Article:
Bolarinwa, IF, Orfila, C and Morgan, MRA (2014) Development and application of an enzyme-linked immunosorbent assay (ELISA) for the quantification of amygdalin, a cyanogenic glycoside, in food. Journal of Agricultural and Food Chemistry, 62 (27). pp. 6299-6305. ISSN 0021-8561

https://doi.org/10.1021/jf501978d

Development and Application of an Enzyme-linked Immunosorbent Assay (ELISA) for the Quantification of Amygdalin, a Cyanogenic Glycoside, in Food

Islamiyat F. Bolarinwa, Caroline Orfila and Michael R.A. Morgan*

School of Food Science and Nutrition,

University of Leeds,

Leeds LS2 9JT,

UK.

***Corresponding author:** email m.morgan@leeds.ac.uk

Keywords: ELISA, Amygdalin, Cyanogenic Glycoside, Food Safety, Analysis

Some of the work reported here was presented at the 245th Meeting of the American Chemical Society, New Orleans, 2013.

ABSTRACT: Amygdalin is a member of the cyanogenic glycoside group of plant secondary metabolites capable of generating hydrogen cyanide under certain conditions. As a consequence, the cyanogenic glycosides have been associated with incidents of acute and sub-acute food poisoning. Specific antibodies were raised against an amygdalin-bovine serum albumin immunogen synthesised using a novel approach. The antibodies were used in a microtitration plate enzyme-linked immunosorbent assay (ELISA) for the quantification, for the first time, of amygdalin in commercially-available foods. Correlation of results with high-pressure liquid chromatography was very high (r = 0.983). The limit of detection of the immunoassay was 200 ± 0.05 pg mL^{-1}, and the 50% inhibitory concentration of amygdalin was 50 ± 0.02ng mL^{-1}, making the ELISA particularly sensitive.

▪ INTRODUCTION

Cyanogenic glycosides are plant natural toxicants that are widely distributed in nature, being found in more than 2500 plant species.[1] Cyanogenic glycosides serve as important chemical weapons in the defence of the plant against herbivores because of the potential to generate toxic hydrogen cyanide.[2] Hydrogen cyanide is released from the plant following tissue disruption as a result of animal chewing, bruising or mechanical damage. When plant tissues are disrupted in this way, the previously inaccessible cyanogenic glycosides located in plant vacuoles are able to come into contact with enzymes in the cell wall (β-glucosidases and α-hydroxynitrile lyases) which catalyse the degradation of cyanogenic glycosides to benzaldehyde and hydrogen

cyanide. When cyanogenic plants are ingested by humans, enzymes produced by the intestinal microflora are also able to hydrolyse intact cyanogenic glycoside to produce hydrogen cyanide in vivo.[3] Cyanide toxicity can occur in humans following the consumption of cyanide (from foods) at doses between 0.5-3.5 mg kg^{-1} body weight.[4] Cases of cyanide toxicity with symptoms such as vomiting, diarrhoea, abdominal cramps, headache, drowsiness, confusion, nausea, hypotension, paralysis and coma have been reported following the consumption of apricot kernels, tapioca cake, cassava-based meal and almond.[5,6,7,8,9]

Several important plant crops, such as cassava, sorghum, millet, barley, almond, cherry, macadamia nut, peach, lima beans, and kidney beans, have been reported to biosynthesize and accumulate cyanogenic glycosides.[1,10,11,12] Traditional and modern food processing techniques such as chopping, grinding, soaking, fermentation, drying, roasting, boiling, and steaming have been used to reduce or eliminate the potential toxicity of dietary cyanogenic plants.[13] These methods can be effective because cyanogenic glycosides are soluble in water and hydrogen cyanide is volatile; bringing together enzymes and substrate can reduce glycoside concentrations substantially. The cyanogenic glycosides, however, are heat stable. Inefficient or inappropriate processing may mean that consumers may be exposed to acute and sub-acute doses of the glycosides.

Amygdalin (D-mandelonitrile-β-D-gentiobioside; Figure 1) is the most common of the cyanogenic glycosides. It is present in abundance in the seeds and kernels of fruits such as apricot, almond, apple, cherry, plum, peach and nectarine. Although the seeds

of some of these fruits are not consumed directly, fruits with high contents of cyanogenic glycosides in their seeds are likely to contain cyanogenic glycosides in the edible portion. Indeed, stewed fruit has been reported by Voldřich & Kyzlink[14] to contain 3-4 mg kg^{-1} HCN equivalents. The same author also reported levels of up to 4 mg kg^{-1} HCN equivalents for canned stone fruits.

Conventional methods for cyanogenic glycoside detection include high performance liquid chromatography (HPLC) with ultra-violet, refractive index or amperometric detection[15,16] and gas chromatography-mass spectrophotometry (GC-MS).[17] An improved reversed-phase HPLC-UV method for detection and quantification of amygdalin in commercially-available food products has been reported recently, utilizing an optimised extraction procedure.[18] Although these methods can be sensitive, they utilise expensive equipment and require complicated sample pre-treatment. There is therefore the need for a method that is simple, rapid, sensitive and cost-effective. Enzyme-linked immunosorbent assay (ELISA) would fulfill these requirements in addition to having the potential for high sample throughput. ELISA methods have been developed for monitoring the levels of herbicides in water, [19] pesticides and estrogenic compounds in agricultural products.[20,21] Antibodies against amygdalin have been described previously[22,23] but have not been fully characterized or used quantitatively. We describe the development, characterization and application (for the first time) of an ELISA for the specific quantification of amygdalin in commercially-available food products.

▪ MATERIALS AND METHODS

Chemicals. Amygdalin, dhurrin, TMB (ready-to-use ELISA substrate), acetone, cyanuric chloride, Tween 20, gelatin, casein and buffer salts were all purchased from the Sigma Chemical Company, Poole, Dorset, UK. Prunasin, mandelonitrile and linamarin were purchased from Santa Cruz Biotechnology, Dallas, Texas, USA. 2, 4, 6-trinitrobenzene sulfonic acid (TNBS; 5%, w/v) was obtained from VWR International Ltd., Leicestershire, UK. Keyhole Limpet Haemocyanin (KLH) was from Merck Chemicals Ltd., Nottingham, UK. Bovine serum albumin (BSA) was purchased from Fisher Scientific Ltd., Leicestershire, UK. Goat anti-rabbit IgG (H+L) horseradish peroxidase conjugate was obtained from Invitrogen Life Technology, Paisley, UK. Microtitration plates (96-well, MaxiSorp) were purchased from Nunc, Rosklide, Denmark. Microtitration plate washing was carried out using a Labsystem 4MK2 Wellwash (Labsystems, Uxbridge, UK) and the optical densities at 405nm were recorded using a plate reader (ThermoFisher Scientific, Vantaa, Finland). Phosphate–buffered saline (PBS, 0.01 M, pH 5.5 and 7.4), phosphate–buffered saline (pH 7.4) containing 0.05% Tween-20 (PBST), sodium carbonate-bicarbonate buffer (0.05 M, pH 9.4 and 9.6) were used.

Food Samples. All fruits and processed products used in this study were purchased from local supermarkets in Leeds, UK. The products were Toasted Almond Kernels (produced in the USA, packaged in the UK by Sainsbury's), EcoMil Almond Cocoa Dessert (8% almond) produced in Spain, Tesco own-brand Almond Flour (produced in the USA, packaged in the UK by Tesco), Morrison's own-brand White

Marzipan (25% almond) produced in the UK, Sainsbury's own-brand Apple Juice (produced in the UK), Copella Apple Juice (hand-picked English apples, produced in the UK), Del Monte Quality Long Life Apple Juice (100% concentrate), squashes, melons and black cherries (from the UK), black plums (from Chile), yellow plums (from South Africa), apples (from Brazil), pears (from Holland), courgettes and cucumbers (from Spain), peaches (from Argentina), and marrows (from Egypt). Three packs from different batches were purchased for each brand or product. All products were stored at 4 ℃ after purchase and extracted as soon as possible.

Synthesis of Hapten-Protein Conjugates. The hapten-protein synthesis route is illustrated in Figure 1. Amygdalin was first activated with cyanuric chloride (CC) using a modification of the method of Abuknesha et al.[24] used for antibody-enzyme conjugations. CC (1 mg) was dissolved in acetone (1 ml) and 0.3 ml of the solution was measured into a glass vial. The content of the vial was allowed to evaporate. Amygdalin was activated by adding a solution of amygdalin (2.3 mg in 0.5 ml sodium carbonate-bicarbonate buffer, pH 9.4) to the CC residue. The mixture was stirred for 6hr at room temperature (20 ± 2 ^{o}C) to produce the activated hapten (amygdalin-CC). BSA (0.1 µmol, 6.6 mg in 0.5 ml sodium carbonate-bicarbonate buffer, pH 9.4) was added to the hapten and the mixture was incubated overnight at 37 ^{o}C and dialysed for 6 hr against 0.01 M phosphate buffer (pH 5.5). The resulting material (amygdalin-BSA conjugate, Figure 1) was freeze-dried to obtain a white powder. The conjugate was stored at –20 ^{o}C until use. The synthesized amygdalin-BSA conjugate was used as the immunogen.

Amygdalin-KLH conjugate used for the immobilized phase of the ELISA was synthesized using the same method as for immunogen production.

Assessment of Hapten-Protein Conjugates. The ratios of coupled hapten to protein in the conjugates were assessed by both spectrophotometric and chemical methods. [25,26,27,28] Firstly, verification of conjugate synthesis and estimation of the number of moles of hapten conjugated per mole of protein was estimated by UV-visible spectrometry. Amygdalin (1 mg), BSA (1 mg) or amygdalin-BSA conjugate (1 mg) were dissolved in sodium carbonate-bicarbonate buffer (0.05 M, pH 9.6, 1 ml) was added. Absorption over 200-400 nm were determined, and the number of moles of hapten in the conjugate was calculated using the maximum absorbance for amygdalin at 262 nm to calculate the molar extinction coefficient. The number of moles of conjugated hapten was calculated using $A = \varepsilon cl$.

Secondly, the trinitrobenzene sulfonic acid assay (TNBS; Habeeb[25]) was used to estimate the molar conjugation ratio of amygdalin-BSA and amygdalin-KLH conjugates. The method is based on the determination of available amino acid groups present in carrier protein before and after hapten conjugation following its reaction with 2,4,6-trinitrobenzenesulfonic acid. The end product of the reaction of TNBS produces trinitophenyl derivatives which can be measured spectrophotometrically. The assay protocol used was as modified by Kemp & Morgan.[26] Carrier protein or conjugate (1 mg) were dissolved in 1 ml of 0.05 M sodium carbonate-bicarbonate buffer, pH 9.4. Standard curves for the proteins were constructed using a total volume of 200µl in the wells of a microtitration plate. To each of the wells, 50 µl of TNBS (0.1%) in carbonate

buffer (4%) were added. The plate was incubated for 2 hr at 37^{o}C for colour development and the absorbance was read at 405 nm on a plate reader. The percentage substitution of amino acid in the carrier protein was used to estimate the conjugation ratio of hapten to carrier protein.

$$\% \text{ Substitution} \equiv \frac{\text{O.D of carrier protein} - \text{O.D of hapten-protein conjugate}}{\text{O.D of carrier protein}} \times 100$$

Immunization Protocol and Polyclonal Antibody Production. Antibodies were raised by Covalab Ltd, Cambridge, UK. Two female New Zealand White rabbits (2-2.5 kg) were immunized with the immunogen (amygdalin-BSA conjugate) to generate polyclonal antibodies. The first injection consisted of the immunogen (50 µg in 0.5 ml of phosphate-buffered saline) emulsified with 0.5 mL of Freund's complete adjuvant and injected intradermally on multiple sites on the rabbits back. The rabbits were further boosted by injecting intradermally with the immunogen (50 µg) dissolved in 0.5 ml of Freund's incomplete adjuvant three times at three weeks intervals, with the final booster injection administered subcutaneously. The antisera were stored at –20 ^{o}C until use.

Enzyme-Linked Immunosorbent Assay (ELISA). For determination of antibody titer, each well of the 96-well microtitration plate was coated with amygdalin-KLH overnight at 4 ^{o}C using 300 µl of a solution of conjugate dissolved in 0.05M carbonate-bicarbonate buffer, pH9.6. The plate was then washed three times with water and twice with 0.01M phosphate-buffered saline containing 0.05% Tween 20 (PBST). Antibody titer was determined by incubating serial dilutions of antisera and pre-immune sera in PBST (200 µl per well, in triplicate). The plate was incubated overnight at 4 ^{o}C, washed

five times with PBST and dried. A species-specific second antibody, goat anti-rabbit horseradish peroxidase IgG conjugate (diluted 1/1000 in PBST, v/v; 200 µl per well) was added. The plate was incubated at 37 ^{o}C for 2 hr, washed and dried as before, and then TMB substrate (200 µl) was added to each well. The plate was further incubated for 30 min at 37 ^{o}C and the reaction was stopped with sulfuric acid (2 M, 50 µl per well). The absorbance of each well was determined at 405 nm. The development of yellow colour was inversely proportional to the amount of antibody present. The mean absorbance values of triplicate determinations were plotted against antiserum dilutions.

For the indirect competitive ELISA (icELISA), standard curves were constructed with100 µl of solution containing amygdalin standard (10 pg/well -10 µg/well) dispensed into appropriate wells of the coated plates prior to adding antibody (100 µl). The plate was then processed as for the antibody titer curve determination. The limit of detection of the assay was estimated as the concentration that corresponds to the optical density at zero binding minus three times the standard deviation of the mean of zero binding.

To optimise the ELISA, the sensitivity of the immunoassay was improved by varying the antiserum dilution, the incubation time, the concentration of the coating antigen and by using different protein buffers. The protein buffers that were evaluated were PBST containing 1% BSA, 1% gelatin or 1% casein. Evaluation of the optimisation process was based on the IC_{50} value and the coefficient of evaluation of the linear equation (R^2) as described by Qian et al.[29]

Cross-Reactions. The optimised assays were applied to cross-reactivity studies using standard solutions of amygdalin and other cyanogenic glycosides. The cross-reactivity of the antibody with some closely related cyanogenic glycosides such as prunasin, dhurrin, mandelonitrile and linamarin was determined by icELISA. The specificity of the polyclonal antibody was determined using:

% Cross-reactivity (CR) = (IC_{50} of amygdalin/ IC_{50} of analogue) x 100

Application of the icELISA to Quantification of Amygdalin in Commercially-available Food Products. The extraction method described previously[18] was used for the extraction of amygdalin from food samples. Solid samples (2 g) were ground in a blender (20 sec; Moulinex Optiblend 2000, France) and 1 g was weighed into a round-bottom flask (500 ml). In the case of liquid samples (10 ml), was added to ethanol (50 ml) in a round-bottom flask, and the mixture was boiled under reflux for 100min. The extracts were filtered (Whatman No. 1 filter paper) and transferred into plastic polypropylene tubes (50 ml). Ethanol was completely evaporated from the filtrate with a rotary evaporator (low BP, 35 ^{o}C, 7 mbar). Diethyl ether (10 ml) was added to the dried sample and the mixture was vortexed (1 min) at room temperature (20 ^{o} ± 2 ^{o}C) to precipitate amygdalin. The diethyl ether was allowed to evaporate overnight and the extracted amygdalin was dissolved in 1%BSA-PBST (1 ml). The food extract (100 µl) was then incubated with antibody on coated plate as previously described for setting up the standard curve. Quantification of amygdalin in the sample was by reference to

standard curves. Samples were also analysed by HPLC as described previously in order to show the correlation of the results from the 2 methods. The HPLC extraction method was, however, different from the ELISA method. In the HPLC method, extracted amygdalin was further purified by dissolving it in water followed by centrifugation (14000 rpm, 22 ^{o}C, 10 min) and filtration with 0.45 µm PTFE filters prior to HPLC analysis.

▪ RESULTS AND DISCUSSION

Immunoassays are now widely used in quantitative analysis for agric-food components for their advantages of cost-effectiveness and simplicity which arise directly from the affinity of the antibody interaction with its target. Surprisingly, perhaps, given that immunoassays can be used away from fully-equipped laboratories to monitor food processing, only 2 previous reports have described antibody production against cyanogenic glycosides, both against amygdalin.[22,23] No antibody characterization was carried out, no assay validation reported and nor was any quantitative data provided. The present report provides antibody characterization, assay validation and quantitative data on commercially-available food products.

Hapten Synthesis. Methods for production of hapten-protein conjugates need to be efficient, be carried out under mild conditions and be effective. In order to produce the amygdalin immunogen we used a method previously used by Abuknesha et al.[24] to synthesise antibody-enzyme conjugates. Cyanuric chloride has reactivity towards hydroxyl groups under mild conditions and the coupling method did not affect the structure of the target analyte (amygdalin). Figure 1 show the synthetic scheme

followed. In order to provide the immobilized phase of the assay, a KLH-amygdalin conjugate was produced using the same chemistry.

Estimation of Hapten-Protein Ratios. Amygdalin exhibited a UV maximum at 262nm; BSA at 278nm. The BSA-amygdalin conjugate had a peak at 278nm and a significant shoulder at 262nm. Similar results were seen with KLH. The magnitude of the 262 absorption was used to calculate hapten-protein ratios, which were found to be 23:1 and 19:1 for the BSA-amygdalin and KLH-amygdalin conjugates, respectively.

Reaction of the proteins with TNBS generated standard curves at 405nm. Theoretically, 59 lysine residues of BSA are available for coupling to hapten.[28] The percentage substitution of amygdalin calculated from the standard curves was 34.4% giving a hapten-protein ratio for the immunogen of 20:1. The same procedure was employed for the KLH coating conjugate and the ratio was calculated to be 17:1.

The hapten:protein ratios obtained for the BSA conjugate (23 and 20:1) and the coating conjugate (19 and 17:1) using the spectrophotometric and chemical methods were very close. This is an indication that the use of cyanuric chloride as a coupling agent for conjugate production was efficient and that the procedure merits wider utilization in future. The hapten:protein ratios obtained in this study were in line with recommended ratios for good antibody production. Kemp & Morgan[26] reported that a conjugate with a high hapten: protein ratio is good as immunogen, while the one with a low ratio may be desirable as a coating antigen. Hapten densities of 15-30 molecules

per carrier protein were also reported to be good for the production of high antibody titers.[28]

Antibody Titer. The BSA-amygdalin conjugate was used in antibody production in rabbits. The presence of anti-amygdalin antibodies was observed through production of titer curves using KLH-amygdalin as the immobilized phase on microtitration plates. Antibody binding as measured by optical density was 2.1 at a dilution of 1:10,000 (v:v) compared to that given by pre-immune serum (0.03 at 1:10,000). Even at dilutions of 1:100,000, the antisera showed significant binding above that of the pre-immune serum. The anti-amygdalin antiserum was used at a dilution of 1:30,000 for subsequent experiments unless otherwise stated. For anti-hapten antisera, such a dilution is at the high end of what is achievable.

Optimisation of the ELISA. The antibody dilution, incubation time, coating conjugate concentration and the use of protein buffers were each investigated for ELISA optimisation. Antibody dilution significantly influenced the sensitivity of the ELISA. A lower dilution (1:10,000) of antibody resulted in higher IC_{50} (7 µg/well) than at a 1:30,000 dilution which gave an IC_{50} of 0.80 µg mL^{-1}. When the antibody dilution was increased beyond 1:30,000, the IC_{50} increased significantly. Consequently, an antibody dilution of 1:30,000 in PBST was used throughout.

The incubation time for anti-amygdalin antibody and amygdalin on the microtitration plate was varied from 1 to 5 hr (at 37 ^{o}C) and overnight at 4 ^{o}C. A higher IC_{50} (5.51 µg/ml) and lower R^2 (0.97) resulted from a 1 hr incubation at 37 ^{o}C. As the

incubation time increased, the IC_{50} decreased and R^2 values increased. Overnight incubation at 4 ^oC resulted in the lowest IC_{50} with $R^2 > 0.9$. Subsequent assays utilised an overnight incubation at 4 ^oC.

The concentration of the coating conjugate, KLH-amygdalin, strongly influenced ELISA performance. As the concentration of the coating conjugate was decreased, the concentration of the free hapten (amygdalin) increases, relatively speaking. Consequently, an increase in the binding of the antibody to the free rather than immobilised amygdalin was evidenced for coating conjugate concentrations of 0.01 to 0.05 µg mL^{-1}. Although the IC_{50} of the standard curve decreased with decreased coating conjugate concentration, the associated R^2 value increased. The assay was most sensitive with a coating conjugate concentration of 0.05 µg mL^{-1}. The assay sensitivity was good at this concentration (50 ng mL^{-1}), possibly because antibody bivalent binding were prevented at this lower concentration of conjugate.[26] A coating conjugate concentration of 0.05 µg mL^{-1} was used for subsequent assays.

The ELISA absorbance and sensitivity was greatly enhanced by using 1% (w/v) BSA-PBST as buffer. A significantly lower IC_{50} value was observed when 1% BSA-PBST was used as a buffer as when compared to the use of PBST or PBST with casein or gelatin. On the basis of the IC_{50} values, R^2 values and the ratios of the maximum and minimum absorbances for the amygdalin standard curves, 1% BSA-PBST was selected as the buffer for subsequent experiments.

Indirect Competitive ELISA (icELISA). The optimised conditions were used to obtain an indirect competitive standard curve for amygdalin (Figure 2), represented by: $Y = A2 + (A1-A2)/(1 + (x/x0)^p)$, $R^2 = 0.9995$. The curve was fitted using OriginPro 8.6 software. The IC_{50} of the assay was estimated to be 50 ± 0.02 ng/ml with a detection limit of 200 ± 0.05 pg mL^{-1} (as determined by the concentration that corresponded to the optical density of zero binding reduced by 3 times the standard deviation of the mean zero binding). The working concentration range of the icELISA was 10 ng mL^{-1} (IC_{20}) and 1 µg mL^{-1} (IC_{80}). The detection limit of the ELISA reported here was significantly lower than previously reported limits using a polyclonal antibody (100 mg mL^{-1})[22] or a recombinant antibody (500 pg mL^{-1}).[23]

Cross-Reactivity. The specificity of the assay was investigated by testing the cross-reactivity of the antibody with other cyanogenic glycosides that are closely related to amygdalin The results obtained (Table 1) showed that the antibody was very specific for amygdalin, with cross-reactions of 1.3% for prunasin, 0.1% for mandelonitrile and <0.01% for linamarin and dhurrin. The differences in structure between amygdalin and prunasin occur at the point furthest from the hydroxyl group used for coupling of hapten to protein. Even prunasin, a metabolite of amygdalin, did not inhibit antibody binding despite the close structural similarity to amygdalin. There was also a lack of reaction of the antibody with dhurrin (0.01%), where the additional hydroxyl group has a significant effect on antibody recognition. It can be assumed that the 2 sugar residues are key factors in antibody recognition. Other slight changes in other parts of amygdalin

structure dramatically reduced antibody recognition for the other cyanogenic glycosides tested.

Quantification of Amygdalin in Commercially-available Food Products. Fruit kernels and processed products were analysed by icELISA and HPLC after extraction of amygdalin. Figure 3 shows the correlation of the results with the 2 methods. The correlation is excellent (r=0.983) across the whole range of amygdalin contents. The sample extraction procedure used for the ELISA is considerably simplified compared to that employed for the HPLC. In addition, the batch-wise nature of the ELISA further contributes to the high rate of sample through-put compared to HPLC.

Table 2 shows the values for amygdalin concentrations as determined by ELISA. Among the food samples analysed in this study, fruit juices had the lowest amygdalin content (0.001 – 0.07 mg g^{-1}) followed by products (0.04 – 0.15 mg g^{-1}) and squashes (0.05 – 0.17 mg g^{-1}). However, high levels of amygdalin were detected in fruit seeds (0.04 – 4.52 mg g^{-1}) and kernels (2.14 – 9.75 mg g^{-1}). Lower levels of amygdalin observed in juices and processed product could be as a result of the effect of processing on amygdalin. Processing methods have been reported to reduce the toxicity of cyanogenic glycosides (see introduction). Although low levels of cyanide would be generated from fruit juices, products and squashes, the level of cyanide that would be generated from fruit seeds (0.005 – 0.6 mg of equivalent HCN) and kernels (0.3 – 1.3 mg of equivalent HCN) are relatively high. Since the lethal dose of cyanide is 0.5-3.5 mg kg^{-1} body weight.[30] Cyanide toxicity could easily occur from the consumption

of fruit seeds and kernels, unless appropriate processing techniques are used and monitored.

Application of the ic-ELISA to Cyanogenic Plants. Extracts of apricot kernels (a natural cyanogenic plant) containing high amygdalin content showed linearity (R^2 = 0.998) of the ic-ELISA assay (result not shown). This further illustrates the detection of amygdalin by the developed ELISA method.

▪ CONCLUSION

In this study, a highly specific polyclonal antibody was raised against amygdalin using a hapten linked to a carrier protein through a very simple conjugation technique. The developed ic-ELISA under the optimised assay conditions was very sensitive (IC_{50} = 0.05 ± 0.02 µg mL^{-1}, limit of detection, 0.2 ± 0.05 ng mL^{-1}). The assay did not show significant cross-reactivity with prunasin, dhurrin, mandelonitrile and linamarin. The antibody was therefore highly specific for amygdalin. The ic-ELISA is a sensitive, cost-effective and simple method for the quantification of amygdalin in food, capable of high sample through-puts.

Further research will investigate the efficiencies of processing techniques used with cyanogenic plants.

REFERENCES

(1) Ganjewala, D.; Kumar, S.; Asha, D.S.; Ambika, K. Advances in cyanogenic glycosides biosynthesis and analyses in plants: A review. Acta Biologica Szegediensis. **2010,** 54, 1-14.

(2) Zagrobelny, M.; Bak, S.; Rasmussen, A. N.; JØrgensen, B.; Naumann, C. M.; MØller, B. L. Cyanogenic glycosides and plant-insect interactions. Review. Phytochem. **2004,** 65, 293-306.

(3) Carter, J. H.; McLafferty, M.A.; Goldman, P. Role of the gastrointestinal microflora in amygdalin (laetrile)-induced cyanide toxicity. Biochemical Pharmacol. **1980**, 29, 301-304.

(4) Speijers, G. Cyanogenic glycosides. WHO Food Additives Series 30.**1993.** Geneva. JECFA.

(5) Suchard, J. R.; Wallace, K. L.; Gerkin, R. D. Acute cyanide toxicity caused by apricot kernel ingestion. Annals of Emerg. Med. **1998,** 32, 742-744.

(6) Geller, R. J.; Barthold, C.; Saier, J. A.; Hall, A. H. Pediatric cyanide poisoning: causes, manifestations, management, and unmet needs. A review. Pediatr. **2006,** 118, 2146-2158.

(7) Akyildiz, B. N.; Kurtoğlu, S.; Kondolot, M.; Tunç, A. Cyanide poisoning caused by ingestion of apricot seeds. Annals of Trop. Paediatr. **2010,** 30, 39- 43.

(8) Sahin, S. Cyanide poisoning in children caused by apricot seeds. J. Health and Med. Inform. **2011,** 2, 106.

(9) Sanchez-Verlaan, P.; Geeraerts, T.; Buys, S.; Riu-Poulenc, B.; Cabot, C.; Fourcade, O.; Mégarbane, B.; Genestal, M. An unusual cause of severe lactic acidosis:cyanide poisoning after bitter almond ingestion. Intensive Care Med. **2011,** 37, 168-169.

(10) Jones, D. A. Why are so many food plants cyanogenic?.Phytochem. **1998,** 47, 155-162.

(11) Francisco, I. A.; Pinitto, M. H. P. Cyanogenic glycosides in plants. Brazilian Archives of Biol. Technol. **2000,** 43, 487-492.

(12) Donald, G. B. (2009). Cyanogenic foods (cassava, fruit kernels, and cycad seeds).Review. Medical Toxicology of Natural Substances, 55, 336-352.

(13) Montagnac, J. A.; Davis, C. R.; Tanumihardjo, S. A. Processing techniques to reduce toxicity and antinutrients of cassava for use as a staple food. Comprehensive Reviews in Food Sci. and Food Safety, **2009,** 8, 17-27.

(14) Voldřich, M.; Kyzlink, V. Cyanogenesis in canned stone fruits. J. Food Sci. **1992,** 57, 161-162.

(15) Wasserkrug, K.; Rassi, Z. E. High performance liquid phase separation of glycosides. I. Reversed phase chromatography of cyanogenic glycosides with UV and pulsed amperometric detection. J. Liquid Chromat. & Related Technol. **1997,** 20, 335-349.

(16) Sornyotha, S.; Kyu, K. L.; Ratanakhanokchai, K. Purification and detection of linamarin from cassava root cortex by high performance liquid chromatography. Food Chem. **2007,** 104, 1750-1754.

(17) Chassagne, D., Crouzet, J. C., Bayonove, C. L., & Baumes, R. L. Identification and quantification of passion fruit cyanogenic glycosides. J. Agric. Food Chem. **1996,** 44, 3817-3820.

(18) Bolarinwa, I. F.; Orfila, C.; Morgan, M. R. A. Amygdalin Content of Seeds, Kernels and Food Products Commercially-available in the UK. Food Chem. **2013,** doi: http://dx.doi.org/10.1016/j.foodchem.2013.11.002.

(19) Watanabe, E.; Hoshino, R.; Kanzaki, Y.; Tokumoto, H.; Kubo, H.; Nakazawa, H. New approach to immunochemical determinations for triclopyr and 3,5,6-trichloro-2-pyridinol by using a bifunctional hapten, and evaluation of polyclonal antiserum. J. Agric. Food Chem. **2002,** 50, 3637-3646.

(20) Shinkaruk, S.; Lamothe, V.; Schmitter, J-M.; Fructus, A.; Sauvant, P.; Vergne, S.; Degueil, M.; Babin, P.; Bennetau, B.; Bennetau-Pelissero, C. Synthesis of haptens and conjugates for ELISA of glycitein: development and validation of an immunological test. J. Agric. Food Chem. **2008**, 56, 6809-6817.

(21) Kondo, M.; Tsuzuki, K.; Hamada, H.; Yamaguchi, Y.; Uchigashima, M.; Saka, M.; Watanabe, E.; Iwasa, S.; Narita, H.; Miyake, S. Development of an enzyme-linked immunosorbent assay (ELISA) for residue analysis of the fungicide azoxystrobin in agricultural products. J. Agric. Food Chem. **2011,** 60, 904-911.

(22) Cho, A-Y.; Yi, K. S.; Rhim, J-H.; Kim, K-I.; Park, J-Y.; Keum, E-H.; Chung, J.; Oh, S. Detection of abnormally high amygdalin content in food by an enzyme immunoassay. Mol. and Cells. **2006,** 21, 308-313.

(23) Cho, A-Y.; Shin, K-J.; Chung, J.; Oh, S. A sensitive enzyme immunoassay for amygdalin in food extracts using a recombinant antibody. J. Food Protec. **2008,** 71, 2048-2052.

(24) Abuknesha, R. A.; Luk, C. Y.; Griffith, H. H. M., Maragkou, A.; Lakovaki, D. Efficient labelling of antibodies with horseradish peroxidase using cyanuric chloride. J. Immunol. Methods. **2005,** 306, 211-217.

(25) Habeeb, A. Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. Anal Biochem. **1966,** 14, 328-336.

(26) Kemp, H.A.; Morgan, M. R. A. Studies on the detrimental effects of bivalent binding in a microtitration plate ELISA and possible remedies. J. Immunol. Methods. **1986,** 94, 65-72.

(27) Szurdoki, F.; Szekacs, A.; Le, H. M.; Hammock, B. D. Synthesis of haptens and protein conjugates for the development of immunoassays for the insect growth regulator fenoxycarb. J. Agric. Food Chem. **2002,** 50, 29-40.

(28) Singh, K. V.; Kaur, J.; Varshney, G. C.; Raje, M.; Suri, C. R. Synthesis and characterization of hapten-protein conjugates for antibody production against small molecules. Bioconjugate Chem. **2004,** 15, 168-173.

(29) Qiant, G. L.; Wang, L. M.; Wu, Y. R.; Zhang, Q.; Sun, Q.; Liu, Y.; Liu., F. A monoclonal antibody-based sensitive enzyme-linked immunosorbent assay (ELISA) for the analysis of the organophosphorous pesticides chlorpyrifos-methyl in real samples. Food Chem. **2009,** 117, 364-370.

(30) Speijers, G. (1993). Cyanogenic glycosides. WHO Food Additives Series 30. Geneva. JECFA.

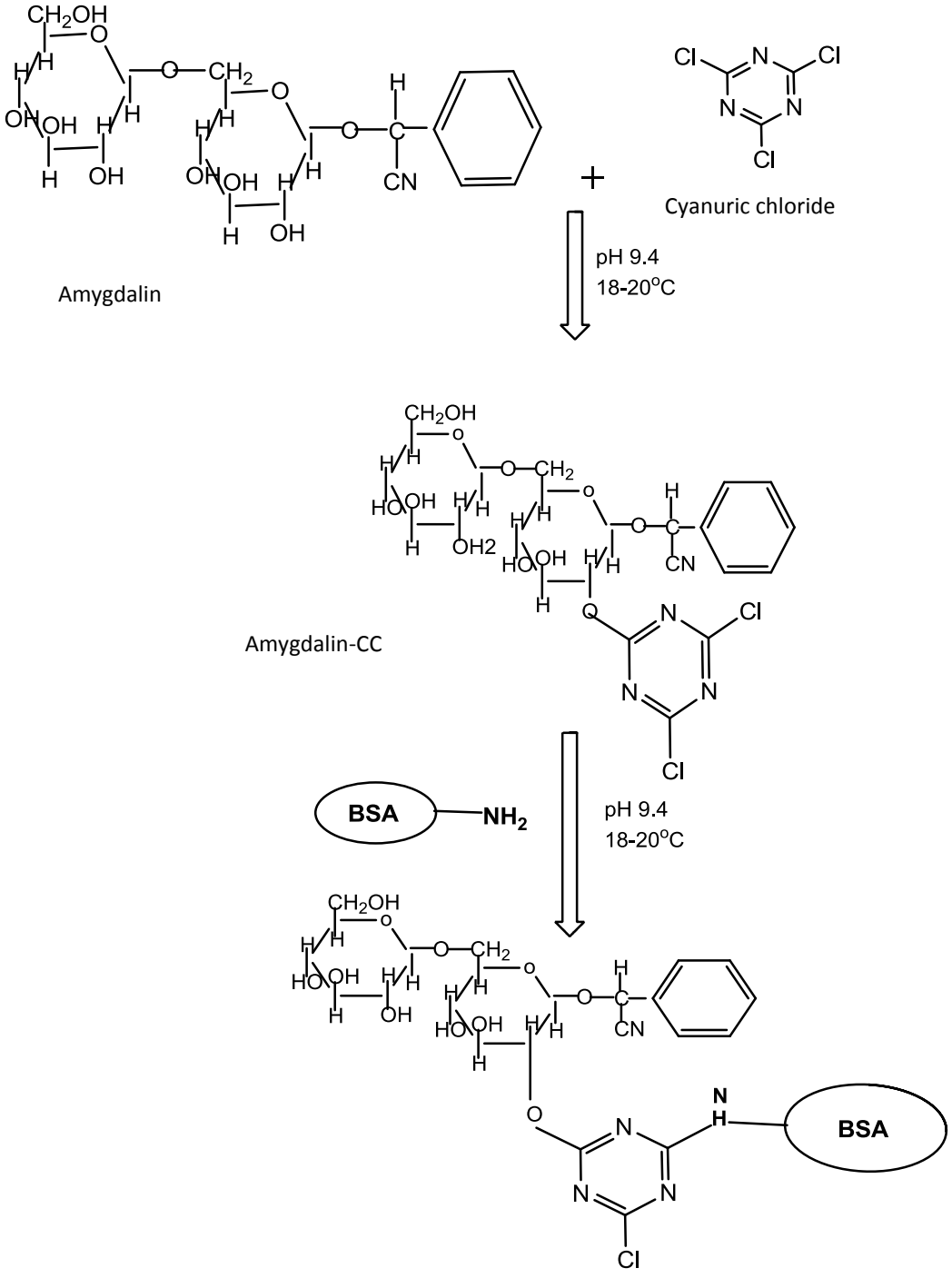
Figure Legends

Figure 1. The structure of amygdalin and the synthetic approach to immunogen production.

Figure 2. ELISA standard curve for amygdalin.

Figure 3. Correlation of results obtained by ELISA and HPLC analysis of food products.

Figure 1


Amygdalin
Cyanuric chloride
pH 9.4
18-20°C
Amygdalin-CC
BSA
NH2
pH 9.4
18-20°C
BSA

1

Figure 2

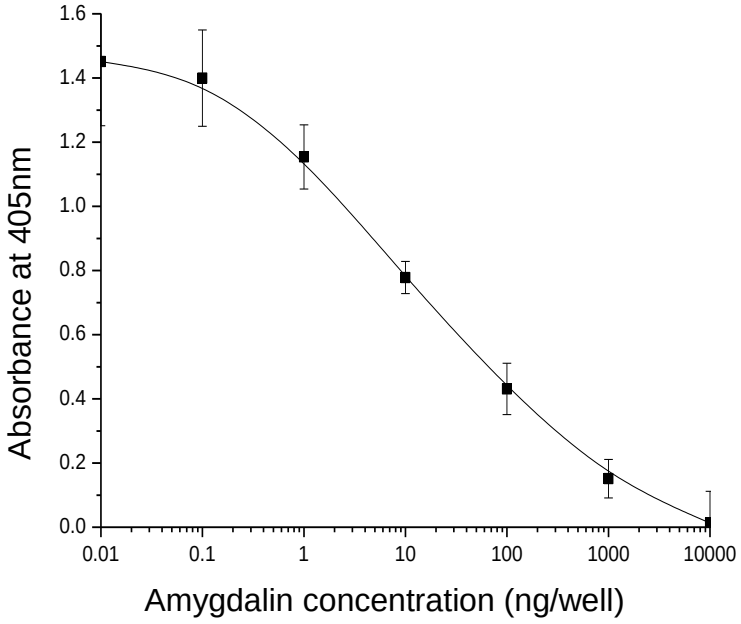


Figure 3

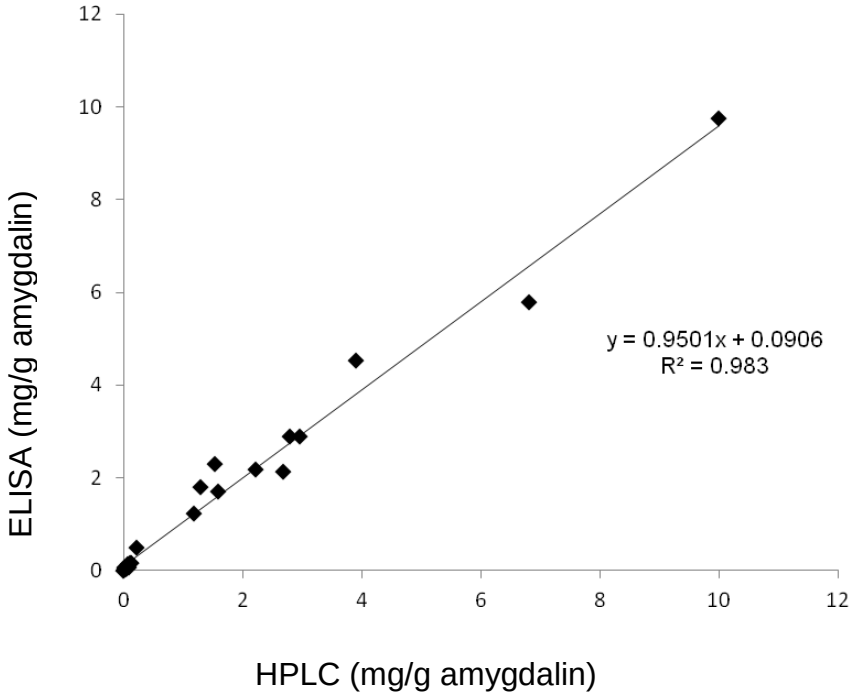
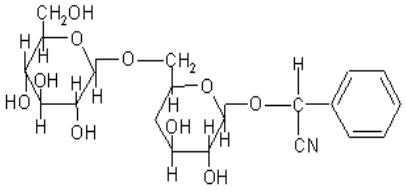
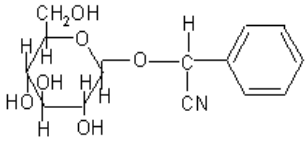
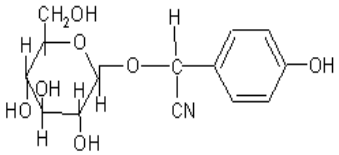
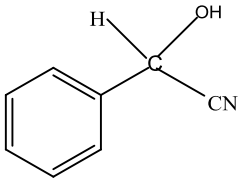
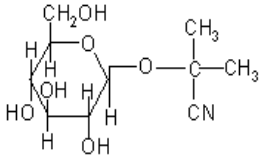


Table 1. Cross-reactivity of the anti-amygdalin antiserum.

Compound	Chemical structure	IC_{50} (µg mL^{-1})	CR (%)
Amygdalin		0.05	100
Prunasin		3.75	1.3
Dhurrin		>500	<0.01
Mandelonitrile		50	0.1
Linamarin		>500	<0.01

IC_{50} = Concentration of the analyte required for 50% inhibition of antibody binding to the coating antigen

Table 2. Quantification of amygdalin in commercially-available food samples as determined by ELISA.

Sample	Amygdalin content (mg g^{-1})
Juices	
Del Monte Apple Juice	0.001 ± 0.001
Tesco Pressed Apple Juice	0.017 ± 0.003
Copella Apple Juice	0.030 ± 0.004
Sainsbury's Pressed Apple Juice	0.034 ± 0.002
Tropicana Apple Juice	0.038 ± 0.002
Sun-Grown Cloudy Apple Juice	0.041 ± 0.005
Bramley Apple Juice	0.070 ± 0.003
Products	
Almond Flour	0.040 ± 0.010
Almond Milk	0.061 ± 0.012
Marzipan	0.060 ± 0.004
Almond Kernel (Toasted)	0.152 ± 0.031
Squashes	
Butternut squash	0.051 ± 0.012
Acorn Squash	0.090 ± 0.022
Red Kabocha Squash	0.131 ± 0.030
Crown Prince Squash	0.172 ± 0.050
Fruit Seeds	
Marrow Seeds	0.041 ± 0.003
Melon (honey dew) seeds	0.170 ± 0.041
Cucumber Seeds	0.110 ± 0.041
Courgette Seeds	0.501 ± 0.020
Braeburn Apple Seeds	1.221 ± 0.510
Cox Apple Seeds	1.711 ± 0.151
Conference Pear Seeds	1.812 ± 0.760
Jazz Apple Seeds	2.180 ± 0.681
Royal Gala Apple Seeds	2.881 ± 0.911
Red Delicious Apple Seeds	2.892 ± 0.621
Golden Delicious Apple Seeds	4.521 ± 1.201
Fruit Kernels	
Black Cherry kernels	2.140 ± 0.151
Yellow Plum kernels	2.303 ± 0.901
Peach kernels	5.792 ± 0.831
Black Plum kernels	9.751 ± 1.322

Each value is expressed as mean ± standard deviation (n = 3 determinations).