Development and Application of an Enzyme-Linked Immunosorbent Assay for Lupin Alkaloids

Brendon N. Greirson,*,† David G. Allen,† Norman F. Gare,‡ and Ian M. Watson‡

Chemistry Centre (W.A.), 125 Hay Street, Perth, Western Australia 6004, Australia, and School of Biomedical Sciences, Curtin University of Technology, Bentley, Western Australia 6102, Australia

An enzyme-linked immunosorbent assay (ELISA) has been developed for the determination of total alkaloids in lupin seed. Polyclonal antialkaloid antibodies from sheep antisera raised against a conjugate of (+)-13-hydroxylupanine hemisuccinate with keyhole limpet hemocyanin were used to construct the indirect ELISA. A bovine serum albumin–hexahydrophthalate–hydroxylupanine conjugate was used as the plate-coating antigen. Different bridge moieties and conjugation techniques were used to overcome unwanted cross-reactivity associated with the production of heterologous antisera. Antisera showed high specificity for (+)-lupanine and (+)-13-hydroxylupanine, the major alkaloids found in the commercial variety of lupin seed grown in Western Australia (*Lupinus angustifolius*). The most sensitive ELISA had a range of 0.1–1000 nmol/mL with 50% inhibition at 8 nmol/mL alkaloid in solution. Comparison between ELISA and capillary gas chromatographic methods of analysis had a correlation coefficient of 0.94 ($p < 0.001$) over the range 10–900 μg/g alkaloid in the seed. Inhibition studies on the alkaloids found in different species of lupin seed were also performed by ELISA.

Although accounting for about 1% of total worldwide legume production, lupin seed grown commercially in Australia is gaining importance with between 70 and 80% of annual production being exported. Although lupin seed is mainly used for stockfeed, it is acquiring increasing importance as a source of vegetable protein for human consumption, particularly in Asian countries. Lupin-based tofu and tempeh are the chief commodities manufactured for human use and offer nutritional advantages over their soybean counterparts. Being a legume, lupins play an important role in Australian agricultural practice by contributing to soil nitrogen.

Lupin seed (*Lupinus angustifolius*) grown commercially in Australia is high in protein and soluble dietary fiber; the alkaloid content appears to be the only antinutritional component of significance, since the levels of hemagglutinins, phytate, α-galactosides, tannins, and trypsin inhibitors are less than those found in other legumes and comparable with those in cereals (Petterson et al., 1986). Toxicity studies on the alkaloids of *L. angustifolius* have so far found them to be neither carcinogenic nor teratogenic in rats at doses of normal dietary intake of seed (Culvenor and Petterson, 1986; Petterson et al., 1987), and their pharmacological effects have been documented (Kinghorn and Balandrin, 1984). High concentrations (>0.1%) impart a bitter taste to the palate. Consequently, considerable effort has gone into the development of suitable analytical methods for determining alkaloid in seed samples of the yearly commercial harvest and breeding programs (Harris, 1988) to ensure the production of "sweet" low-alkaloid cultivars. Currently, capillary gas chromatography is the standard laboratory method (Priddis, 1983; Harris and Spadek, 1986) for lupin alkaloid analysis, but with the advent of some 7000 breeding samples to be introduced annually for analysis, an enzyme-linked immunosorbent assay (ELISA) was developed to provide a rapid screening technique.

Immunoassay techniques are fast becoming acceptable screening and quantitative methods of analysis in agricultural chemistry (Morris and Clifford, 1985; Morris et al., 1986; Vaag and Munck, 1987). Most have been developed to measure small organic molecules (haptens) which are of themselves too small to be immunogenic and therefore need to be conjugated to proteins to invoke an immune response. The lupin alkaloids are of "hapten" size. The alkaloids comprising the commercial variety of lupin seed (*L. angustifolius*) grown in Australia are based on the quinolizidine ring structure and include (+)-lupanine (44%), (+)-13-hydroxylupanine (44%), angustifoline (10%), and α-isolupanine (1%). Minor alkaloids include sparteine, 5,6-dehydrolupanine, 11,12-dehydrolupanine, 17-oxolupanine, tetrahydrorhombifoline, isoangustifoline, and ammodendrine (which is based on a dipyridyl ring structure) (Harris and Spadek, 1986). It was necessary to develop an ELISA to assay for as many of the major alkaloids as possible. This was achieved by raising antibodies capable of recognizing the quinolizidine ring structure and particularly the δ-lactam moiety of the alkaloids (Table I).

MATERIALS AND METHODS

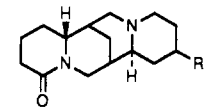
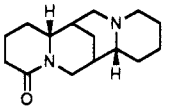
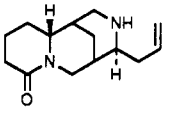
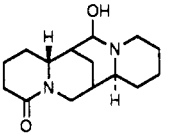
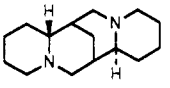
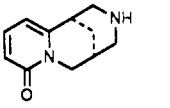
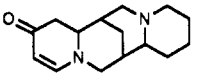
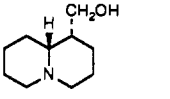
Chemicals. Freund's incomplete adjuvant (FIA) was purchased from Commonwealth Serum Laboratories. Bovine serum albumin (fraction V) (BSA), keyhold limpet hemocyanin (KLH), isobutyl chloroformate, *N*-hydroxysuccinimide, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide were obtained from Sigma Chemical Co. Succinic anhydride was from BDH Chemicals. Tri-*N*-butylamine, disodium 4-nitrophenyl phosphate hexahydrate and *cis*-hexahydrophthalic anhydride were purchased from Fluka. The alkaloids (+)-lupanine, (+)-13-hydroxylupanine, and angustifoline were supplied as standards from the Chemistry Centre (W.A.), and α-isolupanine and 17-hydroxylupanine were obtained from Koch-Light Laboratories. Anion-exchange resin AG1-X8, 200–400 mesh, was supplied by Bio-Rad. Linbro 96-well microtiter plates were purchased from Flow Laboratories. Donkey anti-sheep immunoglobulin G–alkaline phosphatase conjugate was purchased from Silenus Laboratories.

Plant Material. Lupin seed samples were obtained from the Grain Pool of Western Australia and represented the 1989/1990 harvest of the commercially grown sweet (low-alkaloid) varieties of *L. angustifolius*. Other lupin seed were from breeding trials supplied by the Department of Agriculture, South Perth, Western Australia.

† Chemistry Centre (W.A.).
‡ Curtin University of Technology.

0021-8561/91/1439-2327$02.50/0 Published 1991 by the American Chemical Society

Table I. Cross-Reactivities of Lupanine Derivatives and Related Alkaloids for the Antialkaloid Antisera

compound		% cross-reactivity at 50% inhibition
(+)-lupanine	R = H	100
(+)-13-hydroxylupanine	R = OH	100
hemisuccinate ester	R = OCO·$(CH_2)_2$·CO_2H	350
cis-hexahydrophthalate ester	R = OCO·C_6H_{10}·CO_2H	350
α-isolupanine		2
angustifoline		10
17-hydroxylupanine		<0.1
sparteine		<0.1
cytisine		<0.1
multiflorine		<0.1
epilupinine		<0.1

Synthesis of the Immunogen: 13-Hydroxylupanine Hemisuccinate–KLH. A solution of (+)-13-hydroxylupanine (1 mmol) and succinic anhydride (3 mmol) in anhydrous pyridine (3 mL) was incubated for 4 days at room temperature. Reaction progress was monitored by thin-layer chromatography using Dragendorff spray reagent (Munier and Macheboeuf, 1949). When all of the (+)-13-hydroxylupanine had reacted, about 3 mL of deionized water was added to the reaction mixture and the pyridine evaporated under reduced pressure to leave an oil. The oil was dissolved in a minimum volume of deionized water and applied to a column of Bio-Rad AG1-X8, 200–400 mesh, anion-exchange resin (20 g wet weight). The hemisuccinate of (+)-13-hydroxylupanine was eluted with deionized water (20 mL) which was evaporated under reduced pressure to leave an oil; succinic acid was then eluted with 0.1 N HCl. The hemisuccinate was crystallized from the oil with methylene chloride and acetone to give colorless prisms (70% yield). The identity and purity of the product were determined by gas chromatography/mass spectrometry of a methylated derivative (by reaction with diazomethane in diethyl ether): MS (60 eV) m/z (relative intensity) 378 (3, M^+), 248 (14), 247 (89), 246 (100), 245 (27), 148 (21), 134 (43), 112 (25), 55 (27).

The hemisuccinate of (+)-13-hydroxylupanine was conjugated to KLH by employing a mixed-anhydride intermediate (Erlanger et al., 1959; Morgan et al., 1983; Wie and Hammock, 1984). To the hemisuccinate ester (20 mg, 0.05 mmol) dissolved in anhydrous dioxane (1 mL) were added tri-*N*-butylamine (14 μL, 0.06 mmol) and isobutyl chloroformate (14 μL, 0.11 mmol), and the mixture was stirred at room temperature for 1 h. This solution was then added to a cold solution of KLH (25 mg, 4 nmol) in water/dioxane (4 mL, 3:1 v/v) (pH adjusted to 8 by addition of 0.1 N NaOH). The solution was stirred for 4 h at 4 °C with the pH maintained at 8 and then dialyzed against distilled water (3 × 500 mL) at 4 °C for 24 h. The solution was freeze-dried to yield 23 mg of conjugate with a substitution ratio of 420 (assuming a MW of KLH = 6×10^6).

Synthesis of the Plate-Coating Antigen: 13-Hydroxylupanine Hemihexahydrophthalate–BSA. A mixture of (+)-13-hydroxylupanine (1 mmol) and *cis*-hexahydrophthalic anhydride (3 mmol) dissolved in anhydrous pyridine (5 mL) was refluxed for 12 h. Reaction progress was monitored by thin-layer chromatography. Upon completion deionized water was added and the solvent evaporated under reduced pressure to leave an oil. The oil was taken up in a minimum volume of 10% methanol and applied to a column of Bio-Rad AG1X-8, 200–400 mesh, anion-exchange resin equilibrated with 10% methanol. The hemihexahydrophthalate of (+)-13-hydroxylupanine was eluted with water, followed by *cis*-hexahydrophthalic acid with 0.1 N HCl. The hemiester was crystallized from petroleum ether and acetone, and its identity and purity were determined by gas chromatography/mass spectrometry of the methyl ester: MS (60 eV) m/z (relative intensity) 432 (6, M^+), 248 (9), 247 (52), 246 (100), 245 (15), 148 (9), 134 (20), 112 (11), 81 (9), 55 (11).

The *N*-hydroxysuccinimide active ester of the hemihexahydrophthalate was prepared by reacting *N*-hydroxysuccinimide (30 mg, 0.26 mmol) and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (29 mg, 0.15 mmol) with the hemihexahydrophthalate ester (53 mg, 0.13 mmol) in anhydrous dimethylformamide at 4 °C for 24 h. The resulting solution was added to BSA (70 mg, 1 μmol) in phosphate buffer (0.15 M, 4 mL, pH 7.6) and the mixture stirred at 4 °C for 24 h (Anderson et al., 1964; Hassan et al., 1988). The solution was dialyzed against PBS (pH 7.6) for 48 h and distilled water for 12 h. The conjugate was freeze-dried to yield 65 mg of conjugate with a substitution ratio of 8.

Substitution Ratio. Substitution ratio (moles of alkaloid/mole of protein) was determined by gas chromatography of (+)-13-hydroxylupanine following alkaline hydrolysis of the alkaloid–protein conjugates. Conjugates (5 mg) were hydrolyzed with 0.4 N NaOH at room temperature for 2 h, and the liberated alkaloid was extracted three times with methylene chloride at 2.5 N NaOH. The hydrolysis procedure was validated by quantitative recoveries of alkaloid from pure hemiesters added to the hydrolysis mixture.

Preparation of Antisera. One sheep (no. 4201) was injected subcutaneously with 100 μg of immunogen in 1.5 mL of FIA followed by a subcutaneous booster injection of 100 μg of immunogen in 1.5 mL of FIA administered after 28 days. Antisera used to construct the ELISA was from a bleed 57 days after the first injection. Antialkaloid antibodies used were from an ammonium sulfate precipitate of the antisera. Further boosting immunizations were given 10–14 days prior to the collection of antisera from bleeds 3–5.

ELISA Procedure. The coating antigen, alkaloid hexahydrophthalate–BSA, dissolved in alkaline carbonate buffer (0.1 M, pH 9.6, 3 μg/mL) was applied at 200 μL to each well of a microtiter plate. After incubation at 4 °C overnight and washing with Tris-buffered saline (TBS, 0.3 M, pH 7.4), the plate was ready for use. Next, 200 μL of a 3% BSA solution in TBS was added to each well. This acted as an appropriate blocking buffer to reduce nonspecific binding. After incubation for 1 h, the plate was washed three times with TBS. Then, 100 μL of samples or standards in TBS was added to each well followed by 100 μL of antiserum diluted 1:1000 in TBS with 2% BSA. Plates were incubated for 1 h at room temperature after which time they were washed four times with TBS. One hundred microliters of donkey anti-sheep IgG–alkaline phosphatase diluted 1:2000 in TBS with 1% BSA was added to each well followed by incubation for 1 h at room temperature. Plates were washed five times with TBS, and 100 μL of disodium 4-nitrophenyl phosphate hexahydrate (1 mg/mL) in glycine buffer (0.1 M, 0.01 M $MgCl_2$, 0.001 M $ZnSO_4$, pH 10.2) was added to each well. After incubation at room temperature for a suitable length of time (typically 60–120 min or when the maximum absorbance approaches 2.00), the absorbances were read at 405 nm on a Titertek Multiskan Plus Mk II plate reader (Flow Laboratories).

The ELISA followed a competitive inhibition format; alkaloid was quantified by its ability to inhibit the binding of antialka-

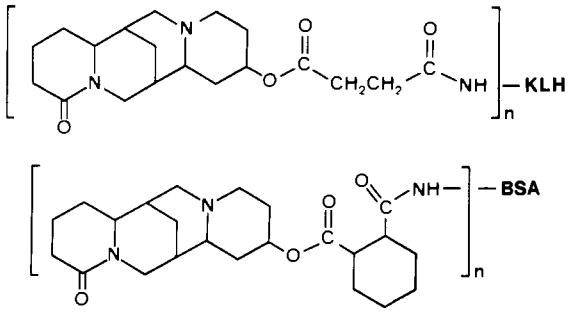


Figure 1. Structures of conjugates used in the ELISA. (Top) Immunogen, 13-hydroxylupanine succinate–KLH; synthesis, mixed-anhydride method. (Bottom) Coating-phase antigen, 13-hydroxylupanine *cis*-hexahydrophthalate–BSA; synthesis, activated ester method.

loid antibodies to the alkaloid–BSA conjugate adsorbed on the solid phase. All absorbances were expressed as a percentage of the maximum absorbance obtained in the absence of inhibition and defined by the relationship $B/B_0 = (A - A_B)/(A_M - A_B) \times 100\%$, where A is the absorbance at 405 nm in the standard of a known amount of alkaloid or in a sample, A_B is the absorbance measured in the absence of coating-phase alkaloid, and A_M is the absorbance in the absence of free alkaloid. Standard curves were obtained from triplicate determinations of standards by plotting $B/B_0\%$ against log [alkaloid] concentration.

Checkerboard titrations were done to determine the optimal concentrations of alkaloid–BSA for plate coating and antibody. Plate-coating conjugate was used at 3 μg/mL and antialkaloid antiserum diluted 1:1000.

Extraction of Alkaloids from Lupin Seed. Various extractants including 70% methanol, TBS (pH 7.4), citrate buffer (pH 2), and 5% trichloroacetic acid (TCA) were compared as extractants for alkaloids; 5% TCA was found to be the most suitable. About 1.00 g of milled lupin seed flour was tumbled overnight in 15 mL of 5% TCA. After centrifugation, 125 μL of extract was neutralized with 75 μL of 10% $NaHCO_3$ and then diluted with 6 mL of TBS. Flours of known alkaloid concentrations (as determined by capillary gas chromatography) were used to construct a standard curve.

Preparation of Standard Solutions. All standard solutions used to determine cross-reactivity and sensitivity of the ELISA were made in either distilled water with 0.1% NaN_3 or TBS (pH 7.4). Standard flours used to construct a standard curve for quantification of unknown flours were prepared in the same procedure as the samples described above.

RESULTS AND DISCUSSION

Being too small to be immunogenic, the lupin alkaloids were conjugated to high molecular weight proteins to invoke an immune response. Hemiesters of (+)-13-hydroxylupanine were prepared containing a reactive substituent group which was used in conjugation reactions with proteins (Figure 1). This procedure resulted in giving the quinolizidine ring structure and particularly the δ-lactam moiety of the alkaloid maximum exposure as the chief antigenic site.

Two hemiesters of (+)-13-hydroxylupanine were employed in the ELISA. The hemisuccinate bridging group was used in the immunogen, while the hemihexahydrophthalate was used as the plate-coating antigen. This protocol eliminated the possibility of cross-reactivities due to antibody recognition of the bridge group (Morgan et al., 1986).

Likewise, two methods of synthesis were used in preparing the different conjugates. The alkaloid succinate–KLH conjugate used as the immunogen was synthesized by the mixed-anhydride method, whereas the alkaloid hexahydrophthalate–BSA conjugate used as the plate-coating antigen was synthesized by the active ester method. This procedure eliminated the cross-reactivity

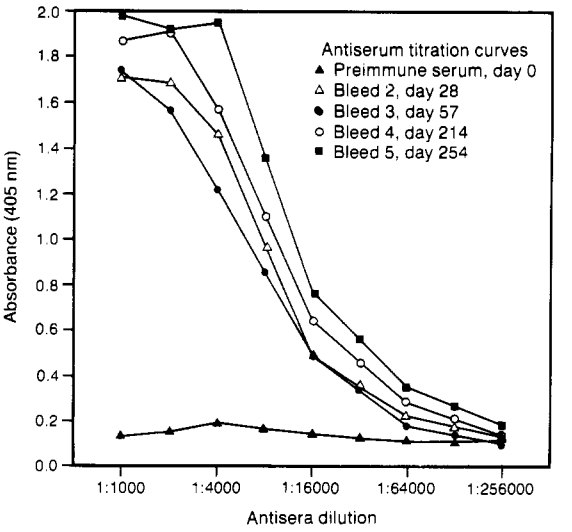


Figure 2. Titration curves of antisera from five successive bleeds at the stated number of days as from the first bleed (sheep 4201). Bleed 1, preimmune sera; bleed 2, 28 days; bleed 3, 57 days; bleed 4, 214 days; bleed 5, 254 days.

encountered in developing the ELISA due to heterologous antisera (Gendloff et al., 1986).

The substitution ratio (moles of alkaloid bonded/mole of protein) of the conjugates was measured by gas chromatography of liberated alkaloid in solvent extracts of conjugate hydrolysates. Quantitative hydrolysis of the conjugates and extraction procedures was proven by spiking experiments. The substitution ratios of the immunogen, alkaloid succinate–KLH, and the plate-coating antigen, alkaloid hexahydrophthalate–BSA, were 420 and 8, respectively.

Antibody titer was determined by titration of serial dilutions (1:1000–1:256 000) of sheep antisera against a fixed concentration of alkaloid–BSA coating antigen of 25 μg/mL (Figure 2). Preimmune sera obtained prior to immunization titrated out to give a constant background of less than 0.14 absorbance unit. Antisera from bleed 3 incubated in wells coated only with BSA gave a constant background absorbance of less than 0.09 AU. These experiments showed that the sheep antisera contain no anti-BSA antibodies and that binding is due to antialkaloid antibodies. Antisera dilutions for the routine screening of alkaloids by ELISA were performed at 1:1000. Sera from subsequent bleeds after boosting immunizations are seen to be of higher titer.

To maximize the sensitivity of the assay, the amount of coating-phase antigen was diluted as far as practical. Figure 3 shows the coating-phase antigen titrated against four different sera concentrations. A coating concentration of 3 μg/mL was shown to give the optimal sensitivity. This was further proven by assaying duplicate determinations of (+)-13-hydroxylupanine by ELISA at 3 and 30 μg/mL of plate-coating antigen (data not presented). An increased sensitivity was gained at 3 μg/mL, and this concentration therefore became the coating concentration used throughout the sample determinations.

Inhibition curves for the alkaloids (+)-lupanine, (+)-13-hydroxylupanine, angustifoline, and α-isolupanine are shown in Figure 4. Table I lists the cross-reactivities of these and other alkaloids relative to (+)-lupanine. (+)-Lupanine and (+)-13-hydroxylupanine demonstrate equal affinities, while angustifoline and α-isolupanine show much reduced affinities.

Development of an ELISA to measure four structurally similar haptens [(+)-13-hydroxylupanine, (+)-lupanine,

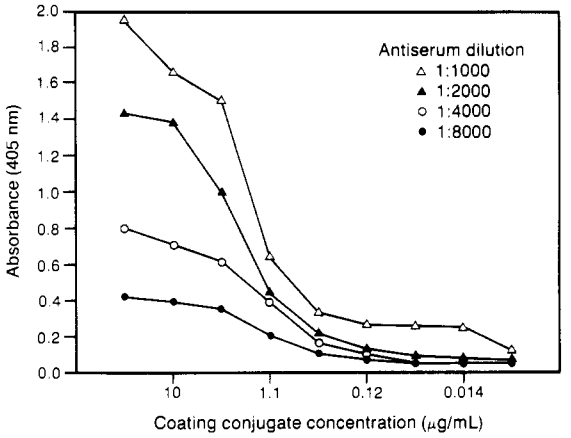


Figure 3. Conjugate titration curves at four different antisera dilutions. Antisera used were from bleed 3.

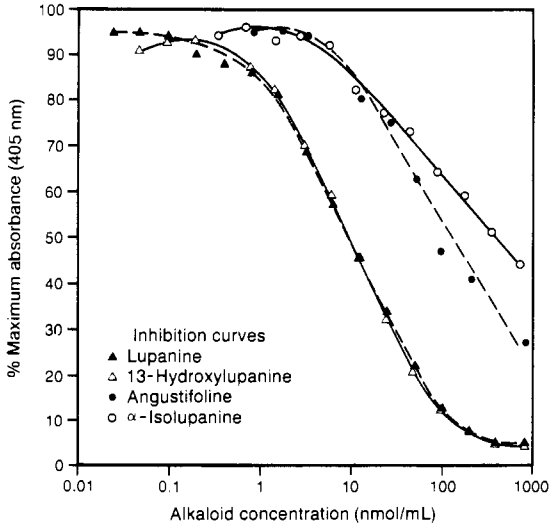


Figure 4. Inhibition curves of the four major alkaloids found in *L. angustifolius*. Results are from triplicate determinations with assay conditions described in text.

α-isolupanine, and angustifoline] in the same extract is fully dependent on the quality of antibodies produced. It was desired that the quinolizidine ring structure with the δ-lactam in the A ring be the chief antigenic site to which antibodies would be produced. This was achieved with the present antisera, but differences in structure of the D ring did affect the affinities the present polyclonal antisera displayed for the haptens. Table I shows the different affinities of various lupin alkaloids tested relative to (+)-lupanine. Alkaloids containing double bonds in the A ring (e.g., cytisine) or lacking the δ-lactam group (e.g., sparteine) in the A ring of the quinolizidine structure showed very little affinity for the antibodies. Likewise, inclusion of a hydroxyl substituent at the 17-position (e.g., 17-hydroxylupanine) showed little affinity for the antibodies, due presumably to steric hindrance effects. Consequently, cross-reactivity with other lupin alkaloids namely, cytisine, sparteine, and 17-hydroxylupanine was not a characteristic of our polyclonal antibodies. The current antibodies are highly specific for the alkaloids of *L. angustifolius*, in particular, (+)-lupanine and (+)-13-hydroxylupanine, both of which are of equal affinity, and to α-isolupanine and angustifoline, which have much reduced affinities relative to (+)-lupanine. The reduced affinity observed for α-isolupanine can be understood when the structural isomerism is considered.

While the polyclonal antisera developed was specific for the four major alkaloids of interest at the exclusion of

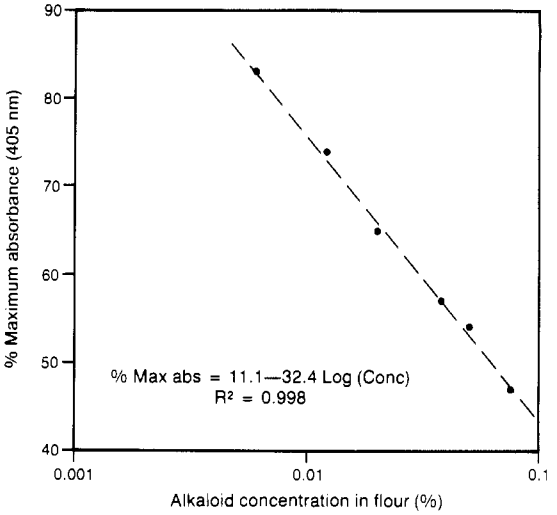


Figure 5. Typical standard curve from six lupin flours used in the routine determination of breeding samples. $R^2 = 0.998$.

other lupin alkaloids, the different affinities displayed between them caused difficulty in analytical quantitation. To overcome this, a set of lupin seed flours of known alkaloid content, as determined by capillary gas chromatography, covering a range from 60 to 760 μg/g were used to construct the standard curve. These standard flours underwent the same preparation, dilution, and ELISA analysis as the samples and generated a standard curve from which unknown samples were quantitated. Standard flours used were also of the *L. angustifolius* species and had alkaloid profiles similar to those of the samples.

The absorbances of the standard flours were approximately linear with the logarithm of the alkaloid concentration over the range 60–760 μg/g alkaloid in the seed (Figure 5). Quantitation of the lupin seed flour extracts fell within this range after the appropriate dilution. A comparison between ELISA and GC methods of determining lupin alkaloids in the same extracts of 398 lupin flour samples gave a correlation equation of GC = 0.002 + 0.919ELISA. The coefficient of correlation was 0.94 ($p < 0.001$) over the range 10–900 μg/g in the seed. This demonstrates the accuracy of the ELISA for these breeding samples. Precision data for both methods on 50 pairs of duplicate samples gave a coefficient of variation of 15% for the ELISA and 12% for the GC procedure.

Inhibition studies were also undertaken on crude alkaloid extracts of other species of lupin including *L. albus* and *L. mutabilis*. Figure 6 shows a comparison of the inhibition curves of these extracts with that of *L. angustifolius* and pure (+)-lupanine. The alkaloid extracts of *L. angustifolius* and *L. mutabilis* demonstrated inhibitory behavior identical to that of pure lupanine. The crude alkaloid extract of *L. albus*, however, showed a reduced inhibition. This behavior cannot be explained by the alkaloid profile of *L albus*, which has (+)-lupanine as the major component (GC/MS data not presented). The ELISA could not be used to determine the alkaloid content of a series of *L. albus* cultivars when the standard curve was constructed using either (+)-lupanine or standard flours of *L. angustifolius*. It is necessary to use *L. albus* flours of known alkaloid content to construct a calibration curve to determine the alkaloid content *L. albus* samples (data not presented). The ELISA cannot be used for other lupin species such as the yellow lupin (*L. luteus*), which do not contain (+)-lupanine or (+)-13-hydroxylupanine as major alkaloids.

Analysis of alkaloids by ELISA in crude lupin flour extracts does not involve extensive purification routinely

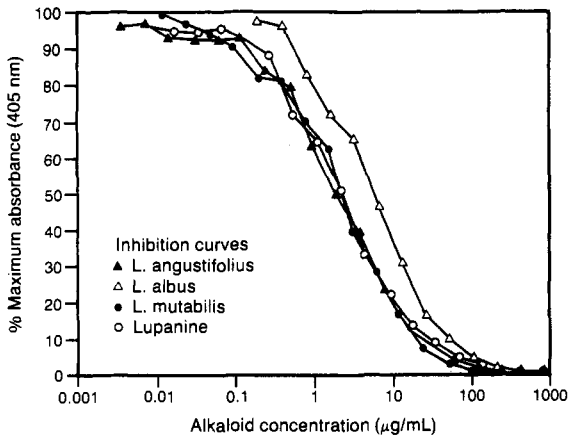


Figure 6. Comparison of inhibition curves of crude alkaloid extracts from *L. angustifolius*, *L. albus*, and *L. mutabilis* with pure (+)-lupanine.

needed for capillary gas chromatography or HPLC. Alkaloids are extracted in 5% TCA, neutralized, and diluted in buffer to a concentration within a linear range of the standard curve. The ELISA is adequate as a screening procedure for lupin seed breeding material, capable of high sample throughput, and is also applicable in assaying plant tissue extracts and plant saps. The benefit to the lupin industry of Australia will be seen in superior varieties of lupin being introduced by breeders for cultivation.

ACKNOWLEDGMENT

We thank the Rural Credits Development Fund of the Reserve Bank of Australia and the Grain Legumes Research Council for financial support of this project. We also thank Z. E. Spadek for supplying (+)-lupanine and (+)-13-hydroxylupanine and R. C. Hansson for GC–MS data. This paper is published with the permission of the Director of the Chemistry Centre (W.A.).

LITERATURE CITED

Anderson, G. W.; Zimmerman, J. E.; Callahan, F. M. The Use of N-Hydroxysuccinimide in Peptide Synthesis. *J. Am. Chem. Soc.* **1964**, *86*, 1839–1842.

Culvenor, C. C. J.; Petterson, D. S. Lupin Toxins—Alkaloids and Phomopsin. In *Proceedings of the Fourth International Lupin Conference*; Department of Agriculture: South Perth, Western Australia, 1986.

Erlanger, B. F.; Borek, F.; Beiser, S. M.; Lieberman, S. Steroid Protein Conjugates. II. Preparation and Characterisation of Conjugates of Bovine Serum Albumin with Progesterone, Decorticosterone, and Estrone. *J. Biol. Chem.* **1959**, *234*, 1090–1094.

Gendloff, E. H.; Casale, W. L.; Ram, B. P.; Tai, J. H.; Petska, J. J.; Hart, L. P. Hapten-protein Conjugates Prepared by the Mixed Anhydride Method. *J. Immunol. Methods* **1986**, *92*, 15–20.

Harris, D. J. Five Year Study of Western Australian Lupinseed. In *Proceedings of the 5th International Lupin Conference*; Twardowski, T., Ed.; PWRiL: Poznan, Poland, 1988.

Harris, D. J.; Spadek, Z. E. Isolation of Standard Alkaloids and the Profile of Bitter L. *angustifolius* Cultivar Fest. In *Proceedings of the Fourth International Lupin Conference*; Department of Agriculture: South Perth, Western Australia, 1986.

Hassan, F.; Rothnie, N. E.; Yeung, S. P.; Palmer, M. V. Enzyme-Linked Immunosorbent Assays for Alkenyl Glucosinolates. *J. Agric. Food Chem.* **1988**, *36*, 398–403.

Kinghorn, D. A.; Balandrin, M. F. Quinolizidine Alkaloids of the Leguminosae: Structural Types, Analysis, Chemotaxonomy, and Biological Activities. In *Alkaloids: Chemical and Biological Perspectives*; Pelletier, W. S., Ed.; Wiley and Sons: New York, 1984; Vol. 2, Chapter 3.

Morgan, M. R. A.; McNerney, R.; Chan, H. W. S. Enzyme-Linked Immunosorbent Assay for Ochratoxin A in Barley. *J. Assoc. Off. Anal. Chem.* **1983**, *66*, 1481–1484.

Morgan, M. R. A.; Kang, A. S.; Chan, H. W. S. Production of Antisera Against Sterigmatocystin Hemiacetal and its Potential for Use in an Enzyme-Linked Immunosorbent Assay for Sterigmatocystin in Barley. *J. Sci. Food Agric.* **1986**, *37*, 873–880.

Morris, B. A., Clifford, M. N., Eds. *Immunoassays in Food Analysis*; Elsevier Applied Science: London, 1985.

Morris, B. A., Clifford, M. N., Jackman, R., Eds. *Immunoassays for Veterinary and Food Analysis: Proceedings of the 2nd International Symposium on Advances in Immunoassays for Veterinary and Food Analysis*; Elsevier Applied Science: London, 1986.

Munier, R.; Macheboeuf, M. *Bull. Soc. Chim. Biol.* **1949**, 1144.

Petterson, D. S.; Allen, D. G.; Greirson, B. N.; Hancock, G. R.; Harris, D. J.; Legge, F. M. The Suitability of *Lupinus angustifolius* for Human Consumption. *Proc. Nutr. Soc. Aust.* **1986**, *11*, 118.

Petterson, D. S.; Ellis, Z. L.; Harris, D. J.; Spadek, Z. E. Acute Toxicity of the Major Alkaloids of Cultivated *Lupinus angustifolius* Seed to Rats. *J. Appl. Toxicol.* **1987**, *7*, 51–53.

Priddis, C. R. Capillary Gas Chromatography for Lupin Alkaloids. *J. Chromatogr.* **1983**, *261*, 95–101.

Vaag, P.; Munck, L. Immunochemical Methods in Cereal Research and Technology. *Cereal Chem.* **1987**, *64*, 59–72.

Wie, S. I.; Hammock, B. D. Comparison of Coating and Immunizing Antigen Structure on the Sensitivity and Specificity of Immunoassays for Benzylphenylurea Insecticides. *J. Agric. Food Chem.* **1984**, *32*, 1294–1301.

Received for review April 15, 1991. Accepted August 29, 1991.