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Determination of vicine and convicine from faba bean with an optimized high-performance liquid chromatographic method

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ABSTRACT

A robust method was implemented to analyze vicine and convicine from faba bean and used to study variation among cultivars and growing years. Due to the different solubilities and stabilities of vicine and convicine, two extraction methods were compared, and conditions for high-performance liquid chromatography (HPLC) were optimized. The chosen method consisted of extraction with 7% perchloric acid with uridine as an internal standard, separation with a C18 column using 0.1% formic acid in water as the mobile phase, and UV detection at 273 nm. The response factors of vicine and convicine relative to uridine were determined using purchased vicine and convicine that was isolated from faba bean fractions by a preparative liquid chromatographic (LC) system coupled with mass spectrometry (MS). The established factors for vicine and convicine were 1.53 and 1.63, respectively. Their similarity makes it possible to use vicine as a standard for quantification of convicine in the absence of a convicine standard and vice versa. Among 10 cultivars grown in the same year and location, vicine and convicine concentrations ranged from 5.2–7.6 mg/g dw and 2.1–3.6 mg/g dw, respectively. Variation among growing years was relatively small but statistically significant ($P < 0.05$).

1. Introduction

Due to their high protein content, grain legumes are valuable for use in feeds and foods. Currently, Europe depends on imported soybean for 70% of the plant protein required to supplement animal feeds (Bues et al., 2013) and Finland is even more heavily reliant on imports (Stoddard, Hovinen, Kontturi, Lindström, & Nykänen, 2009). Soybean components are widely used as food ingredients. Replacement of imported soybean products with domestically produced legume proteins would increase self-sufficiency in plant protein while improving the sustainability of European cropping systems (Bues et al., 2013). At present, less than 2% of the arable land in the European Union is used for grain legumes, in contrast to more than 8% in Canada and Australia (Bues et al., 2013). Faba bean (*Vicia faba* L.) is generally higher in protein than pea (*Pisum sativum* L.) and other cool-season grain legumes, and in Finland it is usually higher-yielding than pea, so it has the highest protein yield of any legume in this country (Lizarazo et al., 2014; Stoddard et al., 2009).

Legumes have evolved a wide range of antinutritional factors to protect themselves, but these compounds restrict the use of legumes in food systems and have health implications for humans. In faba bean, the most limiting antinutritional factors are the pyrimidine glucosides vicine [2,6-diamino-4,5-dihydroxypyrimidine-5-(β-D-glucopyranoside)] and convicine [2,4,5-trihydroxy-6-aminopyrimidine-5-(β-D glucopyranoside)]. They are considered typical of *Vicia* species, especially the faba bean. The glucosides are hydrolyzed in the digestive tract, producing the highly reactive aglycones divicine and isouramil (McMillan, Schey, Meier, & Jollow, 1993). Hydrolysis can also take place when the seeds or flour are treated with strong acids or enzymes such as beta-glucosidase released by microbes used in fermentation (Marquardt, Frohlich, & Arbid, 1989; McKay, 1992). The aglycones cause oxidative stress in human red blood cells that are deficient in glucose-6-phosphate dehydrogenase (G6PD) by oxidizing glutathione (Chevion, Navok, Glaser, & Mager, 1982) that is normally restored to its reduced biologically active form by G6PD in the pentose phosphate pathway. In G6PD-deficient red blood cells, uncontrolled oxidation leads to an acute hemolytic anemia called favism. Different variants of G6PD deficiency exist in human populations originating from certain parts of Africa, Asia, the Mediterranean basin, and the Middle East (Cappelini & Fiorelli, 2008). In addition, among livestock species, laying hens are especially sensitive to vicine and convicine (Muduuli, Marquardt, & Guenter, 1981).

Cultivars with one-tenth the wild-type vicine and convicine concentration have been bred (Duc, Crepon, Marget, & Muel, 2004) but

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reduction to zero has not yet been achieved, and the low-vicine-convicine gene, *vc-*, has not been bred into commercially available cultivars for the Boreal region. Hence, the potential for processing methods to enable the removal of vicine and convicine should be investigated. For this to succeed, reliable methods are needed to measure vicine and convicine concentrations in both raw materials and processed products.

High performance liquid chromatography (HPLC) is widely used to analyze vicine and convicine (Marquardt & Frohlich, 1981; Quemener, 1988). In the method of Marquardt and Frohlich (1981), they are extracted with perchloric acid and separated by reversed phase HPLC using ammonium phosphate buffer (pH 2) as a mobile phase and UV detection at 280 nm. In the alternative approach, to improve stability of the analytes, water was used as both the extraction medium and the mobile phase. Detection was at 273 nm (Quemener, 1988).

Quantification of vicine and convicine is problematic in the HPLC methods because vicine standards are expensive when available and convicine standards are not available. Because the two compounds have similar chemical structures, their isolation as pure compounds from faba bean extract requires efficient separation techniques. Isolation has been based on unspecific crystallization with further recrystallizations and washings (Bien, Salemnik, Zamir, & Rosenblum, 1968; Marquardt, Muduuli, & Frohlich, 1983) or preparative LC with UV detection (Quemener, 1988), but more efficient methods, such as preparative LC with mass spectroscopy (LC-MS), are now available. Determination of the relative responses of vicine and convicine to a commercially available internal standard, such as uridine, would decrease the need for vicine and convicine standards in routine analysis. Response factors were determined in an earlier study (Quemener, 1988), but details of the procedure were not well documented, so reliable factors have yet to be established.

The main aims of this study were to implement a robust and reliable HPLC method for vicine and convicine analysis using uridine as an internal standard, to isolate pure convicine by preparative LC–MS and to validate the improved method by evaluating the variation in vicine and convicine concentrations among cultivars and growing years. The effects of HPLC parameters on responses of vicine, convicine and uridine were evaluated, and relative response factors were elaborated.

2. Materials and methods

2.1. Standards

Convicine for standard purpose was isolated from a protein-rich fraction prepared from dehulled and milled faba beans (Coda et al., 2015) starting as Marquardt et al. (1983). A sample of 250 g of the fraction was mixed with 575 ml of MilliQ-water from the MilliQ Plus system (0.22 μm, Millipore Corporation, Bedford, MA, USA) and 45 ml of 3 N NaOH. The mixture was homogenized for 5 min by a blender (Bamix, Switzerland), then 1250 ml of acetone was added and the solution was further mixed with a spoon for 15 min. The mixture was left to stand for 60 min at room temperature, after which the solution was collected, filtered through a Buchner funnel and concentrated in a rotavapor with a silicone antifoam agent (Serva electrophoresis GmbH, Germany) from 800 ml to 25 ml. The pH of the concentrate was adjusted with 8 N HCl to 7.2 to precipitate vicine and convicine over 72 h. In the method of Marquardt et al. (1983), vicine and convicine were separated from each other and purified by further crystallization. In this study, however, the precipitate was collected and dissolved into ammonia adjusted alkaline water (pH 9) for isolation of pure convicine using automated preparative LC–MS (Waters Corp., Milford, MA, USA).

The preparative LC–MS equipment consisted of a sample manager (Waters 2767), which was coupled with a single quadrupole MS (Waters micromass ZQ) and photodiode array (PDA) (Waters 2996) detectors. Separation was carried out with an Atlantis prepT3 column (10 × 250 mm) with a particle size of 5 μm. Elution was performed with MilliQ-water and acetonitrile, both containing 0.1% formic acid to give pH 2.7. The run started isocratically with formic acid in MilliQ-water for 20 min (separation of the compounds), after which the proportion of acetonitrile with formic acid was raised to 80% (washing of column) in 1 min and stayed there for 10 min. A constant flow of 3.5 ml/min was pumped by a binary gradient module (Waters 2545), and after the column the flow was split to the detectors with an active flow splitter at a 1:100 ratio. MassLynx 4.1 software (Waters) was used for system operation. The volume of the injection was 950 μl. Convicine was collected according to the m/z value of 306. In addition, elution was monitored by UV detection at 273 nm. From 250 g of flour, 15 mg of freeze-dried convicine powder was obtained.

The concentration of isolated convicine was determined spectrophotometrically using molar absorption coefficient 17 400 l mol^{-1} cm^{-1} at 271 nm when measured at pH 1 (Bien et al., 1968). Convicine powder was dissolved in 7% perchloric acid (5 μg/ml) and the absorbance was measured at 271–275 nm in duplicates with a spectrophotometer (Lambda 25 UV/Vis, Perkin Elmer Inc., USA), and absorbance at the obtained maximum wavelength 273 nm was used for calculation. The purity of the convicine was also evaluated with an LC–ESI–MS method with UV detection (Section 2.4).

Vicine standard (≥98%) was purchased from Cfm Oskar Tropitzsch GmbH (Germany) and uridine (≥99%) was purchased from Sigma Aldrich (USA). Their concentrations were checked by spectroscopy using molar absorption coefficient 16 400 l mol^{-1} cm^{-1} at 274 nm (pH 1) for vicine (Bendich & Clements, 1953) and 9800 l mol^{-1} cm^{-1} at 262 nm (pH 7) for uridine (Ploeser & Loring, 1949). Vicine powder was dissolved in 7% perchloric acid (10 μg/ml) and uridine in MilliQ-water (5 μg/ml). In addition, their purity was confirmed by LC–ESI–MS with UV detection (Section 2.4).

2.2. Selection of extraction conditions

Perchloric acid and water extraction were compared using faba bean flour (cultivar 'Kontu' from 2011) as the sample material (Section 2.5). In addition, the extraction efficiency of vicine, convicine and uridine in single, double and triple extraction was compared.

The perchloric acid extraction was modified from the method of Marquardt and Frohlich (1981). Briefly, 0.5 g of faba bean flour was weighed into a 50 ml centrifuge tube (Nalgene, ThermoFisher Scientific, Waltham, MA, USA), and 1 ml of 8 μg/μl uridine was added. In single extraction, 15 ml of 7% perchloric acid (diluted 1:10 from 70% perchloric acid, Merck KGaA, Darmstadt, Germany) was added, and the solution was vortex-mixed for 1 min. After mixing, the extract was centrifuged at 13 000 g for 10 min at 4–8 °C. In double and triple extractions, the solvent volume of 15 ml was divided into two × 7.5 ml and three × 5 ml portions, respectively, and supernatants were combined after centrifugation. An aliquot of the supernatant was filtered through a 0.45 μm filter (Acrodisc Pall, Cornwall, UK) before injection into the HPLC. Water extraction was modified from the method of Quemener (1988). Single, double and triple extractions were carried out similarly to the perchloric acid extraction, except that MilliQ-water was used instead of perchloric acid. After extraction, 60 μl of 1 N HCl was added for protein precipitation, and the tube was vortex-mixed. After centrifugation (13 000 g, 10 min, 4–8 °C), the supernatant was collected and filtered similarly as above.

All extraction experiments were carried out with five replicates, and the extraction behavior of uridine was compared with those of vicine and convicine based on responses as peak areas. Finally, to extract the samples, perchloric acid extraction using three extractions was used.

Each sample was analyzed in triplicate.

2.3. HPLC analysis and quantification

Vicine and convicine were analyzed with an analytical HPLC system consisting of a Waters 2690 Separation Module attached to a diode