



Project Code	
Project Type	

FINAL REPORT 2024

Final reports must be submitted using the online application form at www.sagit.com.au with this Word document attached **within two months** after the completion of the Project Term.

PROJECT CODE	UAD-001123
PROJECT TITLE	
Processing solutions for a novel high-protein food ingredient from vetch	

PROJECT DURATION	<i>These dates must be the same as those stated in the Funding Agreement.</i>					
Project start date	29/06/2023					
Project end date	30/06/2024					
SAGIT Funding						

PRINCIPAL INVESTIGATOR <i>(responsible for the overall project and reporting)</i>		
Title: Dr	First Name: Janine	Surname: Croser
Organisation:	South Australian Research and Development Institute (SARDI)	
Mailing address:	Plant Genomics Centre, Hartley Grove, Urrbrae SA 5064	
Telephone:		Email:
Mobile:		

ADMINISTRATION CONTACT DETAILS <i>(responsible for all administrative matters relating to project)</i>		
Title: Ms	First Name: Michelle	Surname: McBride
Organisation:	The University of Adelaide	
Mailing address:	The University of Adelaide Level 3, Rundle Mall Plaza, 50 Rundle Mall, Adelaide SA 5000	
Telephone:		Email:
Mobile:		

PROJECT REPORT: Please provide a clear description for each of the following:

Executive Summary (200 words maximum)

A number of common foods and food ingredients (such as cassava and almonds) can contain compounds that, when consumed and metabolized, are converted into cyanide. The safety risk to consumers is managed by food regulatory authorities imposing maximum limits for the toxin in the food. Common vetch contains high concentrations of the neurotoxins β -cyanoalanine (BCA) and γ -glutamyl- β -cyano-alanine (ggBCA), that are metabolized to cyanide by humans, pigs, poultry and other monogastric animals, making it unsuitable for consumption. Considerable long-term efforts using genetic strategies to eliminate the toxin have so far been unsuccessful.

In this one-year project, we investigated alternative options for post-harvest processing to remove or reduce the levels of BCA and ggBCA from vetch. A sensitive LC-MS/MS method was developed by project partners at UniSA for quantitative analysis of ggBCA and BCA, and a number of processing treatments were assessed for their effectiveness in toxin removal. Several of the processing methods reduced the concentration of both compounds by 60-99% but have yet to reliably attain levels below 10 mg cyanide kg⁻¹, the accepted limit for cassava flour. Further testing of techniques for post-harvest processing of lower toxin varieties and refining the sample preparation for quantification of toxin levels is required.

Project objectives

The project facilitated initial processing and analytics research with aims to validate and optimise detoxification of vetch. Validation of a robust, cost-effective method of removal of β -cyanoalanine and γ -glutamyl- β -cyano-alanine, which are toxic to humans and monogastric animals, could open new market opportunities for this under-utilised grain legume. The addition of another food or feed source of pulse protein to South Australian industry, in an environment of high market demand, would stimulate grower interest in vetch in cropping rotations and add significant value to the crop. It would offer the opportunity for breeding and release of varieties targeted specifically at grain production.

Overall Performance

The overall objective of this project, to identify, optimise and up-scale post-harvest processing treatments suitable for up to 99% reduction of the cyanide-containing toxins from vetch, was achieved.

An industry-standard assay was developed with our UniSA partners, providing capacity in SA for assessment of toxin levels in vetch for breeding and future pre-breeding projects.

However, further research is required to ensure robust and repeatable assays and combinations of post-harvest treatments sufficient to ensure that toxin levels in treated vetch are safely below the limit of 10 mg kg⁻¹ allowed for a similar cyanogenic flour made from cassava. z

Personnel:

University of Adelaide/SARDI: Janine Croser (SARDI Crop Sciences)

University of Adelaide/University of South Australia/SARDI: Maria Saarela (SARDI Food Sciences)

SARDI: Stuart Nagel, Ruwan Lenorage

University of Adelaide: Julie Hayes

External Consultant: John Carragher

University of South Australia: Leigh Donnellan, Peter Hoffman

KEY PERFORMANCE INDICATORS (KPI)

KPI	Achieved	If not achieved, please state reason.
Quantitative method developed for high-sensitivity detection of both β -cyanoalanine (BCA) and γ -glutamyl- β -cyano-alanine (ggBCA) in vetch flour	Yes <input checked="" type="checkbox"/> No <input type="checkbox"/>	

Wet protein fractionation, extrusion, and fermentation of vetch flours undertaken, and the resulting flours analysed for toxin levels, and subjected to functionality and sensory testing	Yes <input checked="" type="checkbox"/> No <input type="checkbox"/>	
Optimal conditions for steeping removal of BCA toxin from vetch grains identified; the optimized process up-scaled and trialed in dry fractionation for protein enrichment. Functionality and sensory testing completed on the protein-rich and starch-rich fractions	Yes <input checked="" type="checkbox"/> No <input type="checkbox"/>	
Results communicated to stakeholders, final SAGIT report completed	Yes <input checked="" type="checkbox"/> No <input type="checkbox"/>	

TECHNICAL INFORMATION (Not to exceed three pages)

Provide sufficient data and short clear statements of outcomes.

1. **Method development for high-sensitivity detection of β -cyanoalanine (BCA) and γ -glutamyl- β -cyano-alanine (ggBCA).** UniSA partners Dr Leigh Donnellan and Prof Peter Hoffman established a liquid chromatography/mass spectrometry (LC-MS/MS)-based method for analysis of the two forms of the toxin present in common vetch. The method is optimised to enable maximum sensitivity of detection in samples subjected to post-harvest processing. Limits of quantitation (LOQ) were determined as the lowest concentrations with precision <20%, at 25 $\mu\text{g g}^{-1}$ flour for ggBCA and 3 $\mu\text{g g}^{-1}$ flour for BCA.

2. **Analysis of the effectiveness of a range of modern processing methods for vetch detoxification.** Experiments were undertaken to investigate the potential for wet and dry protein fractionation, fermentation, microwave treatment and extrusion, to remove toxin from vetch grain. All processed samples were milled and measured for ggBCA and BCA content using UniSA's quantitative extraction and analysis method. Results are summarised below and compared with de-hulled grain and our best detoxification method (4 h steeping at 60 °C, with hourly water changes). Dry fractionation led to an increase in toxin levels in the protein-rich fraction relative to initial flour. Fermentation of de-hulled vetch using two different commercial fungal starter cultures (Koji (*Aspergillus* spores) and Tempeh (*Rhizobus* spores)) reduced ggBCA levels to around 5% of pre-processed grain, while extrusion across a range of settings also significantly reduced ggBCA. Most treatments resulted in an increase in the less prevalent form of the toxin, BCA. This indicated incomplete degradation of ggBCA and/or incomplete rinsing away of toxin. The best of the processing methods were wet fractionation and microwaving of intact grain followed by dehulling and a short period of steeping at room temperature. Microwave methods warrant further exploration as they may be less energy- and water-consuming than steeping at high temperatures.

Assessments of colour and smell of the processed ingredients were undertaken. There were no major impacts of extrusion, wet or dry extraction, steeping or fermentation on the colour of the processed ingredients (e.g. Figure 3). Fermented product had a strong, unpleasant smell due to fungal growth that would require mitigation by optimising the fermentation time.

Nutritional comparisons between microwaved, steeped and untreated samples indicated that whilst macronutrient composition was not adversely affected, some micronutrients were reduced by steeping: iron (by 95%), potassium (75%), thiamine (60%), folic acid (50%) and niacin (by >25%). Microwave methods resulted in minimal losses of iron and folic acid.

Table 1. Effect of various modern processing methods on levels of γ -glutamyl- β -cyano-alanine (ggBCA) and β -cyanoalanine (BCA) in common vetch (*Vicia sativa* L.). All treatments, except for microwaving, were applied to de-hulled grain, and all samples were milled to a powder after processing and extracted and analysed in triplicate. Values are presented as means. Treatments include a pre-processing treatment (Nil) and a steeping treatment at 60 °C for comparison. Steeping at 60 °C has been our most successful detoxification method.

Treatment	ggBCA content (mg g ⁻¹)	BCA content (mg g ⁻¹)	Total toxin content (mg g ⁻¹)	Change (relative to no treatment)
Nil (de-hulled grain)	19.5 – 23.5	0.028 – 0.054	19.6 – 23.5	
Dry fractionation (protein-rich fraction)	37.7	0.12	37.8	1.9-fold increase
Wet extraction (protein-rich fraction)	0.41	0.039	0.45	98% decrease
Microwave, then dehulling and 3 h soak at room temp	0.34 – 0.85	0.065 – 0.16	0.41 – 1.01	95-98% decrease
4 h steep at 60 °C	<LOQ – 0.03	0.016 – 0.040	0.02 – 0.07	>99% decrease
Fermentation (Koji starter)	1.2	0.20	1.4	93% decrease
Fermentation (Tempeh starter)	1.4	0.30	1.7	91% decrease
Extrusion (range of settings)	4.3 – 7.7	0.052 – 0.22	4.3 – 7.8	60-80% decrease

3. Optimisation of a steeping method for toxin removal. The optimal conditions for steeping to remove toxin from vetch were investigated. We identified 60 °C as the ideal steeping temperature (Figure 1a). This facilitated maximum conversion of the ggBCA precursor to BCA, and maximum leaching of toxin. More frequent water changes led to lower levels of both forms of the toxin, as did longer incubation times (Figure 1b).

Based on food safety guidelines for limits of consumption of cyanide (CN) compounds, an expected ratio of ggBCA:BCA in steeped vetch of 1:2 and the theoretical CN content of the vetch toxins (10.7% ggBCA/ 22.8% BCA), we calculate that a processing method would need to reliably remove the combined ggBCA/BCA content to below 0.05 mg g⁻¹ to be considered safe. Incubation for 4 h at 60 °C, with hourly water changes, was generally sufficient to achieve this level (Table 1).

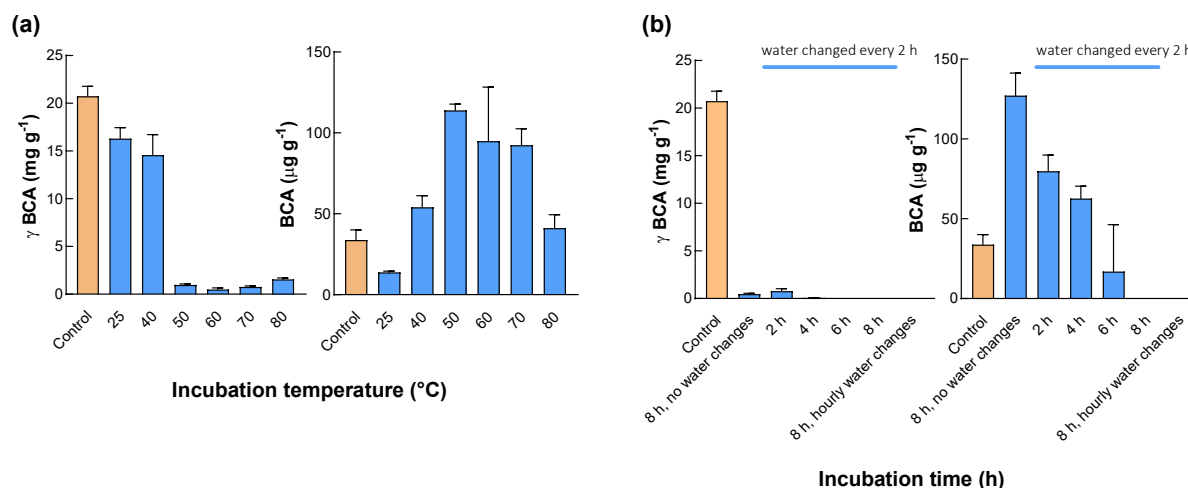


Figure 1. (a) ggBCA and BCA content of dehulled common vetch grains after steeping in a single volume of water (4:1 v/v) for 8 h at a range of temperatures, compared to an untreated control sample. (b) ggBCA and BCA content of dehulled vetch after steeping at 60 °C for different times; The 2 h, 4 h, 6 h and 8 h treatments had water replaced every two hours. Values are presented as means ± s.d. for three biological replicates.

All steeping experiments were performed using a water to grain volumetric ratio of 4:1. Increasing this ratio, and/or establishing a constant flow of heated water through the grain is expected to further increase leaching of the toxin. The leached toxin was detected in water sampled from steeping experiments (Figure 2). This has implications for management of wastewater from grain processing.

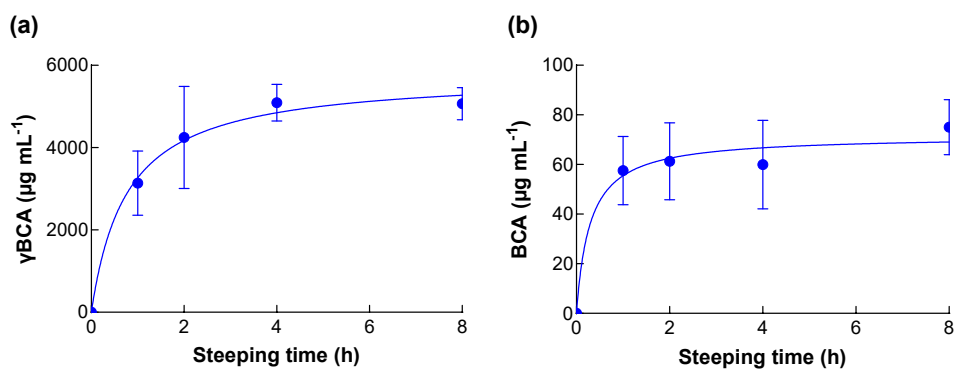


Figure 2. Amounts of (a) γ -glutamyl- β -cyano-alanine (ggBCA) and (b) β -cyanoalanine (BCA) leached from vetch during 8 h incubation at 60 °C into a single volume of water. Values are means \pm s.d of 6 replicate measurements of samples of the steeping water; Michaelis-Menton kinetics curves are fitted to the data.

4. Up-scaling detoxification of vetch, and trialling in dry fractionation. Two of the processing treatments, steeping, and microwave treatment followed by soaking at room temperature, were up-scaled to kg quantities. The resulting flours were jet-milled and subjected to dry fractionation. There was no negative impact of these treatments on downstream processing of the flours, with protein content of the fine fractions enriched by around 2-fold, to around 50% protein (Table 2).

For all flours, the fine fraction was apparently enriched for the toxins (Table 3; by \sim 2-fold in all treatments apart from steeping). However, toxin analyses using these samples revealed a possible influence of particle size on the extraction efficiency of ggBCA. We investigated this further but found that apparent increases in toxin levels in the fine, protein-rich fraction were not simply a consequence of this fraction having smaller particle size. Rather, and somewhat to our puzzlement, jet-milling of the steeped sample specifically, exposed an additional, significant pool of extractable toxins. The effect of steeping on the availability of toxin would need to be comprehensively examined, to ensure steeped vetch can be safely used for downstream processing.

Dry fractionation of steeped sample has called the reliability of steeping for toxin removal into question.

Table 2. Protein enrichment of the fine fractions generated from dry fractionation of milled flour from common vetch, and of flours prepared after microwaving followed by dehulling, microwaving followed by dehulling and soaking, and a steeping treatment. *Protein levels were determined by N combustion, using a conversion factor of 5.4.

Treatment detail	% protein*			Protein enrichment
	Milled flour	Coarse fraction	Fine fraction	
Nil	23.7	15.9	52.2	2.2-fold
Microwave (3 min)	24.2	16.7	50.1	2.1-fold
Microwave + soak (3 h at room temperature, hourly water changes)	24.0	21.5	46.1	1.9-fold
Steep (4 h at 60 °C, hourly changes)	22.2	16.9	51.2	2.3-fold

Table 3. Levels of γ -glutamyl- β -cyano-alanine (ggBCA) and β -cyanoalanine (BCA) in fractionated flours prepared from common vetch, after dehulling (Nil), microwaving followed by dehulling, microwaving followed by dehulling and soaking, and a steeping treatment. All treatments were extracted and analysed in triplicate. Values are presented as means.

Treatment detail	Milled flour	Coarse fraction	Fine fraction
Nil			
ggBCA content (mg g ⁻¹)	19.5	14.4	37.7
BCA content (mg g ⁻¹)	0.054	0.061	0.12
Microwave (3 min)			
ggBCA content (mg g ⁻¹)	7.2	7.5	15.5
BCA content (mg g ⁻¹)	0.63	0.34	0.85

Microwave, then soak (3 h at room temperature, hourly water changes)

ggBCA content (mg g ⁻¹)	0.85	1.25	1.90
BCA content (mg g ⁻¹)	0.16	0.17	0.15

Steep (4 h at 60 °C, hourly water changes)

ggBCA content (mg g ⁻¹)	<LOQ	1.01	2.87
BCA content (mg g ⁻¹)	0.016	0.072	0.11

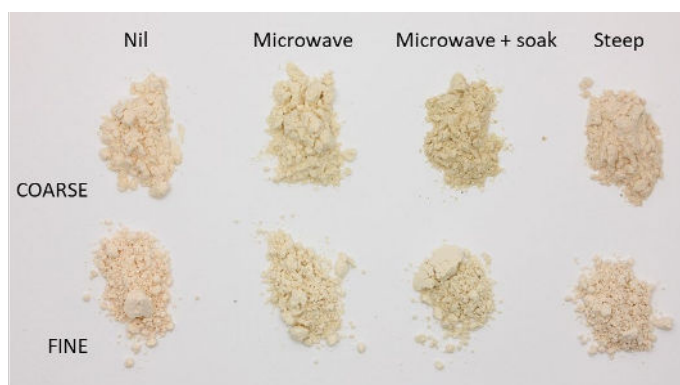


Figure 3. Appearance of a series of dry-fractionated vetch flours after the application of different processing methods for toxin removal.

CONCLUSIONS REACHED &/OR DISCOVERIES MADE (Not to exceed one page)

- Our UniSA partners (Dr Leigh Donnellan and Prof Peter Hoffman) established a liquid chromatography/mass spectrometry (LC-MS/MS)-based method for analysis of the two forms of the toxin present in common vetch. The method was optimised to enable maximum sensitivity of detection in samples subjected to post-harvest processing, achieving lower limits of quantitation in extractable toxins of 25 µg g⁻¹ ggBCA and 3 µg g⁻¹ BCA. It is noted these limits are still not low enough to demonstrate the toxin levels in treated vetch are safe for human consumption.
- A method of steeping vetch grain in water at elevated temperature was optimised to determine the steeping time, temperature and water changes needed to reduce toxin levels to below detection (<0.5% of starting levels). Temperatures either greater than or less than 60 °C were less effective at toxin removal and suggested a temperature-dependent process of conversion between the two forms of the toxin. BCA appeared to be more effectively leached from vetch than the ggBCA form.
- The potential for alternative methods for toxin removal were explored. These were wet and dry protein fractionation, microbial fermentation, microwave treatment and extrusion. Microbial fermentation has traditionally been performed to improve the safety (e.g. by reducing the levels of antinutritional factors), digestibility and nutritional value of food products. Fermentation of de-hulled vetch using two different fungal starter cultures reduced ggBCA levels to around 5% of pre-processed grain, while extrusion across a range of settings also significantly reduced ggBCA. These processes tended to increase the levels of BCA. Wet protein extraction using methods reflecting those used to make faba bean and pea protein isolates, reduced ggBCA to around 2% of pre-processed grain, with little change in the content of BCA.
- A microwave treatment followed by a period of steeping at room temperature reduced toxin levels to 2 – 5% of pre-processed grain. Microwave-based methods warrant further exploration, as they may be less energy- and water-consuming than steeping at high temperatures and have a lesser effect on the levels of iron and folic acid.
- The microwave and steeping methods were up-scaled to kg quantities and tested in milling and dry fractionation. These methods did not appear to affect behaviour of vetch flour during processing. Protein levels were maintained in all flours and could be enriched using dry fractionation, by 2.1-fold.

- With the application of dry fractionation, we made an unexpected discovery with steeped vetch flour after jet-milling having an increase in ggBCA. Further work is required to determine what mechanism is at play here.
- Consistently achieving a vetch-based flour with less than 10 mg kg⁻¹ of cyanogen will be a significant challenge. Our steeping treatment achieved close to this level, but as yet, not reproducibly and not with a satisfactory margin.

INTELLECTUAL PROPERTY

For IP generated see the section above. Of the methods tested for toxin reduction, microwave treatment seems to be the most promising due to it being more sustainable than steeping. Fungal fermentation also demonstrated promising results and the approach could be used to develop a novel fermented food product. Further work is needed to optimise the most promising methods and thus at the moment there is no potential for commercialisation.

We kindly request project IP remains confidential pending further discussions between SAGIT and SARDI.

APPLICATION / COMMUNICATION OF RESULTS

Main findings of the project:

- Despite development of new methods for processing of food ingredients, the best way to remove a cyanogenic toxin from vetch is a 'steeping' method, where grain is soaked in multiple changes of water at a temperature of 60 °C. High temperature appears to convert the more-difficult-to-remove form of the toxin to a form that is more easily leached from the grain. However, with further optimisation of the best performing alternative processing (e.g. microwave treatment), equal efficacy in toxin removal could likely be achieved.
- The main problem of steeping is that toxins remain in leaching water leading to questions about its safe disposal/handling. Steeping also consumes large amount of water, thus being less sustainable than the other methods used.
- Steeping does not affect the technological properties of the milled flour, notwithstanding a possible incremental effect of the process on the concentration of toxin in extremely finely milled, steeped flours.

Publications and extension articles delivered as part of the project:

- Description of the project aims and objectives to GRDC Southern Panel members, during a visit to Waite facilities and researchers on 30th March 2023.
- Oral and poster presentation at the 2023 Australian Pulse Conference, Toowoomba QLD (Vetch: a future source of pulse protein for foods?)
- A short article prepared for the State and Federal Governments in July 2023, as a feature story illustrating how NCRIS-funded organisations (i.e. UniSA's proteomics facility) actively collaborate with research groups in applied projects.
- Hosting of SAGIT members at UniSA proteomics facility on 21st August 2023.
- A project proposal to extend this work, prepared as part of a broader research package placing South Australia as a centre for plant protein-focused R&D. The package was to be presented to GRDC for consideration in early 2024, by UA/SARDI/UniSA, but has been put on hold due to uncertainties for the future of plant protein-related industries in Australia. It is evident that the local plant protein landscape has changed considerably during the last 18 months.
- SAGIT Update presentation on 24th July 2024.

POSSIBLE FUTURE WORK

In the presence of substantial market drivers, recommended future work would include:

- modification of the extraction and analysis method to overcome a possible effect of flour particle size on extraction efficiency/cyanotoxin concentration; synthesis of labelled standards to achieve further reductions in assay limits of detection and quantitation

- application of the quantitative vetch toxin analysis method across current varieties and germplasm in the SARDI vetch breeding program, to benchmark ggBCA/BCA levels in relevant material
- engineering solutions for treatment of steeping water to capture and remove the toxins; design of a minimum-energy, minimum-water use steeping system
- further optimising the microwave treatment and fermentation processes; fermentation will bring about further benefits by potentially improving the nutritional value of vetch.
- market research for consumer acceptance of a vetch-based food ingredient, and establishment of safe limits of the toxin for human consumption
- development of a RIL mapping population between Love2 low-toxin vetch (Prof. M. Tate) and a commercial variety, and genetic analysis for the low-toxin trait (note this can now be achieved rapidly due to SAGIT investment in the development of accelerated single seed descent protocol for vetch).
- Traitomic-based mutant mining ([Traitomic - Empowering Nature](#))