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Toward a high-throughput method for determining vicine and convicine levels in faba bean seeds using flow injection analysis combined with tandem mass spectrometry

Randy W. Purves*, Hamid Khazaei, Albert Vandenberg

Department of Plant Sciences, University of Saskatchewan, 51 Campus Drive, Saskatoon, SK S7N 5A8, Canada

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ABSTRACT

Although faba bean provides environmental and health benefits, vicine and convicine (v-c) limit its use as a source of vegetable protein. Crop improvement efforts to minimize v-c concentration require low-cost, rapid screening methods to distinguish between high and low v-c genotypes to accelerate development of new cultivars and to detect out-crossing events. To assist crop breeders, we developed a unique and rapid screening method that uses a 60 s instrumental analysis step to accurately distinguish between high and low v-c genotypes. The method involves flow injection analysis (FIA) coupled with tandem mass spectrometry (i.e., selective reaction monitoring, SRM). Using seeds with known v-c levels as calibrants, measured v-c levels were comparable with liquid chromatography (LC)-SRM results and the method was used to screen 370 faba bean genotypes. Widespread use of FIA-SRM will accelerate breeding of low v-c faba bean, thereby alleviating concerns about anti-nutritional effects of v-c in this crop.

1. Introduction

Faba bean (*Vicia faba* L.) seeds are a good source of protein, dietary fiber, and other nutritional characteristics (Duc, Marget, Esnault, Le Guen, & Bastianelli, 1999; Crépon et al., 2010). However, consumption of faba bean has been historically limited due to anti-nutritional factors in its seeds, such as vicine and convicine (v-c), that can cause favism in humans deficient in the enzyme glucose-6-phosphate dehydrogenase (Cappellini & Fiorelli, 2008). The ability to rapidly and accurately measure v-c concentration and determine whether a genotype is relatively low or high in v-c content is extremely important, particularly as a tool to accelerate breeding programs targeting removal of v-c in the crop. Such a method would also help to ensure food safety since bees are attracted to faba bean flowers and cross-pollination of low v-c plants with high v-c can re-introduce high v-c levels into low v-c cultivars (Bond & Pope, 1974; Nayak et al., 2015). An ideal method for v-c detection should be reliable, cost-efficient, and can quickly screen a large number of genotypes.

Different methods to detect v-c concentrations have been developed over several decades. Techniques based on spectrophotometric/colorimetric methods (e.g., Collier, 1976; Kim, Hoehn, Eskin & Ismail, 1982; Sixdenier, Cassecuelle, Guillaumin & Duc, 1996; Khazaei et al., 2015) enable a rapid analysis; however, these methods suffer from a lack of specificity. Liquid chromatography (LC) methods with ultraviolet (UV) detection based on the work of Marquardt & Frohlich (1981) and Quemener (1988) have been reported but are time-consuming and not conducive to rapid analysis (Khamassi et al., 2013; Khazaei et al., 2015; Pulkkinen et al., 2015). In addition, the use of LC-UV for quantifying convicine in low v-c genotypes has been problematic because convicine has often been reported as not detected (e.g., Pulkkinen et al., 2015). We recently reported two methods based on hydrophilic interaction liquid chromatography (HILIC) that were capable of quantifying v-c in both low and high v-c faba bean cultivars using a 10 min gradient (Purves, Khazaei, & Vandenberg, 2018). In particular, mass spectrometry (MS)-based detection was much more sensitive and selective than the use of a DAD (diode array detector, UV–vis detection). With DAD detection, the chemical background complicated the analysis for low concentrations, whereas with tandem MS, the chemical background was minimized. Tandem MS, and in particular selective reaction monitoring (SRM) on a triple quadrupole instrument, is a very selective technique as three mass analyzers are used (Yost & Enke, 1979). The first enables only the m/z value of the ion of interest to pass through (first quadrupole), the second fragments this ion using a specific collision energy (second quadrupole), and the third enables only the m/z

* Corresponding author.
E-mail address: randy.purves@usask.ca (R.W. Purves).

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value of a specific fragment ion to reach the detector (third quadrupole).

The very high selectivity of SRM can be exploited to develop a rapid method for analyzing v-c in faba bean seeds. Instead of injecting samples onto a column for analyte separation before MS detection, the column could be by-passed and sample introduced straight into the mass spectrometer. A typical limitation of SRM, known as ion suppression (Tang, Page & Smith, 2004), can arise because of the vast complexity of biological samples. In addition, lower level species can often suffer from endogenous or isobaric interferences (and is why SRM is commonly used with LC). However, when analyte concentrations are relatively large (such as for vicine and convicine), sample dilution and direct analysis using flow injection analysis (FIA) offers a feasible alternative that could result in complete analysis in less than a minute and therefore would be an ideal choice for a rapid analysis method.

Our long-term goal is to develop a high-throughput v-c detection method that minimizes sample preparation time, uses small amounts of plant material, and has an instrumental analysis step of less than a minute to enable screening of a large number of samples in the shortest time possible. Our previous publication details optimization of sample preparation, moving toward a workflow that is more amenable to automation and SRM analysis (Purves et al., 2018). The main objective of this study was to develop a rapid analysis based on FIA-SRM; to assess this method, we refer to results from our previous publication for faba bean genotypes analyzed using LC-UV and LC-SRM (Purves et al., 2018).

2. Experimental

2.1. Reagents and standards

Vicine and uridine standards were purchased from Sigma (St. Louis, MO, USA). Commercial convicine was not available and therefore convicine was extracted from faba bean flour using a previously described procedure (Purves et al., 2018) based on the work of Marquardt, Muduuli, and Fröhlich (1983) and Pulkkinen et al. (2015). The purity of the compounds was confirmed using an HILIC column with DAD and SRM detection and also by using ^{1}H and ^{13}C NMR as described previously (Purves et al., 2018). The convicine purity was estimated at 99.3%. Formic acid, acetonitrile, and acetone were purchased from Thermo Fisher Scientific (Waltham, MA, USA), and purified water was obtained from a Millipore Milli-Q system (Milford, MA, USA).

2.2. Plant material

Seeds from three sets of faba bean genotypes were analyzed. Set A, consisting of 13 faba bean genotypes with known v-c concentrations, was used for method optimization; five are known to be low in v-c and eight are known to be high in v-c. Two larger sets of samples (370 total) were then used to test the method. Set B consisted of 50 genotypes grown under controlled conditions in 2016 that were part of a larger set of recombinant inbred lines that segregate genetically for high and low v-c concentration (Khazaei et al., 2015). Set C included 320 pre-breeding lines from the faba bean breeding program at the University of Saskatchewan (UofS) grown under field conditions in Saskatchewan, Canada in 2016. In set C, low v-c genotypes were used as parents, but no effort was made to select for or against the low v-c phenotype. Details on the genotypes used in this study are presented in Supplementary Table 1.

2.3. Sample preparation and extraction methods

2.3.1. Final sample preparation method

Seeds of all faba bean genotypes were ground to a fine powder using a Cyclone Sample mill (UDY Corporation, Model 3010-030, Fort Collins, Colorado, USA). Powdered samples (30 ± 0.1 mg) were placed into 2-mL Sarstedt micro tubes (Numbrecht, Germany). For method optimization (set A), five biological replicates were used for each cultivar (results compared with LC-SRM). For the study with 50 genotypes (set B), three biological replicates were used. The final high-throughput field study (set C) used only one replicate.

Two different extraction methods were employed based on our prior results (Purves et al., 2018). The first method involved a two-step extraction. First, 500 μL of extraction solvent (70:30 acetone:water) containing 800 μM of the uridine internal standard was added to each tube. After vortexing for 5–10 s (Thermo Vortex Maxi Mix II), the samples were placed into an Eppendorf Thermo mixer R for 20 min at 1400 rpm. Samples were centrifuged using a Thermo Legend Micro 17 for 5 min at 13.8*g* (12,000 rpm) and a 400 μL aliquot of the supernatant was pipetted into an Eppendorf tube (extract). A second extraction from the remaining pellet/ supernatant in the Sarstedt microtube was carried out by adding 500 μL of extraction solvent (70:30 acetone:water) with no internal standard. After vortexing, mixing, and centrifuging as described above, 400 μL from the second extraction was combined with the 400 μL from the first extraction to give 800 μL of extract. After vortexing the combined extract, a 20 μL aliquot was pipetted into an HPLC vial containing 980 μL of 90:10 acetonitrile:water (50× dilution) and vortexed. This vial was used for most FIA-SRM analyses; however, more dilute samples (500× and 1000× dilutions) were prepared by pipetting a 50 (or 100) μL aliquot of the 50× dilution solution into a vial containing 950 (or 900) μL of 90:10 acetonitrile:water.

The second method involved a one-step extraction and used 1 mL of an extraction solvent (1% formic acid) containing 400 μM of the uridine internal standard. After vortexing for 5–10 s (Thermo Vortex Maxi Mix II), the samples were placed into an Eppendorf Thermo mixer R for 15 min at 1400 rpm. Samples were then centrifuged using a Thermo Legend Micro 17 for 5 min at 13.8*g* and a 20 μL aliquot of the supernatant pipetted directly into a vial containing 980 μL of 90:10 acetonitrile:water to give a total dilution of 50×.

2.4. Analytical methods

An FIA method was employed to quantify the vicine and convicine in the faba bean samples. The feasibility of the method was tested by comparing with results obtained from faba bean genotypes using LC-SRM for the same cultivars (Purves et al., 2018). For LC-SRM, an Agilent 1290 Infinity UPLC (Santa Clara, CA, USA) equipped with a G4226A autosampler, a G4220 A binary pump, and a G1316 TCC was used along with an Agilent Poroshell 1290 HILIC (2.1 × 100 mm, 2.7 μm) column, as described previously (Purves et al., 2018). A Thermo Fisher TSQ Vantage (San Jose, CA, USA) was used for all mass spectrometric analyses (SRM).

2.4.1. LC-SRM using a HILIC column

The SRM transitions used in the LC-SRM analysis were the same as reported previously (Purves et al., 2018), specifically 303 → 141 (collision energy, CE = 19 eV) for vicine, 304 → 141 (CE = 23 eV) for convicine, and 243 → 200 (CE = 13 eV) for uridine.

2.4.2. FIA-MS analysis

For FIA analyses, ~1 m of polyetheretherketone (PEEK) tubing was used to bypass the G1316 TCC and send the sample directly from the outlet of the sample loop to the inlet of the mass spectrometer. In line with the PEEK tubing was a back-pressure regulator (Western Analytical Products, Boise, ID, USA), operated at ~800 psi, used to dampen fluctuations in pressure and ensure signal stability. To analyze the samples (prepared in ~90:10 acetonitrile:water so as to facilitate a comparison with LC-SRM) and ensure a rapid method, an isocratic