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Occurrence of vicine and convicine in faba bean and their removal by hydrolysis

Marjo Pulkkinen

ACADEMIC DISSERTATION

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Abstract

Legumes are a sustainable source of plant protein, and their production could be increased in Europe. The use of faba bean (*Vicia faba* L.) is limited in part due to the presence of the pyrimidine glycosides vicine and convicine. Vicine and convicine, and particularly their aglycones, can cause a form of haemolytic anaemia called favism in individuals who have genetic deficiency in the glucose-6-phosphate dehydrogenase (G6PD) enzyme. Different processing methods have reduced the vicine and convicine contents to varying levels, but the formation of the aglycones have not been studied. Practical processing methods for removal of vicine, convicine and their aglycones are still required.

The main aim of this study was to implement methods for the total elimination of vicine and convicine, with a focus on the aglycones. The compounds of interest were quantified using reversed phase high performance liquid chromatography (RP-HPLC) with ultraviolet (UV) detection against an internal standard uridine. The contents of vicine and convicine were determined in cultivars grown in Finland, and the contents were compared within a selected growing year and among three growing years. Vicine and convicine were hydrolysed in faba bean extracts, in vicine and convicine fractions and in faba bean suspensions by using β-glucosidase to study the formation and stability of the aglycones. Finally, the formation and stability reactions were studied under selected model conditions and in sourdoughs and breads.

The performance of the HPLC method was suitable for the analysis of vicine and convicine. The levels in the studied cultivars varied from 5.2–7.6 mg/g dry matter (DM) and 2.1–3.6 mg/g DM within one growing year for vicine and convicine, respectively. Cultivar comparison showed that the cultivar 'Kontu', the commonly grown cultivar in Finland, contained high amounts of vicine and convicine. No extensive variation was noted among three studied growing years, even though the weather conditions varied markedly.

The aglycones were detected and monitored with the HPLC method used for vicine and convicine analysis. The aglycones formed in the vicine and convicine fractions decreased in amount and finally lost their UV absorptivity after 2 h at pH 5 at 37 °C. The need for an external enzyme source was confirmed, as losses of vicine and convicine were rather small in faba bean suspensions. Selected lactic acid bacteria (LAB) strains were able to hydrolyse vicine and convicine in faba bean sourdoughs at 25 °C, 24 h; the hydrolysis depended on the fermentation temperature. Sourdoughs lost up to 82–85% of the vicine and up to 34–47% of the convicine. The amounts of vicine and convicine in wheat breads, containing 30% faba bean, were comparable to the amounts that were in sourdoughs after 24 h of fermentation. The aglycones were measured from sourdoughs fermented at 25 °C and from the corresponding doughs, but not from the breads.

This study showed that vicine and convicine can be analysed simultaneously with their aglycones with an RP-HPLC-UV method, which provides the benefit of estimation of the total elimination of these compounds. The aglycones were found to disappear in all the studied matrices. Fermentation can induce losses of vicine and convicine, but the efficiency of hydrolysis depends on the selection of strains and the fermentation conditions. Furthermore, controlled acidification is necessary for maintain acceptable sensory quality.

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Helsinki, May 2019

Marjo Pulkkinen

Contents

List of original publications

This thesis is based on the following publications:

I Pulkkinen M., Gautam M., Lampi A.-M., Ollilainen V., Stoddard F. L., Sontag-Strohm T., Salovaara H., Piironen V. 2015. Determination of vicine and convicine from faba bean with an optimized high performance liquid chromatographic method. Food Res Int, 76, 168–177.

II Pulkkinen M., Zhou, X., Lampi A.-M., Piironen V. 2016. Determination and stability of divicine and isouramil produced by enzymatic hydrolysis of vicine and convicine of faba bean. Food Chem, 212, 10–19.

III Pulkkinen M., Coda R., Lampi A.-M., Varis, J., Katina K., Piironen V. 2019. Possibilities of reducing amounts of vicine and convicine in faba bean suspensions and sourdoughs. Eur Food Res and Technol. Published online. DOI: 10.1007/s00217-019-03282-4.

The publications are referred to in the text by their roman numerals.

Contribution of the author to papers I to III:

I-II Marjo Pulkkinen planned the studies, together with the other authors. She had the main responsibility for experimental work and for interpreting results, and she was the corresponding author of the papers.

III Marjo Pulkkinen planned the study, together with the other authors, and performed most of the analyses. She had the main responsibility for interpreting results, and she was the corresponding author of the paper.

Abbreviations

cfu	colony forming units
DAD	diode array detector
DM	dry matter
DTT	dithiothreitol
ESI	electro spray ionisation
ESTD	external standard
G6PD	glucose-6-phosphate dehydrogenase
GSH	glutathione
GSSG	glutathione disulfide
HCl	hydrochloric acid
HILIC	hydrophilic interaction liquid chromatography
HPLC	high performance liquid chromatography
ISTD	internal standard
LAB	lactic acid bacteria
LC	liquid chromatography
MS	mass spectrometry
NADPH	nicotinamide adenine dinucleotide phosphate
NMR	nuclear magnetic resonance
PCA	perchloric acid
PDA	photodiode array
PDCAAS	protein digestibility-corrected amino acid score
pNPG	p-nitrophenyl-β -D-glucopyranoside
RBCs	red blood cells
RP	reversed phase
RSD	relative standard deviation
SCX	strong cation exchange chromatography
TTA	total titratable acidity
UHPLC	ultra high performance liquid chromatographic methods
UV	ultraviolet
vc-	vicine-convicine lowering gene

1 Introduction

Grain legumes (Family Fabaceae) are important agricultural crops alongside cereals (Zander et al. 2016). The Fabaceae family covers wide range of legumes used for grain and forage, such as soybean (*Glycine max* L.), pea (*Pisum sativum* L.), faba bean (*Vicia faba* L.) and *Lupinus* species. Legumes have a long cultivation history, yet only small portion of arable land in Europe is currently used for legumes (de Visser et al. 2014; Zander et al. 2016). However, legume production could be enhanced with changes in policies. Legumes are viewed as a sustainable option for food production, because their ability to fix atmospheric nitrogen in symbiosis with *Rhizobium* microbiota can decrease the use of fertilisers (Köpke and Nemecek 2010). The use of legumes in intercropping and rotational cropping can also help nutrient intake and break disease cycles. Legumes have a high protein content, so they are considered as a good source of plant-based protein (Day, L. 2013). Legume crops are primarily produced for use as livestock feed, and imported soybean meal is the main protein source for the cattle in many parts of Europe (Zander et al. 2016; Halmemies-Beauchet-Filleau et al. 2018). However, increasingly favourable consumer attitudes towards plant-based diets and heightened awareness of ecological issues have raised the possibility of increasing the use of plant protein directly as human food as well (Day, L. 2013; Vainio et al. 2016).

Faba bean is one of the legume crops that is grown in several different climatic zones. It is primarily a warm temperature and subtropical crop, and it originated as a crop in the Near East and the Mediterranean area (Jensen et al. 2010). The largest producers of faba bean in the years from 2007–2017 were China, Ethiopia, France and Australia (FAOSTAT 2017). Large faba beans (*V. faba major*) are consumed as food in the Middle East, the Mediterranean region, Southern China, Northwest India, Pakistan and Ethiopia (Crépon et al. 2010; Jensen et al. 2010). Small-grain faba beans (*V. faba minor*), which are cool-season crops that can be grown in wider regions, are used as livestock feed and consumed as food (Crépon et al. 2010; Link et al. 2010).

Faba beans have an overall good nutritive value, especially because of the high protein content (Crépon et al. 2010; Multari et al. 2016) which, in Finnish grown cultivars, was over 30% (Lizarazo et al. 2015). However, the nutritional quality of legume proteins is reportedly lower than that of animal proteins (Boye et al. 2012). The lysine content is higher in faba bean than in cereals, whereas the contents of sulphur-containing amino acids (methionine and cysteine) and tryptophan are lower (Duc et al. 1999; Day, L. 2013). The protein digestibility-corrected amino acid score (PDCAAS) for soybean protein isolate is equivalent to that of animal proteins, but the PDCAAS scores for other plant proteins are lower (Boye et al. 2012; Day, L. 2013). The nutritional quality of legume proteins can be enhanced by combining legumes with cereals in a balanced diet. The fat content in most pulses like faba bean is low (1–3%) when compared to oilseeds (soybean and canola) (Hall et al. 2017). Legumes contain 60–65% carbohydrates, mainly starch in most legumes. Pulse starch has a low glycaemic index that indicates slow digestion (Ofuya and Akhidue 2005; Oomah et al.

2011). The starch content was ca. 40% in faba bean (Duc et al. 1999). Legumes are also good sources of minor nutrients, such as minerals and vitamins (Ofuya and Akhidue 2005; Oomah et al. 2011).

The drawback in the use of legumes in human nutrition is the presence of antinutrients and other harmful compounds. Legumes are still minor crops, so their properties have not been as extensively developed when compared to cereals. Plant breeding programmes for faba bean have focused on the reduction of certain harmful compounds, i.e. tannins and the pyrimidine glycosides vicine and convicine (Duc et al. 1999; Ray et al. 2015). The contents of harmful compounds can also be decreased by processing, while simultaneously improving techno-functional properties (Azarpazhooh and Boye 2013). Heating is a traditional processing method and can inactivate heat-labile antinutrients, such as lectins and trypsin inhibitors (Patterson et al. 2017), as well as the enzymes responsible for beany off-flavour (Jiang et al. 2016). However, certain antinutrients are more challenging to remove and require a combination of processing methods. In faba bean, the main harmful compounds are the vicine and convicine, which occur only in *Vicia* species. Their reactive aglycones, divicine and isouramil, can damage red blood cells (RBCs) by participating in oxidative-reductive cycling; thus, these compounds have haemotoxic activity in people who carry a certain genetic disorder called glucose-6-phosphate dehydrogenase deficiency (G6PD) (Cappelini and Fiorelli 2008). A form of haemolytic anaemia called favism may develop after digestion of faba beans by G6PD-deficient individuals.

The total amount of vicine and convicine is approximately 10 mg/g per ripened seeds of faba bean. Cultivars with lower vicine and convicine contents have been developed with the help of the mutant vc- gene found in faba bean in the 1990s (Duc et al. 1989). However, the available cultivars are not yet optimal for boreal cropping systems. Processing methods, such as soaking, heating, germination and fermentation, have shown varying efficiency at removing vicine and convicine. However, these studies have focused only on removal of vicine and convicine without studying the release of the aglycones in food systems. The aglycones have been only studied in model conditions (Chevion et al. 1982; Marquardt, Frohlich et al. 1989; Marquardt, Arbid et al. 1989; McMillan et al. 1993), and have revealed their potential instability. Tools for removal of vicine and convicine need to be developed, with a focus on both vicine and convicine as well as their aglycones.

In this thesis, the main aim was to study the elimination of vicine, convicine and the aglycones from faba beans. The literature review covers the properties of vicine, convicine and the aglycones, the analytical methods for their analysis, the occurrence of vicine and convicine in faba bean and the attempts at removal of these compounds by both genetic and processing methods. The experimental part deals with the implementation of the methods, cultivar variation in Finnish grown faba beans from selected years, investigations on the aglycone formation and stability in different models and the losses of vicine and convicine in suspensions, sourdoughs and breads.

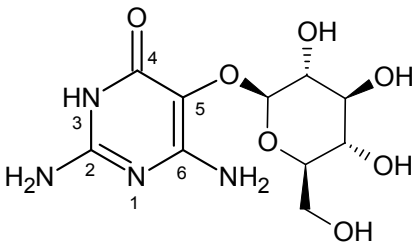
2 Review of the literature

2.1 Vicine and convicine

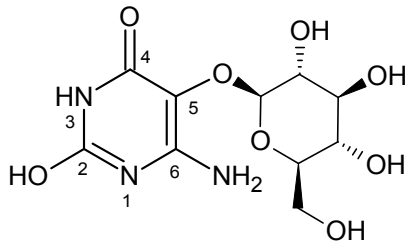
2.1.1 Structure and properties

Vicine [2,6-diamino-4,5-dihydroxypyrimidine 5-(β-D-glucopyranoside)] and convicine [2,4,5-trihydroxy-6-aminopyrimidine 5-(β-D-glucopyranoside)] are pyrimidine glycosides (Figure 1). These compounds occur almost solely in *Vicia faba,* which belongs to the vetch group of legumes. Small amounts of vicine and convicine (< 0.1 mg/g) have been also found in other species of the *Vicia* genus, such as *Vicia narbonsensis* (Pitz et al. 1980; Griffiths and Ramsay 1992). Only in *Vicia bithynica* are the levels of vicine and convicine comparable to those in *Vicia faba*. Vicine was originally found in the common vetch (*Vicia sativa*), but at low amounts (Griffiths and Ramsay 1992).

Pyrimidine nucleotides have an important function in the synthesis of DNA and RNA, and their metabolism and catabolism are controlled by different pathways (Kafer et al. 2004). Nitrogen-containing secondary metabolites are likely to be derived from these compounds. The pyrimidine part of convicine is similar to that of uracil, except that the functional groups in positions 5 and 6 are lacking. Vicine and convicine are possibly formed via the orotic acid pathway (Brown and Roberts 1972).



vicine



convicine

Figure 1 The structures of vicine and convicine.

The molecular weights of vicine and convicine are 304.2 and 305.2 g/mol, respectively. The only difference between these two forms is a functional group in position 2 in the pyrimidine ring (Figure 1). The functional group of vicine is an amino group (-NH_2), whereas it is a hydroxyl group (-OH) in convicine. Both compounds are soluble in water, but the solubility depends on the pH value. Marquardt et al. (1983) showed that the solubility of vicine is highest at acidic pH, whereas that of convicine is highest under alkaline conditions. In a pH

range of 4–8, solubilisation was less efficient. Depending on the pH value, both compounds can be protonated, neutral or deprotonated. The pKa values differ slightly for vicine and convicine. The pKa1 and pKa2 values for vicine are 9.56 and 3.16, respectively, and the corresponding values for convicine are 11.11 and 2.71, respectively, according to the ACD/Labs Software V11.02 (© 1994-2012, ACD/Labs). Pyrimidine compounds are considered weak bases because of the amino groups in their structures.

Vicine and convicine are compounds that absorb ultraviolet (UV) light, and their UV spectra and molar absorption coefficients have been defined. The absorption maxima for vicine occur at 274, 275 and 269 nm at pH 1, 6.8 and 13, respectively (Bendich and Clements 1953; Olsen and Andersen 1978). The corresponding absorption maxima for convicine are 271, 271 and 273 nm (Bien et al. 1968).

The structures of vicine and convicine also enable the formation of keto-enol and amine-imine tautomers (Donath and Kujawa 1991). Keto-enol tautomerism occurs at positions 3 and 4 in vicine and convicine, and may also occur at positions 1 and 6 in convicine. The keto forms are usually present to a major extent in the equilibrium state (McMurry 2011). Vicine and convicine are shown as keto forms in Figure 1. The enol forms are more reactive and are formed by protonation in acidic conditions or by deprotonation in alkaline conditions (McMurry 2011).

2.1.2 Hydrolysis of vicine and convicine to their aglycones

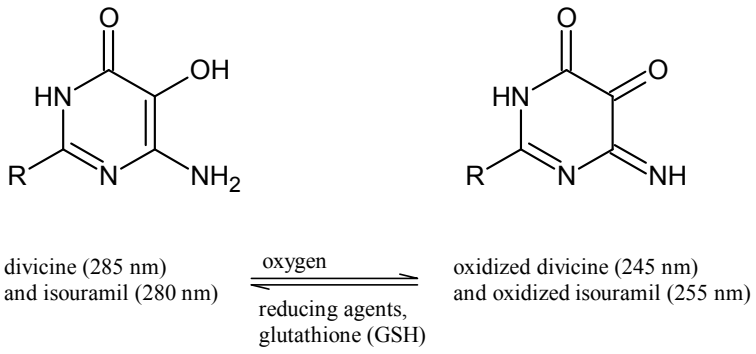
The non-carbohydrate moiety of vicine or convicine, i.e. the pyrimidine aglycone, is attached to D-glucose via a β-glycosidic bond at position 5. The glucose unit can be released enzymatically or by treatment with strong acid at high temperature. Several studies have demonstrated acid hydrolysis to aglycones by treatment with sulphuric acid or hydrochloric acid (HCl) at 100 °C (Benatti et al. 1984; Pedersen et al. 1988; Marquardt, Frohlich et al. 1989; Marquardt, Arbid et al. 1989; Winterbourn et al. 1989; McMillan et al. 1993). Enzymatic hydrolysis using β-glucosidase is more relevant for food systems, and the enzyme can originate from several sources. Commercial β-glucosidase (from almonds) has been the enzyme used to produce and to study the stability of divicine (Baker et al. 1984; Pedersen et al. 1988; McMillan et al. 1993).

In the human body, hydrolysis of vicine and convicine occurs either in the gastrointestinal tract or in the liver. Animal experiments with young chickens and rats showed that the actual hydrolysis of vicine and convicine occurred in the large intestine and caecum, but not in the small intestine (Frohlich and Marquardt 1983; Hegazy and Marquardt 1984). However, deglycosylation of flavonoid and isoflavonoid glycosides has been demonstrated in cell-free extracts of human intestine and liver by a cytosolic broad-specificity β-glucosidase (Day, A. J. et al. 1998; Nemeth et al. 2003). Many bacterial strains resident in the human gut also have the ability to produce β-glucosidases (Dabek et al. 2008).

The aglycone formed after hydrolysis is the reactive part of the molecule. The aglycones of vicine and convicine, called divicine and isouramil, respectively, are shown in Figure 2. The molecular weights of divicine and isouramil are 143.1 and 144.1 g/mol, respectively, and the pKa values are 4.49 and 3.36, respectively.

2.1.3 Reactivity of divicine and isouramil

Divicine and isouramil are reducing agents (Chevion et al. 1982). The reductive–oxidative potency of divicine at physiological pH was proved in cyclic voltametry experiments (McMillan et al. 1993). The aglycones have been reactive and unstable in model systems, because both divicine and isouramil were rapidly oxidised within hours (Chevion et al. 1982; Baker et al. 1984; Marquardt, Arbid et al. 1989; Marquardt, Frohlich et al. 1989; McMillan et al. 1993). As described in Figure 2, changes in UV absorption indicate the oxidation and regeneration of the reduced forms of the aglycones (Chevion et al. 1982; Marquardt, Arbid et al. 1989; Marquardt, Frohlich et al. 1989). In the reduced state, divicine or isouramil show absorption maxima at 280–285 nm at pH 7 (Chevion et al. 1982; Benatti et al. 1984; Marquardt, Arbid et al. 1989; Marquardt, Frohlich et al. 1989). When the aglycones are oxidised, the absorption maxima shift to 245–255 nm (Chevion et al. 1982; Benatti et al. 1984; Marquardt, Arbid et al. 1989).



R= NH_2, divicine,

R = OH, isouramil

Figure 2 The suggested oxidation pathway for divicine and isouramil at pH 7 (adapted from Chevion et al. 1982).

The prevention of aglycone oxidation was investigated in a number of studies (Chevion et al. 1982; Benatti et al. 1984; Marquardt, Arbid et al. 1989; Marquardt, Frohlich et al. 1989).

Replacement of air or oxygen with inert gases delayed the oxidation of the aglycones (Pedersen et al. 1988; Marquardt, Arbid et al. 1989; Marquardt, Frohlich et al. 1989; McMillan et al. 1993). Divicine was regenerated from oxidised divicine by adding reducing agents, such as sodium hydrosulphite, sodium borohydride, cysteine, 3-mercaptoethanol or dithiothreitol (DTT), and these agents also prevented changes in divicine (Chevion et al. 1982; Marquardt, Frohlich et al. 1989; McMillan et al. 1993).

The stability of the reduced forms of divicine and isouramil also depended on the pH, because the aglycones were more stable in acidic medium than under neutral or alkaline conditions (Chevion et al. 1982; Baker et al. 1984; Marquardt, Arbid et al. 1989; Marquardt, Frohlich et al. 1989). Changes were observed in the UV spectra of divicine and isouramil in less than an hour in the presence of oxygen at slightly acidic and neutral pH (Chevion et al. 1982; Marquardt, Arbid et al. 1989; Marquardt, Frohlich et al. 1989; McMillan et al. 1993). At low pH (1–2), the stability of the aglycones was prolonged for several hours (Chevion et al. 1982; Marquardt, Frohlich et al. 1989).

Further reactions of oxidised divicine and isouramil were suggested to lead to the formation of end-products with low UV absorption, as the UV maxima dropped to values lower than 230 nm (Chevion et al. 1982) or 250 nm (Marquardt, Frohlich et al. 1989). The end-products were reported to remain stable for a longer time (at least 1–2 h) when compared to oxidised divicine or divicine itself (Chevion et al. 1982; Marquardt, Frohlich et al. 1989). The loss of UV absorptivity has been explained by an irreversible opening of the pyrimidine ring structure. The structures of the decomposition products of divicine and isouramil are still not known. The reactions of the pyrimidine ring itself have been studied in model systems (Katritzky et al. 2010a); however, divicine and isouramil are substituted pyrimidines, and their substituents are likely to contribute to their stability.

The hydroxyl group in position 5 of the pyrimidine ring is exposed when glucose is hydrolysed. Electronegative nitrogen atoms cause an irregular distribution of electron density to the heterocyclic pyrimidine ring, and this may partially explain the reactivity of the aglycones (Katritzky et al. 2010b). Carbonyl groups are reactive and can undergo keto-enol tautomerism, oxidation and nucleophilic addition (McMurry 2011). The formation of a divicine-glutathione adduct has been reported (Chevion et al. 1982; Benatti et al. 1984); however, the adduct formation and stability were not studied further.

Radical-induced oxidation of divicine has also been reported (Pedersen et al. 1988; Winterbourn et al. 1989; Arese and De Flora 1990; Ta-Shma et al. 2006). A semiquinone radical was suggested to form as an intermediate of the hydroquinone and quinone of divicine, as shown in Figure 3, which illustrates the formation of a semiquinone radical for the keto form of divicine. (The reaction products of the enol form of divicine are not presented.) The formation of a semiquinoid radical was demonstrated in many previous studies by electron spin resonance spectroscopy (Baker et al. 1984; Pedersen et al. 1988; Winterbourn et al. 1989). The radical signal was only detected when oxygen was present,

and the intensity of the signal was also pH dependent (Baker et al. 1984; Pedersen et al. 1988). The intensity of the radical signal was maximal under alkaline conditions and lower under slightly acidic conditions, in line with earlier stability studies. The authors suggested that the higher pH values promoted the deprotonation of the semiquinoid form into a more reactive radical (Baker et al. 1984; Pedersen et al. 1988).

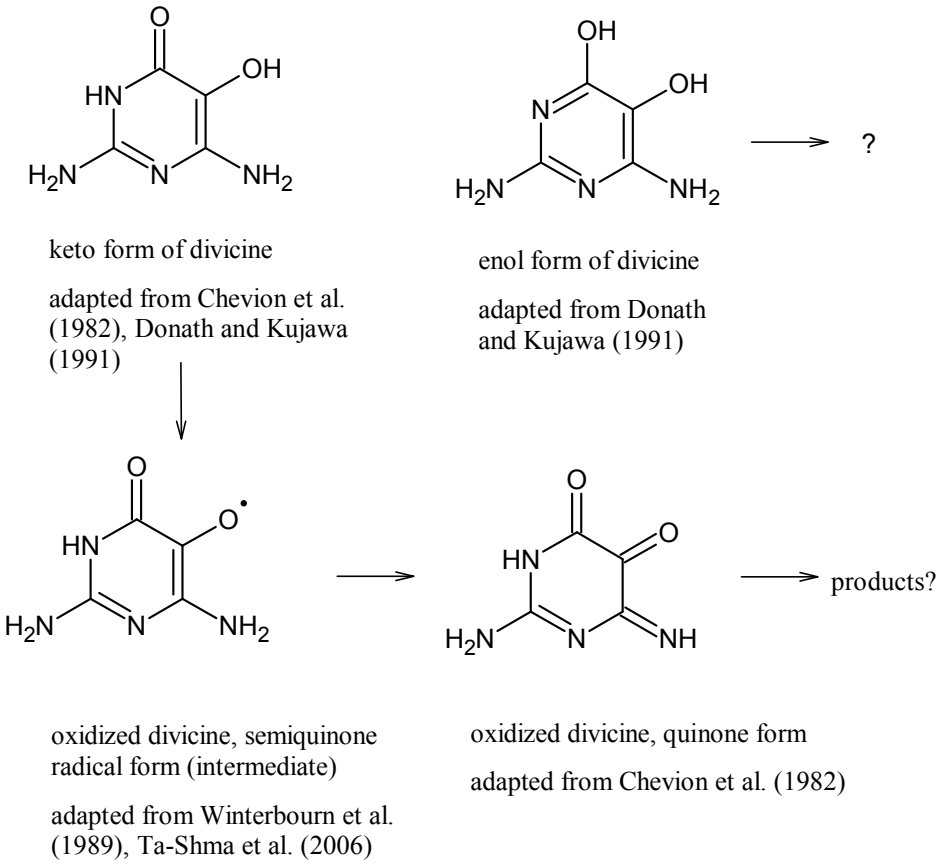


Figure 3 The suggested reaction pathways for divicine, according to Chevion et al. (1982), Winterbourn et al. (1989), Donath and Kujawa (1991) and Ta-Shma et al. (2006).

Overall, the aglycones are compounds that are able to oxidise and reduce back to their original form; in other words, they can regenerate. They were unstable, as confirmed by the loss of UV absorption. Their degradation was delayed by the removal of oxygen and the addition of reducing agents. Low pH and reduced temperature also slowed down the degradation. The decomposition of the ring structure is possible caused by radical attacks.

The reaction routes, except for the oxidation, are unknown, and the final degradation products also remain uncharacterised.

2.1.4 Biological effects

Vicine and convicine are biologically reactive compounds that may have a primary role in plant defence. The hypothesis regarding a protective role of vicine and convicine has been supported in a few studies (Desroches et al. 1997; Pavlik et al. 2002). For example, divicine has shown fungicidal and fungistatic properties against plant pathogens (Pavlik et al. 2002). Mortality increased in the larvae of bruchid beetles when vicine was digested (Desroches et al. 1997). By contrast, lower amounts of vicine and convicine in faba beans did not increase the susceptibility of the beans to diseases (Duc et al. 1989).

The harmful effects of vicine and convicine are observed in monogastric animals. Feed supplemented with faba bean was not harmful to pigs (Jezierny et al. 2011), but negative effects of vicine and convicine were noticed in laying hens. Hens fed diets supplemented with faba bean showed reduced egg weights (Olaboro et al. 1981; Koivunen et al. 2014; Lessire et al. 2017), as well as erythrocyte haemolysis and decreased GSH concentrations (Muduuli et al. 1981). Laying hens that consumed vc- faba bean cultivars did not show these adverse effects (Lessire et al. 2017). Vicine and convicine were not harmful to broiler chickens in the diets studied (Koivunen et al. 2016).

In humans, vicine and convicine can also be haemotoxic in certain people. In humans, the digestion of faba beans can cause a severe haemolytic anaemia termed favism (Arese and De Flora 1990; Cappelini and Fiorelli 2008; Arese et al. 2012). This favism is caused by a deficiency in the G6PD enzyme in susceptible people. The deficiency was originally identified in the 1950s, when the use of an antimalarial drug (primaquine) as an oxidative drug led to haemolytic anaemia in certain individuals (WHO 1989). This disorder is one of the most prevalent enzyme defects in humans that leads to severe illness. G6PD deficiency is a hereditary X-chromosome linked mutation, so hemizygous males are the most vulnerable group for the disorder (Cappelini and Fiorelli 2008). The deficiency is highest in populations in tropical Africa (16%) and in subtropical Asia (7%), and lowest in Europe (1%) (WHO 1989). Overall, approximately 7% of the human population has this genetic disorder worldwide. Furthermore, the evolution of G6PD-deficiency in humans is most likely connected to tolerance of malaria, because the malfunction in the RBCs protects against malaria caused by *Plasmodium falciparum* (Ruwende and Hill 1998).

G6PD deficiency is especially crucial for RBCs, because these cells do not contain mitochondria for energy metabolism (Cappelini and Fiorelli 2008). Thus, the only way to regenerate nicotinamide adenine dinucleotide phosphate (NADPH) is via the activity of the G6PD enzyme in pentose phosphate pathway. In regular RBCs, the activities of G6PD-enzyme are sufficiently high to regenerate NADPH and prevent attacks from oxidative

species, i.e. the aglycones of vicine and convicine of faba bean (Baker et al. 1984; Arese and De Flora 1990; Cappelini and Fiorelli 2008). However, when the oxidative reactions are not controlled, a massive number of RBCs go through rapid senescence and removal from the circulation through phagocytosis by monocytes (Cappelini and Fiorelli 2008; Arese et al. 2012). The first symptoms of favism, which include nausea, vomiting, headache, abdominal pain and fever, appear a few hours to 24 h after digestion of faba beans (Cappelini and Fiorelli 2008; Arese et al. 2012). At the more severe stage, jaundice appears and the spleen and liver may be enlarged. Haemoglobinuria (i.e. haemoglobin in the urine) is also a common symptom. In severe cases of this anaemia, blood transfusion may be necessary.

The main reason for development of favism is the rapid drop in GSH levels (Arese and De Flora 1990). Regeneration of GSH happens rapidly after a decrease in GSH in healthy individuals, but in G6PD-deficient humans, this regeneration is too slow. Under oxidative conditions, the amount of oxidised haemoglobin (met-Hb) increases, and erythrocytes undergo deleterious changes, such as the cross-bonding and formation of Heinz bodies, which are denatured haemoglobin precipitates (McMillan et al. 2001). At this point of favic crisis, acute haemolysis has started and the RBC counts are low due to phagocytosis (Arese et al. 2012).

The cellular function of GSH is to maintain the activity of cell-protecting enzymes by protecting sulfhydryl (-SH) groups (Arese and De Flora 1990; Cappelini and Fiorelli 2008). Oxidants turn GSH into its inactive form, namely glutathione disulfide (GSSG), so oxidants like the aglycones accelerate this oxidation via oxidative-reductive cycling (Chevion et al. 1982). The aglycones are first oxidised and then regenerated by taking hydrogen atoms from GSH, reducing agents or other molecules (Fig 2). Hydrogen peroxide (H_2O_2) and other oxidants are formed in these reactions. GSH participates in the detoxification of H_2O_2 by GSH-peroxidase, which turns H_2O_2 into water (2 H_2O) and oxygen and consumes two GSH molecules in that reaction.

The regeneration of GSH (1) involves the conversion of one GSSG molecule into two GSH molecules by glutathione reductase (Chevion et al. 1982; Cappelini and Fiorelli 2008). NADPH is oxidised in this reaction. To restore the reduced NADPH, the G6PD enzyme converts glucose-6-phosphate to 6-phosphoglucono-omega-lactone (2). This cycle keeps a balance between the oxidised and reduced forms of several compounds, and if the G6PD enzyme activity is insufficient, then protective NADPH-mediated reactions cannot occur.

$$\mathrm{GSSG + NADPH + H^+ + \xrightarrow[\text{reductase}]{\text{glutathione}} 2\,GSH + NADP^+} \quad (1)$$

$$\mathrm{Glucose\text{-}6\text{-}phosphate + NADP^+ \xrightarrow{G6PD} 6\text{-}phospho\text{-}glucono\text{-}omega\text{-}lactone + NADPH + H^+} \quad (2)$$

The safe amounts of faba beans for consumption by G6PD-defient persons cannot be predicted (Cappelini and Fiorelli 2008). Developing an acute haemolytic crisis after digestion of faba beans is rather unpredictable, because the responses are partly dependent on the genetic variant, and thus the severity, of the G6PD deficiency. If the activity of the G6PD enzyme is not sufficient to eliminate the harmful effects of the aglycones of vicine and convicine, then favism can develop. Generally, male children are the high risk group for favism (Arese and De Flora 1990; Reading et al. 2016). However, depletion of RBCs was not seen after consumption of vc- faba bean cultivars by G6PD-deficient individuals (Gallo et al. 2018).

2.2 Analysis of vicine and convicine and the aglycones

2.2.1 Standard preparation

Vicine and convicine standards are not widely available, and convicine is especially limited in its availability. To produce the standards, vicine and convicine were extracted under alkaline conditions, followed by acetone treatment (Marquardt et al. 1983; Purves et al. 2018a), or they were extracted with an ethanol-water mixture (75:25, v/v) (Arbid and Marquardt 1985a). The extracts were concentrated, and vicine and convicine were crystallised from solution. The compounds were separated from the crystallised mixture based on the solubility differences of vicine and convicine, as described by Marquardt et al. (1983), and the same separation protocol was later used in the studies by Arbid and Marquardt (1985a) and Purves et al. (2018a).

The compounds were also extracted for standard preparation with ethanol-water (80:20, v/v) and the resulting solution was deproteinised using Carrez reagents after concentration (Quemener 1988). A more complicated extraction protocol was introduced by Pavlik et al. (2002), who used light petroleum ether, ethyl acetate, methanol, methanol-water (50:50, v/v) and water for extraction and 1-butanol for the final washing. Vicine and convicine were separated from the extract by high performance liquid chromatography (HPLC) at a larger scale with a C18 column using water or water-methanol as an eluent and detected with a UV detector (Quemener 1988; Pavlik et al. 2002).

The aglycones are not available as standards due to their instability. Production of the aglycones was reported from vicine and convicine by acid hydrolysis, followed by crystallisation and storage as dried crystals (Marquardt, Frohlich et al. 1989; Marquardt, Arbid et al. 1989). Divicine was also synthesised from 6-hydroxy-2,4,5-triaminopyrimidine sulphate by McMillan et al. (1993). The most practical approach was to release the aglycones from vicine or convicine by acid or enzymatic hydrolysis (Marquardt, Arbid et al. 1989; Marquardt, Frohlich et al. 1989; McMillan et al. 1993). Stabilisation, such as by

removal of oxygen or using reducing agents, was required until determination to avoid decomposition of the aglycone of interest.

2.2.2 Sample preparation

The first analysis methods to determine vicine and convicine contained many steps and were time-consuming. Different extraction solutions have been applied, such as trichloroacetic acid and metaphosphoric acid (Higazi and Read 1974; Collier 1976; Kim et al. 1982), ethanol-water (75:25, v/v) mixtures (Olsen and Andersen 1978) and alkaline solutions (Quemener et al. 1982). Phenolic compounds interfere with the analysis (Higazi and Read 1974; Collier 1976; Kim et al. 1982), so neutral alumina was used to purify the solutions (Kim et al. 1982).

Alternative extraction methods have included in HPLC methods (Table 1), which have been later mainly used for vicine and convicine. Many HPLC studies have used 5–6% (0.8–1 M) perchloric acid (PCA) for extraction, following the protocol described by Marquardt and Frohlich (1981). Water extraction, followed by pH adjustment with HCl to the isoelectric point of the main faba proteins (pH 4), was first presented by Quemener (1988). The same extracts were used for analysis of raffinose family oligosaccharides, but Carrez reagents were added for the further removal of interfering substances for different HPLC systems. The extraction method was further modified by Gutierrez et al. (2006), who soaked two seeds from each parental line of interest in water for 3.5 h at 90 °C, followed by acidification with HCl to remove proteins. The extraction method of Gutierrez et al. (2006) was also used by Ivarsson and Neil (2018) and Mattila et al. (2018). Methanol-water (50:50, v/v) was also used for extraction (Lattanzio et al. 1982). Various extraction solvents were compared by Purves et al. (2018a); these included acetone-water (70:30, v/v), methanol-water (70:30, v/v), and water with and without 0.1% formic acid. The compared solutions gave similar results, and acetone-water (70:30, v/v), which had been used previously for an extraction of polyphenols, was selected for vicine and convicine extraction.

Sample preparation has either focused on vicine and convicine or the aglycones, but not on both simultaneously. In contrast to the earlier sample preparation methods presented, studies on the aglycones were carried out under model conditions. The aglycones were analysed in aqueous solutions (i.e. in acids, buffers and alkaline solutions) (Chevion et al. 1982; Marquardt, Frohlich et al. 1989; Marquardt, Arbid et al. 1989; McMillan et al. 1993).

2.2.3 Instrumental methods for analysis of vicine and convicine

The first methods for analysis of vicine and convicine were based on their UV absorptivity. Extracts were measured directly with a spectrophotometer at 273 nm (Collier 1976) or they were measured at 480 nm after formation of a coloured titanium tetrachloride complex from

hydrolysed vicine and convicine (Kim et al. 1982). The compounds were separated by thin layer chromatography on cellulose plates (300MN, 0.5 mm) using ethanol, methanol-ammonia-water solution, or methanol and phosphate buffer (pH 5.8) at a ratio 70:30 as elution solvents (Olsen and Andersen 1978). Ion-exchange chromatography (microcrystalline cellulose, 200 × 25 mm column) with the same eluents was used for separation of vicine and convicine. Vicine and convicine were also silylated and analysed by gas chromatography (Pitz et al. 1981; Sosulski and McCurdy 1987). However, vicine and convicine have mostly been separated by HPLC, because it is practical and effective. No ultra high performance liquid chromatography (UHPLC) methods have yet been applied to separate vicine and convicine, probably because adequate analysis can be already obtained with HPLC systems. Reversed phase (RP) HPLC methods are suitable for these compounds and were used by Marquardt and Frohlich (1981) and Quemener (1988). Methods from earlier publications are presented in Table 1.

The most often used eluent compositions in RP-HPLC systems have been ammonium phosphate buffer at pH 2, as described by Marquardt and Frohlich (1981), and water, as described by Quemener (1988) (Table 1). Vicine and convicine can be separated in an isocratic run. Organic solvents are not necessarily required in the eluent composition, albeit methanol or methanol-water (70:30, v/v) was used for washing the column (Marquardt and Frohlich 1981).

Depending on the column dimensions and elution systems, the reported retention times on C18 columns were only 2–3 min (Marquardt and Frohlich 1981) or 5–6 min for vicine and 5–8 min for convicine (Lattanzio et al. 1982; Goyoaga et al. 2008; Cardaror-Martinez et al. 2012; Rizzello et al. 2016). The retention of vicine and convicine depends on the pH of the eluent, because vicine and convicine can exist in ionised or neutral forms. The protonated NH_3^+, as functional group, is more polar than NH_2; therefore, the retention times on a C18 column may be shortened. The compounds have better retention on C18 columns in the neutral state than as charged forms. Good separation was obtained in the study by Marquardt and Frohlich (1981) when the pH of the eluent was 2. The retention time of convicine was shortened and that of vicine was prolonged when pH of the eluent was changed from pH 2 to 3. A similar pH effect was also reported by Zhang et al. (2003). Even longer retention times (10–15 min) were reported by Quemener (1988), who used water as an eluent, and convicine eluted before vicine (Lattanzio et al. 1982; Quemener 1988). Convicine also eluted before vicine in a gradient run with 90% MilliQ-water and 10% acetonitrile, with both eluents containing 0.1% formic acid (Rizzello et al. 2016).

Based on these earlier studies, retention on a C18 column was considered good and retention times were moderate. In addition to RP-HPLC systems, a hydrophilic interaction liquid chromatography (HILIC) column was recently applied as a part of a high-throughput analysis method developed for cultivar screening in plant breeding (Purves et al. 2018a). Retention times were short (2.5 min for convicine and 3.0 min for vicine), and the whole run time was 10 min. Formic acid (0.1%) was required in the eluent to obtain good

separation of convicine and vicine, and the compounds were eluted with water containing 10 mM ammonium acetate and 0.1% formic acid. Acetonitrile-water (90:10, v/v) containing the same modifiers was used only for column washing.

The separated vicine and convicine are typically detected by UV absorption (Table 1), with confirmation and further analysis of the structure conducted using mass spectrometry (MS) coupled with liquid chromatography (LC) system. Nuclear magnetic resonance (NMR) was also used in a few studies for identification of vicine and convicine, and chemical shifts were published for ^{13}C NMR (Delfini et al. 1990) and ^{13}C NMR and ^{1}H NMR for convicine (Purves et al. 2018a). The UV wavelengths for detection have typically been 273–280 nm. Quantification is carried out with calibration curves or with an internal standard. Uridine was proposed as an internal standard by Quemener (1988) and proved to be a better option than either cytosine, cytidine, adenosine, 2-deoxythymidine or uridine monophosphate. Uridine was later used by Helsper et al. (1993) and Purves et al. (2018a). Quemener (1988) established relative response factors for vicine and convicine to uridine as 0.8 and 0.6, respectively.

MS can be used for structural analysis, but some recent studies have also used it for quantification (Rizzello et al. 2016; Purves et al. 2018a). Due to the presence of both positive and negative charges in vicine and convicine, both positive and negative ESI ionisations can be used. Rizzello et al. (2016) used positive mode in ESI, whereas Purves et al. (2018a) used the negative mode for less background. When compared to UV detection, MS is more selective and sensitive. However, UV detection is generally linear and repeatable. Lack of selectivity is the main concern in UV detection but, depending on conditions, interferences can be avoided.

All these studies have focused on analysis of vicine and convicine. The hydrolysis of vicine and convicine was only mentioned when the identity of peaks was confirmed by hydrolysing them with almond β-glucosidase. The formation and identity of the aglycones were not confirmed in these studies on vicine and convicine.

2.2.4 Instrumental methods for analysis of the aglycones

The methods used for the analysis of the vicine and convicine aglycones have based on their UV absorptivity (Chevion et al. 1982; Marquardt, Frohlich et al. 1989; Marquardt, Arbid et al. 1989; Winterbourn et al. 1989; McMillan et al. 1993) and on complex formation (Kim et al. 1982). Only a few studies have used HPLC methods to study the formation and stability of the aglycones (Marquardt, Frohlich et al. 1989; Marquardt, Arbid et al. 1989; McMillan et al. 1993). Marquardt and Frohlich (1981) suggested that the aglycones can be followed, along vicine and convicine, on a C18 column, but harsh conditions (2 N sulphuric acid, 90 °C) were used to induce the formation of the aglycones. Acid hydrolysis was also used in later studies that investigated the aglycones and their further products more deeply.

Indications of the aglycones were also seen with C18 column in an LC-MS system, but they did not properly separate from vicine and convicine (Rizzello et al. 2016).

Cation-exchange chromatography methods with UV detection were applied to detect either divicine or isouramil and further oxidation products (Marquardt, Arbid et al. 1989; Marquardt, Frohlich et al. 1989; McMillan et al. 1993). Further details are shown in Table 1. Hydrolysis mixtures were injected to HPLC immediately after incubation (Marquardt, Arbid et al. 1989; Marquardt, Frohlich et al. 1989). One product was an oxidised form of divicine or isouramil, as confirmed by the addition of a reducing agent. The other products were not affected by the reductant and were therefore considered as further products. McMillan et al. (1993) showed that acid hydrolysis can induce the formation of further, and possibly new, products that were seen with HPLC. They decided to use enzymatic hydrolysis instead, and found oxidised divicine and two further products from divicine after hydrolysis.

The introduced analysis methods focused only on the presence of either divicine or isouramil and their reaction products in model conditions, separately from non-hydrolysed forms. In order to study the aglycones in real food systems, the aglycones should be studied together with vicine and convicine.

Table 1. HPLC methods used for the analysis of vicine, convicine and their aglycones.

Form	Matrix	Extraction	Separation (column)	Mobile phase	Flow rate (ml/min)	Detection	Quantification	Reference
Glycosides	faba beans	5% perchloric acid	Ultrasphere ODS C18 (250 × 4.6 mm, 5 µm)	50 mM ammonium phosphate buffer (pH 2)	2	UV (280 nm)	ESTD	(Marquardt and Frohlich 1981)
		methanol:water (50:50)	µBondapak C18 (300 × 4 mm, 10 µm)	water	1.5	UV (280 nm)	nr	(Lattanzio et al. 1982)
		water, acidification with HCl to pH 4	Rosil C18 (150 × 4.6 mm, 3 µm)	water	1	UV (273 nm)	ISTD (uridine)	(Quemener 1988)
		according to Marquardt and Frohlich (1981)	Serva octadecyl-Si 100 (250 × 4.6 mm, 5 µm)	according to Marquardt (1981)	0.8	UV (280 nm)	nr	(Wang and Ueberschär 1990)
		modified from Quemener (1988)	C18 Hypersil ODS (250 × 4.6 mm, 5 µm)	methanol-water (5:95)	nr	UV (273 nm)	nr	(Griffits and Ramsay 1992)
		according to Marquardt and Frohlich (1981)	C18 Spherisorb ODS 2 Cis (125 × 4 mm, 3 µm)	50 mM sodium citrate (pH 6.8)	1	UV (265 nm)	ISTD (uridine)	(Helsper et al. 1993)
		according to Marquardt and Frohlich (1981)	C18 Ultrasphere ODS (250 × 4.6 mm, 5 µm)	50 mM ammonium phosphate buffer (pH 2)	nr	UV (280 nm)	ESTD	(Burbano et al. 1995)
		modified from Quemener (1988)	RP-18 Lichrospher 100 (5 µm)	water	1	UV (276 nm)	ESTD	(Gutierrez et al. 2006)

		according to Marquardt and Frohlich (1981)	C18 Spherisorb ODS (250 × 4.6 mm, 5 µm)	50 mM ammonium phosphate buffer (pH 2)	1.2	UV (280 nm)	ESTD	(Goyaga et al. 2008)
		according to Marquardt and Frohlich (1981)	C18 Spherisorb ODS (250 × 4.6 mm, 5 µm)	50 mM ammonium phosphate buffer (pH 2)	1	UV (280 nm)	ESTD	(Cardaror-Martinez et al. 2012)
	faba beans, protein isolate	according to Marquardt and Frohlich (1981)	C18 Ultrasphere ODS	50 mM ammonium phosphate buffer (pH 1.4)	2	UV (280 nm)	nr	(Vioque et al. 2012)
	faba beans	acetone-water (70:30, v/v), diluted with acetonitrile:water (90:10, v/v)	Agilent Poroshell 1290 (HILIC) (100 × 2.1 mm, 2.7 µm)	A (water) and B (acetonitrile:water, 90:10 v/v), both with 10 mM ammonium acetate and 0.1% formic acid	nr	UV (278 nm), ESI-MS (negative mode)	ISTD (uridine)	(Purves et al. 2018a)
		according to Gutierrez et al. (2006)	Atlantis T-3 (150 × 2.1 mm, 3 µm)	gradient run with 50 mM phosphate buffer and methanol	0.2	UV (280 nm)	ESTD	(Ivarsson and Neil 2018; Mattila et al. 2018)
Aglycones	vicine or convicine	acid-hydrolysis	Ultrasil-CX (250 × 4.6 mm)	10 mM hexanesulphonic acid 25 mM ammonium phosphate buffer (pH 2)	1.8	UV (250, 282 nm) for divicine, UV (220, 281 nm) for isouramil	nr	(Marquardt, Arbid et al. 1989; Marquardt, Frohlich, et al. 1989)
	vicine	enzyme and acid hydrolysed	Radial-Pak Partisil SCX (100 × 8 mm)	35 mM ammonium citrate, 1 mM EDTA, (pH 3.3)	1.5	UV (254 nm)	nr	(McMillan et al. 1993)

ESTD = external standard, ISTD = internal standard, SCX = strong cation exchange chromatography, nr = not reported.

2.3 Occurrence of vicine and convicine in faba bean

Vicine and convicine are compounds found only in vetches (*Vicia* species), and faba bean (*Vicia faba* L.) is the only vetch that is used for human consumption. The vicine and convicine levels are notably lower (< 0.01%) in most of the wild vetches when compared to faba bean. The total vicine and convicine levels are around 1% of weight in faba bean (Pitz et al. 1981; Griffiths and Ramsay 1992).

Vicine and convicine are present in seeds even at the early stage of development (Pitz et al. 1981; Duc et al. 1989; Burbano et al. 1995). The vicine and convicine contents were at the highest in young fresh green seeds (80% moisture), decreased with maturity and then stabilised at a certain level. Ripened seeds then contained similar amounts of vicine and convicine despite the harvest time. The total vicine and convicine contents were approximately 3–4% per dry weight in fresh green seeds; thus, the contents were 3–4-fold higher than in ripened seeds, but the water content is much lower in ripened seeds than in fresh green seeds.

2.3.1 Location in seeds

The faba bean consists of a seed coat (testa), a cotyledon, a radicle, a hypocotyl and an epicotyl, as shown in Figure 4. The radicle, the hypocotyl and the epicotyl are called as the embryonic axis (embryo). The growth of the seedling starts from the embryo during germination. In contrast to cereal grains, the cotyledon and the embryo cannot be separated. The calculated proportions of each part of faba bean were seed coat testa (13%), cotyledon (86%) and embryo (1%) (Ramsay and Griffiths 1995).

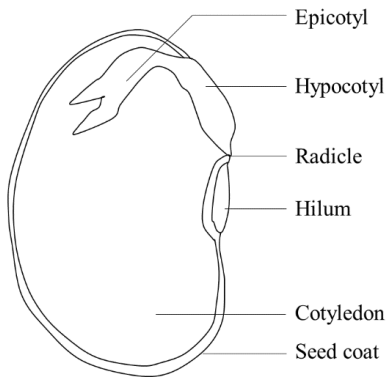


Figure 4 Structure of a dry bean seed cotyledon. Adapted from Uebersax and Siddiq (2013).

Dissection of a faba bean showed that vicine and convicine were found in all parts of the cotyledon (Ramsay and Griffiths 1996; Goyoaga et al. 2008). The seed coat did not contain vicine and convicine (Pitz et al. 1981), or their amounts were low (Griffiths and Ramsay 1992; Burbano et al. 1995). The embryo radicle had the highest content of vicine and convicine, at 10-fold compared to whole seed (Griffiths and Ramsay 1992; Burbano et al. 1995; Goyoaga et al. 2008). In cultivars with lower amounts of vicine and convicine, dissection showed that the contents in cotyledon were decreased, but the embryos still contained high amounts of vicine and convicine (Ramsay and Griffiths 1996).

Study at the whole plant level revealed an accumulation of vicine and convicine in the seeds and roots (Ramsay and Griffiths 1996). Vicine was not detected in the pod walls (Ramsay and Griffiths 1992; Burbano et al 1995). The synthesis routes leading to vicine and convicine are not well known (Ramsay and Griffiths 1996); however, they are likely related to pyrimidine or nitrogen metabolism (Griffiths and Ramsay 1996).

2.3.2 Genetic selection of cultivars

Plant breeding programmes have aimed for a reduction in contents of condensed tannins (proanthocyanidins) and of vicine and convicine (Duc et al. 1989; Duc et al. 1999; Crépon et al. 2010). Discovery of a mutant allele, vc-, has enabled the development of faba bean cultivars with lowered contents of vicine and convicine (Duc et al. 1989). A change in a single gene has lowered vicine and convicine contents in the low vicine-convicine cultivars, e.g. known as ‘Melodié’ and ‘Divine’, to 10–30% of the content in wild-type cultivars. The gene is maternally inherited and recessive (Duc et al. 1989; Ramsay and Griffiths 1996; Khamassi et al. 2013; Khazaei et al. 2015).

Genetic maps are lacking for faba bean because its diploid genome is large (~13 Gbp) and complex (Gutierrez et al. 2006; Khazaei et al. 2015; Ray et al. 2015). The size of the faba bean genome is one of the largest among legumes. Screening of cultivars based on chemical analyses is time-consuming; therefore, molecular markers have been developed to switch off the gene that regulates the production of vicine and convicine (Gutierrez et al. 2006; Torres et al. 2010; Khazaei et al. 2015). This marker-assisted selection is an efficient approach for developing new cultivars, but the complexity of the faba bean genome remains a challenge.

2.3.3 Content and variation among cultivars

Faba bean can be grown in the varying climates of Europe. In areas that experience only mild frosts during the winter (subtropical areas), such as in the Mediterranean and Atlantic climatic zones, faba bean is grown as an autumn sown crop (Link et al. 2010). In the northern

parts of Europe, it is grown as a spring sown crop. Growing seasons are long enough for flowering and seed maturation. Faba bean is sensitive to drought, especially during early seed development (Jensen et al. 2010). Heavy-textured soils, such as loam and clay soils, are favoured over light sandy soils. Acidic soils may impair nodulation.

The cultivars that are aimed for northern growing conditions require early flowering and maturity (Stoddard et al. 2009). In Finland, the cultivars now in use ('Ukko' and 'Kontu') were introduced in the 1990s, but early maturing vc- cultivars are still lacking (Koivunen et al. 2016). The low vicine-convicine vc- cultivars ('Melodié', 'Divine' and 'Disco') originate from France (Duc et al. 1999; Crépon et al. 2010).

Table 2 The contents of vicine and convicine in faba bean cultivars grown in different origins.

Origin of faba beans, Cultivars (n)	Content (mg/g) Vicine	Convicine	Reference
Denmark, 5	6.9 – 7.7	2.6 – 3.3	(Olsen and Andersen 1978)
Canada, 36	5.1 – 8.0	1.9 – 6.0	(Pitz et al. 1981)
Canada, 78	4.5 – 9.0	1.5 – 5.4	(Gardiner et al. 1982)
France, 10	6.0 – 9.2	1.9 – 4.0	(Quemener et al. 1982)
Egypt, 29	2.9 – 8.7	0.9 – 2.8	(Hussein et al. 1986)
France, 5	5.0 – 6.6	1.4 – 4.3	(Quemener 1988)
France, 918	2.2 – 10.1	0.7 – 9.6	(Duc 1989)
France, vc- , 1	0.4	0.04	
Germany, 20	4.4 – 8.7	1.3 – 3.5	(Wang and Ueberschär 1990)
UK, 56	4.2 – 10.8	0.3 – 5.1	(Griffits and Ramsay 1992)
Netherlands, 6	4.3 – 11.7	1.5 – 5.8	(Helsper et al. 1993)
France, 9	3.4 – 10.4	1.7 – 4.3	(Duc et al. 1999)
France, vc- , 3	0.2 – 0.6	0.1 – 0.2	
Spain, 2	4.2 – 4.7	2.0 – 2.8	(Goyaga et al. 2008)
several origins, 5	5.6 – 7.2	2.8 – 3.7	(Jezierny et al. 2011)
France, vc- , 1	0.3	0	
Mexico, 10	2.9 – 6.1	0.6 – 1.7	(Cardaror-Martinez et al. 2012)
Canada, 8	4.5 – 6.6	1.2 – 6.3	(Purves et al. 2018a)
Canada, vc- , 5	0.1 – 0.6	0.02 – 0.04	
Sweden, 16	6.4 – 7.9	2.2 – 4.4	(Ivarsson and Neil 2018)
Canada, 313	1.8 – 15	1.0 – 8.4	(Purves et al. 2018b)
Canada, vc- , 7	0.5 – 0.9	0.06 – 0.16	

Variation in the contents of vicine and convicine has been rather wide among cultivars, as the contents have ranged from 1.8–15 mg/g and 0.3–9.6 mg/g for wild-type cultivars and 0.1–0.9 mg/g and 0–0.3 mg/g for vc- cultivars, respectively (Table 2). However, not all the cultivars screened are widely used for cultivation. The variation was more pronounced

among cultivars than between two years (Pitz et al. 1981), and the growing years differed when genotypes were screened for four years (Duc et al. 1989).

Correlations in vicine and convicine contents would indicate that their formation is genetically interdependent (Gardiner et al. 1982; Duc et al. 1989). Indeed, interconversion of vicine to convicine by transamination has been considered (Griffiths and Ramsay 1992). However, vicine and convicine are most likely independently synthesised in plants, because correlation in the contents has not been confirmed (Gardiner et al. 1982; Duc et al. 1989; Purves et al. 2018a; Purves et al. 2018b).

2.4 Removal of vicine and convicine by processing

Vicine and convicine are water-soluble and heat-stable compounds, which makes them a challenge to remove. Reduction in the contents of vicine and convicine has been investigated in a few studies that mainly focused on soaking (Hussein et al. 1986; Abd Allah et al. 1988; Jamalian 1999; Jamalian and Ghorbani 2005); cooking, heating and roasting (Cardaror-Martinez et al. 2012); and fractionation (Olsen and Andersen 1978; Sosulski and McCurdy 1987; Vioque et al. 2012; Coda et al. 2015). Certain processing methods, such as enzyme treatments (Arbid and Marquardt 1985b), germination (Goyoaga et al. 2008) and fermentation (McKay 1992; Coda et al. 2015; Rizzello et al. 2016), may induce hydrolysis of the β-glycosidic bond. Hydrolysis causes the disappearance of vicine and convicine, but the simultaneous release of the aglycones. The focus of most studies has been generally on the removal of vicine and convicine, so the release of the toxic aglycones has not been well investigated.

2.4.1 Conventional processing

Soaking The solubility of vicine and convicine in water makes soaking an effective method for reducing their levels and, in practice, removes all vicine and convicine (Table 3). Soaking of bean flours removed vicine and convicine at all tested pH conditions from pH 3.5 to 13 (Jamalian 1999). Continuous soaking in an acidic (1%) solution or water (flow rate 0.5 ml/min, 48–72 h at 50 °C) removed vicine and convicine from whole beans (Jamalian and Ghorbani 2005). Soaking is a traditional and easy processing method, but it is not practical for dry products, and microbes may grow in high-moisture environment. Valuable water-soluble nutrients may also be lost.

Thermal treatments Heating is another traditional processing technique that can improve nutritional quality and digestibility of dry legume seeds by inactivating or eliminating heat-labile antinutrients (Champ 2002; Azarpazhooh and Boye 2013; Patterson et al. 2017). Vicine and convicine tolerate heating relatively well and their levels are reduced by only up to 50% for vicine and 60% for convicine (Table 3). Boiling was more efficient than roasting,

probably due to solubilisation in water and then enhanced exposure to heat (Cardaror-Martinez et al. 2012). The efficiency of roasting and boiling treatments varied among 10 cultivars (Cardaror-Martinez et al. 2012). Food-type preparation of faba beans, including soaking for 12 h at 25 °C, rinsing and cooking for 45 min, yielded vicine losses of 35% and convicine losses of 33% (Khalil and Mansour 1995). Heating should be combined with other processing methods to obtain an optimal reduction of all harmful compounds (Patterson et al. 2017).

Wet and dry fractionation Depending on the fractionation process, the contents of vicine and convicine are either increased or decreased. In air classification, vicine and convicine seem to move along with protein when separated from starch (Olsen and Andersen 1978; Sosulski and McCurdy 1987; Coda et al. 2015). The vicine and convicine contents were 2.6 and 2.2-fold higher in protein concentrates containing 63% protein than in flour (Sosulski and McCurdy 1987). In concentrates that contained 51% protein, the contents of vicine and convicine were 1.4-fold higher than in flour (Coda et al. 2015). Starch-rich fractions also contained vicine and convicine, and the amount was approximately 45% of the contents of flour (Coda et al. 2015). Protein isolates produced by alkaline extraction and precipitation at the isoelectric point at pH 4 were nearly completely free of vicine and convicine (Sosulski and McCurdy 1987; Vioque et al. 2012). The protein content of the isolates was high, at 86–92% (Sosulski and McCurdy 1987; Vioque et al. 2012). Vioque et al. (2012) first washed flours with acetone-water (75:25) to remove polyphenols, and possibly some vicine and convicine was also soaked away that further assisted the removal process.

2.4.2 Bioprocessing

Enzymatic modification Vicine and convicine are known to be hydrolysed by β-glucosidase enzyme, but this enzyme has only been used in a food system by Arbid and Marquardt (1985b), who added almond β-glucosidase or almond powder to faba bean paste. The hydrolysis was efficient only when the pH was adjusted to pH 5 with lemon juice; however, at the optimal pH for the enzyme, 90% of the vicine and convicine were lost in a relatively short time (Arbid and Marquardt 1985b). Arese and De Flora (1990) stated that faba bean had endogenous β-glucosidase activity, although this claim was not supported in other studies (Arbid and Marquardt 1985b; McKay 1992). Rizzello et al. (2016) detected β-glucosidase activity in faba bean flours; however, the endogenous β-glucosidase of faba bean has not been studied thoroughly.

Germination Vicine is mainly located in the embryo, where it may have a protective role in the growing seed (Griffiths and Ramsay 1996; Goyoaga et al. 2008). During germination, vicine and convicine were either almost totally lost or the contents decreased 30–50% (Table 3). The most efficient reduction was observed in the whole seeds that were germinated for 7 days at low temperature (17 °C) (Jamalian 1999). Germination for 3 days at 25 °C was less efficient for the reduction of vicine and convicine (Khalil and Mansour 1995). However,

long soaking (12–16 h) before germination may have also reduced the contents of vicine and convicine. The vicine and convicine contents decreased by 30–40% and 50%, respectively, in the cotyledons during germination for 9 days (Goyoaga et al. 2008). The greatest change was noticed in the embryos when they were separated from germinated cotyledons, because the vicine levels markedly dropped (from 51–57 mg/g to 10 mg/g) and the convicine levels increased (2-fold higher) during germination. However, the relative size of the embryo is small, accounting for about 1% of the total weight of the cotyledon.

Fermentation Production of a β-glucosidase enzyme is the key factor in removal of vicine and convicine in a fermentation process. *Lactobacillus plantarum* has been grown efficiently in faba bean suspensions and enabled the hydrolysis of vicine and convicine (McKay 1992; Coda et al. 2015; Rizzello et al. 2016). Vicine and convicine were almost completely lost in a long (48 h) fermentation of faba bean with *L. plantarum* (McKay 1992; Coda et al. 2015). The *L. plantarum* strain used in fermentation was proved to have an active β-glucosidase (Di Cagno et al. 2010; Rizzello et al. 2016). The β-glucosidase enzymes produced by lactic acid bacteria (LAB) are most likely intracellular, and they may be related metabolisation of cellobiose (McKay 1992; Michlmayr et al. 2010). Production of extracellular β-glucosidase is also possible, as the β-glucosidase enzyme produced by *Aspergillus oryzae* completely eliminated vicine and convicine from faba bean suspensions (McKay 1992). Filamentous fungal biomass may also produce vicine- and convicine-hydrolysing β-glucosidases. Fermentation has provided good results, but using fermentation for enzymatic hydrolysis is not straightforward, when the mechanisms underlying the production of β-glucosidase depend on multiple factors. Acceptable sensory quality is an important criterion for food, so it too must be taken into account when developing fermented products.

Table 3 Elimination of vicine and convicine from faba bean by different treatments and their efficiencies.

Treatment	Details	Type	Time (h)	Temperature (°C)	Removal (- %) Vicine	Convicine	Reference
Dry fractionation	air classification	protein concentrate			0 (+)	0 (+)	(Sosulski and McGurdy 1987; Coda et al. 2015)
Heating	boiling	flours	0.33	121	30	61	(Cardaror-Martinez et al. 2012)
	roasting		0.17	120	12	40	
	autoclaving, after soaking (48 h)		0.5	121	50	50	(Jamalian and Ghorbani 2005)
Soaking	acid or alkaline (0.01 M), water		4	25	94–100	100	(Jamalian 1999)
Continuous soaking	1% acid (flow 0.5 ml/min)	whole beans	72	50	100	100	(Jamalian and Ghorbani 2005)
	water (flow 0.5 ml/min)	whole beans	48	60	100	100	
			72	50	100	100	
Wet fractionation	alkaline extraction (pH 10.5)	protein isolate			99	99	(Vioque et al. 2012)
Germination	after soaking	whole seed	168	17	84	100	(Jamalian 1999)
	after soaking	whole seed	72	25	28	30	(Khalil and Mansour 1995)
		cotyledons	216	20	30–40	50	(Goyoaga et al. 2008)
Fermentation	*Lactobacillus plantarum*	suspension	48	30	100	75	(McKay et al. 1992)
	Fusarium graminearum		72	25	100	100	
	Aspergillus oryzae		2	60	100	100	
	L. plantarum	sourdough	48	30	95	91	(Coda et al. 2015)
Food	almond powder + lemon juice	faba bean paste	3	30	90	90	(Arbid and Marquardt 1985b)

3 Aims of the study

Faba bean is a grain legume that can be grown in the boreal conditions of Europe. Faba bean has a good nutritional value, with the main advantage being its high protein content (25–30%). Cultivation and consumption of faba bean is still minor when compared to cereals, even though interest is growing in legumes. Plant breeding techniques have not yet totally removed vicine and convicine from faba bean. The amounts of vicine and convicine can be reduced by processing, but this requires further research.

Bioprocessing enables the effective removal of vicine and convicine by utilising endogenous or added enzymes or fermentation. However, hydrolysis of vicine and convicine in these kinds of processes leads to the release of the toxic aglycone forms, divicine and isouramil. Little is known regarding the fate of the aglycones in real food systems. The overall aim of the study was therefore to study possibilities for eliminating vicine, convicine and the aglycones by food processing based on the hydrolysis of vicine and convicine.

The more detailed objectives of the individual studies were:

1. To introduce a method for analysing vicine, convicine and the aglycones from faba beans and processed materials. A practical quantification method was to be implemented for vicine and convicine using internal standard **(I-III)**.

2. To study vicine and convicine levels and their variation in faba bean cultivars grown in Finland in different harvesting years **(I)**.

3. To induce enzymatic hydrolysis of vicine and convicine in selected models to observe the formation and stability of the aglycones **(II)**.

4. To investigate the losses of vicine and convicine and the simultaneous formation and stability of the aglycones in model conditions and during fermentation by lactic acid bacteria (LAB) and in baking processes **(II, III)**.

4 Materials and methods

4.1 Materials

4.1.1 Faba beans

The faba bean cultivars were grown at the Viikki Experimental Farm of the University of Helsinki in the years 2010–2015. Detailed information on the cultivation is given by Lizarazo et al. (2015). The cultivars are presented in Table 4. The vicine and convicine contents in the cultivars from year 2011 were compared in study I. Four cultivars ('Alexia', 'Fatima', 'Kontu' and 'SSNS-1') were selected to study the effect of growing year (2010–2012) on the vicine and convicine contents. The same cultivars, from years 2011 and 2015, were used in study III for the incubation experiments. In studies I and III, the beans were not dehulled before analysis, whereas dehulling was done prior to vicine and convicine isolation in study II for the cultivar 'Kontu' from the year 2011. All faba beans were first pooled and then milled with a 0.5 mm sieve in a centrifugal mill (ZM200; Retsch, Haan, Germany).

Table 4 List of faba bean cultivars, cultivar type and growing years.

Cultivar	Growing years	Cultivar type	Used in study (I, II, III)
Alexia	2010, 2011, 2012, 2015	wild	I (2010–2012), III (2011, 2015)
Aurora	2011	wild	I
Babylon	2011	wild	I
Disco	2010	vc-	I
Divine	2011, 2012	vc-	I
Fatima	2010, 2011, 2012, 2015	wild	I (2010–2012), III (2011, 2015)
GLA 1103	2011	wild	I
Kontu	2010, 2011, 2012, 2015	wild	I (2010–2012), II (2011), III (2011, 2015)
Melodié	2010, 2011	vc-	I
SSNS-1	2010, 2011, 2012, 2015	wild	I (2010–2012), III (2011, 2015)
Taifun	2011	wild	I
Tangenta	2011	wild	I
Witkiem Manita	2011	wild	I

Faba bean flour from the cultivar 'Kontu' from the year 2011 was also used as an in-house reference sample during the study. A commercial Italian faba bean flour (Cereal Veneta,

San Martino di Lupari, PD, Italy), called Flour_It, from the year 2014, was used in study III for incubations and in the fermentation and baking experiments.

4.1.2 Reagents and micro-organisms

MilliQ water (Millipore Corporation, Bedford, MA, USA), acetone, ethanol (Altia) and perchloric acid (70–72%, Merck KGaA, Darmstadt, Germany), KOH, HCl, NaOH, sodium phosphate, acetic acid and sodium acetate were used in extraction solutions. Acetonitrile and formic acid were used in HPLC analysis. The reagents used in hydrolysis were β-glucosidase (EC 3.2.1.21, from almonds, Sigma Aldrich, Israel) and (+)- sodium L-ascorbate (purity ≥ 99%, Sigma Aldrich, USA). The standards purchased were vicine (≥ 98%, Cfm Oskar Tropitzsch GmbH Germany) and uridine (≥ 99%, Sigma Aldrich, USA).

Three LAB strains were used for preparation of sourdoughs. *Lactobacillus plantarum* DPPMA B24W/VTT E-133328 (B24W), *L. plantarum* VTT E-78076 (E76) and *Pediococcus pentosaceous* I02 (I02) from different culture collections had been used earlier for the fermentation of faba bean and cereals and were confirmed as β-glucosidase positive.

The β-glucosidase activities of the strains B24W, E76 and I02 were 0.043 U ± 0.001 U, 0.021 U ± 0.000 U and 0.028 U ± 0.000 U, respectively. One unit (U) is defined as the activity that releases 1 μmol of p-nitrophenol from the substrate p-nitrophenyl-β-D-glucopyranoside (pNPG) per ml per min. The values were measured as described by Di Cagno et al. (2010).

4.2 Analysis of vicine, convicine and their aglycones

4.2.1 Preparation of the convicine standard (I)

No convicine standard was commercially available; therefore, it was isolated from a protein-rich fraction prepared by an air-classification method after dehulling and milling of faba bean seeds (cultivar 'Kontu'). The extraction was carried out until crystallisation step according to the study of Marquardt et al. (1983). Precipitate containing both vicine and convicine was redissolved and convicine was separated by automated preparative HPLC-UV/MS (Waters Corp., Milford, MA, USA).

The preparative HPLC equipment was equipped with a photodiode array (PDA) (Waters 2996) and a single quadrupole mass spectrophotometric (MS) detector (Waters micromassZQ). Convicine was separated on a C18 Atlantis prepT3 column (250 × 10 mm; 5 μm). The compounds were eluted with MilliQ-water containing 0.1% formic acid (eluent

A, pH 2.7) with a flow rate of 3.5 ml/min. Acetonitrile containing 0.1% formic acid (eluent B) was used in gradient washing, and the total run time, with stabilisation, was 40 min. Formic acid (0.1%) was required in the preparative HPLC system to protonate convicine. The flow was split before the detectors with an active flow splitter at a 1:100 ratio. The m/z value of convicine as the protonated $[M+H]^+$ ion, m/z 306, was used for detection of convicine to initiate collection. The UV absorption was also monitored at 273 nm. The injection volumes were 500–950 µl. The system was operated and data were collected using MassLynx 4.1 software (Waters Corp., Milford, MA, USA).

4.2.2 Extraction of vicine, convicine and their aglycones (I, III)

Development of extraction procedure (I) Vicine and convicine were extracted with 7% PCA, using a protocol modified from the study of Marquardt and Frohlich (1981) or with MilliQ-water as described by Quemener (1988). Water extracts were adjusted to pH 4 with 1 N HCl to precipitate proteins. Faba bean flour (cultivar 'Kontu' from 2011) was used for comparison of the extraction conditions. A 0.5 g sample of flour was weighed into a centrifuge tube and 1 ml of uridine (8 mg/ml), used as an internal standard, was added. The extraction solution was added either once (15 ml) or in two (2 × 7.5 ml) or three cycles (3 × 5 ml) to test the extractability. The flour was extracted by mixing with a vortex mixer for 1 min and then centrifuging at 13 000 g for 10 min at 4−8 °C. The extracts were collected, filtered (0.45-µm GH Polypro, GHP, filter; Acrodisc Pall, Cornwall, UK) and injected into the HPLC system.

Selected extraction conditions (I, III) Three repeated (3 × 5 ml) extractions with 7% PCA were used as the final extraction protocol. Extraction with water was applied for the aglycone analysis of sourdoughs, doughs and breads by weighing 0.1–0.3 g sample and vortexing with 10 ml of MilliQ-water. Solutions were filtered through Amicon Ultracel® 0.5-ml filters (Merck Millipore, Darmstadt, Germany) to remove high-molecular-weight substances.

4.2.3 Optimisation and implementation of the HPLC-UV method (I, II, III)

Selection of HPLC conditions (I) Vicine and convicine were analysed in studies I and II with an analytical HPLC system equipped with a PDA detector (Waters, Milford, MA, USA). In later experiments, an Agilent 1200 instrument (Agilent Technologies, Santa Clara, CA, USA) with a diode array detector (DAD) was used.

The compounds were separated with an Atlantis T3 C18 column (100 × 4.6 mm, 3 µm; Waters, Milford, USA) equipped with a guard column (20 × 4.6 mm). In study I, HPLC conditions were optimised by testing selected factors (i.e. eluent composition and detection wavelength). For eluents, MilliQ-water containing 0.1% formic acid was compared to pure

MilliQ-water. Similarly, the C18 column was washed with either acetonitrile containing 0.1% formic acid or acetonitrile alone. During the analyses, the column was kept at 30 °C at a flow rate 0.8 ml/min. Selected chromatographic parameters (retention factors k', resolution Rs and separation factor α) were determined with an in-house reference sample for both eluents. Detection responses for vicine, convicine and uridine were compared with both tested eluent compositions at 273 nm and at 280 nm. Calibration curves were also constructed at both wavelengths. UV absorption was measured from 210–400 nm with a Waters system for identification purposes. Waters Empower 2 software or Agilent 1200 online software were used for data collection. The peak areas were recorded as μV × sec in the Waters software, and as mAu × sec in the Agilent software.

Applied conditions and quality parameters (I) A mobile phase containing 0.1% formic acid was selected for analysis of vicine and convicine with detection wavelength 273 nm. Both gradient and isocratic runs were used in studies. An isocratic run followed by a gradient step was used in the experiments of study I and III. The studied compounds eluted in 10 min, and the total run time was 35 min, including a washing step with acetonitrile up to 70%, followed by stabilisation. In study II, only a 15 min isocratic run was used for extracts and the vicine and convicine fractions. An isocratic run for 30 min was used to study the aglycones in suspensions in study II and in the sourdoughs, doughs and breads in study III.

The limits of detection and determination were determined with uridine solutions in 7% PCA. Linearity was tested in conjunction with solutions containing from 250 ng to 7.5 μg uridine. The linearities of the vicine and convicine responses were tested using 0.125–5 μg and 0.043–1.7 μg, respectively. The in-house reference sample (section 4.1.1) was used in a repeatability test and in a recovery test for uridine.

Identification of vicine and convicine (I, II) The identities of vicine and convicine were first confirmed by LC-ESI-MS with UV detection from the extracts obtained using the analysis method in study I. The extracts were diluted and acidic extracts were neutralised, and then filtered prior to injection. The purity of the isolated convicine standard was also confirmed with the LC-ESI-MS method. The identities of the aglycones were also confirmed from the vicine and convicine fractions in study II. The HPLC system (HP/Agilent 1100 HPLC system; Santa Clara, CA, USA) was coupled with a PDA detector and with a Bruker Esquire quadrupole ion trap MS detector (QIT-MS, Bremen, Germany). An Atlantis T3 C18 column (100 × 2.1 mm) with particle size of 5 μm was used to separate compounds at 25 °C at a flow rate of 0.2 ml/min. The HPLC conditions were similar as described above.

An ESI interface was used in the positive ion mode for vicine and convicine in study I and for the aglycones in study II at a scanning range of m/z 100−400. The MS parameters were first optimised with a vicine solution (0.04 μg/μl, in water containing 0.1% formic acid) using direct injection. The conditions after optimisation were: nebuliser (nitrogen) 60.0 psi,

dry gas (nitrogen) 8.0 l/min, dry temperature 350 °C, capillary 4500 V, end plate offset 500 V, trap drive 37.0, skim 1 15.0 V and skim 2 6.0 V.

Total ion chromatograms were recorded for the extracts, the convicine standard and the vicine and convicine fractions in the full scan MS mode. UV absorption was additionally followed at wavelengths of 210 and 273 nm. Protonated molecular ions of vicine and convicine were also fragmented with tandem mass spectrometry (MS/MS) for the extracts and for the convicine standard. Data were processed with Bruker Daltonics Data Analysis software.

Quantification of vicine and convicine (I, III) In study I, the contents of vicine and convicine were quantified with an internal standard method. The standard solutions (in 7% PCA) were prepared at a range of 0.0125−0.50 µg/µl and 0.0043−0.17 µg/µl for vicine and convicine, respectively, with a constant level 0.25 µg/µl of uridine. The relative response factors were calculated from linear regression equations in which the area ratios were plotted against the amount ratios. In study III, standard solutions of vicine ranged from 0.015–0.3 µg/µl, and the constant level of uridine was 0.3 µg/µl. Convicine content was calculated from the standard curve of vicine in study III. The in-house reference sample was used to follow daily analytical levels of analysis in every sample set (in studies I and III). For study II, the analytical levels were monitored with a uridine solution (0.25 mg/ml).

The concentrations of vicine, convicine and uridine standards were confirmed spectrophotometrically using molar absorption coefficient values. The molar absorption coefficient values were 16 400 l mol^{-1} cm^{-1} at 274 nm (pH 1) for vicine (Bendich and Clements 1953), 17 400 l mol^{-1} cm^{-1} at 271 nm (pH 1) for convicine (Bien et al. 1968) and 9800 l mol^{-1} cm^{-1} at 262 nm (pH 7) for uridine (Ploeser and Loring 1949). Vicine and convicine powders were dissolved in 7% PCA and diluted to concentrations of 10 µg/ml and 5 µg/ml for vicine and convicine, respectively. Uridine powder was dissolved in MilliQ-water to a concentration of 5 µg/ml. The absorbance was measured at 271−275 nm with a spectrophotometer (Lambda 25 UV/Vis, Perkin Elmer Inc., USA). For convicine, the purity of the isolated powder was calculated at a maximum wavelength at 273 nm. The purity of the isolated convicine was 66.9%, whereas the purities of purchased vicine and uridine were 100.8% and 101.2%, respectively. The standard curves of convicine were corrected according to the obtained purity.

Analysis and identification of the aglycones (II, III) The aglycones were detected in studies II and III in a simultaneous run with vicine and convicine under the selected HPLC conditions described above and using the same detection wavelength of 273 nm. Detection at a wavelength of 210 nm was also used to monitor for further degradation products. Samples for the aglycone analysis were injected into the HPLC directly after preparation.

Divicine and isouramil were identified from hydrolysed vicine and convicine fractions according to their UV and MS spectra in study II. For the LC-UV-MS analysis, the vicine and convicine fractions were hydrolysed for 15 min at pH 3. After hydrolysis, the samples were filtered and injected into the LC-UV-MS. The aglycones were separated by an isocratic HPLC run for 15 min. The MS detection was conducted under the conditions described above.

4.3 Enzymatic hydrolysis of vicine and convicine to their aglycones

4.3.1 Hydrolysis with added β-glucosidase (II)

Faba bean extract containing vicine and convicine was prepared and used for hydrolysis with β-glucosidase to release their respective aglycones, divicine and isouramil. The extract was also used to produce fractions containing only vicine or convicine, which were also hydrolysed. The hydrolysis and the formation of the aglycones was also studied in suspensions. The stability of the aglycones was studied in all sample types, as presented in Figure 5.

Both the hydrolysis of vicine and convicine and the formation of divicine and isouramil were monitored simultaneously. The formation of an oxidised form of divicine and indications of further degradation products at lower wavelengths were also observed.

Vicine and convicine extracts were prepared from dehulled and milled faba beans with an isolation method modified from Arbid & Marquardt (1985a) and Quemener (1988). Faba bean flour was mixed with ethanol-water (70:30, v/v) and the mixture was centrifuged at 10,000 g and filtered. The supernatant was concentrated, the pH was adjusted to 4 to precipitate proteins and the solution was centrifuged. The obtained extract was directly used for hydrolysis experiments, as well as diluted with MilliQ-water (1:10) for separation of vicine and convicine in preparative LC-MS. Conditions used in preparative HPLC were same as presented earlier (section 4.2.1), except both vicine and convicine were collected. The collection was based on the m/z values of vicine and convicine as protonated $[M+H]^+$ ions. The suspensions were prepared by mixing 0.2 g of dehulled faba bean flour with 10 ml of 0.05 M sodium phosphate buffer (pH 5).

Different levels of β-glucosidase from almonds (5 units/mg of activity) were used for hydrolysis of vicine and convicine in the extracts, fractions and suspensions. Enzymatic hydrolysis was carried out at pH 5 in 0.05 M sodium phosphate buffer at 37 °C in a water bath. Three levels of activity were tested for the extracts and for the vicine fractions (Figure 5). The convicine fractions and suspensions were hydrolysed with one selected level of

activity. Control samples from buffer, enzyme and substrate solution were also prepared. The extracts and fractions were injected into the HPLC directly after incubation, but the suspensions were filtered through Amicon Ultracel® filters prior to injection (section 4.2.3).

The stability of the aglycones after enzymatic hydrolysis was monitored for 60 min for extracts and 120 min for vicine fractions and convicine fraction (Figure 5). The time points were 0, 15, 30, 45, 60, 90 and 120 min. The suspensions were hydrolysed from 15 min up to 360 min (6 h). Changes in the amounts were compared as peak areas.

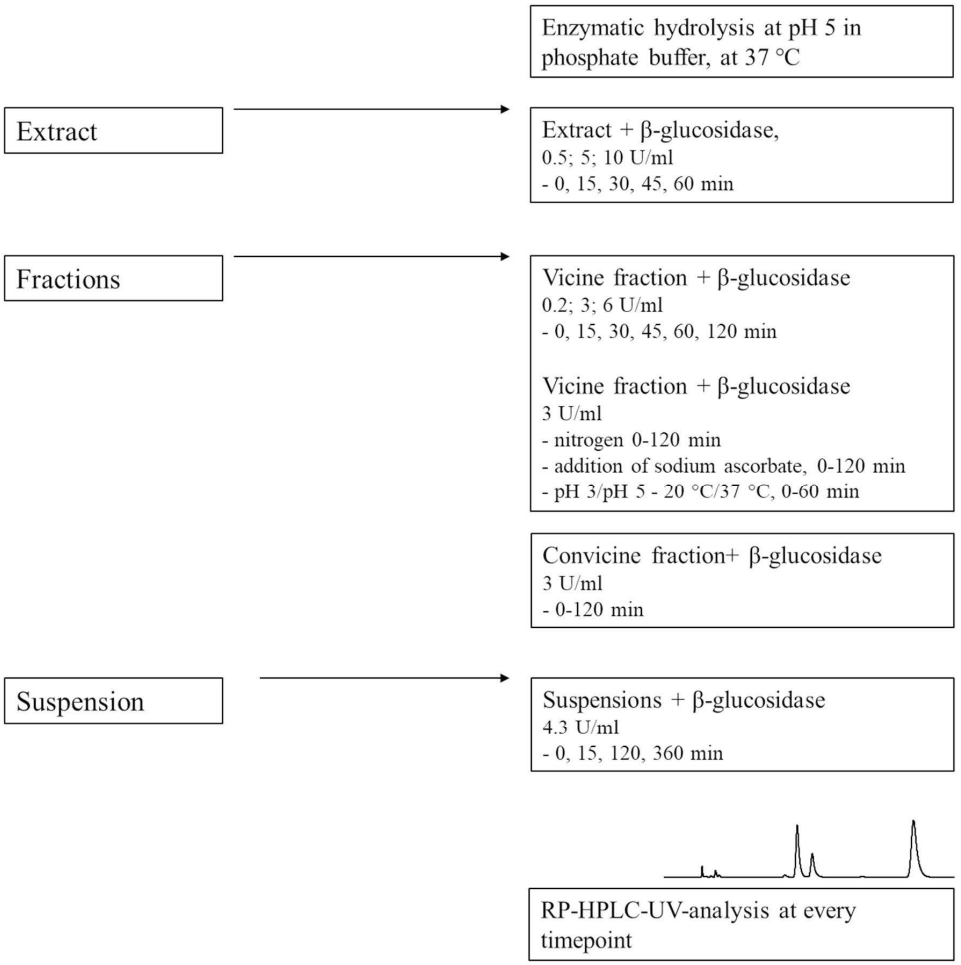


Figure 5 Protocol for study II. Enzymatic hydrolysis was carried out on extracts, fractions and suspensions at the conditions described.

The stability of divicine was also studied in vicine fractions under stabilising conditions (Figure 5) using several selected factors aimed at altering the stability of divicine. Air was replaced with nitrogen in test tubes by purging them for 1 min. The effect of a reducing agent (i.e. sodium ascorbate; 100 µg/ml in solution) was also tested. The total incubation time was 120 min in these experiments. The effects of pH and temperature (two temperatures and two pH values) on the stability of divicine were also evaluated for 60 min. The combinations of pH and temperature were pH 3.0, 20 °C; pH 3.0, 37 °C; pH 5.0, 20 °C; and pH 5.0, 37 °C. In contrast to earlier incubation experiments, the vicine solution was first hydrolysed for 15 min at 37 °C at pH 3, and then the solution was diluted to adjust pH, divided into test tubes, and incubated at either 20 °C or 37 °C. Incubations were continued for 24 h.

4.3.2 Determination of β-glucosidase activity from faba beans (III)

Faba bean suspensions were incubated at different pH values to see if vicine and convicine are hydrolysed by endogenous β-glucosidase activity (Figure 6). In suspensions, 0.5 g of flour was mixed with 5 ml of either buffer (0.1 M sodium acetate buffers) or MilliQ-water. The suspensions were incubated at 30 °C and 37 °C for short incubations of 4 h or at 37 °C for long incubations of 24 h. Cultivars from two years were tested at pH 5 and 37 °C for 4 h (Figure 6). The incubated suspensions were compared to the non-incubated ones. In addition, the flours of the cultivar 'Kontu' and Flour_It were autoclaved and suspensions were prepared from autoclaved flours for long incubations as a control for native flours.

After incubation, the suspensions were acidified with PCA for deproteinisation. The suspensions incubated at pH 1 and 2 were not acidified further. The suspensions were then centrifuged, diluted to a ratio of 1:50 and filtered prior to HPLC analysis with the method described above (section 4.2.3).

4.3.3 Analysis of vicine and convicine in fermented doughs and breads (III)

Fermentation conditions Before fermentation, the LAB strains (I02, E76 and B24W) were propagated using routine culture conditions, as described by Coda, Varis et al. (2017). Faba bean flour and tap water were mixed in a ratio of 40:60 (w/v) to prepare sourdoughs. Cell densities (log cfu/g) for the LAB were calculated using DeMan-Rogosa-Sharpe agar medium. The initial cell density of the LAB cells was approximately log 6.0 cfu/g of dough. Fermented sourdoughs were used for wheat composite breads made from flours containing 30% faba bean flour. The doughs were raised in total for 1 h at 30 °C, and baked at 200 °C for 15 min. Four different types of breads were obtained, including three fermented and one control bread. The control bread contained non-fermented faba bean flour instead of sourdough.

Determination of losses of vicine, convicine and their aglycones The contents of vicine and convicine were analysed by the described HPLC analysis method (sections 4.2.2, 4.2.3). The results were calculated as losses of vicine and convicine by comparing the treated samples to non-treated ones. The losses were presented as percentage values (%).

For the aglycone analysis, the presence and stability of the aglycones was monitored in selected sourdoughs, doughs and breads. The stability of the aglycones was also evaluated in extracts of sourdoughs after 0.5, 2 and 20 h of incubation at room temperature.

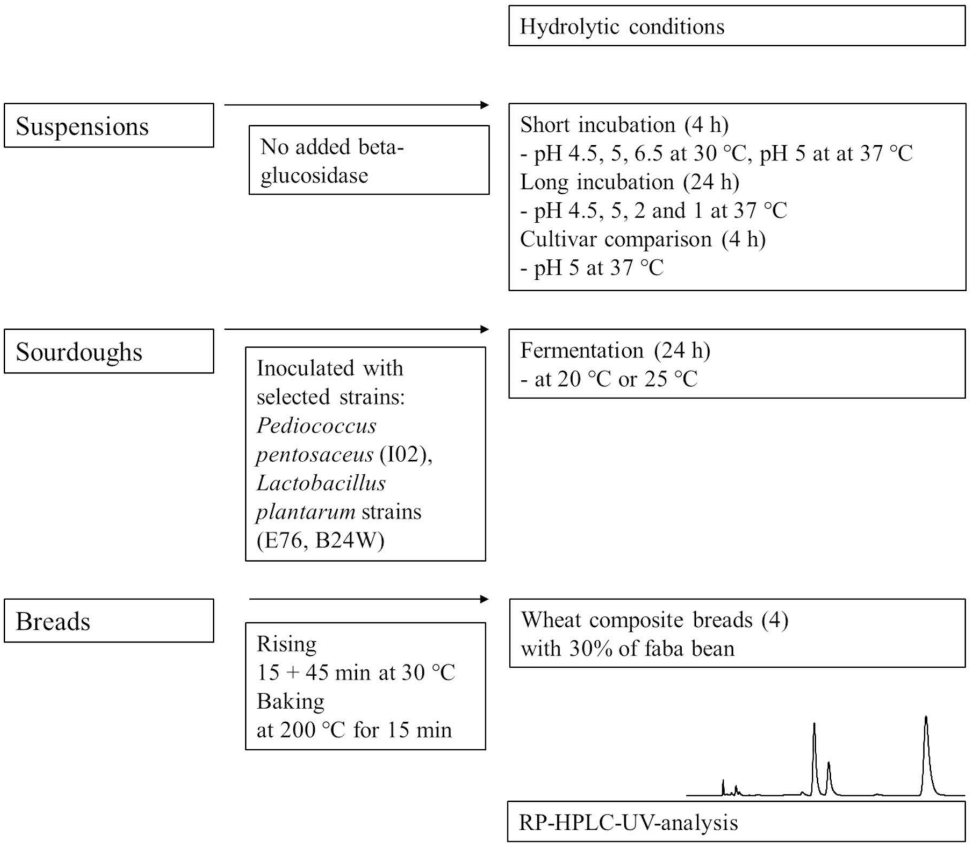


Figure 6 Scheme of study III. The hydrolysis of vicine and convicine was first studied at different conditions and finally in sourdoughs and breads. The aglycones were additionally analysed from sourdoughs, doughs after rising, and breads.

Determination of acidification parameters from sourdoughs and breads A titrimetric method was used for determination of the pH and total titratable acidity (TTA) values. Thawed

sourdoughs and breads were suspended in water (at a 1:10 ratio, w/v) and titrated with 1 N NaOH in Mettler Toledo EasyPlus Titration with a pH meter (Mettler Toledo, DGi115-SC) to pH 8.5. The pH value before titration was also recorded.

Lactic acid content was determined with an HPLC-UV method from sourdoughs fermented at 20 °C. Sourdough samples were centrifuged at 10,000 g, diluted and filtered before injecting into the HPLC system. The Agilent Hi-Plex H (300 × 7.7 mm, 8 µm; Agilent Technologies, Santa Clara, CA, USA) column was used at 50 °C at a flow rate of 0.4 ml/min and an eluent of 5 mM sulphuric acid. A lactic acid kit (Megazyme, Ireland) was used for sourdoughs fermented at 25 °C. Sourdough samples were centrifuged and diluted (1:50-100, v/v) before use.

4.4 Data analysis

Treatments were mainly done twice. The analyses were carried out with three replicates for most of the analyses and with two replicates for the aglycone and lactic acid analyses. The results were reported mainly as averages with standard deviations, but for the aglycone analyses with two replicates, the differences between replicates were presented.

Statistical differences among cultivars and treatments were evaluated by one-way analysis of variance (ANOVA) in studies I and III. Fischer's least significant difference (LSD) tests were used to differentiate samples. Two-way ANOVA was used for evaluation of both cultivar and year variation in study I. Data were analysed using either Statgraphics Centurion IV software (Statpoint Technologies. Inc., USA) or SPSS (SPSS version 22.0, IBM SPSS Statistics, Chicago, IL). Differences were considered statistically significant when $p < 0.05$. For the standard curves, simple linear regression was used.

5 Results

5.1 Determination of vicine, convicine and their aglycones (I, II, III)

Vicine and convicine were determined with an RP-HPLC-UV method in studies I, II and III, and their aglycones were determined in studies II and III. The RP-HPLC-UV method was optimised for separation and quantification of vicine and convicine, and it was further applied for the aglycone forms.

5.1.1 Comparison of the extraction conditions (I)

Extraction was first optimised by testing two different extraction solutions (water and 7% PCA) with the extraction solution (15 ml) divided to 1–3 parts. Comparison showed that difference in extraction efficiency between acidic and water extraction was rather small. The internal standard uridine was extracted similarly with both extraction solutions. Vicine was slightly better extracted in acidic media than in water, while convicine favoured water. The peak areas of vicine were 8−12% higher in acidic media than in water, while the peak areas of convicine were 7−14% higher in water than in acidic media in all three compared extraction options.

Peak areas of vicine, convicine and uridine were slightly increased when the extraction solvent was divided into 2–3 parts. The peak areas showed no notable increases when the extraction was repeated three times (5 ml of solvent) rather than twice. Repetition in the acidic extraction mostly improved the extractability of convicine, as 15% more convicine was obtained when the extraction was repeated three times rather than once. The peak areas of uridine were also higher (ca 10%) following repeated extractions.

Based on these findings, extraction with 7% PCA was selected as the final method. Three repeated extractions were selected as the final extraction method, because the extractability of convicine was slightly improved.

5.1.2 Optimisation of the HPLC-UV method (I)

The HPLC-UV method was optimised in terms of eluent composition and detection wavelength. Chromatographic parameters and quality parameters were determined to evaluate the analysis method. Samples were quantified against an internal uridine standard and relative response factors were established.

The retention times were 5.40 ± 0.02 min, 6.05 ± 0.02 min and 10.30 ± 0.04 min for vicine, convicine and uridine, respectively, with water containing 0.1% formic acid as an eluent (A). Even though vicine and convicine eluted close to each other, the values for resolution and separation factor were good, 2.67 and 1.19, respectively. Retention factors were 2.51, 2.93 and 5.69 for vicine, convicine and uridine, respectively. The dead volume elution time, analysed with $NaNO_3$, was 1.54 min. The retention times were slightly longer when water was used as an eluent (B), at 6.20 min ± 0.01 min, 6.76 min ± 0.02 min and 10.55 min ± 0.02 min for convicine, vicine and uridine, respectively. Convicine also eluted before vicine with a water as an eluent. Retention factors with this eluent (B) were 3.03, 3.39 and 5.85 for convicine, vicine and uridine, respectively. Resolution and separation factors for vicine and convicine were 2.07 and 1.14, respectively, with eluent (B). Eluent (A) was chosen for use due to the buffering capacity of formic acid and because addition of acid was required for protonation in the LC-MS analyses. Even though the retention times were 1.5 min and 0.15 min shorter for vicine and convicine, respectively, with eluent (A) than with (B), the difference was not significant for analysis.

After selection of the eluent composition, the detection wavelength was chosen. When vicine and convicine were analysed under the selected conditions, the absorption maxima were recorded as 275, 273 and 261 nm for vicine, convicine and uridine, respectively. The wavelengths considered for detection were 273 nm and 280 nm. The wavelength selection mostly affected the response of the internal standard uridine; its response was lower at 280 nm than at 273 nm. The peak areas were 50%, 92% and 75% at 280 nm when compared to the peak areas at 273 nm for uridine, vicine and convicine, respectively. The decision was made to detect vicine, convicine and uridine at 273 nm. A PDA detector was also used for collection of data from 210 nm to 400 nm for identification purposes. An HPLC chromatogram showing separation of vicine, convicine and uridine with the selected eluent (A) and detection wavelength (273 nm) is presented in Figure 7. The UV absorption maxima of the studied compounds are also presented.

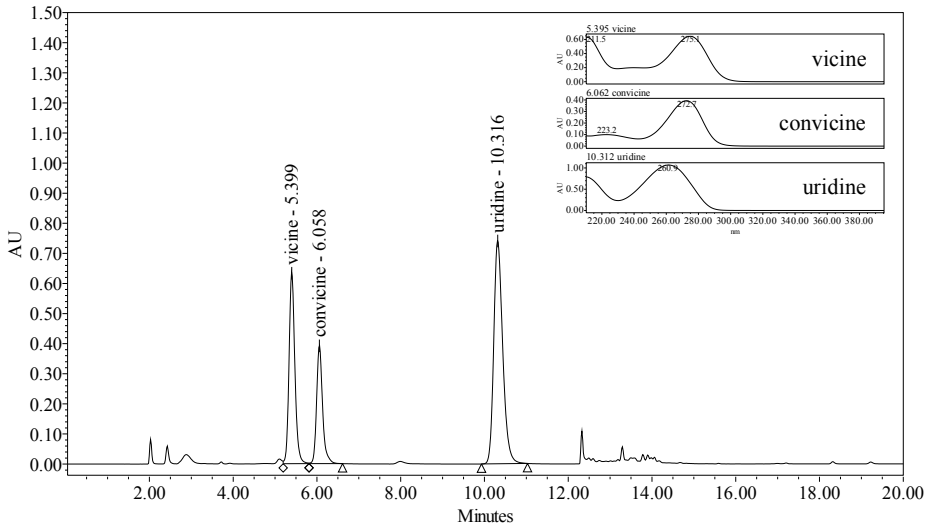


Figure 7 An HPLC chromatogram (λ = 273 nm) of vicine, convicine and uridine in a faba bean extract using an eluent of water containing 0.1% formic acid for separation and acetonitrile containing 0.1% formic acid for a gradient wash. Injection volume was 10 µl. UV spectra of each compound are shown in the upper right inset.

Analysis of the vicine and convicine extracts with the internal standard by LC-MS revealed the protonated $[M+H]^+$ ions of vicine, convicine and uridine at m/z 305, m/z 306 and m/z 245, respectively, for each particular analyte in the total ion chromatograms. The $[M+H]^+$ ions of vicine and convicine were further fragmented, yielding fragment ions $[M+H-162]^+$ m/z 143 and $[M+H-162]^+$ m/z 144 for vicine and convicine, respectively. The presence of $[M+H-162]^+$ in the MS spectra indicated the release of the glucose unit from vicine and convicine.

Calibration curves were calculated from the responses of vicine and convicine at both 273 and 280 nm. Depending on the selected wavelength, the slopes for the calibration curves differed slightly, due to the lower response of uridine at 280 nm than at 273 nm. The correlation coefficients (R^2) for the standard curves for vicine and convicine were 0.9857–0.9999 and 0.9864–0.9970, respectively. The slopes of the curves were considered as relative response factors since the intercepts of calibration curves were close to zero. The relative responses were 1.53 and 1.63 at 273 nm and 2.78 and 2.42 at 280 nm for vicine and convicine, respectively.

Internal standard uridine was used in evaluation of some quality parameters. The values for the limits of detection and determination defined by uridine were 0.2 ng and 0.6 ng, respectively. Uridine showed a linearity from 50 ng up to 7.5 µg/injection, and the correlation coefficients (R^2) for the curves were 0.9995–0.9997. The recovery of added

uridine in extracts was good, at 99.9%–102.8% on separate days. The retention times were constant within each day; however, slight variation in retention times occurred between days over the long term. The repeatability of the method was good, because the relative standard deviation values (RSD%) over a long time period for the in-house reference sample were 3.1% and 6.6%, respectively, for vicine and convicine contents.

5.1.3 Applicability of the method for analysis of the aglycones and further products (II)

After optimisation of the method for vicine and convicine, the method was further applied for the detection of the aglycones. The aglycones were identified according to their UV spectra, as well as by LC-MS.

The aglycones (divicine and isouramil) eluted earlier than vicine and convicine in the HPLC conditions used (Figure 8). The retention times were 2.16 ± 0.04 and 2.50 ± 0.03 min for divicine and isouramil, respectively. The corresponding retention factors were 0.4 and 0.6. The absorption maxima for divicine and isouramil were 282 nm and 280 nm, respectively.

In addition to divicine and isouramil, indications of further products were also observed. A new peak was observed at 1.75 ± 0.04 min in the chromatograms of the vicine fractions and extracts (Figure 8). This peak was considered to be oxidised divicine, because it was related to the formation of divicine. The absorption maximum was at 262 nm, which was lower than the maximum for divicine. Some indications of further reactions products were seen at 1.9 min, because a peak with UV absorption was detected at low wavelengths (< 230 nm). No peaks were seen at the beginning of the chromatograms in the controls of the vicine and convicine fractions, but an additional peak at 2.32 ± 0.02 min from faba bean extract was noticed. However, this peak did not interfere with the detection of divicine and isouramil. Apparently, the sodium phosphate buffer used in the suspensions caused a small peak at 1.7 min at 273 nm.

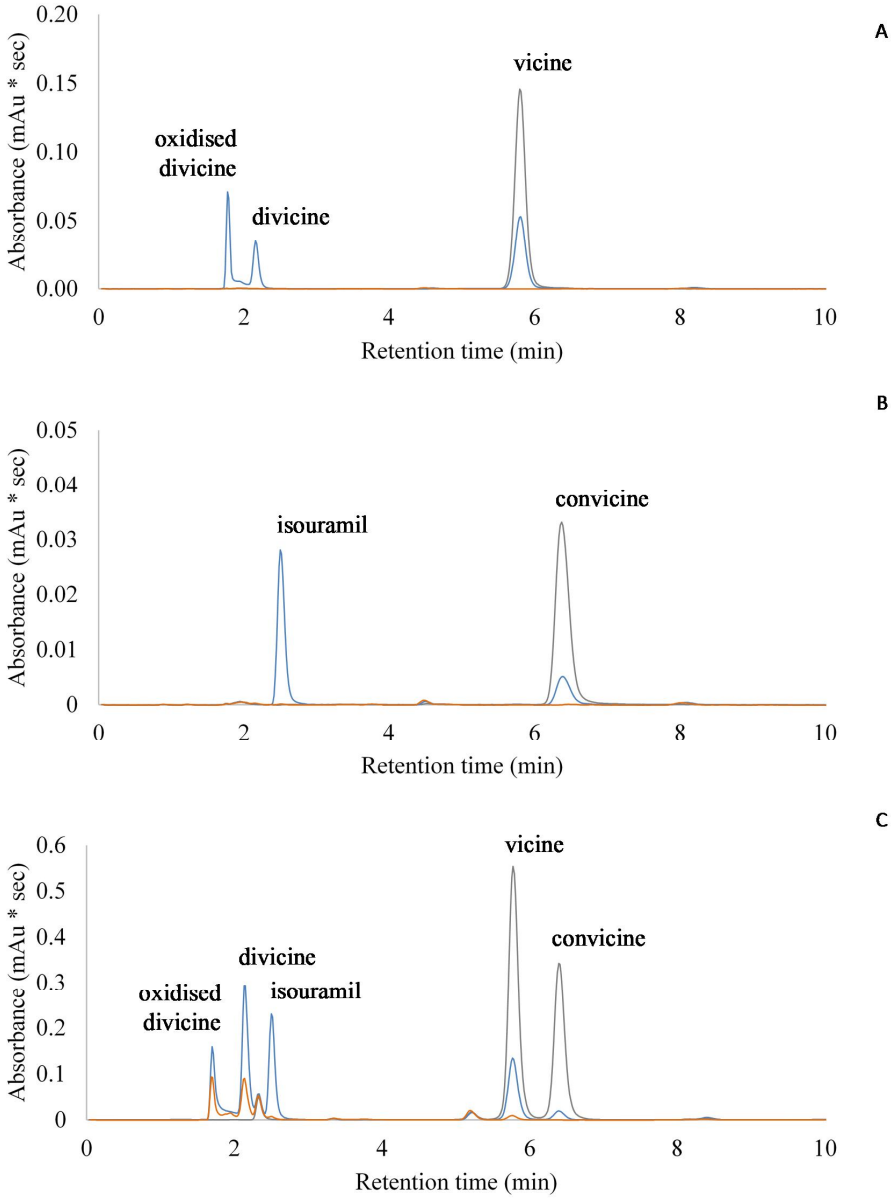


Figure 8 Chromatographic separation (λ = 273 nm) of the aglycones and oxidised divicine after hydrolysis of the vicine fraction (A), the convicine fraction (B) and a faba bean extract (C). The β-glucosidase activities were A) 10, B) 6 and C) 3 units in hydrolysis mixtures. Controls are presented with grey colour, and materials hydrolysed for 15 min with blue colour. The orange line represents hydrolysed materials after 120 min (A, B) and 60 min (C).

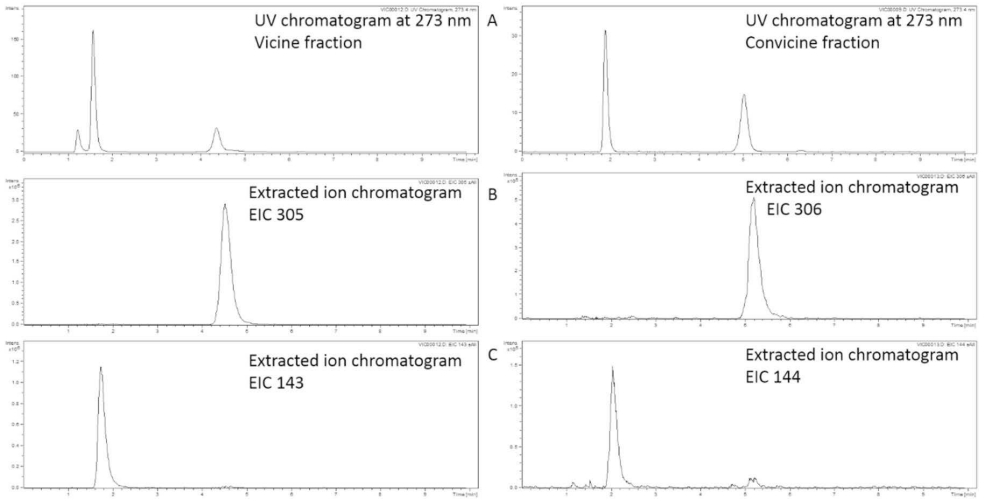


Figure 9 Confirmation of the identification of divicine and isouramil in hydrolysed vicine and convicine fractions by LC-MS. A) UV detection at 273 nm, B) extracted ion chromatograms at m/z 305 for vicine and 306 for convicine and C) extracted ion chromatograms at m/z 143 for divicine and m/z 144 for isouramil.

Identification of the aglycones was confirmed by LC-MS. The particular m/z ions for divicine and isouramil, m/z 143 for divicine and m/z 144 for isouramil, were seen in the EIC chromatograms (Figure 9). These m/z values arise from the pyrimidine structure after release of the glucose unit from vicine and convicine. The identity of further oxidation products was not fully confirmed, unlike the aglycones, because the compound named oxidised divicine was not detected by LC-MS.

5.2 Vicine and convicine in domestic faba beans (I)

The contents of vicine and convicine varied from 5.16–7.59 and 2.09–3.62 mg/g dry matter (DM) for vicine and convicine, respectively, in the wild-type cultivars (Figure 10). The contents were therefore higher for vicine than for convicine in all cultivars. Variation among cultivars was statistically significant ($p < 0.05$). The highest content of vicine was found in the cultivar 'Babylon' and the lowest in the cultivar 'Witkiem Manita' (Figure 10). The cultivar 'Kontu' had the highest content of convicine, and therefore the highest total content of vicine and convicine, at 10.42 mg/g DM. The vicine content in the low vicine-convicine cultivars ('Divine', 'Melodié') was approximately one tenth that of the vicine contents in the wild-type cultivars, and no convicine was detected.

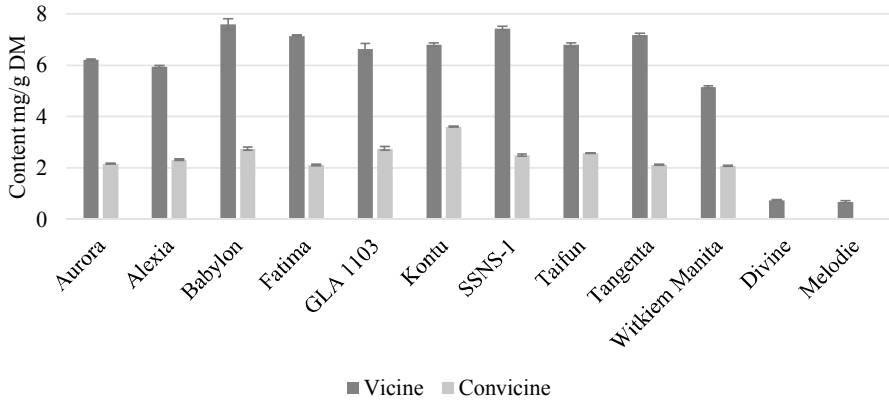


Figure 10 Comparison of vicine and convicine contents presented as mg/g per dry matter (DM) in faba bean cultivars from the same growing year (2011).

Variation among the growing years was relatively small but was statistically significant ($p < 0.05$). Vicine and convicine contents were lower in year 2012 than in year 2011 (Table 5). The contents were not consistent in year 2010 with other two years. The contents of convicine were consistently higher in the cultivar 'Kontu' than in the other cultivars. Apart from 'Alexia', the ratio of vicine to convicine was similar among the three years. The vicine contents were approximately 2–3-fold higher than the convicine contents. The highest ratio was found for the cultivar 'Fatima' (3.3–3.4) and the lowest ratio for the cultivar 'Kontu' (1.9–2.0).

Table 5 The contents (mg/g DM) of vicine and convicine in four cultivars grown in southern Finland in three subsequent years (2010-2012). Values represent the average ± standard deviation (n = 3).

Compound	Cultivar	Year		
		2010	2011	2012
Vicine	Alexia	6.37 ± 0.05	5.96 ± 0.04	5.62 ± 0.11
	Fatima	7.41 ± 0.12	7.15 ± 0.03	6.51 ± 0.13
	Kontu	6.27 ± 0.10	6.81 ± 0.07	6.40 ± 0.15
	SSNS-1	6.98 ± 0.06	7.43 ± 0.07	6.82 ± 0.09
Convicine	Alexia	1.93 ± 0.02	2.33 ± 0.03	2.24 ± 0.03
	Fatima	2.18 ± 0.04	2.12 ± 0.03	1.99 ± 0.01
	Kontu	3.14 ± 0.05	3.62 ± 0.02	3.14 ± 0.10
	SSNS-1	2.38 ± 0.02	2.50 ± 0.05	2.44 ± 0.04
Total	Alexia	8.30	8.29	7.86
	Fatima	9.58	9.27	8.49
	Kontu	9.42	10.43	9.54
	SSNS-1	9.36	9.93	9.26

5.3 Enzymatic hydrolysis of vicine and convicine (II, III)

5.3.1 Hydrolysis by added β-glucosidase (II)

Vicine and convicine were hydrolysed by treating faba bean extracts containing both vicine and convicine, vicine fractions, convicine fractions and suspensions with β-glucosidase from almonds at pH 5 and 37 °C. Different levels of the β-glucosidase enzyme were used to evaluate the formation and stability of the aglycones in extracts and vicine fractions.

The hydrolysis rates for vicine and convicine depended on the activity of the enzyme. At the lowest enzyme activity (0.5 U/ml) tested for extracts, only 23% of vicine and 41% of convicine were lost after 60 min at pH 5 and 37 °C (Figure 11 A). Increasing the activity 10-fold resulted in 59% and 84% losses of vicine and convicine, respectively, in 15 min and 94% and 100% losses of vicine and convicine, respectively, in 60 min. The highest level of enzyme activity (10 U/ml) resulted in 73% and 93% of vicine and convicine losses in 15 min, and almost total hydrolysis in 30–45 min.

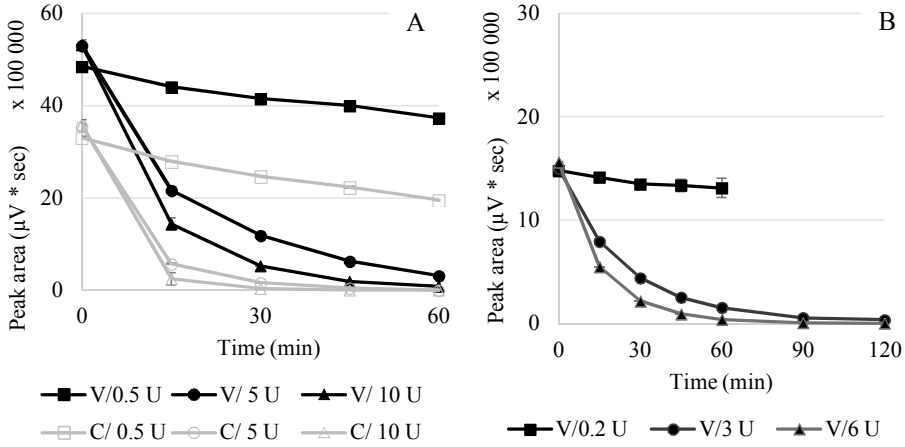


Figure 11 Hydrolysis of vicine (V) and convicine (C) in faba bean extracts (A) and in vicine fractions (B) at pH 5 by added almond β-glucosidase (extracts containing 0.5–10 U/ml, vicine fractions containing 0.2–6 U/ml) during 60 or 120 min at 37 °C, respectively. Values are shown as average of peak areas. The difference between replicates (n = 2) was mostly less than 10%.

Similarly, as was observed with the extracts, higher enzyme activity caused faster hydrolysis of vicine in hydrolysis mixtures of vicine fractions at pH 5 at 37 °C. With the lowest enzyme activity (0.2 U/ml), only 11% was lost in 60 min (Figure 11 B). By increasing the enzyme

activity, 47% and 65% of vicine were lost in 15 min with 3 U/ml and 6 U/ml, respectively. The hydrolysis was almost complete (96–97%) in 90 min and 60 min with these activities.

When hydrolysis was carried out at a lower pH (pH 3), vicine in the vicine fractions was completely hydrolysed in 15 min at the highest enzyme level (6 U/ml). An enzyme activity of 3 U/ml was used for convicine fractions, and 72%, 91% and 99% of convicine was lost in 15, 30 and 120 min, respectively (Figure 15).

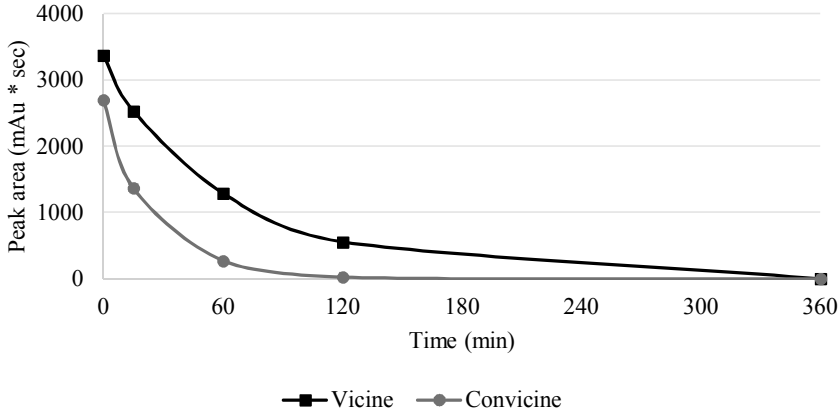


Figure 12 Hydrolysis of vicine and convicine in faba bean suspensions with added almond β-glucosidase (4.3 U/ml) during 6 h. (n = 3).

Hydrolysis was also observed in the suspension model, in which one selected enzyme activity was used (Figure 12). After 15 min of hydrolysis, 25% of the vicine and 49% of the convicine were lost, and 62% and 83% of the vicine and 90% and 99% of the convicine were lost by 60 and 120 min, respectively. After 6 h, vicine and convicine were completely disappeared.

5.3.2 Vicine and convicine losses in suspensions, sourdoughs and breads (III)

After experiments with added β-glucosidase, faba bean suspensions without added enzyme were prepared at different pH values to observe the losses of vicine and convicine induced by endogenous enzyme activity. Fermentations with selected β-glucosidase-active LAB were also prepared at two fermentation temperatures. Sourdoughs were used in bread baking to obtain wheat composite breads containing 30% faba bean flour. Losses of vicine and convicine were monitored in the sourdoughs and breads.

Table 6 Losses (%) of vicine and convicine in suspensions of different faba bean flours in selected experimental conditions (pH, temperature, time). Losses were calculated by comparing incubated suspensions (4 or 24 h) to starting point. (n = 6).

Cultivar/ Flour	Cultivation year	pH	Temperature (°C)	Time (h)	Loss (%)	
					Vicine	Convicine
Kontu	2011	4.5	30	4	13.5	5.4
		5			9.6	11.3
		6.5			6.0	7.4
		5	37	4	10.6	5.8
Kontu (autoclaved)	2015	5	37	24	0	0
Kontu		5			19.6	24.5
		4.5			20.1	21.5
		2			9.5	16.3
		1			8.5	66.7
Flour_It	2014	4.5	30	4	12.7	8.6
		5			9.1	6.1
		6.5			2.4	0
		5	37	4	8.2	7.9
Flour_It (autoclaved)		5	37	24	1.4	14.2
Flour_It		5			26.3	21.5
		4.5			18.6	20.7
		2			9.8	17.7
		1			8.2	66.4
Alexia	2011	5	37	4	8.2	8.7
Fatima					6.4	8.8
Kontu					7.6	6.6
SSNS					9.0	9.3
Alexia	2015	5	37	4	11.0	13.6
Fatima					7.2	3.8
Kontu					5.2	6.7
SSNS					7.0	7.4

The endogenous β-glucosidase activity towards vicine and convicine in faba bean suspensions was rather small. Incubation at different pH and temperature combinations resulted in losses of vicine and convicine in 'Kontu' and commercial (Flour_It) flours of only up to 13% and 11% in 4 h (Table 6). Prolonging the incubation to 24 h enhanced hydrolysis slightly, up to 25–26% for both vicine and convicine. Suspensions from autoclaved flours showed that hydrolysis did not occur when endogenous enzymes and microbes were eliminated. Low pH (1 and 2) did not cause a marked decrease in vicine levels (10% loss compared to starting point). At a low pH of pH 1, convicine was notably decreased, as losses were 66–67% for both flours. Cultivar comparison showed no marked

differences among cultivars from two years (Table 6). The losses among cultivars were up to 11% for vicine and 14% for convicine after a 4 h incubation at the selected pH and temperature (pH 5, 37 °C).

The losses of vicine and convicine following fermentation with selected LAB strains depended on the strain and fermentation temperature (Figure 13). At the lower fermentation temperature of 20 °C, only sourdoughs inoculated with strain I02 showed a small decrease (10–13%) in vicine and convicine levels (Figure 13 A). Fermentation at 25 °C yielded in greater losses of vicine and convicine, especially with the strains I02 and B24W (Figure 13 A, C). The losses were up to 82–85% for vicine and up to 34–47% for convicine in smaller batches (**). The losses in sourdoughs were comparable to the losses in breads. For example for the strain I02, the vicine losses were approximately 45% and convicine losses 10–20% in both sourdough and breads in smaller batches (*) (Figure 13 A).

The performance of the LAB strains was good and acidification in sourdoughs was successful. All strains grew efficiently in faba bean. Overall, the acidification was similar among the different strains (Table 7). The most similar growth rates (Δlog) were observed for the strains I02 and B24W at 20 °C and at 25 °C, and fermentation with these strains also yielded the highest losses of vicine and convicine. The growth rate for the strain E76 was slightly increased at 25 °C when compared to 20 °C.

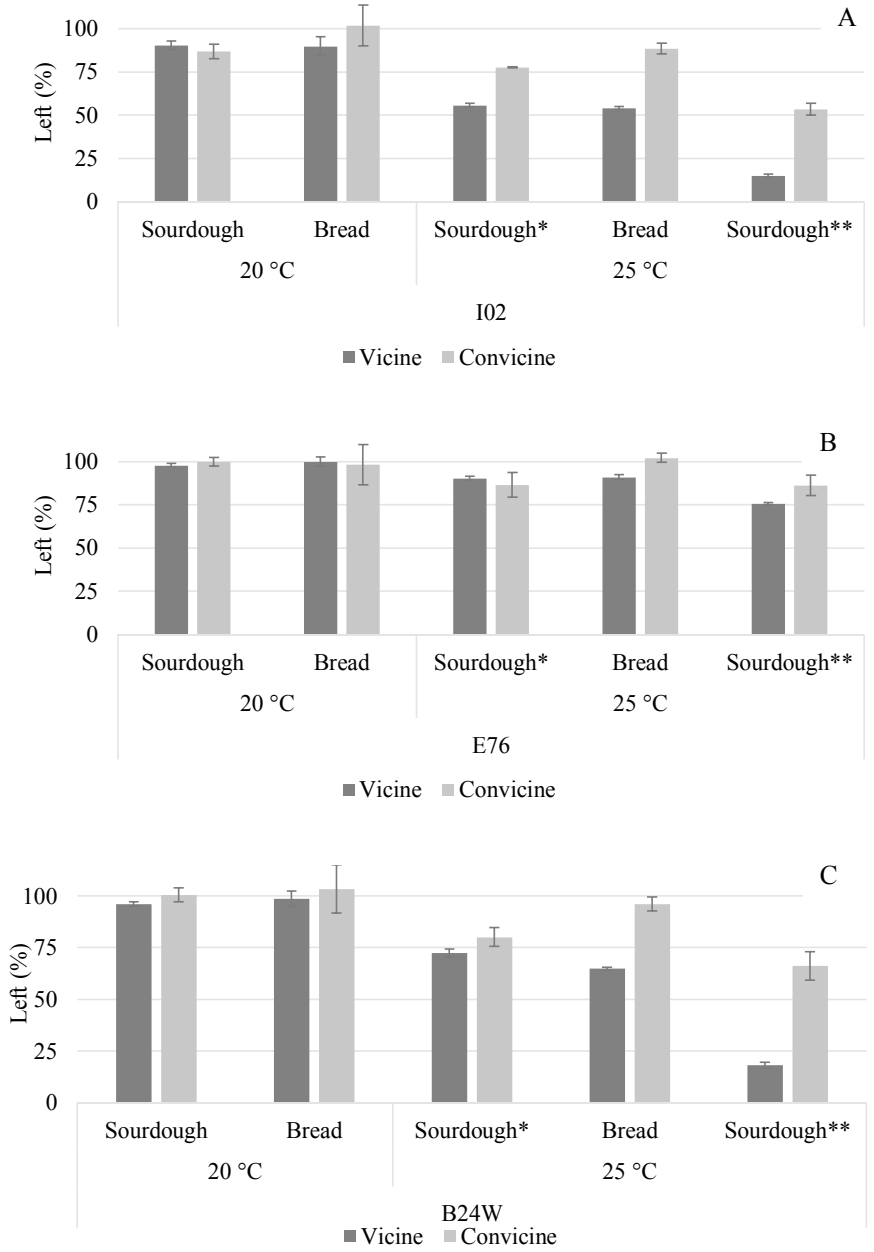


Figure 13 Vicine and convicine left (%) in sourdoughs fermented with three LAB strains, I02 (A), E76 (B) and B24W (C) at 20 °C and 25 °C and in the corresponding breads (n = 3–9). Sourdoughs were compared to the starting point, and breads were compared to breads containing non-fermented faba bean flour. Sourdoughs at 25 °C were prepared three times, once (*) in a larger batch and two times (**) in smaller batches.

Table 7 The properties of sourdoughs used for preparing composite wheat breads containing 30% of faba bean. (n = 4–6). [a)]

	(°C)	Strain	Cell density (log cfu/g)	Δlog	pH	ΔpH	TTA [c)] (ml)
Sourdough	20	I02	9.00 ± 0.07	3.05	5.3 ± 0.2	0.86	8.6 ± 0.4
		E76	8.90 ± 0.40	2.51	5.4 ± 0.4	0.82	10.0 ± 2.7
		B24W	8.95 ± 0.20	2.45	5.3 ± 0.5	0.93	8.5 ± 0.8
	25	I02	9.25 ± 0.07	3.00	4.7 ± 0.0	1.74	13.8 ± 1.20
		E76	9.65 ± 0.07	3.15	4.4 ± 0.1	1.91	14.9 ± 1.90
		B24W	9.10 ± 0.20	2.56	4.8 ± 0.2	1.64	13.0 ± 0.10
Bread (30% faba bean flour)		NF [b)]	n.d	n.d	5.9 ± 0.0	n.d	4.3 ± 0.2
	20	I02	n.d	n.d	5.2 ± 0.0	n.d	6.7 ± 0.0
		E76	n.d	n.d	5.0 ± 0.0	n.d	7.8 ± 0.3
		B24W	n.d	n.d	5.3 ± 0.0	n.d	6.7 ± 0.0
	25	I02	n.d	n.d	4.9 ± 0.0	n.d	7.4 ± 0.1
		E76	n.d	n.d	4.7 ± 0.0	n.d	8.6 ± 0.0
		B24W	n.d	n.d	4.9 ± 0.0	n.d	7.6 ± 0.0

a) Measurements were made per fresh weight and conducted in duplicate from each process replicate. n.d = no data.
b) NF = bread containing non-fermented faba bean
c) TTA = total titratable acidity

The pH values were 5.3–5.4 for sourdoughs after fermentation at 20 °C, and 4.4–4.8 at 25 °C (Table 7). Correspondingly, the TTA values were higher (13.0–14.9 ml 1 M NaOH) for fermentation at 25 °C than at 20 °C (8.5–10.0 ml). The lactic acid content correlated with pH and TTA, being higher at lower pH and ranging from 0.41–1.32 g/100 g of dough. Hydrolysis of vicine and convicine was only observed at the higher fermentation temperature (25 °C) in conditions of higher acidity when compared to fermentation at 20 °C. The highest acidity was noted during fermentation with strain E76, but the vicine and convicine losses were the lowest. Breads containing 30% sourdough showed similar trends in pH and TTA values, as the higher acidity in sourdough was seen in the lower pH and higher TTA value in the bread. The differences were smaller in breads than in sourdoughs.

5.3.3 Formation and stability of the aglycones (II, III)

The aglycones were formed after enzymatic hydrolysis of vicine and convicine. Three compounds (i.e. divicine, oxidised divicine and isouramil) were detected in the fractions and faba bean extracts, and these were followed under selected conditions. The stability of divicine was further studied in varying conditions in the vicine fractions. The stability of the aglycones was also studied in food systems as suspensions and in sourdoughs, doughs and breads.

The aglycones were detected at the first measurement point at 15 min in extracts at pH 5 at 37 °C, with all three enzyme activities applied for hydrolysis. In the extracts with the lowest enzyme activity, divicine was constantly formed, while the amount of isouramil was at the highest after 15–30 min and then started to decrease (Figure 14 A). The use of higher enzyme activities (5 U and 10 U/ml) resulted in the highest amounts of divicine and isouramil at the timepoints of 15–30 min, and then the levels decreased (Figure 14 C, E). The amounts of divicine and isouramil were slightly higher with higher enzyme activity (10 U/ml). Divicine was still present at the last time point at 60 min. A degradation product of divicine (i.e. oxidised divicine) appeared immediately with divicine. The amount of oxidised divicine was higher than that of divicine at the lowest enzyme activity (0.5 U/ml), whereas it was lower with a higher activity of enzyme (5 and 10 U/ml). The course of divicine oxidation in extracts depended on the enzyme activity, because the levels of oxidised divicine decreased only with higher enzyme activities (5 and 10 U/ml) during 60 min.

Divicine was found after 15 min in the vicine fractions at pH 5 at 37 °C, and the amounts subsequently decreased, although the formation and decrease were slow with the lowest enzyme activity (0.2 U/ml). The amount of divicine then remained constant from 15 to 45 min (Figure 14 B). The use of higher enzyme activities (3 U and 6 U/ml) caused a more rapid decrease in divicine amounts. The amounts of divicine gradually decreased until divicine was completely lost by 90 min with the enzyme activities of 3 and 6 U/ml (Figure 14 D, F). Oxidised divicine was formed alongside divicine, and the amounts of oxidised divicine showed a similar decrease to that of divicine during 120 min. In contrast to the observations for extracts (5 and 10 U/ml), the amount of oxidised divicine was higher than the amount of divicine during the incubations.

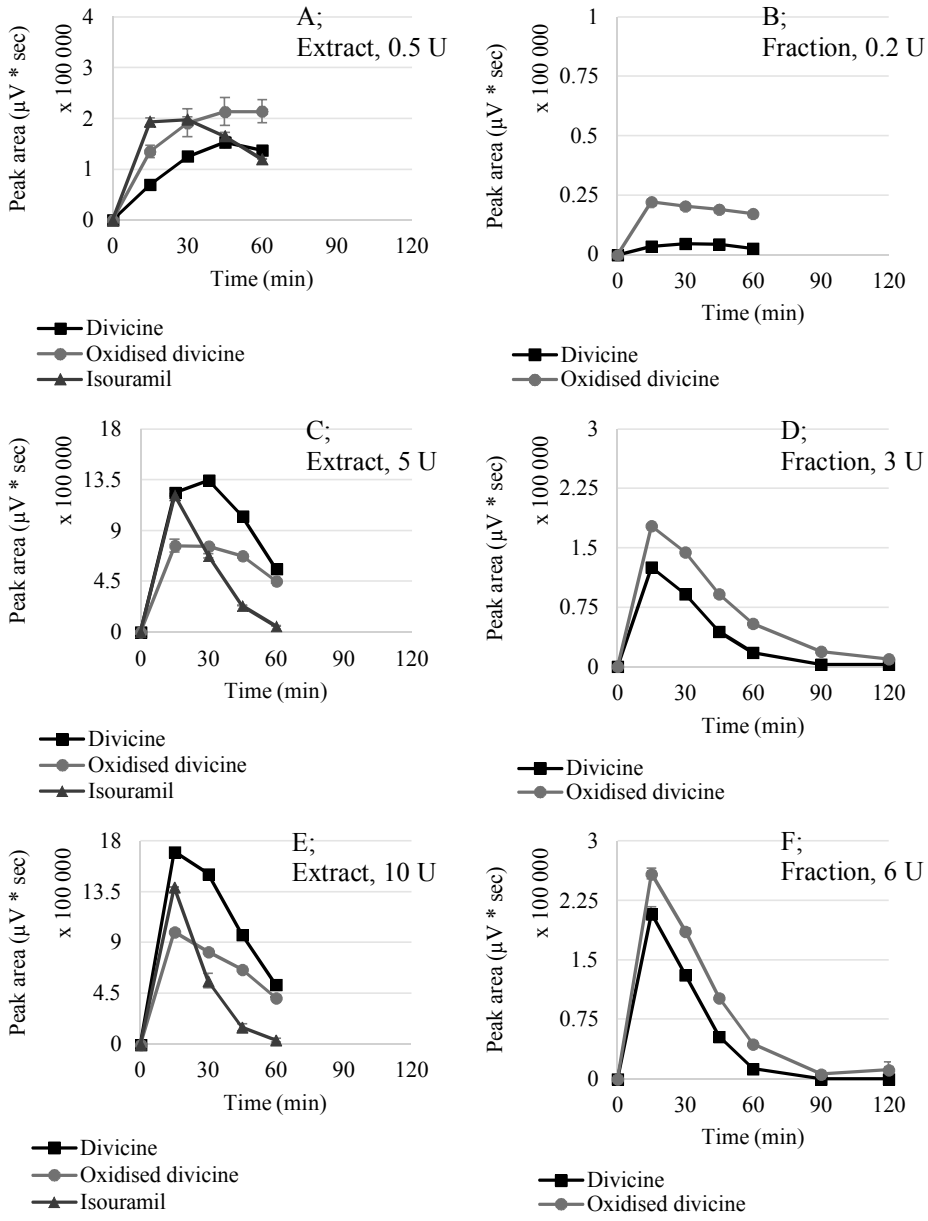


Figure 14 Formation and stability of the aglycones and oxidised divicine in hydrolysis mixtures at pH 5, 37 °C. The amounts of divicine, oxidised divicine and isouramil in extracts (A, C, E) after hydrolysis with 0.5, 5 and 10 U/ml of almond β-glucosidase; the amounts of divicine and oxidised divicine in vicine fractions (B, D, F) after hydrolysis with 0.2, 3 and 6 U/ml. (n = 2).

In the convicine fractions with enzyme activity of 3 U/ml, isouramil was the only product followed in hydrolysis. The amount of the aglycone isouramil was highest at 15 min, maintained the same level for 15–30 min and then started to decrease (Figure 15). All isouramil was lost by 120 min.

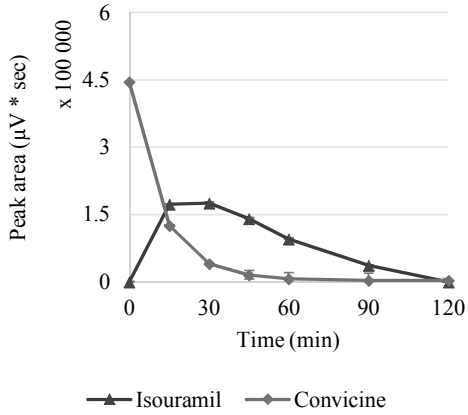


Figure 15 Hydrolysis in the convicine fraction and formation of isouramil (3 U/ml) at pH 5 at 37 °C. (n = 2).

In summary, the aglycones were not stable under the model conditions studied. The amounts of the aglycones decreased in all the conditions presented above. The most complete losses of divicine and isouramil were measured in the vicine and convicine fractions. Based on these findings, the stability of divicine in vicine fractions was further studied by changing the incubation conditions. The experiment showed that replacement of air with nitrogen or addition of sodium ascorbate delayed the disappearance of divicine (Table 8). Sodium ascorbate, in particular, maintained the divicine levels from 60 to 120 min. The formation of oxidised divicine was not completely hindered under the stabilising conditions used. The amount of oxidised divicine was first lower in the stabilised conditions than in the non-stabilised condition (Table 8), and then, during hydrolysis, the decrease in oxidised divicine was slower.

In addition to these experiments, hydrolysis was compared in vicine fractions at two pH values and two temperatures. Incubations under the selected conditions showed a slower disappearance of divicine at 20 °C than at 37 °C (Table 8). At both studied pH values (pH 3 and 5), the amounts of divicine and oxidised divicine decreased notably at higher temperature (37 °C), while the amounts stayed at somewhat same level at 20 °C during a 60 min incubation. Even though divicine and oxidised divicine were present after 75 min at pH 3 and 5 at 20 °C, they had disappeared from the hydrolysis mixtures after 24 h.

Table 8 Stability of divicine in selected conditions (n = 2). The values describe the peak areas of divicine and oxidised divicine when hydrolysis mixtures at pH 5, 37 °C were prepared as untreated (air), after purging with nitrogen (nitrogen), with ascorbate addition (ascorbate) and at altered pH and temperature conditions.

Time							
				Divicine			
(min)	Air [a]	Nitrogen [a]	Ascorbate [a]	pH 3/20 °C [b]	pH 5/20 °C [b]	pH 3/37 °C [b]	pH 5/37 °C [b]
15	212	452	591	624	457	584	449
30	134	529	744	567	411	307	115
45	53	450	883	499	444	123	23
60	12	397	988	435	414	30	3
75	na	na	na	376	380	0	0
90	4	152	976	na	na	na	na
120	6	82	978	na	na	na	na
Time				Oxidised divicine			
(min)	Air [a]	Nitrogen [a]	Ascorbate [a]	pH 3/20 °C [b]	pH 5/20 °C [b]	pH 3/37 °C [b]	pH 5/37 °C [b]
15	260	241	107	203	341	215	324
30	187	255	131	207	324	180	193
45	103	211	136	206	247	113	69
60	45	178	158	197	230	57	22
75	na	na	na	188	210	21	7
90	6	115	153	na	na	na	na
120	11	66	154	na	na	na	na

[a] peak areas as μV * sec * 0.001
[b] peak areas as mAu * sec

na not analysed

After the formation of the aglycones was confirmed in the models, the reactions of the aglycones were monitored in food systems. Faba bean suspensions were studied first with added β-glucosidase. Divicine and isouramil were measured in the suspensions after a 15 min hydrolysis, and they had disappeared after 2 h (Figure 16 A). Oxidised divicine was present as well, in amounts higher than the amounts of divicine and isouramil. All hydrolysis and reaction products had disappeared by 6 h.

Different sourdoughs were analysed, and the aglycones were observed from those in which the hydrolysis of vicine and convicine had occurred, namely the sourdoughs fermented at 25 °C. In sourdoughs fermented at 20 °C, no aglycones or oxidised divicine were observed. The aglycones were also present in the corresponding doughs, but not in the breads. The formation of the aglycones is shown in Figure 16 B. The amount of oxidised divicine was lower than that of divicine. The compounds were not stable in extracts of sourdoughs, because the amounts decreased when the extracts were kept at room temperature (Figure 16 B). The amount of divicine was first higher than that of oxidised divicine, but after 0.5 h and 2 h it was lower. Oxidised divicine, divicine and isouramil had disappeared after 20 h.

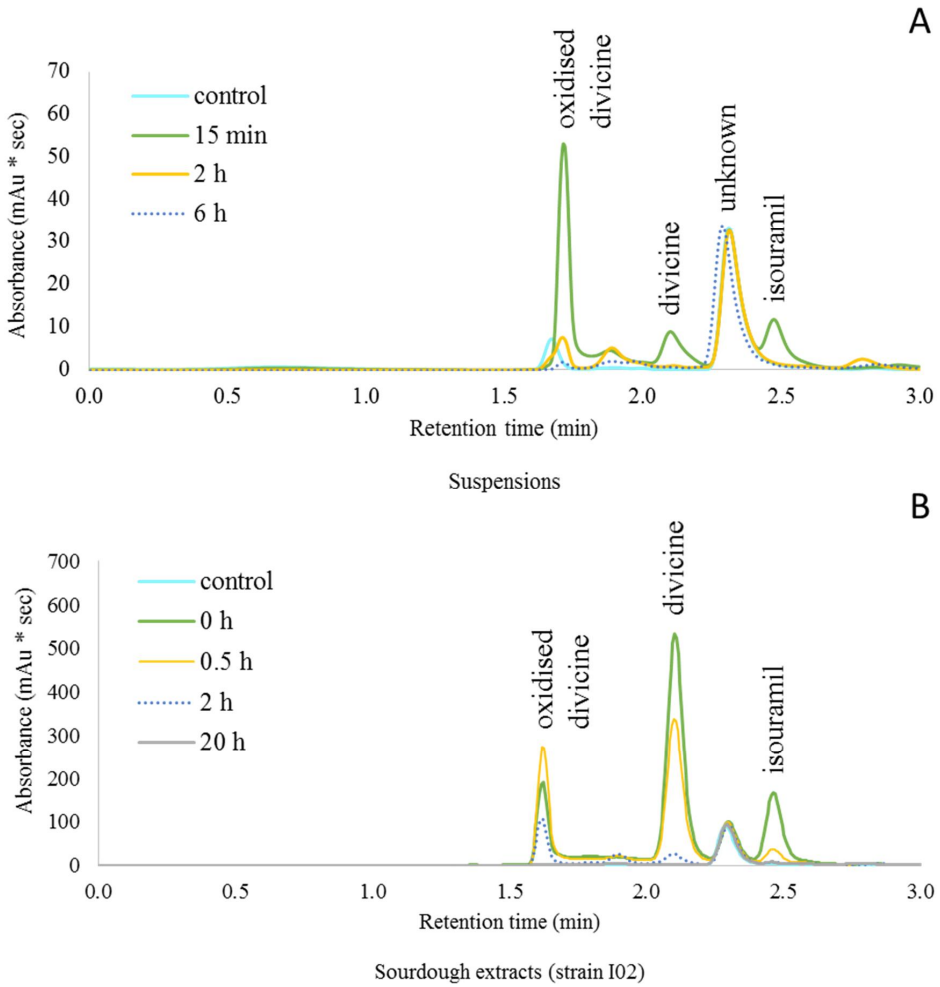


Figure 16 HPLC chromatogram (λ = 273 nm) showing the formation and loss of the aglycones in A) suspensions and B) extracts of sourdoughs.

To conclude, the same compounds were detected in the food systems and in the model conditions, and the disappearance was similar in both food systems and model conditions. The stability of the aglycones was not improved in food systems when compared to the model conditions.

6 Discussion

6.1 Evaluation of the method for analysis of vicine, convicine and their aglycones

6.1.1 Extractability of vicine, convicine and their aglycones

Vicine and convicine were extracted from faba bean flours with two extraction solutions (7% PCA and water) and 1–3 repeated extractions in a total volume of 15 ml. Deproteinisation was one criterion for extraction, because faba bean contains high amounts of proteins that are either soluble in water (albumins) or in salt solutions (globulins, as storage proteins). Extracts were deproteinised either with PCA or by adjusting the pH to 4, which is the known isoelectric point of most faba bean proteins (Bhatty 1974). The comparison showed that differences in extractability were small; however, certain differences were found. Repeated extraction (3 times) with 7% PCA was selected as the final method.

Slightly more vicine was measured in acidic extracts, whereas convicine was somewhat better extracted by water; however, the differences were small between the two extraction solutions. Marquardt et al. (1983) reported that vicine was more soluble at low pH and convicine at high pH values. The solubilities of these two compounds were lowest at pH values 4–8. Marquardt et al. (1983) also reported that 3–3.6 mg/ml of vicine could be solubilised at pH values 4–9 and 0.4–1.2 mg/ml of convicine at pH values 1–8. In the present faba bean analysis, the vicine and convicine concentrations were lower (0.1–0.2 mg/ml) in the extraction solution than their given solubilities; therefore, the differences in solubilities did not limit the analysis. The extraction can be carried out at pH values of 4–8, and acidic extraction is not necessary. Possible acid-induced degradation was not a limitation in the acidic extraction, because the results were repeatable and comparable between the two solutions.

Vicine and convicine were readily dissolved from faba bean flour, but repetition of the extraction as two or three cycles slightly increased the peak areas of the analytes. Repeated extraction particularly improved the extractability of convicine in acidic conditions, so the decision was made for a three-step extraction (3 × 5 ml) as the final method. The peak areas for uridine were also slightly increased by repeated extraction. A single extraction gave acceptable results, and would therefore be sufficient for faster screening of vicine and convicine levels.

The internal standard uridine was added to faba bean flours before the extraction solution, and its recovery was good. It was previously used as an internal standard by Quemener

(1988) and Helsper et al. (1993). The suitability of cytidine as an internal standard was estimated by Purves et al. (2018a), but cytidine was converted to uridine by cytidine deaminase in water solutions, indicating that enzyme inactivation would be required during the extraction. Both uridine and cytidine could be used as internal standards in vicine and convicine analysis, because they occur mainly as intermediate products in plant metabolism and are therefore not detected in analysis of vicine and convicine. Uridine has similar properties and extractability to vicine and convicine, so it is a good choice as an internal standard.

The formation and stability of the aglycones was studied by conducting the extraction with either 0.05 M sodium phosphate buffer or water, and large molecular weight components, such as proteins and polysaccharides, were removed by centrifuging through regenerated cellulose membrane Amicon® filters. The β-glucosidase was presumably also retained by the filter to stop the hydrolysis. The aglycone extraction was kept as simple as possible to prevent changes induced by pH or temperature. However, vicine, convicine and the aglycones could all be extracted either with buffer or water, as concluded above. The solubilities of divicine and isouramil were similar to the solubilities of vicine and convicine (Marquardt, Frohlich et al. 1989; Marquardt, Arbid et al. 1989); thus, their analysis alongside vicine and convicine is feasible.

6.1.2 Performance of the HPLC analysis for vicine and convicine

The optimised RP-HPLC-UV method was suitable for the analysis of vicine and convicine. The analytes were separated on a C18 column at 30 °C, and the separation was good with both tested eluents (water with 0.1% formic acid and water alone) at flow rate of 0.8 ml/min.

Vicine and convicine contain amino and hydroxyl groups that affect their properties. Vicine has one more amino group than convicine, so it is more basic than convicine. The pKa_1 values for vicine and convicine are 3.16 and 2.71, respectively, according to (ACD/Labs) Software V11.02 (© 1994-2012 ACD/Labs), whereas the pKa value for unsubstituted pyrimidine is 1.3. At an acidic pH, amino groups are protonated. The pH of the eluent containing 0.1% formic acid was 2.7, and the pH of the MilliQ-water eluent was close to neutral. In general, retention on C18 column is better when compounds are in their neutral form. Based on the pKa values, vicine and convicine are partly protonated and neutral in the acidic eluent (pH 2.7). The retention time was shorter for vicine than for convicine with an acidic eluent, but was longer for vicine and slightly shorter for convicine with water as an eluent. Zhang et al (2003) also reported an effect of pH on retention time of vicine. The retention times of the internal standard uridine were less affected by the pH of the eluents. The pH of the eluent was slightly lower (pH 2) in the previous studies, that used 0.05 M ammonium phosphate buffer (Burbano et al. 1995; Goyoaga et al. 2008; Cardaror-Martinez et al. 2012) as an eluent, following the protocol of Marquardt and Frohlich (1981). Water was used first used as an eluent by Quemener (1988) and then by Griffiths and Ramsay

(1992). Overall, the separation was good with both tested eluents. The eluent containing 0.1% formic acid was selected in the present study for the final method, because formic acid (pKa value 3.75) has a buffering capacity to retain retention times, and it enables protonation of analytes for the LC-MS-analysis.

The published absorption maxima for vicine (275 nm), convicine (271 nm) and uridine (261 nm) were used to support the identifications of the studied compounds (Ploeser and Loring 1949; Bendich and Clements 1953; Bien et al. 1968). In addition, MS detection was used to confirm the identity of vicine and convicine based on their known MS spectra. The protonated molecular ions for vicine and convicine, m/z 305 and m/z 306, respectively, were detected in positive ionisation with LC-ESI-MS.

The analytes were quantified by UV detection and showed a wide linear range, as the response was linear for injections of 50 ng up to 7.5 µg. No interfering UV absorbing compounds were detected in the chromatograms. The detection wavelength was selected to be suitable for the internal standard uridine. Quantification using uridine was a practical and precise choice, and standard curves of vicine and convicine were not needed in every analysis set. By contrast, the relative response factors were needed because the response of uridine differed from the responses of vicine and convicine. The relative response factors of uridine to vicine and convicine, as established in study I, were close to each other in acidic media at 273 nm, at 0.65 for vicine and 0.61 for convicine. The earlier established relative response factors vicine and convicine to uridine showed a slightly higher value for vicine (0.8) and a similar value for convicine (0.6) in water at 273 nm when compared to the values found in acidic media in study I (Quemener 1988). Marquardt and Frohlich (1981) stated that the response of convicine would be a bit lower than the response of vicine at concentrations of 52 µg/ml at 280 nm. However, the similarity of the relative response factors of vicine and convicine is reasonable because their molar absorption coefficients are close to each other (Bendich & Clements, 1953; Bien et al, 1968). Based on this finding, both compounds could be quantified using only a vicine or convicine standard, as done in study III.

Overall, the performance of the RP-HPLC-UV analysis method was good, as it was similar or slightly better when compared to earlier methods (Marquardt & Frohlich, 1981; Quemener 1988; Burbano et al, 1995). The responses were linear across a wide range, and RSD% values presenting the repeatability of the method were 3–7% for vicine and convicine for the in-house reference over a longer time (n=20).

6.1.3 Simultaneous HPLC analysis of the aglycones

The analysis method was first taken in use for quantification of vicine and convicine and later in study II, and its suitability for analysis of the aglycones was confirmed. The aglycones were determined, and further oxidation products were indicated. The main

advantage of the method was the capability for simultaneous analysis of vicine, convicine and the aglycone forms.

The aglycones were analysed with the RP-HPLC-UV method, which has not been previously used for that purpose. The identities of divicine and isouramil were confirmed based on their known UV absorption maxima and mass spectra. In the early study by Marquardt and Frohlich (1981), indications of the aglycones were seen when the formation of new peaks was noticed after acid hydrolysis of vicine and convicine. However, the peaks were not identified. An HPLC method with a strong cation exchange system (SCX) was instead applied for analysis of the aglycones, which were studied separately as pure compounds. An SCX-HPLC method was also used to study divicine by Marquardt, Frohlich et al. (1989) and McMillan et al. (1993) and to study isouramil by Marquardt, Arbid et al. (1989). Further degradation products were also observed with the SCX-HPLC methods.

Separation of the aglycones was slightly better with the SCX-HPLC methods used in the previous studies than with the RP-HPLC method applied in study II, but the SCX-HPLC system was more complicated and would not be compatible with subsequent MS detection for identification. The retention times for divicine were 2–4 min longer with the SCX-HPLC system (Marquardt, Frohlich et al. 1989; McMillan et al. 1993) than in this work with the RP-HPLC system. The oxidised divicine was detected and identified based on the decrease of the divicine peak area after addition of a reducing agent (Marquardt, Frohlich et al. 1989; McMillan et al. 1993). Oxidised divicine was also determined in study II. Apart from oxidised divicine, one unknown peak was reported by Marquardt, Frohlich et al. (1989) and two unknown peaks by McMillan et al. (1993). In study II, oxidised divicine was the main oxidation product of divicine. In contrast to the findings of Marquardt, Arbid et al. (1989), no further oxidation products were detected from isouramil in the present study. However, the enzymatic hydrolysis used in this work was a milder treatment than the acid hydrolysis used in most of the earlier studies. For example, acid hydrolysis was suggested to induce the formation of deaminated products from divicine (Pedersen et al. 1988; Winterbourn et al. 1989; McMillan et al. 1993). Therefore, the findings cannot be directly compared. In study II, other further products of divicine and isouramil were seen at retention times 1.6-1.9 min, which is not the most optimal for the determination of reactions products because buffers and other slightly retained compounds can interfere with the analysis.

The further reactions of the aglycones could be studied by implementing new methods, such as the HILIC-MS system recently developed for vicine and convicine (Purves et al. 2018a). An HILIC column was also used for determination of pyrimidines and purines (Marrubini et al. 2010). The retention times for vicine and convicine were short, as they eluted in 3 min with the selected conditions (Purves et al., 2018a). The retention of the aglycones of vicine and convicine on an HILIC column has not yet been studied. MS detection could be used for structure analysis of further reaction products of the aglycones when coupled with suitable chromatographic methods.

To conclude, the selected conditions in the analysis method were suitable for the simultaneous study of vicine, convicine, the aglycones and oxidised divicine. This benefit was further utilised in studies II and III to observe the hydrolysis of vicine and convicine and the formation and stability of divicine and isouramil. When studying the aglycones, samples should be immediately analysed or the aglycones should be handled in the absence of oxygen or in the presence of effective reductants.

6.2 Occurrence and variation of vicine and convicine in domestic faba beans

The vicine (5.16–7.59 mg/g DM) and convicine contents (2.09–3.62 mg/g DM) in the Finnish-grown wild-type cultivars from the year 2011 were comparable with the contents in cultivars grown elsewhere (Quemener 1988; Wang and Ueberschär 1990; Jezierny et al. 2011; Ivarsson and Neil 2018). The overall variation in vicine and convicine contents has, however, been wider, because the vicine contents were reported from 1.8 to 15.0 mg/g and convicine concentrations from 0.3 to 8.4 mg/g in some studies (Gardiner et al. 1982; Duc et al. 1989; Griffiths and Ramsay 1992; Helsper et al. 1993; Duc et al. 1999; Purves et al. 2018a; Purves et al. 2018b). The cultivar 'Kontu' is currently the most commonly grown cultivar in Finland; therefore, it was of particular interest in the present study. The total content and particularly convicine content measured in the present study was high compared to other studied cultivars.

The vicine contents in the vc- cultivars ('Divine' and 'Melodié') were around 0.7 mg/g DM, or about one tenth that found in the studied wild-types cultivars. No convicine was detected in the low-vc cultivars. The low-vc cultivars were originally developed in France and are not yet optimal for Finnish growing conditions. The vicine contents were still comparable with earlier studies, as the vicine levels ranged from 0.1 to 0.9 mg/g DM in the vc- cultivars (Duc et al. 1999; Jezierny et al. 2011; Purves et al. 2018a). The contents in 'Divine' and 'Melodié' were lower than in the present study, at 0.3 mg/g DM (Jezierny et al. 2011; Purves et al. 2018a). In contrast to study I, convicine was also found at 0.02–0.2 mg/g DM in previous studies (Purves et al. 2018a). A notable factor that limits the use of faba bean cultivars in the north is their requirement for early flowering and ripening during short growing season of around 120 days (Lizarazo et al. 2015). The vc- cultivars are constantly being developed for earlier ripening.

A comparison of four cultivars ('Alexia', 'Fatima', 'Kontu' and 'SSNS-1') from three growing years (2010–2012) showed that the variation was less pronounced among the growing years than among studied cultivars, although the variation in both cases was statistically significant ($p < 0.05$). Pitz et al. (1981) came to the same conclusion when comparing two growing years. Among the growing years studied in the present work, the weather conditions notably varied. The growing season was dry in year 2010, while the year 2012 was cool and wet. A cool and wet season requires a greater number of growing degree-

days to maturity than does a dry and warmer season (Lizarazo et al. 2015). These environmental factors could have had an effect on the vicine and convicine contents. Nevertheless, the differences among years were relatively small, so varying weather conditions did not have a clear influence on the contents. The vicine and convicine contents were higher in year 2011 than year 2012, whereas the contents for the year 2010 did not show any clear trend. The type of cultivar and growing conditions are factors that can cause variation in vicine and convicine contents, whereas variation after ripening is reported to be small (Pitz et al. 1981; Burbano et al. 1995).

A few individual cultivars from study I were also analysed in other studies (i.e. in the same cultivars grown elsewhere) (Purves et al. 2018a; Ivarsson and Neil 2018). The vicine contents were 6.64 and 7.11 mg/g DM and the convicine contents were 2.50 and 2.90 mg/g in the cultivars 'Alexia' and 'Taifun', respectively (Ivarsson and Neil 2018), compared with the corresponding contents from study I, which were 5.62–6.37 and 6.80 for vicine and 1.93–2.33 and 2.58 for convicine, respectively. Comparison of the cultivar 'SSNS-1' revealed vicine and convicine contents of 6.57 ± 0.17 and 2.78 ± 0.06 mg/g, respectively, in earlier work (Purves et al. 2018a), compared to 7.43 ± 0.07 and 2.50 ± 0.05 mg/g, respectively, in study I. The contents were close to each other, yet slightly different. The differences were presumably caused by growing conditions and harvesting practices.

To summarise, the vicine and convicine contents of Finnish-grown faba bean cultivars were comparable to the contents reported for cultivars grown elsewhere. The contents remained at a certain level from year to year, even though the weather conditions varied markedly. The cultivar 'Kontu' which has high vicine and convicine levels, is still the most commonly grown cultivar in Finland, but new cultivars are being developed and will be available in future.

6.3 Elimination of vicine and convicine by enzymatic hydrolysis

6.3.1 Hydrolysis of vicine and convicine

Enzymatic hydrolysis was conducted in study II for 1–2 h at pH 5 and 37 °C with added β-glucosidase to produce the aglycones. As expected, the higher activity of the added enzyme resulted in higher losses of vicine and convicine from the faba bean extracts and the vicine and convicine fractions. A significant decrease in levels was noted in a short time (15–30 min). The almond β-glucosidase has been used previously for hydrolysis of vicine and convicine (Baker et al. 1984; Arbid and Marquardt 1985b; McMillan et al. 1993; Pedersen et al. 1988), mainly as an enzyme preparation, but Arbid and Marquardt (1985b) treated faba bean paste with non-heated almond powder to hydrolyse vicine and convicine. Another possible source of β-glucosidase for vicine and convicine hydrolysis could be an

extracellular fungal enzyme produced by *Aspergillus* strains (McKay 1992; Sewalt et al. 2016). To the best of the present author's knowledge, food-grade β-glucosidases are not commercially available, although other enzyme preparations, such as pectinase and cellulase preparations, may contain β-glucosidases as side activities (de Andrades et al. 2019). However, the substrate specificity of β-glucosidases should be also considered, because the activities vary depending on the substrate (Sestelo et al. 2004; Michlmayr and Kneifel 2014).

In contrast to the fractions, faba bean extracts and suspensions contained other components in addition to vicine and convicine, and yet hydrolysis was still efficient with the selected enzyme activities. The other compounds in faba bean suspensions, if they contain β-glycosidic bonds, may also act as substrates, thereby possibly increasing the amount of enzyme required. The hydrolysis in suspensions could have been accelerated with a higher enzyme activity in study II. Vicine and convicine losses were 80% and 100% after 2 h for suspensions, and the hydrolysis of vicine continued beyond the 2 h time point. Arbid and Marquardt (1985b) reported notably different hydrolysis efficiencies depending on their treatment choice. For example, 80% of the vicine and all the convicine in faba bean paste were hydrolysed in 10 min by added β-glucosidase at pH 5 at 30 °C, whereas approximately 90% of the vicine and convicine in faba bean paste was hydrolysed with almond powder at pH 5 at 30 °C in 3 h.

Hydrolysis conditions were not optimised for food processing in the present work; however, the conditions could also be applied to food preparation processes. In study II, almond β-glucosidase was used at pH 5 and 3. This enzyme has a reported pH optimum of pH 5 (Heyworth and Walker 1962). Arbid and Marquardt (1985b) found that almond β-glucosidase efficiently hydrolysed vicine and convicine in faba bean paste (67–80%) in 30 min at low pH values (3.5–4.5) at 30 °C. This hydrolysis was hindered at pH values above 6; thus, the hydrolysis was not effective at neutral conditions. In the present work, the optimal pH 5 was mainly used for the hydrolysis, but hydrolysis was also successful at a lower pH of pH 3.

The hypothesis proposed in study III was that faba beans contain endogenous β-glucosidase activity can hydrolyse vicine and convicine. The presence of β-glucosidases in faba bean could be explained as a defence mechanism that releases reactive toxic aglycone forms. When the cell wall is disrupted, the enzyme and its substrates can come together to release the active toxic form. This is a known mechanism for the release of cyanide from cyanogenic glycosides in almonds, sorghum and cassava (Morant et al. 2008; Gleadow and Moller 2014). It is not yet clear if the endogenous β-glucosidase activity of faba bean can act on vicine and convicine. The activity of the β-glucosidase of faba bean was reported by Arese and De Flora (1990), and the activity was reportedly higher in mature seeds than in young seeds. Rizzello et al. (2016) also reported that faba bean flour used for dough making contained β-glucosidase activity when tested against an artificial p-nitrophenyl glucoside (pNPG) substrate.

Flours from the same company were used in study III. However, incubation of flours at pH 4.5–6.5 at 30–37 °C did not result in notable losses of vicine and convicine, which were only approximately 10% after a short time in all the studied cultivars and conditions and were only slightly higher (20–25%) after 24 h using the selected materials and conditions. Some spontaneous fermentation can occur during a 24 h incubation, as reported by Coda et al. (2015) and Rizzello et al. (2016). The results in the present work confirmed that no notable hydrolysis of vicine and convicine occurs under conditions relevant for food processing in the absence of added enzyme or production of enzyme by LAB fermentation.

Enzymatic hydrolysis can be a practical way to remove vicine and convicine. Optimisation of the process is required, because many factors, such as including pH, temperature, enzyme activity and incubation time, are crucial for optimal hydrolysis. However, the hydrolysis can be carried out in a reasonable time under optimal conditions. Hydrolysis with added β-glucosidase in study II enabled the investigation of the stability of the aglycones. The endogenous β-glucosidase activity of faba bean was not sufficiently active to hydrolyse vicine and convicine in study III.

6.3.2 Challenges in removal of vicine and convicine in LAB fermentation

The incubation experiments aimed at studying endogenous enzyme activity in faba bean confirmed that the addition of an external enzyme source was required for efficient hydrolysis of vicine and convicine. Fermentation is a practical and low-cost approach for modification of faba bean raw material, and it can simultaneously enhance faba bean nutritional quality (Coda, Varis et al. 2017). However, the use of fermentation for the hydrolysis of vicine and convicine can be challenging. The vicine and convicine losses were moderate with the selected LAB strains (*Pediococcus pentosaceus* strain I02, *Lactobacillus plantarum* strains E76 and B24W), which were all confirmed as β-glucosidase positive towards a pNPG substrate. The strains were selected from a collection of strains based on their high β-glucosidase activities (Di Cagno et al. 2010; Coda, Kianjam et al. 2017; Verni et al. 2017). The best fermentation outcome for faba bean raw material was seen with the I02 and B24W strains, which had higher β-glucosidase activity than the E76 strain.

In addition to strain selection, the fermentation conditions were also crucial for vicine and convicine hydrolysis. Hydrolysis did not occur when fermentation was conducted at 20 °C for 24 h; only fermentation at a higher temperature (25 °C) induced hydrolysis. Vicine and convicine losses were at their highest, at 82–85% and 34–47%, respectively, in sourdoughs (25 °C, 24 h) from strains I02 and B24W, while the losses were 24% and 14% in sourdough from strain E76. Had fermentation continued for a longer time at the higher temperature, the vicine and convicine losses would presumably have been higher than those reported in study III, as indicated earlier by Coda et al. (2015) and Rizzello et al. (2016), who showed that fermentation at 30 °C for 48 h with strain B24W resulted in greater than 90% hydrolysis

of vicine and convicine. However, their acidification was more intense than in study III, and the higher acid content (lactic and acetic acids) produced by LAB is perceived as an acidic taste. Sourdoughs fermented at 25 °C for bread making were already slightly too high in acidity for an acceptable bread flavour.

To conclude, β-glucosidase-positive LAB strains can produce β-glucosidases that hydrolyse vicine and convicine in sourdoughs, but the obtained hydrolysis efficiency depends on the β-glucosidase activity, other properties of the strain and the fermentation conditions. The β-glucosidases produced by LAB are most likely intracellular, while certain fungi are also able to produce extracellular forms of this enzyme (McKay 1992; Michlmayr et al. 2010). The β-glucosidase activity from LAB was able to hydrolyse other β-glycosides, i.e. isoflavones in soy milk (Otieno et al. 2005; Donkor and Shah 2008; Di Cagno et al. 2010) and to alter the flavour of musts and wines (Sestelo et al. 2004; Michlmayr et al. 2010). Bacteria are known to produce β-glucosidases for degradation of cellulose, and especially cellobiose (Singhania et al. 2017). Determination of the best possible way to induce hydrolysis of vicine and convicine will require further study of the metabolic pathways of LAB microbiota to understand the mechanisms behind their utilisation of different carbon sources. The production of glucosidases like β-glucosidase in LAB is directed by carbohydrate metabolism (Michlmayr and Kneifel 2014). Production of glucosidases is likely related to the release of sugars for energy metabolism, so these enzymes may be activated only when these organisms require sugars to be released from more challenging sources. The sourdoughs in study III contained only faba bean flour, so it was the only energy source available for the LAB.

Vicine and convicine were not notably further lost during baking. Losses could have occurred by fermentation with baker´s yeast, which also has β-glucosidase activity (Inamdar and Kaplan 1966; Riedl et al. 2005). The β-glucosidase activity increased in soy-wheat dough in 2 h when incubated at 22–48 °C (Riedl et al. 2005). However, the yeast fermentation period in baking is usually short, as it was in the present study. The heat stability of vicine and convicine (Arbid and Marquardt, 1985b; Cardaror-Martinez et al., 2012) suggests that these compounds are not effectively lost during baking.

Fermentation was challenging in the conditions selected from the perspective of baking breads with a pleasant taste. Sensory quality is one criteria for fermentation, so the hydrolysis of vicine and convicine was not as successful under the selected conditions as previously reported for longer fermentations. To enhance the hydrolysis, β-glucosidase could be added during the fermentation, because the lowered pH may be favourable for certain enzymes. The total loss of vicine and convicine under moderate fermentation conditions could also be possible with the vc- cultivars like ‘Divine’ or ‘Melodié’.

6.3.3 Formation and stability of the aglycones in models

Formation and stability of the aglycones The aglycones were produced in study II by enzymatic hydrolysis at conditions (pH 5, 37 °C) optimal for the β-glucosidase enzyme. Their formation was seen at the first time point of 15 min in faba bean extracts and in vicine and convicine fractions and, for most of the experiments, the highest amounts of the aglycones were already present by then. Aglycone formation depended on the activity level of the β-glucosidase: constant formation of divicine was seen with the lowest activity of β-glucosidase in faba bean extracts (0.5 U/ml) and vicine fractions (0.2 U/ml). When the enzyme activity was increased by 10–15 or 20-fold, divicine and isouramil were formed more rapidly in extracts and divicine was formed faster in vicine fractions. The formation of the aglycones has not been previously studied to the extent it has been studied with the chromatographic analysis method presented in study II. Previous studies induced the formation of either divicine or isouramil by acid or enzyme hydrolysis, and aglycone stability was studied only at a single time point (Marquardt, Frohlich et al. 1989; Marquardt, Arbid et al. 1989; McMillan et al. 1993). In study II, different levels of β-glucosidase were used and time-dependent observations provided information about the efficiency of the formation of aglycones.

The amounts of the aglycones gradually decreased during 60 min for the faba bean extracts and 120 min for the vicine and convicine fractions. A rapid decrease in the aglycones was also noticed in previous stability studies (Chevion, et al., 1982; Benatti, et al., 1984; Marquardt, Frohlich, et al., 1989; McMillan, et al., 1993). The previous incubation conditions (pH, temperature) were somewhat different and the experiments were carried out at neutral pH or at physiological pH under air. At these conditions, half or more of the divicine and isouramil disappeared in under 20 min at room temperature or 37 °C (Chevion, et al., 1982; Marquardt, Arbid, et al., 1989; McMillan, et al., 1993). At pH values closer to the pH values of 3 and 5 used in study II, divicine decreased by 36% in 30 min at pH 5.5 and isouramil by 79% in 10 min at pH 3.7 under air at room temperature (Marquardt, Arbid, et al., 1989; Marquardt, Frohlich, et al., 1989). The results obtained in study II were in line with these previous studies, because the decrease in aglycone levels occurred in 15–30 min from the beginning of incubation. The decrease in the aglycones depended on the enzyme activity. Follow-up reactions with different enzyme activities showed a steeper decrease in isouramil levels than in divicine levels from the faba bean extracts, indicating a faster degradation of isouramil under the study conditions. The aglycones were not totally eliminated from extracts in 60 min, but 50–70% of the divicine and 95–98% of the isouramil were lost by the last time point. The follow-up reactions showed that the aglycones were completely lost in fractions by 90–120 min following treatment with 3 and 6 U/ml of enzyme. In study II, the formation of the aglycones was shown in relation to the hydrolysis and their decrease was followed during time.

Further reaction products of the aglycones While the amounts of the aglycones decreased, indications of oxidation products were noticed. The compound called oxidised divicine

appeared with divicine and was followed in the extracts and in the vicine fractions. The amount of a compound that eluted at 1.9 min and had UV absorption at low wavelengths (< 230 nm) also increased during time. This compound ultimately disappeared, and no UV absorbing (> 210 nm) compounds remained in vicine and convicine fractions at the last time point.

The ratio between divicine and oxidised divicine differed in the studied models. The amount of oxidised divicine was higher than the amount of divicine in the vicine fractions. By contrast, the amount of divicine was higher than that of oxidised divicine in the faba bean extracts treated with higher enzyme activities (5 and 10 U/ml). The lowest enzyme activity (0.5 U/ml) resulted in constant formation of divicine and the amount was higher than that of oxidised divicine. The vicine fractions did not contain components that could have slowed down the oxidation, whereas oxidation was possibly retarded in the extracts.

The UV absorption maximum of oxidised divicine at 262 nm was slightly higher than the previously reported value of 250–255 nm (Chevion et al. 1982; Marquardt, Frohlich et al. 1989). The identity of oxidised divicine was not fully confirmed, as the amounts obtained were presumably too low for confirmation of the identity with ESI-LC-MS. Further studies are required to optimise the MS determination of the oxidation and decomposition products of divicine and isouramil. However, stabilisation experiments supported the identity of oxidised divicine. The formation of oxidised divicine was diminished when ascorbate was added and when the hydrolysis mixture was purged with nitrogen, while the amount of divicine was slightly increased. Other reductants, such as sodium hydrosulphite, sodium borohydride, cysteine, 3-mercaptoethanol or DTT, have been used previously to show regeneration of divicine from its oxidised form (Chevion, et al., 1982; Marquardt, Frohlich, et al., 1989; McMillan, et al., 1993). Regeneration of divicine from oxidised divicine also routinely occurs in RBCs during reductive-oxidative reactions. Ascorbic acid is a natural antioxidant and a reducing agent that occurs widely in foods. The amount of ascorbate added in study II was higher than is naturally found in faba bean, so the stabilising effect cannot be directly compared to food systems. Nevertheless, the finding that a natural reductant can participate in reactions of divicine was interesting, and the role of ascorbic acid in faba bean could be studied further in greater detail.

As mentioned above, oxygen played a role in the stability of divicine and oxidised divicine, because purging the hydrolysis mixture with nitrogen improved the stability of divicine. Reactivity of the aglycones is greatly enhanced when oxygen is present, thereby confirming that oxidation of the aglycones occurs. Nitrogen or another inert gas atmosphere retarded the disappearance of divicine for 60 min at pH 7 (Chevion, et al., 1982) and isouramil for 20 min at pH 3.7 (Marquardt, Arbid, et al., 1989). In study II, the total removal of oxygen was not attained, but stability was improved even with a partial replacement of air with nitrogen.

The degradation of divicine was slower at 20 °C than at 37 °C at both studied pH values (3 and 5). The effect of pH value on stability was seen at the beginning of the incubation, when the amounts of divicine were higher at pH 3 than at pH 5. Conversely, the amounts of oxidised divicine were lower at pH 3 than at pH 5. By the end of the incubation, temperature became a more important factor for the stability of divicine. A lower temperature slowed down the reactions, so amounts of divicine and oxidised divicine remained after 75 min at 20 °C. Despite the better stability at 20 °C, divicine and oxidised divicine ultimately disappeared from the hydrolysis mixtures by 24 h. Divicine may be more stable at low pH than at high pH values, and more stable at low temperatures (Chevion et al. 1982; Baker et al. 1984; Marquardt, Frohlich et al. 1989). By contrast, isouramil was less stable than divicine at low pH values (Marquardt, Arbid, et al., 1989). However, the pH values and temperatures in study II were not sufficiently low to attain any notable increase in the stability of divicine, and even lower pH values are not relevant for food.

In contrast to oxidised divicine, oxidised isouramil was not seen in the experiments conducted in study II. Isouramil was the only product detected in the convicine fractions and faba bean extracts. An intermediate form of isouramil, presumably oxidised isouramil, was seen with a spectrophotometric method by Chevion et al. (1982) and with an HPLC method by (Marquardt, Arbid et al. 1989). The possibility that isouramil is more reactive than divicine and reacts more rapidly under the studied conditions should be further investigated.

When the levels of the studied compounds decreased, an increase in peak areas was observed at lower wavelengths (< 230 nm). Ultimately, all the UV absorptivity was lost from the vicine and convicine fractions, whereas the reaction products were still observed in the faba bean extracts. The reaction pathways for the degradation of divicine and isouramil are not yet known, and other reactions than oxidation may be occurring concomitantly. The loss of UV absorption could be a result of opening the pyrimidine ring structure in the final end products. Increased absorption at low wavelengths was first reported by Chevion et al. (1982), when UV absorbance of divicine and isouramil shifted from an initial reading of approximately 280 nm to 245–255 nm, and then finally to less than 230 nm. The final products of the aglycone decomposition should be characterised in detail in future studies because the reactions have not yet been established.

Overall, the instability of the aglycones was demonstrated in experiments that examined the formation of the aglycones in response to varying enzyme activities under selected conditions. These hydrolysis conditions and models were a new approach for the study of aglycone stability. The follow-up of the reactions occurring over time provided information about the disappearance of the aglycones and the formation and disappearance of oxidised divicine as a reaction product. Even though the reaction products were not identified, the complete disappearance of UV-absorbing compounds from the vicine and convicine fractions confirmed the instability of the aglycones. Results from the hydrolysis experiments

of study II provided the background information for further study of the reactivity of the aglycones in food systems.

6.3.4 Formation and stability of the aglycones in food systems

The knowledge obtained from studying the aglycones under model conditions was further utilised to study the aglycones in food systems. The stability of the aglycones in food systems is essentially unknown because the previous research focus has been only on the removal of vicine and convicine. The vicine and convicine contents have been reduced by soaking (Jamalian and Ghorbani 2005), heating (Cardaror-Martinez et al. 2012), germination (Goyoaga et al. 2008) and fermentation (McKay 1992; Coda et al. 2015), but the response of the aglycones to these treatments has not been studied. Some investigations have indicated the presence of aglycones, but without experimental confirmation, in faba bean paste (Arbid and Marquardt 1985b) and sourdoughs (Rizzello et al. 2016). Studies II and III confirmed the presence and loss of the aglycones of both vicine and convicine.

The aglycones were detected, although in small amounts, from faba bean suspensions treated with added β-glucosidase for 15 min. The enzyme activity was sufficiently high to hydrolyse vicine and convicine in about 2 h, but higher enzyme activity might have yielded more aglycones at the beginning of the incubation. When suspensions were incubated, the aglycones disappeared. Interestingly, the amount of oxidised divicine was higher than the amount of divicine, which showed that divicine was rapidly oxidised. The same ratio was noticed in the vicine fractions, while it was opposite in faba bean extracts. The reasons behind the differing stabilities of divicine should be studied further to provide a better understanding of the factors that affect stability. Reactions were obviously ongoing in the suspensions, and the rapid disappearance of the aglycones again showed their instability. The identity of the aglycones in food systems was first confirmed in this study, because Arbid and Marquardt (1985b) indicated that the new peaks that formed and disappeared in faba bean paste treated with β-glucosidase were possibly aglycones but did not confirm this possibility.

The presence of the aglycones was noticed in the doughs and breads containing faba bean flour only when losses of vicine and convicine occurred. The aglycones were therefore found only in fermentations at 25 °C and in faba bean-wheat doughs (i.e. not from fermentations at 20 °C). The aglycones were also not detected from breads that were the final products. The ratio of oxidised divicine and divicine in extracts of sourdoughs was similar to that in the faba bean extracts, while the vicine fractions and suspensions had an opposite ratio. The pH values in sourdoughs (4.4–4.8) were close to the pH values used in the hydrolysis experiments (5.0). The peak area of oxidized divicine increased during a 30 min incubation of sourdough extracts, so the ratio of divicine and oxidised divicine subsequently changed. The vicine and convicine losses and the formation of the aglycones in sourdoughs were studied by Rizzello et al. (2016), by using the same strain B24W that

was used for fermentation at 30 °C for 48 h. In that study the aglycones were not properly separated. However, in the present study, the aglycones were followed from extracts of sourdoughs, doughs and breads, and the aglycones were confirmed to disappear from sourdough extracts in 20 h.

This study was one of the first to demonstrate the presence of the aglycones in a food system and to evaluate their stability. The previous studies focusing on the stability of the aglycones were carried out in model conditions (Chevion et al. 1982; Marquardt, Frohlich et al. 1989; Marquardt, Arbid et al. 1989; McMillan et al. 1993). In suspensions and sourdoughs, the aglycones were initially detected and then subsequently disappeared. Disappearance of the aglycones and formation of oxidised divicine proved that the aglycones reacted further and the studied food matrices did not protect the aglycones from oxidation.

6.3.5 Further challenges in evaluation of the aglycone degradation

The disappearance of the aglycones and their instability was confirmed in different conditions in this work, but the degradation products should be studied further. The degradation of divicine and isouramil has been explained by opening of the pyrimidine ring structure (Chevion et al. 1982). The small molecular weights of the decomposition products might pose a challenge for their detection, because suitable and sensitive methods (e.g. MS methods) are required for their identification. Uracil, the suggested precursor of vicine and convicine, is degraded in a reductive pathway by dihydropyrimidine dehydrogenase to β-alanine, carbon dioxide and ammonia during pyrimidine catabolism (Kafer et al. 2004; Zrenner et al. 2006; Loh et al. 2006). The degradation of divicine and isouramil could therefore give rise to similar products. However, degradation reactions induced by dihydropyrimidine dehydrogenase do not explain the decomposition in the conditions used in study II, because enzymes were likely to have been deactivated during the preparation of the faba bean extract and the vicine and convicine fractions.

An alternative explanation is that radical-induced decomposition of the pyrimidine ring could be a possible degradation route for divicine and isouramil. Compounds that can form quinones are suggested to undergo radical-induced oxidation reactions (Winterbourn et al. 1989; Kehrer 2000), and oxidation of divicine to a semiquinoid free radical has been described (Baker et al. 1984; Pedersen et al. 1988; Winterbourn et al. 1989). Radical signals from divicine were especially high at alkaline pH values (Baker et al. 1984; Pedersen et al. 1988), and the degradation of divicine was also rapid at alkaline conditions (pH 8 and 13) (Marquardt, Frohlich et al., 1989). Thus radical-induced reactions could cause the degradation of the pyrimidine ring in divicine and isouramil.

The possibility that the aglycones react with other compounds should be also considered. The adduct formation of divicine with GSH was suggested when the absorption maximum of divicine changed from 280 nm to 305 nm (Chevion et al. 1982). The haemotoxicity of

divicine is also related to the oxidation of GSH. However, the adduct formation with GSH was not confirmed by later studies. The ability of the aglycones to form complexes was shown by Kim et al. (1982), because the aglycones formed a stable complex with titanium tetrachloride. Overall, the ability of divicine and isouramil to form new bonds, e.g. in condensation reactions, is not established. When compounds are studied under model conditions, reactions with other compounds should not occur. The decrease in divicine and isouramil levels has been shown in various model conditions (Chevion et al. 1982; Marquardt, Frohlich et al. 1989; Marquardt, Arbid et al. 1989; McMillan et al. 1993) as well as in this work in the vicine and convicine fractions.

To conclude, the pyrimidine ring is most likely either opened in radical-induced reactions or the aglycones react with other compounds. The best supported hypothesis is a radical-induced oxidation to smaller compounds, because this could occur in all the studied conditions. Decomposition by radical reactions, unlike reactions with other compounds, would also be irreversible and would support the total loss of the aglycones observed in the present work.

7 Conclusions

Removal of vicine and convicine from faba bean has previously focused on the compounds as such, while formation of the aglycones has not been considered. Analysis methods have been applied only for studying vicine and convicine. Understanding the occurrence and stability of the aglycone forms is especially necessary for the total elimination of vicine and convicine.

In this work, the RP-HPLC-UV analysis was first optimised in terms of selected factors, including the extraction solvent, eluent composition and quantification. An internal standard method was used for quantification, and the relative response factors of vicine and convicine to uridine were shown to be close to each other, thereby enabling quantification using only vicine or convicine. The main improvement in the method was the development of simultaneous determination of both vicine and convicine and the aglycones to enable more comprehensive evaluation of their removal. The method also provided preliminary information about further reaction products. The compound considered as oxidised divicine was detected with divicine, and a peak with low UV absorption was seen in chromatograms during enzyme incubations.

The vicine and convicine contents in the Finnish-grown cultivars were close to the average contents previously reported for faba bean. The variation among cultivars was more remarkable than was the variation among growing years. Although the weather conditions varied notably in the study years 2010–2012, the vicine and convicine contents were relatively constant from year to year, and the total contents were high in the cultivar 'Kontu'. Plant breeders are developing early cultivars that are free from or contain low amounts of vicine and convicine for the boreal growing conditions. The cultivars with high vicine and convicine contents (e.g. the cultivar 'Kontu') will remain in use until the new cultivars are released.

The focus of this work was in the elimination of vicine and convicine, and this was studied from the viewpoint of enzymatic hydrolysis. Almond β-glucosidase was used to hydrolyse vicine and convicine to their aglycones. Vicine and convicine levels were decreased within a short time of 1–2 h at pH 5 at 37 °C. The enzyme was active at pH 3 and 5, and these low pH values are reasonable for certain foods, e.g. fermented foods. The activity of the endogenous β-glucosidases of faba bean was investigated under selected conditions, but the activity was low and did not result in significant declines in vicine and convicine content. Thus, the hydrolysis of vicine and convicine in faba bean requires the addition of an external enzyme or production of β-glucosidases, e.g. in LAB fermentation.

LAB fermentation with selected β-glucosidase positive bacteria induced hydrolysis of up to 82–85% for vicine and up to 34–47% for convicine, i.e. complete hydrolysis was not obtained in moderate conditions. The challenge posed by LAB fermentation is to attain efficient hydrolysis while avoiding intense acidification to maintain good sensory quality of

the treated food. The hydrolysis was dependent on the fermentation conditions, because the hydrolysis occurred only at a higher fermentation temperature of 25 °C, and did not occur at 20 °C. The hydrolysis did not notably continue during baking. The breads as final products still contained some vicine and convicine.

The aglycones were formed during enzymatic hydrolysis using different activities of β-glucosidase. The follow-up of the reaction showed that divicine, oxidised divicine and isouramil were unstable, and their levels decreased rapidly during incubation. These compounds completely disappeared from the vicine and convicine fractions during a 120 min incubation. The ratio between divicine and oxidised divicine varied in the different models as a consequence of differences in the stability of divicine. Further products appeared in the chromatograms, but by the end of the incubations of the fractions and faba bean suspensions, all UV-absorbing compounds had disappeared. The stability of divicine in the vicine fractions was improved by partial removal of oxygen, by addition of sodium ascorbate and by lowering the temperature. At the same time, the amount of oxidised divicine was diminished. The instability of the aglycones was confirmed by the hydrolysis experiments, but studying the reaction routes in more detail is recommended.

The occurrence of the aglycones was studied for the first time in conjunction with vicine and convicine in food systems. Divicine and isouramil were formed after addition of β-glucosidase to faba bean suspensions, and they disappeared during incubation. The same sourdoughs that showed hydrolysis of vicine and convicine, contained divicine, oxidised divicine and isouramil, but these compounds disappeared as well during incubation of sourdough extracts. The aglycones were not detected in breads.

Overall, the aglycones were unstable in all the studied materials, indicating that other compounds present in the food matrix did not stabilise the aglycones. In the future, the toxicity of fermented materials and breads could be estimated by in vitro experiments on RBCs exposed to the food materials. The degradation products of the aglycones are poorly studied, but the ring opening has been suggested to occur by radical-induced reactions. The products may be challenging to study, because they are relatively small, but advanced LC-MS methods could provide useful structural information on aglycone degradation products.

This work provided information about the occurrence of vicine and convicine in Finnish-grown *Vicia faba* cultivars and investigated the possibilities for removal of vicine and convicine and their hydrolysed forms. Removal of vicine and convicine by hydrolysis is promising, thus slightly challenging, and the aglycones may be eliminated as well due to their instability. In addition, cultivars free from vicine and convicine may be available in the future, as vc- cultivars are already available for climate areas warmer than the boreal region.

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