction solution and gel pH gradient 3-10 (numbers 1-7 indicated the position of each PPB).

Table 7. Number of polymorphic bands from pairwise comparisons of seed proteins of 10 Thai hot-chilli cultivars from *Capsicum annuum* and *C. frutescens* by UTLIEF using H_2O as extraction solution and gel pH gradient 3-10.

Species / Cultivars		*C. annuum*					*C. frutescens*				
		HRY	MN	GW	PM	YSL	MG	SDK	PP	SKR	KNS
C. annuum	HRY	0									
	MN	1	0								
	GW	1	0	0							
	PM	2	3	3	0						
	YSL	2	3	3	0	0					
C. frutescens	MG	4	3	3	4	4	0				
	SDK	4	3	3	4	4	0	0			
	PP	5	4	4	5	5	0	0	0		
	SKR	6	5	5	6	6	2	2	1	0	
	KNS	5	4	4	5	5	1	1	2	1	0

* HRY = ‘Hua reur yon’; MN = ‘Mun’; GW = ‘Gaw’; PM = ‘Pama’; YSL = ‘Yod son lek’; MG = ‘Mong’; SDK = ‘She deuy kai’; PP = ‘Pop puang’; SKR = ‘Som ka rieang’; and KNS = ‘Kee noo suan’.

The varietal verification of hot-chilli cultivars within species and between species of *C. annuum* and *C. frutescens* using the sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) technique was reported earlier by Odeigah *et al*. (1999). It was found that the verification of hot chilli cultivars by using the presence and absence of polymorphic protein bands was only possible when comparing the cultivars that came from different species but not for within species. This might suggest that the UTLIEF technique that separates the proteins by the difference in their pI values was probably more effective than the SDS-PAGE technique using the difference in molecule weight to separate among different proteins.

Seed proteins are the primary products of structural genes (Srivalli *et al*., 1999). The differences in seed proteins among plant cultivars can indicate the magnitude of genetic diversity among them. In this study we found the greater genetic diversity between hot chilli cultivars from different species than within species as indicated by the number of polymorphic protein bands found in pairwise comparisons. The greatest number of polymorphic bands (11) was found when comparing *C. annuum* cultivar YSL from *C. frutescens* SKR. Therefore, these two cultivars may have the greatest genetic difference among the 10 cultivars tested. The crossing between these two cultivars should provide a high genetic diversity which is desirable for hot chilli breeding. Thus, the UTLIEF technique can also support plant breeding programmes by providing another effective method for assessing genetic diversity.

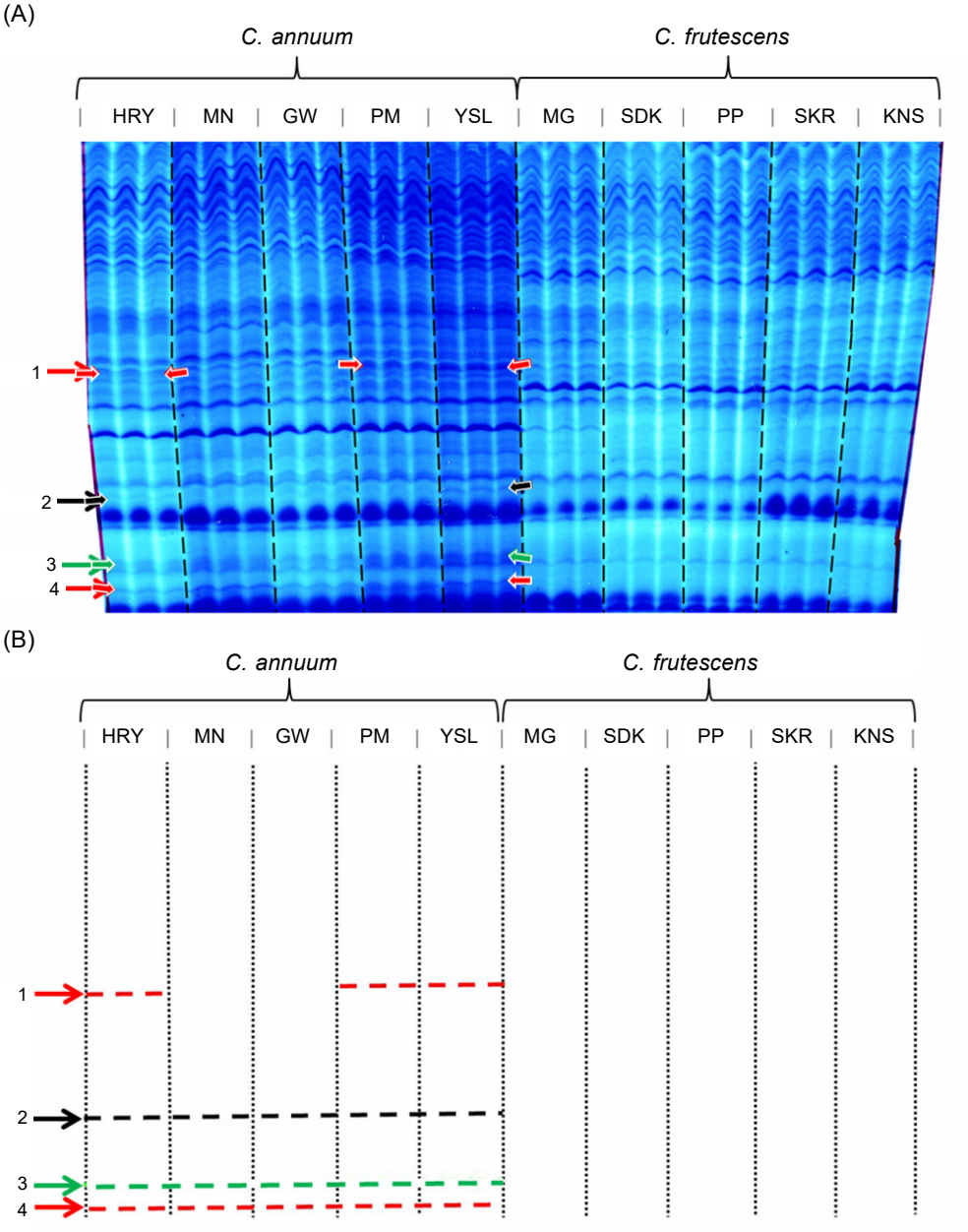

Figure 8. Electrophoretogram (**A**) and diagram (**B**) four polymorphic protein bands (PPB) derived from performing UTLIEF of seed protein of 10 Thai hot-chili cultivars of *Capsicum annuum* and *C. frutescens* using H_2O solution as extraction solution and gel pH gradient 5-8/2-11 (numbers 1-4 indicate the position of each PPB).

Table 8. Number of polymorphic bands from pairwise comparisons of seed proteins of 10 Thai hot-chilli cultivars from *Capsicum annuum* and *C. frutescens* by UTLIEF using H_2O as extraction solution and gel pH gradient 5-8/2-11.

Species / Cultivars		*C. annuum*					*C. frutescens*				
		HRY	MN	GW	PM	YSL	MG	SDK	PP	SKR	KNS
C. annuum	HRY	0									
	MN	1	0								
	GW	1	0	0							
	PM	0	1	1	0						
	YSL	0	1	1	0	0					
C. frutescens	MG	4	3	3	4	4	0				
	SDK	4	3	3	4	4	0	0			
	PP	4	3	3	4	4	0	0	0		
	SKR	4	3	3	4	4	0	0	0	0	
	KNS	4	3	3	4	4	0	0	0	0	0

* HRY = ‘Hua reur yon’; MN = ‘Mun’; GW = ‘Gaw’; PM = ‘Pama’; YSL = ‘Yod son lek’; MG = ‘Mong’; SDK = ‘She deuy kai’; PP = ‘Pop puang’; SKR = ‘Som ka rieang’; and KNS = ‘Kee noo suan’.

Table 9. Number of polymorphic protein bands, number of pairs that could be differentiated in pairwise comparisons and efficacy of varietal verification of 10 Thai hot-chilli cultivars extracted by different solvents in combination with different gel pH gradient.

Treatments	Number of polymorphic protein bands	Number of pairs that could be differentiated in pairwise comparison	Efficacy of varietal verification (%)
EX 3 with pH 3-10	13	45	100
EX 3 with pH 5-8/2-11	5	34	76
PO_4 with pH 3-10	4	36	80
PO_4 with pH 5-8/2-11	5	41	91
HEPES with pH 3-10	3	25	56
HEPES with pH 5-8/2-11	6	29	64
H_2O with pH 3-10	7	40	89
H_2O with pH 5-8/2-11	4	31	69

In conclusion, this study demonstrated that the UTLIEF technique is a highly effective method to verify Thai hot chilli cultivars. The efficacy of the verification depended on the chosen protein extracting solution and gel pH range. The EX3 solution and gel pH range of 3-10 showed the highest efficacy (100%), enabling verification of all 45 pairs in pairwise comparisons among the 10 Thai hot chilli cultivars. Water can also be a promising alternative protein extracting solution when testing hot chilli cultivars of *C. annuum*.

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References

Arus, P. (1983). Genetic purity of commercial seed lots (Part A). In *Isozymes in Plant Genetics and Breeding*, (eds. S.D. Tanksley and T.J. Orton), pp. 415-423, Elsevier Science Publishers, Amsterdam.

Cooke, R.J. (1995). Gel electrophoresis for the identification of plant varieties. *Journal of Chromatography A*, **698**, 281-299. <https://doi.org/10.1016/0021-9673(94)00649-T>

Dou, X.Y., Yan, M., Xu, Y.F., Hussain, K., Liu, Y.Z. and Lin, F. (2012) Identification and purity testing of maize hybrids with one parent in common by ultrathin-layer isoelectric focusing of seed salt-soluble proteins. *Turkish Journal of Agriculture and Forestry*, **36**, 267-273. <https:// doi:10.3906/tar-1012-1530>

ISTA (2016). *International Rules for Seed Testing*, International Seed Testing Association, Bassersdorf, Switzerland.

Jiamtae, P., Onwimol, D., Chanprasert, W., Thongket, T. and Prasertsak, A. (2017). Varietal verification of Thai rice using ultrathin layer isoelectric focusing of seed proteins. *ScienceAsia*, **43**, 145-154. <https:// doi: 10.2306/scienceasia1513-1874.2017.43.145>

Kraikruan, W., Sukprkarn, S., Mongkolporn, O. and Wasee, S. (2008). Capsaicin and dihydrocapsaicin contents of Thai chili cultivars. *Kasetsart Journal (Natural Science)*, **41**, 611-616.

Manum, P. (2008). *Varietal Identification of Sweet Corn using Seed Protein Analysis via Ultrathin Layer Isoelectric Focusing*, MSc Thesis, Kasetsart University, Nakhon Pathom, Thailand.

McDonald, M.B. (1998). Seed quality assessment. *Seed Science Research*, **8**, 265-275.

Nantareeyawatn, C. (2006). *Mungbean Cultivars Identification by Seed Proteins using Ultra-Thin Layer Isoelectric Focusing Technique*, MSc Thesis, Kasetsart University, Kamphaengsaen, Thailand.

Odeigah, P.G.C., Oboh, B. and Aghalokpe, I.O. (1999). The characterization of Nigerian varieties of pepper, *Capsicum annuum* and *Capsicum frutescens* by SDS-polyacrylamide gel electrophoresis of seed proteins. *Genetic Resources and Crop Evolution*, **46**, 127-131.

Onwimol, D., Chanpreme, S. and Thongket, T. (2010). Seed storage protein extraction and gel pH gradient for cucumber varietal identification via ultrathin-layer isoelectric focusing technique. *Kasetsart Journal (Natural Science)*, **44**, 902-911.

Onwimol, D., Chanpreme, S. and Thongket, T. (2014). Hybridity test of cucumber *via* ultrathin layer isoelectric focusing technique using water as the extraction buffer. *Kasetsart Journal (Natural Science)*, **48**, 689-695.

Srivalli, T., Lakshmi, N. and Gupta (1999). Analysis of seed proteins by polyacrylamide gel electrophoresis (PAGE) in diploids, tetraploids and tetraploid hybrids of Capsicum. *Capsicum Eggplant Newsletter*, **18**, 48-51. <https://doi.org/10.1023/A:102355623319>

Thai Seed Trade Association. (no date). Thailand' seed export in 2018. Available at <https://www.thasta.com/index.php/>Accessed May 2019. [In Thai.]

Thongket, T. and Chanpreme, S. (2006). Varietal verification using seed proteins *via* UTLIEF technique. *The Seed News Letter*, **13**, 11-12. Available at <http://seed.or.th/documents/seedmags/may&june06.pdf> Accessed May 2019. [In Thai.]

Tu, H., Zhang, J.Q. and Wang, X.F. (2012). Variation in seed protein within and among species of Cucurbita by ultrathin-layer isoelectric focusing. *Seed Science and Technology*, **40**, 113-117. <https://doi.org/10.15258/sst.2012.40.1.13>

Van den Berg, B.M. (1990). Inbred testing of tomato (*Lycopersicon esculentum* L.) F1 varieties by ultrathin-layer isoelectric focusing of seed protein. *Electrophoresis*, **11**, 824-829. <https://doi.org/10.1002/elps.1150111009>

Van den Berg, B.M. (1991). A rapid and economical method for hybrid purity testing of tomato (*Lycopersicon esculentum* L.) F1 hybrids using ultrathin-layer isoelectric focusing of alcohol dehydrogenase. *Electrophoresis*, **12**, 64-69. <https://doi.org/10.1002/elps.1150120112>

Vladova, R., Tsanev, V. and Petcolicheva, K. (2004). Seed storage proteins in Solanaceae and Cucurbitaceae species. *Biologia Plantarum*, **48**, 601-603.

Wang, X.F., Knoblauch, R. and Leist, N. (2000). Varietal discrimination of tomato (*Lycopersicon esculentum* L.) by ultrathin-layer isoelectric focusing of seed protein. *Seed Science and Technology*, 28, 521-526.

Yan, M. (2013). Genetic purity testing of pumpkin hybrid seed by ultrathin-layer isoelectric focusing. *Crop Breeding and Applied Biotechnology*, **13**, 200-202. <https://doi:10.1590/S1984-70332013000300008>

Zhao, T., Yan, M., Lu, Y.P., Yang, F., Huang, J. and Wang, X.F. (2003). Genetic purity testing of two-line hybrid rice seeds by ultrathin-layer isoelectric focusing of proteins. *Seed Science and Technology*, **33**, 45-52. <https://doi.org/10.15258/sst.2005.33.1.05>