academicJournals

Vol. 9(12), pp. 476-489, December 2015
DOI: 10.5897/AJPS2015.1294
Article Number: 0C5653E56047
ISSN 1996-0824

http://www.academicjournals.org/AJPS

African Journal of Plant Science

Full Length Research Paper

Re-evaluating $TiCl_4$ and UV assays for detection of vicine and convicine in high-throughput screening of immature and mature seeds of faba bean

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Received 11 March, 2015; Accepted 6 October, 2015

Genomic resources have just started to focus on the faba bean; the genes for synthesis of vicine and convicine (V-C) have not been determined, and recently developed genetic markers for these anti-nutritionals have not been used to examine these traits in very large scale in faba bean. Simple, rapid and cost-effective technologies are crucial in crop breeding programs, especially in the developing world, and in some cases, traditional methodologies are used in combination with genetic markers to assess agronomic traits and the value of gene markers. Here, two methodologies (TiCl4 assay and 274 nm absorption) are re-evaluated for their application in detection of V-C in faba beans. In comparison with TiCl4 assay, the method of 274 nm UV absorption without an HPLC analysis offers more reliable analysis for detection of V-C in immature and mature seeds of faba bean. Its application in high throughput screening by 60 min agitation of immature seeds or mature seed flour in 2% trichloroacetic acid (TCA) allows quick screening of low V-C faba beans. The level of V-C was maximum when seed moisture was 80% and V-C level was measured as 0.92% in CDC Fatima flour. Though V-C from 2% TCA extract of mature and immature seeds of CDC Fatima was detected by 274 wavelength in the TiCl4 assay reaction, a Ti-aglycone complex was not clearly detectible at 480 nm as previously suggested.

Key words: Vicine, convicine, TiCl4 assay, UV assay, high throughput, seed, faba bean.

INTRODUCTION

Faba bean (*Vicia faba* L.) is the third most important feed grain legume after soybean and pea. The largest producer is China, followed by Australia, France, the Americas, Egypt, Sudan, Morocco and Ethiopia (FAO, 2013). In North America, faba bean has been grown on a limited basis on the Canadian prairies since the 1970s (Gade, 1994). Production has expanded since 1997 and the annual growth rate was 5.82% between 2003 and 2013 in Canada (FAO, 2013).

Faba bean is rich in protein (Burstin et al., 2011) and essential amino acids (Alghamdi, 2009; Mortuza et al., 2009; Sosulski and Hold, 1980). It has potential as a meat substitute in many parts of the world where there is demand for non-animal protein sources. Faba bean produces the highest percentage of protein (24–38%) per seed (Burridge, 1999; Burstin et al., 2011) and per unit

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land area as compared to all other temperate and tropical pulse crops, including lentil (*Lens culinaris*), common bean (*Phaseolus vulgaris*), chickpea (*Cicer arietinum*) and pea (*Pisum sativum*) (Broad beans world yield, FAOSTAT 2013). Mature faba beans provide high-quality carbohydrates (58%), fiber (25%) (USDA Nutritional Database, http://www.nal.usda.gov/fnic/foodcomp/search), antioxidants, and nutrients, with a comparatively low glycemic index (GI) (40 and 48 GI per 150 g serving for baked bean and dark durum wheat pasta, respectively [GI database, University of Sydney]). They could be an alternative to wheat in the food industry.

Faba bean flour alone and mixed with wheat flour at 100 and 70% provides a gluten-free option for the bread and pasta industries (Sozer, personal communication, VTT Technical Research Centre of Finland, 2014). Immature faba beans used as vegetable are a significant source of vitamin A (350 iU per 100 g) and vitamin C (0.033%) (http://www.nal.usda.gov/fnic/foodcomp/search, Kmiecik et al., 1990) and polyphenols with antioxidant activity (Baginsky et al., 2013). Green pods of faba bean are a significant source of L-DOPA (Burbano et al., 1995) which is used for treating Parkinson disease (Seeberger and Hauser, 2009) and for overcoming low libido in human (Hulse, 1994). Faba beans contain significant amounts of flavanoid glycosides which inhibit xanthine oxidase (XO) (Spanou et al., 2012), XO inhibitors are beneficial in vascular disorders (George and Struthers, 2008).

Presence of some anti-nutritional factors limits faba bean use in the food and feed industry**.** In humans, aglycones of vicine (2,6-diamino-4,5-dihydroxypyrimidine-5-[*β*-D-glucopyranoside]) and convicine (2,4,5-trihydroxy-6-aminopyrimidine-5-[*β*-D-glucopyranoside]), divicine and isouramil, respectively, are responsible for the favism in individuals who carry specific alleles that cause deficient, non-functional or low functional G6P dehydrogenase (G6PD) enzymes (Mason et al., 2007). Favism is a potential life-threatening oxidative damage that results from the ingestion of faba beans. These individuals are unable to regenerate reduced glutathione (GSH) which results in deposition of toxic substances in the membranes of red blood cells, causing cell apoptosis or destruction (Arese et al., 2012). This X-linked gene is known to be the most common human enzyme defect (> 400 million people) worldwide (Mason et al., 2007; Cappellini and Fiorelli, 2008) and natural variants are expected to be generally found in both animal and human populations. The negative effects of toxic glycosides were also reported in animal models such as poultry (Muduuli et al., 1982).

Earlier studies of faba bean tissue analysis for vicine and convicine (V-C) showed it could be detected in seeds from 13-15 cm long pods but not from younger pods. The substrate [6-14C], orotate incorporation into the pyrimidine ring of V-C in pod and seed showed V-C synthesis synthesis in both tissues, but with accumulation in seeds and not in pod tissue (Brown and Robbins, 1972). A contradicting study that analyzed V-C content during seed development showed that vicine content was highest when seed contained 80% moisture but was present even in seeds of new developing pods (Burbano et al., 1995). Another study showed the highest amount of V-C in mature resting seed, and lesser amounts in roots during vegetative growth. The V-C was present in root tissues until seed maturation and also in stem before the start of seed development (Ramsay and Griffiths, 1996). Among faba bean flour extracts that have commercial value, the amount of V-C was higher in protein concentrate than in starch and flour of faba bean (Kim et al., 1982). Reduction of V-C in faba bean is of ongoing interest for its breeders (Abd Allah et al., 1988; Jamalian et al., 2006), processes are ineffective and costly for the food and feed industry. The V-C compounds are thermally stable [melting point: 242-244°C (Olaboro et al., 1981; Marquardt et al., 1983) and the cooking and boiling processes do not remove V-C by more than 50%, although natural genetic variation in V-C reduction by these processes does exist (Martinez et al., 2012). Ultimately V-C has to be removed by crop development programs.

The lack of genomic data was limited until now by the enormous nuclear genome of *V. faba* (predicted as ~13 000 mb, which is 30 times greater than the sequenced *Medicago truncatula* genome (Bennett et al., 1982; Johnston et al., 1999), and the small size of sequence database (579 assembled genome nucleotide, 75870 transcript, 64079 un-annotated nucleotide from various resources) at public domain (Vicia faba nucleotides, NCBI 2015) have eliminated gene based marker development for V-C content screening in faba bean. The low V-C gene locus has originally been discovered from Polish collection by Duc et al. (1989), by discovery of low V-C mutant (with 0.046% V+C of seed dry matter) which led to development of genetic markers (Gutierrez et al., 2006 and Khazaei et al., 2015) for V-C trait or concentration analysis but neither genes for their synthesis nor the wide use of genetic marker application has been determined. Genetic markers, generally speaking, are valuable when tightly linked to agronomic traits in breeding programs, ideally they are very valuable when whole genome is known.

Acid, alkaline and alcohol extractions have been used for V-C estimation (Higazi and Read, 1974; Jamalian, 1978; Sosulski and Pitz, 1979; Hegazy and Marquardt, 1983). Three different spectrophotometric methods have been used for measuring V-C in faba bean samples. One is based on UV (273.5 nm) spectrophotometry of protein free (Collier, 1976) while the others three are colorimetric methods. One is based on the formation of a Ti-complex with V-C, detected at 480 nm (Kim et al., 1982), the second is based on the reduction of Folin-Ciocalteau phenol reagent reads at 650 nm (Higazi and Read, 1974), and in

Table 1. Faba bean cultivars and characteristics.

Genotypes and definitions	Flower color	Vicine – convicine	Hilum color	Seed coat color-tannin-total phenolic of mature seed
Disco* (D)	White	Low	light brown	Light-low- low
Snowbird* (SN)	White	Normal	light brown	Light-high-high
CDC Fatima* (F)	Normal type (white with black wing spots)	Normal	Black	Dark-high-moderate
CDC Snowdrop (SD)	White	Normal	light brown	Light-unknown-unknown
CEB04928 (CEB)	White	Normal	light brown	Light-unknown-high

Source: *Oomah et al. (2011) Phenolics, phytic acid, and phytase in Canadian-grown low-tannin faba bean (*Vicia faba* L.) genotypes, J. Agric Food Chem. 2011 Apr 27; 59(8):3763-71. doi: 10.1021/jf200338b. Epub 2011 Mar 10.

the third method, the reduction of o-ferriphenanthroline to o-ferrophenanthroline by V-C aglycones is read at 610 nm (Chevion and Novak, 1983). The visual color forming Folin-Ciocalteau phenol and $TiCl_4$ assays are also used for analysis of phenolic compounds [example: sinapic acid (Ismail and Eskin, 1979). Additionally, TiCl4 is used for analyzing H_2O_2 (Gupta and Eskin, 1977). More accurately, V-C content measuring analytical methods include C18 reverse phase HPLC connected to a uv-vis detector (Marquardt and Frohlich, 1981; Zhang et al., 2003) and GC-MS (Sosulski and Pitz, 1979) which are quite costly and the TLC (Jamalian and Bassiri, 1978), which is cost effective but very slow technique. Low-cost, reliable, simple, less time-consuming tests are needed for routine analysis of V-C in faba bean breeding programs. Here, we aimed to re-evaluate TiCl4 and UV spectrophotometric assays of immature and mature seeds.

Improving crops using genomic tools is still in its infancy, especially in legumes (Varshney et al., 2015). There is little evidence on the types of cases in which they work, and their application on very large scales is still limited (Varshney et al., 2015). Utilization of the faba bean genome by its breeders or for any other application, especially in developing countries, takes a long time. Costly and time-consuming V-C screening strategies can be overcome with quick, cheap, simple, practical phenotypic markers and analytical tools. Here, TiCl4 and UV assays were compared to clarify whether any of these methods could be a better option in large-scale low V-C faba bean analysis.

MATERIALS AND METHODS

Plant material

Mature and immature seeds of the faba bean cultivars listed in Table 1 were obtained from the Crop Development Centre (CDC) of the University of Saskatchewan. Dry seeds were from the 2014 harvest of field research plots located at Preston, Saskatoon, whereas immature seeds were collected from plants at the CDC greenhouses within the same year. Dry seeds were kept at room temperature and fresh seeds in a –20°C freezer until analysis. V-C was determined in mature and immature seeds and immature seed tissues, including cotyledon, radical, seed coat and pod.

Mature seeds were manually de-hulled and then ground in a coffee mill at the day of analysis. An unknown cultivar protein concentrate from dry milling process was obtained from Parrheim Foods (PF) (Saskatoon, SK, CA) and was used in purification and analysis of V-C.

Other materials

All reagents were of analytical grade. Titanium tetrachloride was obtained from Sigma-Aldrich Canada Co. (Oakville, Ontario, CA). V-C standards were not commercially available; therefore they were purified from faba bean protein concentrate of PF using the method of Marquardt et al. (1983). Purity of the extracted V-C was determined by reversed phase analytical HPLC (RP-HPLC) and mass spectrophotometer (HP1100 series of Agilent, Germany) equipped with UV detection coupled to a Quattro LC (Waters, UK) triple quadrupole mass spectrometer (MS) equipped with an electrospray ionization (ESI) interface. Identifications of V-C were initially made by specific absorbance spectra (Merck Index, 2006) and then by MS.

All HPLC analyses were performed using a C18 column (CAPCELL C18 AG120, 25 × 4.6, 5 micron, Phenomenex, Torrance, CA, USA) connected to a UV detector that was adjusted to 274 nm. Extracts were diluted ten times in 2% TCA (trichloroacetic acid), then were applied to the column equilibrated and run with water (Quemener, 1988) at 0.8 µl/min.

HPLC mass spectrophotometer conditions were a short RP-HPLC column (Genesis C18 2.1 × 100 mm, 3.5 µm, Germany) with the 97% A (5% formic acid) and 3% B (95:5 methanol : water with 5% formic acid) as a mobile phase at 0.6 ml/min, for 20 min.

Extraction and UV spectrophotometer analysis of V-C

Percentage V-C analysis in 2% TCA extracts of mature and immature seed

Ground, mature, dehulled seed samples (100 mg) were extracted in duplicate with 2% TCA (0.6 ml), and the aqueous sample solution was suspended, then sonicated (5 min, two times). For immature seeds, a single seed from each of three pods was extracted with extraction solvent

Table 2. V-C content of faba bean with 2% TCA extract.

Sample type	Fresh seed weight (%)	Total absorbance at 274 nm (%)		
Immature seed- CDC Fatima (weight 0.5±0.07 g, mortar pestle extract)		**Convicine (C)**	**Vicine (V)**	**V+C**
Seed coat	31	17±2	69±3	86±4
Cotyledon	67	17±2	73±3	86±4
Seed radicle	2.5	4±0.5	5±0.5	9±0.5
Green pod	357	none	none	none
Mature seed flour, sonicated	**Total absorbance at 274 nm, relative to CDC Fatima**			
CDC Fatima	1	17±3	67±4	84±5
Snowbird	1	15±3	71±3	86±4
Disco	0.33	18±4	47±2	65±3
Protein concentrate	2.36	12±3	59±3	71±3
Immature green seed (weight (gram), mortar pestle extract)	**Total absorbance at 274 nm, relative to CDC Fatima**			
CDC Fatima (0.45±0.12)	1	26±3	56±3	83±3
Snowbird (0.70±0.26)	1	16±3	67±2	86±6
Disco (0.47±0.1)	0.5	-	-	-

(0.4 ml, 2% TCA/100 mg), as described in the dehulled mature seed sample analysis, by mortar and pestle disruption. Immature seed sections including cotyledon, seed coat, radical and pod were also extracted as in the whole immature seeds in the triplicate sample set. All sample extracts were suspended and then centrifuged (12,000 g for 15 min). The V-C percentage (% V+C) in all samples was determined by comparing the V and C peak areas to the whole run area in chromatograms obtained from the HPLC analysis described above; data are presented in Table 2. Identification of V and C peaks on chromatograms was determined by an HPLC run of analytical standards. A typical HPLC chromatogram of V and C in immature seed tissue is shown in Figure 1.

CDC Fatima V-C quantification

CDC Fatima was used as a control sample and its V-C level was determined in dehulled ground mature seeds by duplicate sample analysis. The 100 mg samples were consecutively extracted three times with 2% TCA in a sonicator and centrifuged; consecutive extracts were combined. Another set of duplicate samples was extracted from the same source of CDC Fatima by soaking two seed cotyledons in 30 ml of hot water for 3.5 h, then adding 100 ul HCl followed by centrifugation (12,000 g for 15 min) (Gutierrez et al., 2006). Analytical standards (V and C) were also prepared in the same buffer (2% TCA) and read by UV assay. Extinction coefficients (ε) of both V and C were taken as 16.400 at 274 nm (Merck Index, 2006) and Beer's law (Absorbance = ε L c) was applied to determine the concentration of standard solutions. The amount of standard solutions injected on HPLC corresponding peak areas was used in quantification of V+C in HPLC runs and extracts.

CDC Fatima buffer tests to maximize V-C extraction

CDC Fatima immature seeds (~0.45 g) were extracted in triplicate with mortar and pestle as mentioned above, with the aqueous solutions including acetone : water (70:30 ratio), 2% TCA, water, 0.01 N NaOH, or ethanol : water (70:30; 0.6 ml). Supernatants were used immediately for the 220-300 nm (UV spectrum) data collection by UV plate reader (FLUOStar Omega). Mean data were used in plotting wavelength versus absorbance (OD) (Figure 2A).

Assessing developing seed for V-C accumulation

The V+C level of CDC Fatima developing seed (Figure 6A) was determined by UV assay (274 nm absorbance), and samples were extracted as in immature seeds as earlier mentioned. Mean OD data ± standard deviation (SD) versus seed moisture levels as seed stage determinant was plotted (Figure 2B). Moisture level versus wet weight of Fatima immature seed was assayed, and the related mean was plotted (Figure 2C). The V+C level of Snowbird, Disco and CDC Fatima genotype seeds was determined similarly by UV assay during development (assayed by seed wet weight), and mean ± SD of samples is presented in Figure 3.

Quick extraction of V-C from immature seeds

Single seeds from three pods split into two cotyledons were sonicated for 5 min in microcentrifuge tubes, each containing a single seed, or were shaken (200 rpm) at room temperature for 60 min in 2% TCA in 96 well plate samples. Samples were then centrifuged at 4000 g. All quick extractions were processed by UV plate reader, which was set to 220-300 nm (FLUOStar Omega). Sample means of 274 nm absorbance were used for plotting minutes versus absorbance (OD274; Figure 4).

Titanium tetrachloride assay

Greenhouse-grown developing immature seeds (~82% moisture

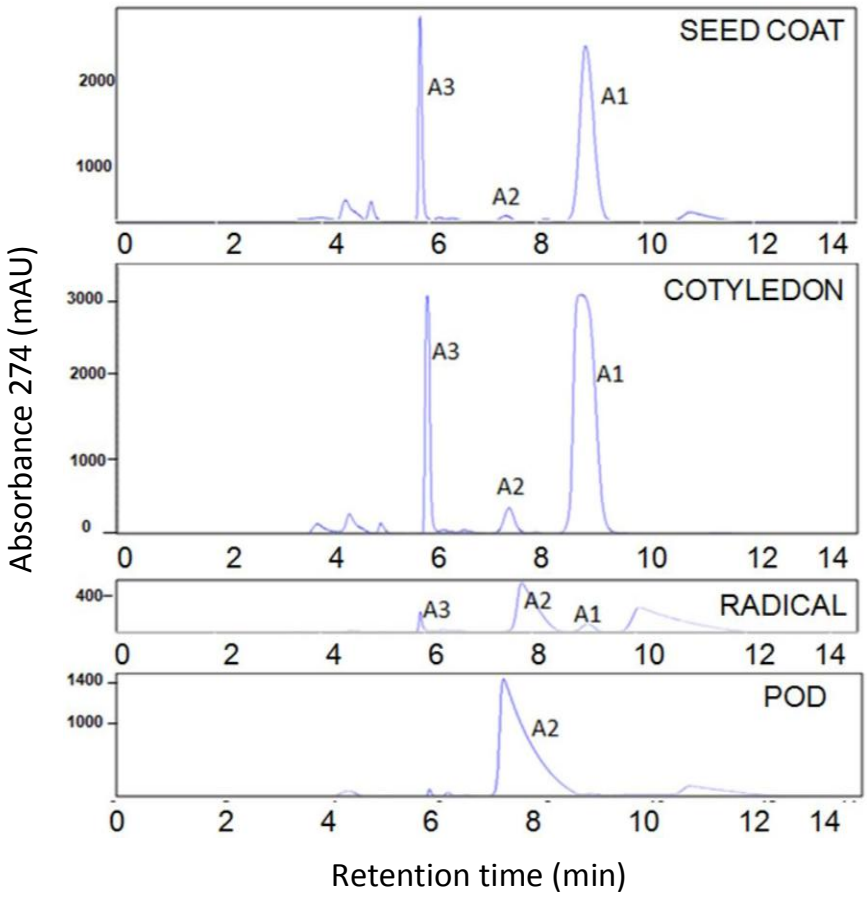

Figure 1. Typical HPLC chromatograms of vicine and convicine from 100 mg of single seed coat, cotyledon, radicle and pod tissue of CDC Fatima (0.5±0.07 g seed fresh weight) extract with 400 µl of 2% TCA. Eluents: Water. Flow: 0.8 ul/min. Injection: 80 ul/min of 1/10 dilution of extract. Absorbance 274 nm versus retention time for vicine (A1), unknown (A2), convicine (A3).

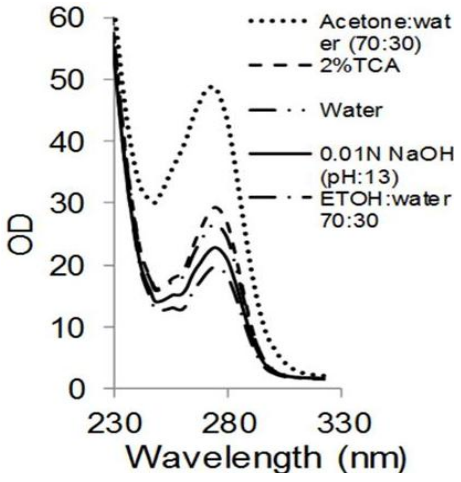

Figure 2A. Optical density (OD) in relation to 220-300 nm wavelength of CDC Fatima immature seed extracted with five solvents.

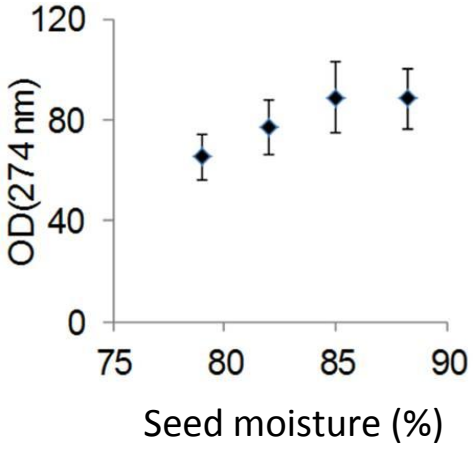

Figure 2B. Wavelength 274 nm in relation to % seed moisture of CDC Fatima immature seed extracted with 2% trichloroacetic acid (TCA).

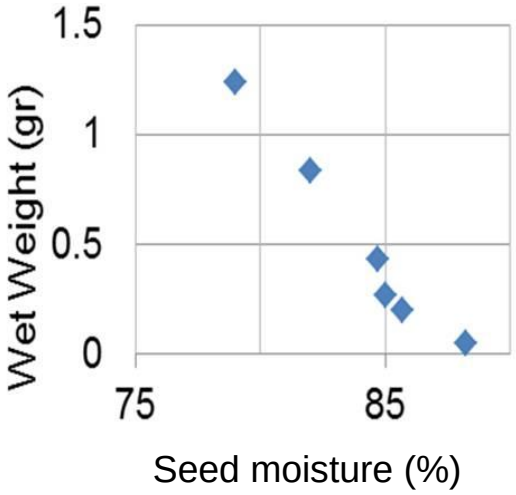

Figure 2C. Wet weight in relation to % seed moisture of CDC Fatima immature seed extracted with 2% trichloroacetic acid (TCA).

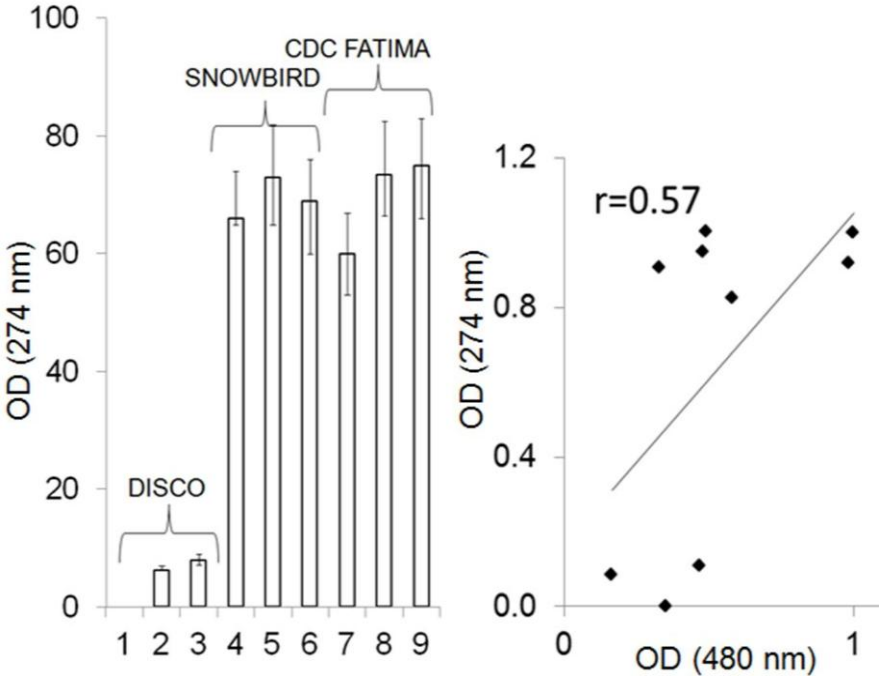

Figure 3. UV and $TICl_4$ assay in V-C detection from Disco, Snowbird, and CDC CDC Fatima green seeds. A: Disco (1-3), Snowbird (4-6), CDC Fatima (7-9) immature seed development and corresponding UV assay (274 nm) profile. Weight of seeds: 1, 4, 7=0.1±0.03; 2, 5, 8=0.2±0.05; 3, 6, 9=0.4±0.07. 6. B). B. shows correlation of UV (274 nm) and $TiCl_4$ assay (480 nm) in green seed samples from faba bean varieties Disco, Snowbird and CDC Fatima. Expressions of ODs are relative to CDC Fatima seed (0.5 g).

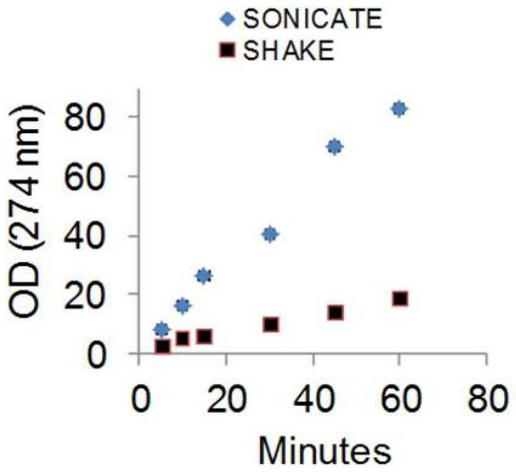

Figure 4. Wavelength 274 nm in relation to extraction time of CDC Fatima immature seed extracted with 2% trichloroacetic acid (TCA). Seed shaking and sonication extraction time in quick extraction process.

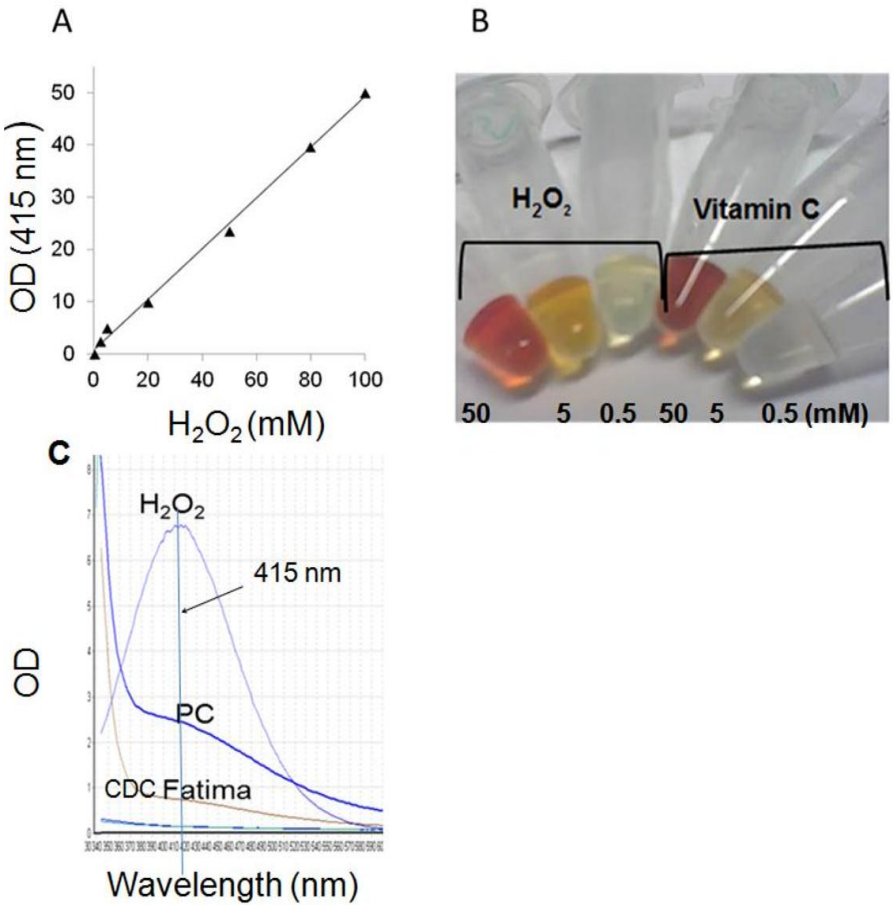

Figure 5. A) Standard curve (absorption plot) for Ti-peroxy complex ($TiCl_4$+ H_2O_2). Absorbance (415 nm) versus H_2O_2 concentration. B) Ti-peroxy and ascorbate color scale. H_2O_2 and vitamin C concentrations (left to right): 50, 5 and 0.5 mM. C) PC, CDC Fatima, H_2O_2 indicate faba seed protein concentrate, CDC Fatima flour and Ti-peroxy complex λ_{max}: 415 nm, respectively.

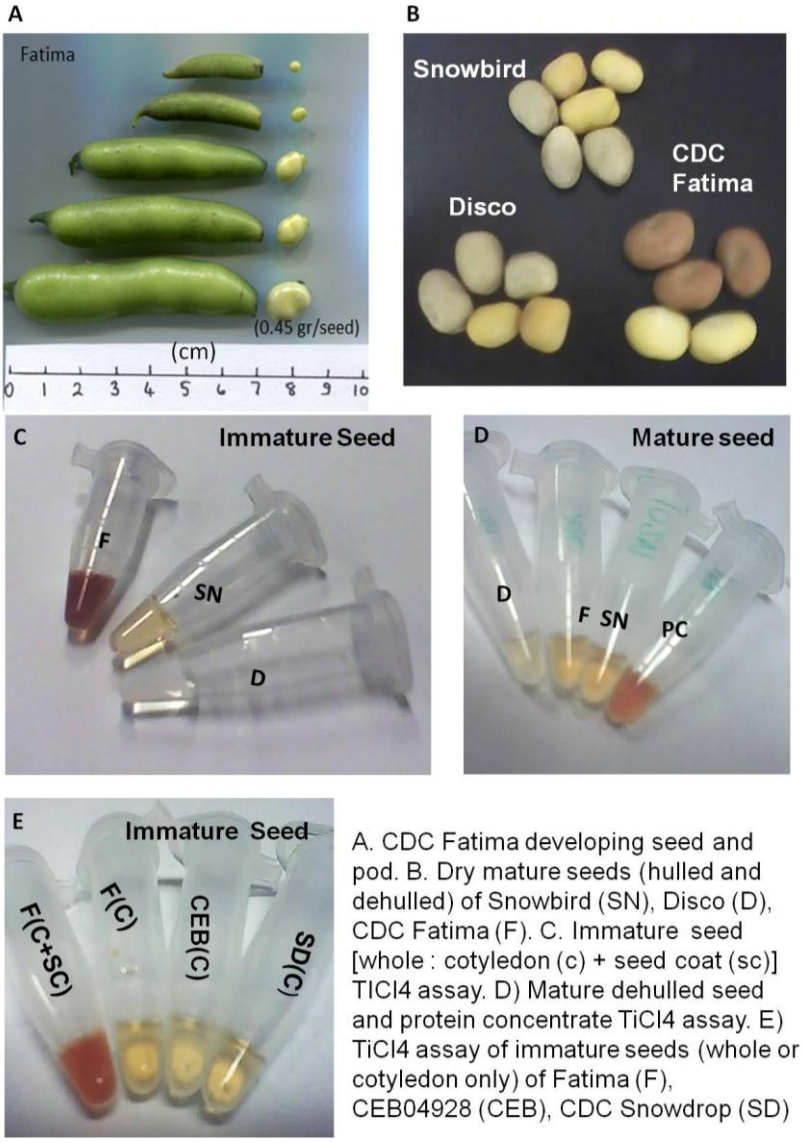

Figure 6. Faba bean seeds and $TiCl_4$ assay color formation.

and 0.58 ± 0.13 g) that reached maximum levels of V+C according to the earlier assay (Figures 2B, C and 3), and ground, mature, dehulled seed samples (100 mg) were extracted in duplicate in 2% TCA, as mentioned in the percentage V-C analysis of mature and immature seeds, then TiCl4 assay was applied to precentrifuged aqueous solutions. Data are presented in Figures 5C, 6C, D and E. All color assays were done twice using the extracts. A 15 % titanium tetrachloride (TiCl4) in concentrated HCl (15 ml of $TiCl_4$ in 85 ml of 20% HCl) was prepared in an ice bath in a fume hood and by adding $TiCl_4$ (99%) into 20% HCl slowly, drop by drop. $TiCl_4$ reaction was measured with minor modifications of the rapid colorimetric method (Kim et al., 1982) by adding 50 µl $TiCl_4$ (15%) and 50 µl of extract from mature or green seeds and then heating the reaction at 80°C for 5 min since application of Kim's method had not formed any color of the indicator product [Ti-aglycone (λmax_{480}) (Kim et al., 1982), Ti-peroxy (λmax_{415}) (Gupta and Eskin 1977), or any major peak that is in the visible spectrum. Initial modifications of the assay (Kim et al., 1982), included directly heating 2% TCA V-C extract (0.6 ml /100 mg and 0.4 ml/100 mg tissue for mature and immature seed, respectively) in HCl (4 N) for 5 min at 80°C and then adding $TiCl_4$ (15%) for color formation. V-C hydrolysis was monitored by spectrophotometer and disappearance of V-C peak was evident in acid hydrolysed samples as similar event was evident at earlier studies. For comparative purpose, theTiCl4 assay was applied to H202 and ascorbic acid dissolved in 2% TCA (Figure 5B).

British research lines V+C from HPLC analysis

Areas of v-c (V+C) obtained from HPLC runs of 159 recombinant inbred lines (RILs) including the single plant progeny parents Melodie (LVC), normal VC parent and the LVC parent Betty (Gutierrez et al., 2006) were used in the calculation of correlation coefficient (**r**=Pearson correlation) with the data obtained from the same sample set (mature faba bean seed ground flour provided by Donal O'Sullivan University of Reading, Berkshire, UK) but extracted and analyzed differently. Our ground mature seed

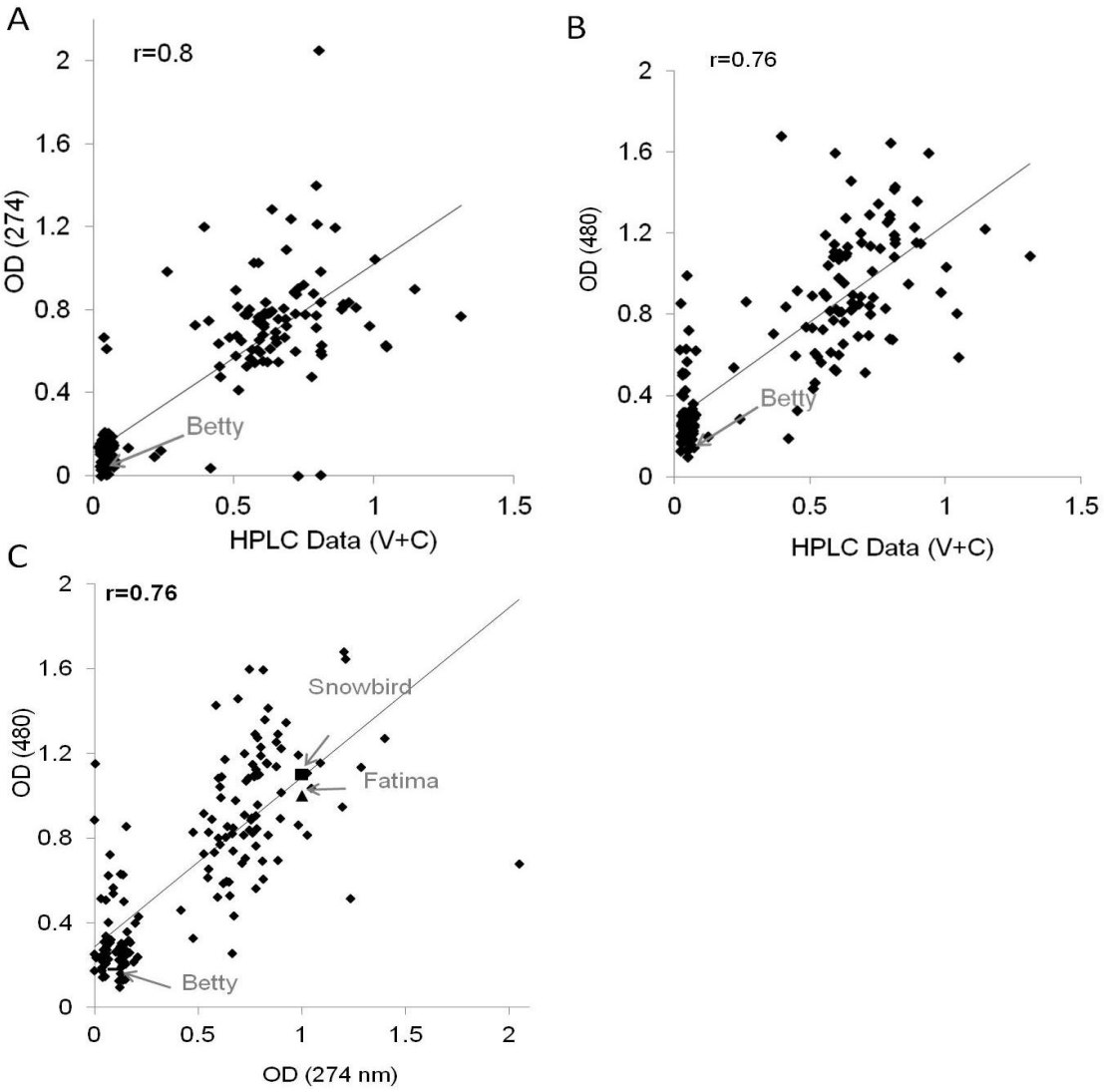

Figure 7. Mature seed flour from 159 Faba bean RILs and V-C negative (Betty, zero V-C) and positive control varieties (Snowbird and CDC Fatima, normal v-c). A) UV assay (OD 274 nm) versus HPLC data (V-C), B) $TICl_4$ assay (OD 480 nm) versus HPLC data (V-C), C) $TiCl_4$ assay versus UV assay.

analysis was done as described in the method section of this study by 2 × 5 min sonication in 2% TCA (100 mg flour/600 µl 2% TCA) of duplicate samples and centrifugation at room temperature. Data were collected at 274 nm wavelength at UV plate reader (FLUOStar Omega) and duplicate sample means were used plotting of HPLC data (V+C) versus absorbance (274 nm) . The r was determined using the Microsoft Excel function Pearson tool. The same extract was used for the TiCl4 assay and data were used in r calculation with the both HPLC and OD (274 nm) data of Britain Research lines (Figure 7).

RESULTS AND DISCUSSION

UV assay in V-C detection and analysis

V-C was detectible in seed coat (maternal tissue), seed radicle and cotyledons of immature seed but not in maternal seedpod tissue of test variety CDC Fatima (Table 2). In earlier studies, trace amounts of V-C were de-tected in shells of young (4-cm pod) and mature pod of low V-C and normal V-C genotypes in addition to seeds (Duc et al., 1989). It has not been quantified, but a significant amount (86% of 274 nm absorbed extract) of V-C was detected in seed coats of immature seeds (Figure 1 and Table 2), though trace amounts of V-C existed in dry mature seed coats (data not shown), which is similar to earlier studies (Burbano et al., 1995). Mature seeds of the low V-C variety Disco (not single-plant progeny) contained about 25% V-C (expressed as the percentage of CDC Fatima total absorbance and of V+C of total area absorbed at 274 nm) of the normal V-C va-rieties CDC Fatima and Snowbird (Table 2), and the V-C

content of protein concentrate of an unknown variety was about twice that of the flour V-C of CDC Fatima and Snowbird.

The V-C content of dry mature seed flour of the CDC Fatima variety was predicted to be 0.92% according to the two extraction methods (2% TCA and hot-water soaking HCl). Similar results were observed by Collier (1976), Hussein et al. (1986) and Khamassi et al. (2013). Khamassi et al. (2013) reported V-C from 0.02 to 1.32% (by weight) of dry seeds in normal commercial varieties and UK research lines, whereas Hussein et al. (1986) reported ~0.83% V-C in Egyptian-grown varieties.

Varieties of aqueous extraction buffers were studied in immature seed analysis. Using alcohol : water (70:30) at high pH in extraction enhanced the UV (274 nm) absorption (Figure 2A) but also reduced the specificity of the TiCl4 assay (data not shown); therefore, 2% TCA was the better option. In earlier studies, acetone: water enhanced the isolation of V-C from mature faba bean protein concentrate (Marquardt et al., 1983). The result from water was not better than the results from 2% TCA. Zhang et al. (2003) extracted vicine with water from bitter melon seed and leaves, and the HPLC run of these extracts (methanol: 0.025 mol/L phosphate [10:90 v/v, pH 3.0]) contained a clear vicine chromatogram in seed tissue but minor impurities in leaf.

V-C was detected in immature seeds as small as 2 mm (0.1 g seed) (Figure 2C) and their presence was at its maximum (85% of total absorbance at 274 nm) when seed moisture was ~85% and wet seed weight was 0.4 g (Figure 2B and C), which is in agreement with Burbano's studies using different genotypes (1995). In variety Snowbird, as compared to CDC Fatima, accumulation of V-C seems to start earlier (Figure 3), and seeds as small as 0.1 g have showed V-C differences between low and normal V-C varieties, as was the case for the Disco, Snowbird and Fatima genotypes (Figure 3).

Seed shaking and sonication for 60 and 5 min, respectively, provided enough signal for UV analysis in immature green seeds (Figure 4) of CDC Fatima and Snowbird (80-85% moisture), and mature seeds contained ~85% V-C of total extract in both types of extraction samples, according to HPLC analysis (data not shown).

$TICl_4$ assay re-evaluation, modification

The standard curve for H_2O_2 levels by $TiCl_4$ is shown in Figure 5A. A linear relationship was evident for the Ti-peroxy complex, which was detectable at 415 nm and by the naked eye at 0.5 mM concentration (Figure 5A and B). The same visible color was observed in 0.35 and 0.5 mM concentrations of vicine and ascorbic acid, respectively. Darker colors were formed in 5 and 50 mM concentrations of both H_2O_2 and vitamin C (Figure 5B), and the corresponding lambda max was observed at 415 (Figure 5C) and 410 nm (not shown), respectively. In contrast to the results of Kim et al. (1982), divicine and isomural at 480 nm were not detectible in purified vicine and protein concentrate (Figure 5C). Pedersen et al. (1988) stated that the chemical cleavage of vicine is not divicine, but actually the deamino divicine later formed the H_2O_2 in a non-air-sealed environment. There is no previous study on color assay (Ti-complex), if the suggested air-sealed environment is necessary, it is not easily doable on a large scale. On a small scale, this was mimicked by creating a medium containing extracted V-C, then adding $TiCl_4$ directly before or after heating for divicine and isomural formation. N_2 bubbled (air-free) V-C extract was tested in our modified $TiCl_4$ assay, but in none of these attempts were we able to detect the proposed Ti complex (Kim et al., 1982).

Our attempt to use $TiCl_4$ and UV assays comparatively in V-C analysis had poor to medium correlation (Pearson r = 0.57) of both analysis methods for immature seeds of CDC Fatima and Snowbird (Figure 3). In another set of immature seeds (weight: 0.43 g) of faba bean research lines, the correlation was very poor (-0.25; data not shown).

$TiCl_4$ analysis of immature seeds showed that CDC Fatima (normal-type flower) had the darkest color seed, which contained a high-tannin seed coat (Figure 6B). Though CDC Fatima and Snowbird contain similar percentages of V-C in immature seeds (Table 2), the color formed in the $TiCl_4$ reaction was much richer and darker for CDC Fatima than for Snowbird (Figure 6C). The white-flowered cultivars, Snowbird, CDC Snowdrop (SD), CEB04928 (CEB) and CDC Fatima, had identical colors by $TiCl_4$ assay when the seed coat of immature seeds of CDC Fatima was removed (Figure 6E). This shows $TiCl_4$ interacts with phenolic compounds and possibly also with tannins in the seed coats of fresh cotyledons of CDC Fatima. Baginsky et al. (2013) reported significant amounts of phenolic compounds (anthocyanins, flavonoids and other compounds structurally similar to V-C) in immature faba bean seeds. Our analytical studies (not published) done later showed significant amounts of phenolic compounds (for example, cathechin) in normal-type flowers of *V. faba* (Ferhatoglu et al., unpublished).

The $TiCl_4$ assay has been suggested for analyzing V-C in mature seeds (Kim et al., 1982). In mature seed analysis, we observed a color difference between the low V-C, low phenolic, zero-tannin variety Disco (zt-2 white flower genotype) (Khamassi et al., 2013; Pedersen et al., 1988) and the normal V-C, normal phenolic, tannin-containing seed varieties Snowbird (zt-1 white flower gene) and CDC Fatima (normal flower color) (Figure 6D) (Pedersen et al., 1988). The protein concentrate had the most intense color using the $TiCl_4$ assays, as expected by the corresponding high V-C values (Table 2) and possible high tannins and phenolic compounds (Figure 6D).

A single 2% TCA extract from a mature CDC Fatima seed contains enough V-C for visual determination of the

Ti-aglycone complex, which should carry a color lighter than the 5 mM Ti-peroxy and Ti-Vit C complex (Figures 5B and 6D) because the Ti-alyocone complex concentration was predicted to be about 1.6 mM in colorimetric assays from corresponding UV quantitation (data not shown).

In earlier studies, $TiCl_4$ assay had not been applied on a large scale to genotypically different faba bean lines or immature seeds. In this study, mature seed flour of faba bean Melodie × Betty RIL lines was analyzed for V-C using the modified $TiCl_4$ assay and two extraction methods (solvent and method wise) and HPLC chromatography. When both types of data were plotted (Figure 7), fair levels of correlation (r ~ 0.76) existed between $TiCl_4$ data (OD480) and HPLC data (Figure 7B). This means that the $TiCl_4$ assay without significant prepurification may be applied in detecting V+C in mature seeds, but outliers do exist, as shown in Figure 7B. A slightly better correlation between UV assay and HPLC data was evident (Figure 7A).

This study, especially given a choice of Disco (white-flowered, low V-C, low phenolic, zero tannin), CDC Fatima (white with black wing spots, normal V-C, high phenolic, tannin), or Snowbird (white-flowered, normal V-C, high phenolic, tannin), appears to provide enough information on the interaction between unspecific compounds and $TiCl_4$ in addition to V-C in immature seeds. The $TiCl_4$ assay is simple and can be evaluated by the naked eye (colorimetrical) at ~1% w/w V-C seed content with 600 µl 2% TCA/0.1 g extract, but specificity is low, possibly because of the complexity of the plant metabolic system in immature seeds. The $TiCl_4$ assay can and should be used together with the UV assay for selecting low V-C but high phenolic/tannin (nutritionally valuable, rich in antioxidants) genotypes in cotyledons and/or whole seeds that are immature or mature. Oomah et al. (2011) determined a very high correlation between tannins and phenolic compounds in screened faba bean varieties. They measured higher levels of phenolic compounds and tannin in Snowbird relative to CDC Fatima, similar to the results observed in our $TiCl_4$ assay (480 nm absorbance; Figure 7). Here, we were able to identify normal and white-colored flower varieties by simple visualization of $TiCl_4$ assay and phenolic differences of Snowbird and CDC Fatima by $TiCl_4$ assay at 480 nm absorbance.

Removal of the unwanted metabolites with neutral alumina prior to $TiCl_4$ assay, as previously suggested (Kim et al., 1982), will be incomplete, as this was observed in the analysis of sinapic acid samples (Fenwick, 1981) and in our attempts in V-C analysis in protein concentrate, even though the extract was passed through a neutral alumina column (Supelclean LC-Alumina, Supelco, PA), according to Kim et al. (1982). The extraction of faba bean flour V+C with simple 2% TCA and its analysis by UV assay (274 nm absorbance) and data correlation (by Pearson correlation) with the HPLC quantified V-C (extracted differently) from the same sample set (Figure 7) has shown that UV assay (r = 0.80 for OD 274 nm versus V+C area from HPLC) can be applied directly to the high-throughput platform for the selection of low V-C seeds (Figure 7). Nucleosides, nucleotides and peptides absorbed by UV must be at a very low level in the freshly prepared 2% TCA extract, which was prepared at room temperature because the correlation is very high between both types of UV-based V-C analysis with or without HPLC. The solvent/extract ratio is low; therefore, 2% TCA should precipitate most of the interference compounds. The $TiCl_4$ assay with the dry flour extract had poorer correlation (r = 0.76) relative to the UV assay (Figure 7). The 2% TCA extraction makes V-C extraction and detection simple for low V-C faba bean. There is no report on V-C degradation by 2% TCA (0.125 N). HCl, whose acid strength is about the same as that of TCA at 0.7 N, is known to hydrolyze V-C (Marquardt et al., 1983).

Supporting this study, our most recent low-cost low–V-C screening studies in faba bean breeding programs during seed development suggest that both $TiCl_4$ and UV assay on 2% TCA extracts can be applied successfully to large-scale sample sets in selecting low V-C and low to high tannin/phenolic seed genotypes (Ferhatoglu et al., unpublished).

Appendices

Supplementary data on UV (280 nm) and mass analysis of vicine and convicine by HPLC mass spectrophotometer are shown in supplementary Figure 1. Vicine (ppt#2) is pure and convicine (Xtal yellow ppt#1) contains some minor impurities.

Conflict of interests

The authors have not declared any conflict of interest.

ACKNOWLEDGEMENTS

The authors greatly acknowledge Mahla Mirali for technical assistance on MS analysis of V-C and funding source SASKPULSE for supporting this research.

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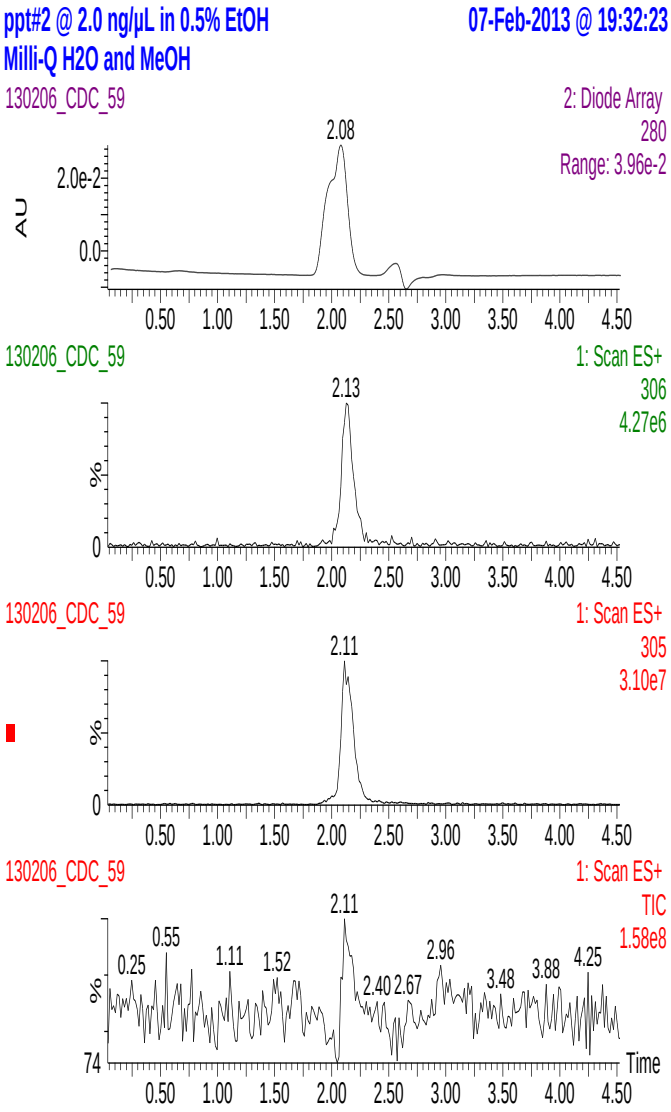
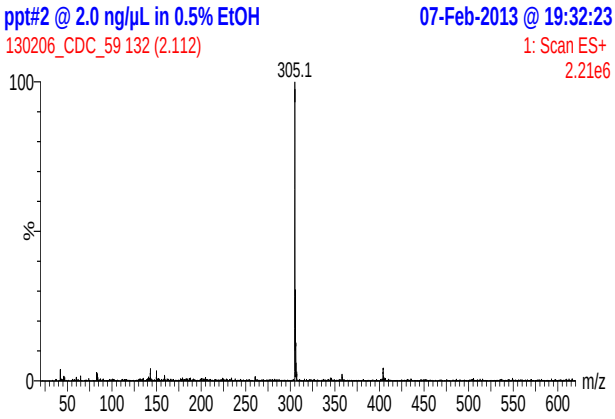
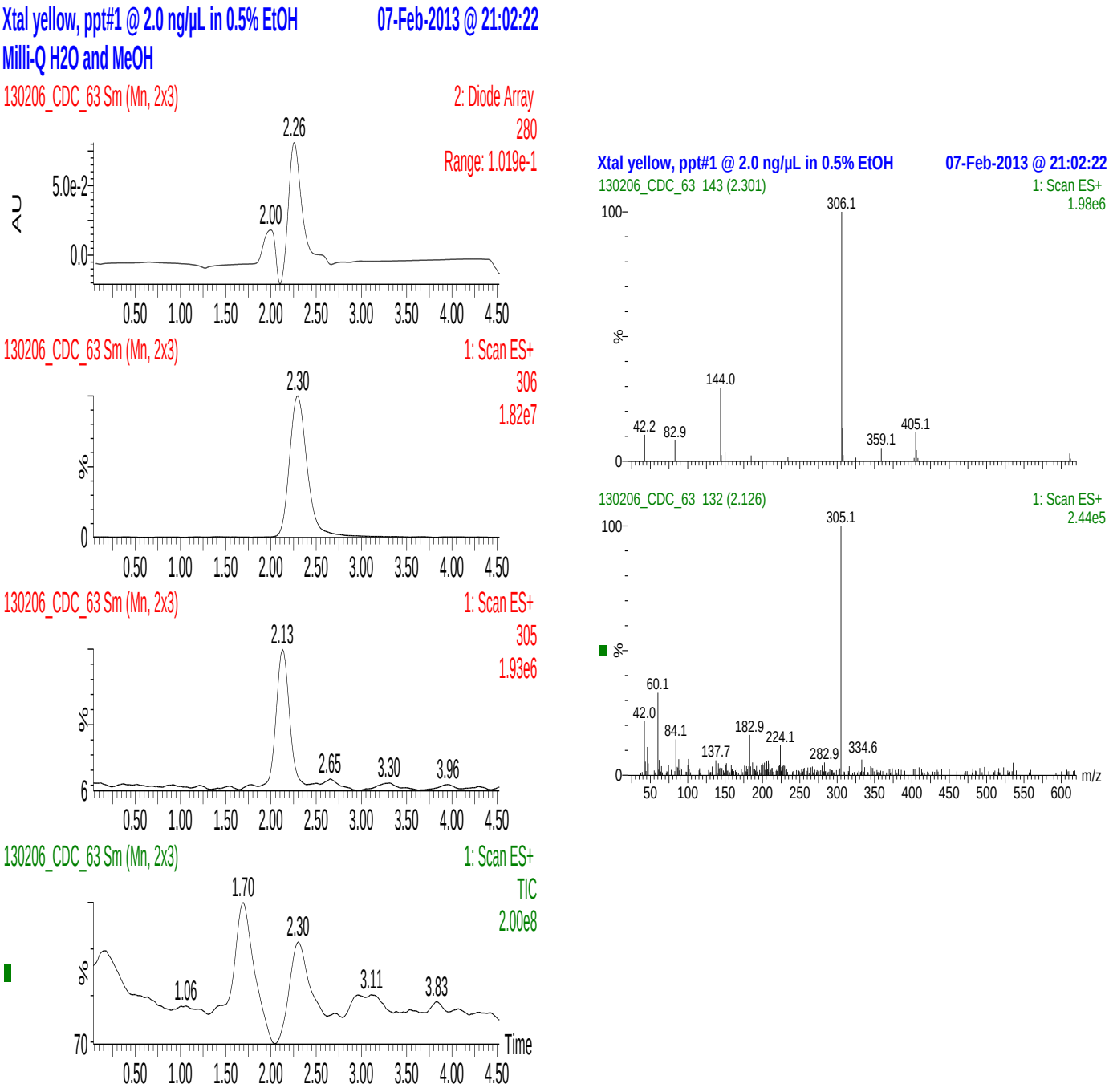




Supplementary Figure 1A. UV (280 nm) and mass analysis of vicine by HPLC mass spectrophotometer.



Supplementary Figure 1B. UV (280 nm) and mass analysis of convicine by HPLC mass spectrophotometer.