sciendo

Hellenic Plant Protection Journal **18:** 40-49, 2025
DOI 10.2478/hppj-2025-0005

An easy analytical method for the determination of L-Canavanine in legumes and pulse-based fish feed

C. Anagnostopoulos[1]*, A.C. Charalampous[1] and K. Liapis[1]

Summary L-Canavanine is a naturally occurring non-protein amino acid found in certain legumes, where it plays a significant role in plant defence acting as an anti-herbivory compound by disrupting the biology of insect herbivores and pulse-based fish feed. In this study, a simple, easy and robust analytical method was developed and validated to monitor the compound accurately and reliable. The developed method includes a simple quick and low-cost extraction procedure using acidified acetonitrile. Chromatographic determination is based on Hydrophilic Interaction Liquid Chromatography coupled to tandem mass spectrometry operating in positive electron spray ionization. The method was successfully validated by assessing accuracy, precision, linearity of response (gradient of the calibration curve) at a range of concentrations 1.25 - 25 μg L^{-1}, matrix effect and limit of quantitation according to European Guidelines. One way analysis of variance (ANOVA) was employed for estimating the contribution to overall uncertainty and finally to the reported value/concentration, of homogenization and sub-sampling step.

Additional keywords: Fish feed, HILIC, L-Canavanine, legumes, mass spectrometry

Introduction

L-Canavanine, (*L-2-amino-4-(guanidinooxy) butyric acid*), is a non-protein amino acid, structural analog to L-arginine (Fig. 1), and possess potentially toxic properties for both plant and animal systems, including humans (Rosenthal, 2001; Staszek *et al*., 2017).

L-Canavanine is a common ingredient, naturally occurring in alfalfa sprouts (*Medicago sativa* L), broad beans, jack beans, sword beans and other legume products (Brown, 2005). Owing to this structural similarity, L-Canavanine role in food production revolves around its mistakenly incorporation into organism's own proteins in place of L-arginine, leading to the production of structurally aberrant proteins (EFSA 2020). Canavanine has also been reported to induce a condition that mimics systemic lupus erythematosus (SLE) in primates (Malinow, 1982; Prete, 1985), to increase antibodies to nuclear components and promote SLE-like lesions in auto immune-susceptible (Moulton, 2017). L-Canavanine can also act as a feed-inhibitor for pigs at concentrations 13 mM kg^{-1}, reducing feed intake up to 25% (Enneking *et al*., 1993).

Up to 2024, the references in public literature search (Scopus 2024) by using the keywords 'canavanine', 'determination', 'analytical methods' is scarce. To our knowledge the first reference for the determination of Canavanine is from 1954, where the detection was carried out with a colour reaction of pentacyanoferrate derivatives (Fearon and Bell, 1955; Bell, 1958). In more recent studies, analytical methods involved pre-column derivatization step to form either dansylated derivatives (Ekanayake *et al*., 2007) or diethyl ethoxymethylenemalonated derivatives (Megías *et al*., 2015), extraction with strong acids in hydrophilic interaction chromatography coupled to diode array detector (Irakli *et al*., 2018). In addition to the above, a dual fluorescent protein assay system also developed this compound and other DNA-damaging substances in the yeast Saccharomyces cerevisiae (Lu *et al*., 2015).

Liquid chromatography in combination

[1] Laboratory of Pesticides Residues, Scientific Directorate of Pesticide's Control and Phytopharmacy, Benaki Phytopathological Institute, 8 St. Delta Street, GR-145 61 Kifissia, Greece.
* Corresponding author: c.anagnostopoulos@bpi.gr

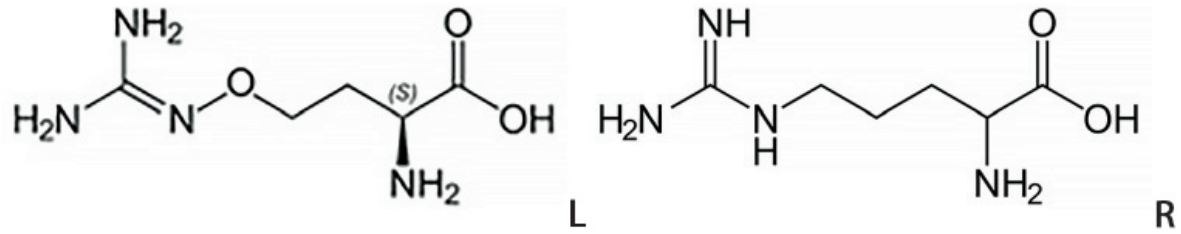

Figure 1. Chemical structure of L-Canavanine (Left) and L-Arginine (Right)

with mass spectrometry (LC-MS/MS) is a powerful analytical technique that enables the determination of analytes at low levels with minimum, in most cases, sample preparation procedures. In this study, a simple, quick and low-cost extraction procedure in combination with LC-MS/MS was developed and validated for the determination of Canavanine in pulses and related animal feed, with a low limit of quantification (LOQ).

Materials and methods

Reagents and Chemicals

Analytical standard of L-Canavanine (>98%) was purchased from Sigma – Aldrich/ Merck. Reagents used were acetonitrile (ACN, >99,9%, HPLC gradient, Sigma Aldrich), methanol (MeOH, >99.9%, LC-MS grade, Fischer Scientific), water (H2O, LC-MS grade, Fischer Scientific), formic acid (98/100%, Analytical Reagent, Fischer Scientific), ammonium formate (99%, Acros Organics) and C18 Bondesil (400 μm, Varian), a hydrophobic bonded silica sorbent used for dispersive solid phase extraction (d-SPE) to remove fat and other interferences from the extract.

Preparation of Analytical standards

Canavanine stock solution of 1000 mg L^{-1} was prepared by accurately weighing 0.02551 g of Canavanine neat standard and dilute in a grade A volumetric flask with 25 mL MeOH:H_2O (1:1 v/v). Working solutions were prepared with appropriate dilutions. In particular, 3 series of calibration standards were prepared within the range of 1.25 – 25 μg L^{-1} by serial dilution in acetonitrile, lentil extract and animal feed extract. The lentil and animal feed extracts were prepared as described below.

Extraction Procedure

The extraction procedure is based on a previously published work for the determination of Aloin (Anagnostopoulos and Ampadogiannis, 2020) applied successfully on pulses and related animal feed with slight modification on the clean-up step.

In particular, an aliquot of 5 (±0.05) g of homogenized sample was weighted in a 50mL polypropylene centrifuge tube, 10 mL of H_2O were added, and the mixture was shaken at a Vortex mixer, until a homogenized slurry was formed. The analyte was extracted by adding 10mL of ACN/H_2O (80:20, v/v) acidified with 1% of HCOOH, and then shaken intensively at a Vortex mixer for 2min. After centrifugation at 4000 rpm for 5 min, an aliquot of 2 mL of the supernatant was transferred into a 15mL polypropylene centrifuge tube with 2 mL of ACN and 200 mg of octadecyl sorbent (C_{18}). The mixture was shaken at a Vortex mixer for another 2 min and centrifuged at 4000 rpm for 5 min. The final extract was filtered through a disposable PTFE syringe filter, 0.45 *μm* and transferred into a screw cup storage vial and stored in the freezer until analysis. A graphical presentation of the sample preparation procedure in presented in Figure 2. Following this extraction procedure, the concentration C_s (mg kg^{-1}) of the analyte in the sample corresponds to 8 x C_{vial} (*μg* mL^{-1}) of the analyte in the extract.

Chromatographic analysis

Due to the physicochemical properties (log Pow -4.26 and pKa values 2.1 and 9.31), L-Canavanine, exists as zwitterion in pH

range 2-9, so Hydrophilic Liquid Interaction Chromatography (HILIC) coupled to mass spectrometry was chosen.

LC analysis was conducted by a Varian liquid chromatography system consisting of two Varian Prostar 210 pumps and a Prostar 420 autosampler using a 100 *μL* syringe, combined with triple quadrupole mass spectrometer (Varian model 1200L). Chromatographic separation was achieved in the absence of guard column, using a HILIC column (ZIC-pHILIC, 150 mm × 4.6 mm, i.d.= 5 *μm*, SeQuant) at 35°C and a gradient program of a binary mobile phase system consisting of solvent A (ACN/H_2O, 90:10, v/v) and solvent B (50 mM $HCOONH_4$ in H_2O). Flow rate was constant at 0.25 mL min^{-1} and the elution program was as follows: After an isocratic step at 10% B for 5 min, eluent B was increased by a linear gradient from 10 to 95% in 10 min and held at 95% for 5 min. At 20.1 min the eluent B was lowered to 10% and held for 5 min to re-equilibrate the column. As to avoid carry-over phenomenon, the needle was washed with a 0.1% HCOOH in MeOH/H_2O (50:50, v/v) after each injection. The injection volume was 10μL and the total run-time was 25 min.

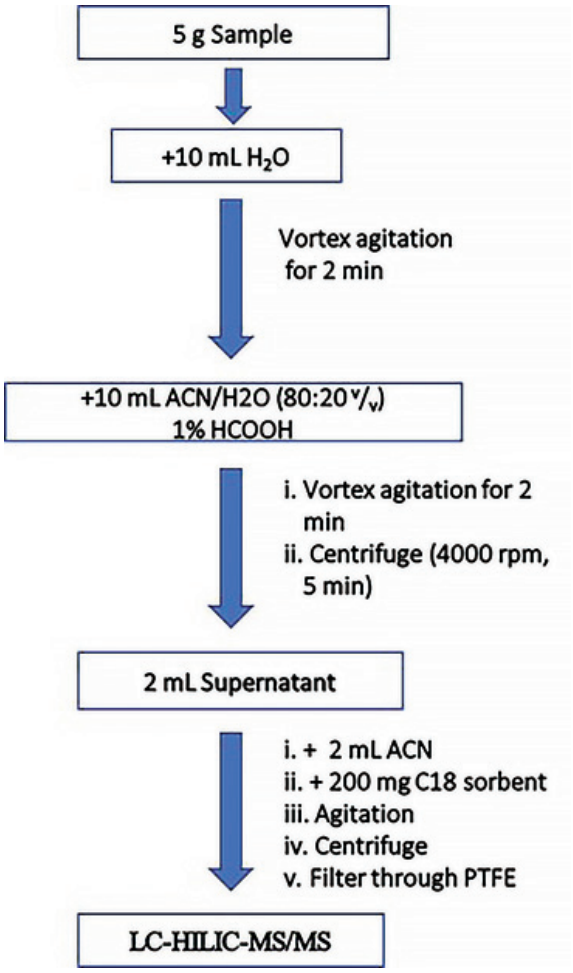

Figure 2. Abbreviated flow diagram describing extraction procedure.

Mass Spectrometry

Mass spectrometer was a Varian 1200L triple quadrupole equipped with an electrospray ionization interface operating in the positive mode. The theoretical fragmentation pattern of L-Canavanine under ESI-(+)-MS/MS conditions is presented in Figure S1. Selected values for capillary voltage (V) and collision energy (eV) used for each transition are given in Table S1. For optimizing the above, a working solution of Canavanine at 0.5 *μg* mL^{-1} in solvent was injected in the absence of chromatographic column using a loop, under Flow Injection Analysis (FIA) conditions, firstly for optimized fragmentor voltage and secondly for optimized collision cell energy. Typical source parameters were as follow: source temperature was set at 250°C and drying gas temperature at 250°C. Drying and nebulizing gas was nitrogen generated from a high purity generator and their pressures were set at 18 and 55 psi respectively. For the operation in MS/MS mode, Argon 99.999% was used as collision gas with a pressure of 1.5 mTorr. The multiple reaction monitoring experiments were conducted with a dwell time of 100 msec. For instrument control, data acquisition and processing, Varian MS Workstation software version 6.8 was used. In Figure 3, the Total Ion Chromatogram (TIC) of blank sample, and matrix match calibration standards are presented.

Confirmation criteria

For initial identification of the analyte, the retention time (t_R) criterion was used. The t_R of the analyte was matched based on an external calibration standard at a tolerance of ± 0.2 min. The final confirmation was done by

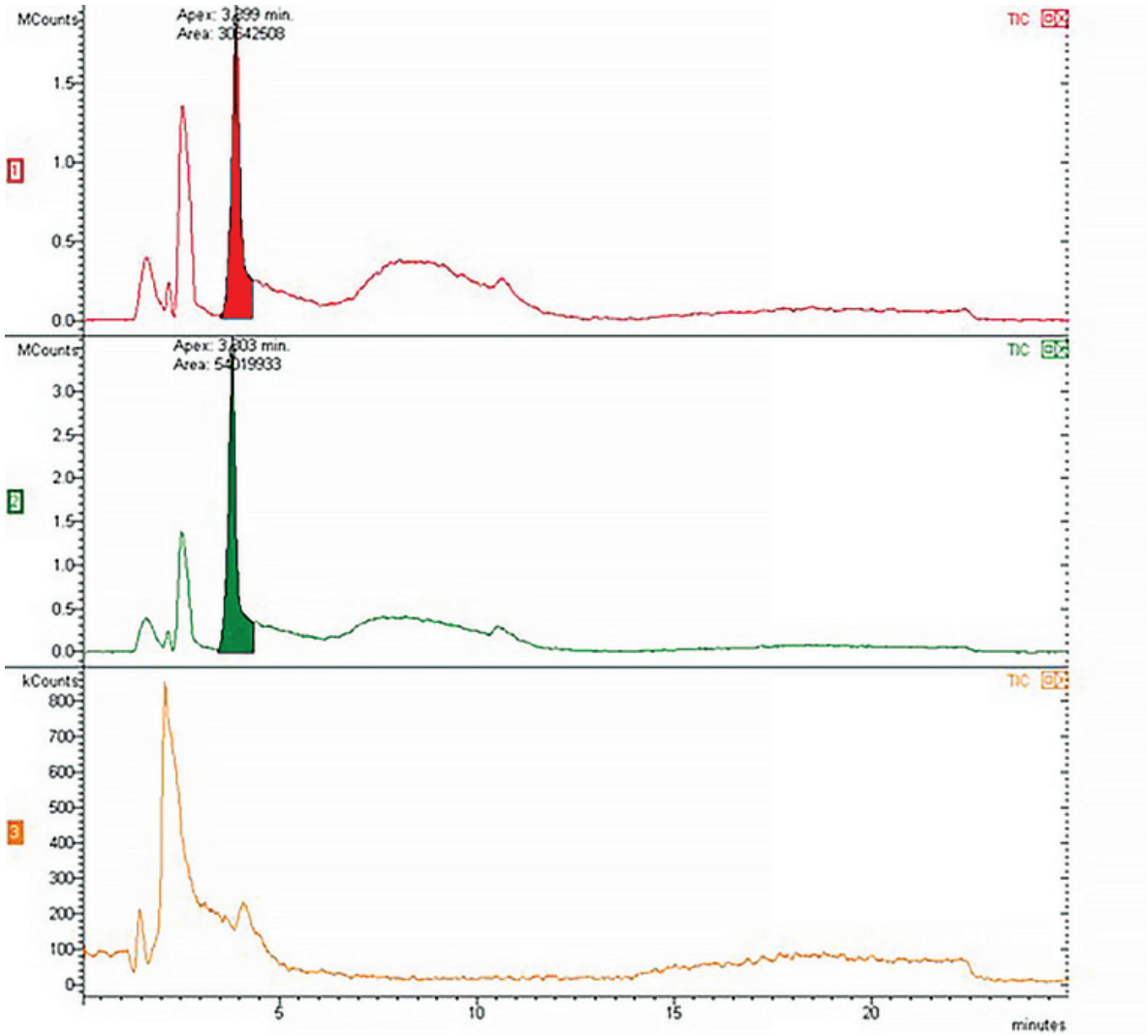

Figure3. TIC chromatogram for matrix matched calibration standards of Canavanine (at 0.25 μg mL^{-1} (1^{st} row) and 0.5 μg mL^{-1} (2^{nd} row) and for blank sample.

implementing the ion ratio criterion, according to SANTE/11312/2021, whereas ion ratio must be in the range of ±30% of external standard ratio (European Commission 2024).

Method validation

The following parameters were assessed during validation procedure: accuracy and precision based on recovery experiments, linearity, limit of quantification, (LOQ), matrix effects (ME). In addition, the overall uncertainty (U) of the method was also estimated. Homogeneity during sub-sampling was also assessed as to exclude any results variation due to too small sample size. Accuracy was evaluated by calculating the attained mean recovery (% REC) at each fortification level and the deviation of results expressed as percent relative standard deviation (% RSD) was used for estimating precision. Homogenized samples of lentils and animal feed based on dried beans, previously checked for the presence of Canavanine, were used for recovery experiments (and the whole validation process) at 5 concentration levels (0.01, 0.02, 0.05, 0.1 and 0.2 mg kg^{-1}) with 6 replicates at each.

Uncertainty of subsampling

For estimating the contribution to overall uncertainty and finally to the reported value/concentration, of homogenization

and sub-sampling step in the laboratory, a 5 kg of blank animal feed sample (the one used in fortification experiments), was spiked at 1 mg kg^{-1} and homogenized using a knife-blender. Ten (10) subsamples of 5 g were prepared/extracted according to the already described in house developed method and were in duplicate analyzed/measured. One-way ANOVA at 95% confidence level, which tests the null hypothesis (samples in two or more groups are drawn from populations with the same values) was employed for assessing distribution of results. As it is already known, ANOVA produces an *F-statistic* (ratio between the variance of mean values, to the variance between samples) which is compared to *F-criterion* (value produced when null hypothesis is valid). According to results, sub-samples were homogeneous (*F-statistic* < *F-criterion*), and the 5g sample is considered proper/enough for the specific method.

Results and Discussion

Accuracy and Precision

Accuracy was evaluated by calculating the attained mean recovery (% Rec) for Canavanine at each fortification level, and the deviation of results expressed as percent relative standard deviation (RSD) was used for estimating precision. Homogenized samples of lentils and animal feed based on dried beans, previously checked for the presence of Canavanine, were used for recovery experiments (and the whole validation process) at 5 concentration levels (0.01, 0.02, 0.05, 0.1 and 0.2 mg kg^{-1}) with 6 replicates at each. Results for both accuracy and precision are shown in Table S2. Recovery values varied between 74.6 - 90.2 % and RSD values were well below 20%, meaning that they were in accordance with SANTE criteria for method validation (European Commission 2024).

Linearity

Linearity of response was assessed for both calibration standards in solvent and in matrix (lentils and animal feed). Regression analysis was performed at 95% confidence level using least squares, for concentration varying from 1.25-25 μg L^{-1} (5 different concentrations) and correlation coefficient R^2 was above 0.99 for each case (solvent, lentils, animal feed). Results are listed in Table S3.

Limit of Quantitation (LOQ)

LOQ was determined as the lowest fortification level for which accuracy and precision are acceptable according to SANTE (European Commission 2024), thus 0.01 mg kg^{-1}. Taking into consideration that LOD equals to (LOQ/3), then LOD is set at 0.0033 mg kg^{-1}.

Uncertainty estimation

The uncertainty of the method was calculated based on the Eurachem/Citac Guidelines (Ellison 2012) as described below.

Type A Uncertainty

For the calculation of type A uncertainty, uncertainty associated with the use of calibration curve and the least square fitting $U_{calCurv}$ is combined with uncertainty associated with bias $U_{(bias)}$ according to Eq.1

$$U_{calcurv} = \frac{Sy}{b}\sqrt{\frac{1}{N} + \frac{1}{n} + \frac{(yo - y)^2}{b^2 + \sum_{i=1}^{N}(xi - x)^2}} \rightarrow \text{(Eq.1)}$$

where Sy is the residual standard deviation, b the slope of the calibration curve, N the number of calibration levels (x), n the number of values per level, yo the experimental value of y for which the concentration is xo, y the average of y values and $\Sigma_{i=1}^{N}(xi - x)^2$- the sum of the obtained concentration (given by the calibration curve) minus the average concentration of the standards used in the calibration curve.

U_{bias}

The uncertainty related to bias, is estimated through recovery experiments at different fortification levels (Eq. 2).

$$U_{bias} = \sqrt{RMSbias + uCrecovery^2} \rightarrow \text{(Eq. 2)}$$

where $RMS_{bias} = \sqrt{\frac{\Sigma(bias)^2}{n}}$

and $u_{Crecovery} = \sqrt{u(\mathrm{RefMat})^2 + uvol^2} \rightarrow$(Eq. 3)

where u(RefMat) the uncertainty from the reference material and Uvol the uncertainty from the use of automated micropippetes (see Type B uncertainty).

Type B Uncertainty

For the calculation of type B uncertainty, all parameters that contribute to the overall uncertainty of the final result, and which are the result of use of weighing scale (U_{mass}), mechanic pipettes (U_{vol}), use of analytical standard stock solution (U_{stock}) are combined as to calculate total type B uncertainty (Eq. 4).

$$U_{total} = \sqrt{Umass^2 + Uvol^2 + Ustock^2} \rightarrow \text{(Eq. 4)}$$

where:

U_{mass}: Based on the calibration certificate of the balance ($U_{balance}$) the relative uncertainty associated with mass measurement is: $U_{mass} = u_{mass}/m_{sample} = (0.02+3.7319x10^{-5}x5)/5 = 0.004$.

U_{vol}: For the uncertainty associated with the use of automated micropipettes, a rectangular distribution is assumed. Taking into consideration the standard deviation *S* from the calibration certificate of the mechanical pipette, the uncertainty from the addition of the extraction solvent is:

i. Addition of 10 mL: $U_{vol} = u_{vol}/Vol = (S/\sqrt{3})/10 = 0.001156$
ii. Addition of 2 mL: $U_{vol} = u_{vol}/Vol = (S/\sqrt{3})/2 = 0.138672$

U_{stock}: For the uncertainty rising from the use of analytical standard, a rectangular distribution is for once more assumed. Taking into consideration the S from manufacturer's certificate, uncertainty is equal to:

$$U_{stock} = (S/\sqrt{3}) = 0.00289$$

$$U_{total} = \sqrt{Umass^2 + Uvol^2 + Ustock^2} = 0.138769 = 13.87\%$$

The impact of type B uncertainty is minimal in comparison to type A, highlighting that achieving reliable measurements primarily depends on the method's performance within the laboratory.

Matrix Effects (ME)

Matrix Effects, is the effect of the components of a matrix to the response of a detector to a certain analyte, compared to it's response in the absence of matrix. Matrix components can suppress or increase detector's response. ME was calculated as the percentage relative difference of the slopes (Eq. 5), of two calibration curves in the above-mentioned range, one by using matrix matched calibration standards and one in solvent.

$$\% ME = 100 \times [(b_{solvent} - b_{matrix})/b_{solvent}] \rightarrow \text{(Eq. 5)}$$

According to regression analysis data, |ME| was >20%, meaning that the use of matrix matched calibration standards deemed necessary.

Conclusions

A simple quick and easy method was developed and validated for the determination of L-canavanine in pulse-based fish feed and legumes. The method is simple, low cost, with excellent validation data and low overall uncertainty of the measurement at the validated range. The LOQ of the method is 0.01 mg kg^{-1}. The extraction procedure can be considered as universal, since it does not include any specific analytical step or reagent dedicated for canavanine. The use of LC-MS/MS chromatography is also an asset, since it is already known to be the method of choice when it comes to multi residue methods. This gives the opportunity, for future work, since other analytes sharing similar physicochemical properties (polar compounds in zwitterion form), like polar pesticides (Anastassiades *et al.*, 2023), polar veterinary drugs, melamine (Molognoni *et al.,* 2024), aminoglycosides (Yang *et al*., 2022) or other natural toxins in food could be extracted along with canavanine simultaneously. Finally, the above described method could be useful in the investigation of chemical composition of plant matrices.

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Authors' contributions

Chris Anagnostopoulos: Conception and design; All authors: Validation; Chris Anagnostopoulos and Angeliki Charalampous: Material preparation, data collection and analysis, preparation of the first draft; All authors read and approved the final manuscript.

Funding

Part of the work was funded by the project "Development of analytical methodology for the determination of L-Canavanine in animal feed" which was implemented at Benaki Phytopathological Institute.

Literature Cited

Anagnostopoulos, C. and G. Ampadogiannis, G. 2020. Development and Validation of an Analytical Method for the Determination of Aloin Using Liquid Chromatography Tandem Mass Spectrometry. *Food Analytical Methods,* 13: 1409-1420.

Anastassiades, M., *et al.* (2023). Quick Method for the Analysis of Highly Polar Pesticides in Food Involving Extraction with Acidified Methanol and LC- or IC-MS/MS Measurement I. Food of Plant Origin (QuPPe-PO-Method), V12.1, EU Reference Laboratories for Residues fo Pesticides- Single Residue Methods.

Bell, E.A. 1958. Canavanine and related compounds in Leguminosae. *The Biochemical Journal* 70(4): 617-619.

Brown, D.L. 2005. Canavanine-induced longevity in mice may require diets with greater than 15.7% protein. *Nutrition and Metabolism* (Lond) 2.

Enneking, D., Giles, L.C., Tate, M.E. and Davies, L.R.L. 1993. L-canavanine: A natural feed-intake inhibitor for pigs (isolation, identification and significance). *Science of Food and Agriculture,* 61(3): 315-325 https://doi.org/10.1002/jsfa.2740610305.

EFSA 2020. Safety of a botanical extract derived from *Panax notoginseng* and *Astragalus membranaceus* (AstraGinTM) as a novel food pursuant to Regulation (EU) 2015/2283 EFSA Journal 5.

Ekanayake, S., Skog, K. and Asp, N.G. 2007. Canavanine content in sword beans (*Canavalia gladiata*): Analysis and effect of processing. *Food and Chemical Toxicology,* 45(5): 797-803.

Ellison, S.L.R. and Williams, A. 2012. EURACHEM/CITAC Guide: Quantifying Uncertainty in Analytical Measurement, Second edition, EURACHEM.

European Commission 2024. Guidance document on analytical quality control and method validation procedures for pesticide residues and analysis in food and feed. SANTE 11312/2021 v2. E. Commission.

Fearon, W.R. and Bell, E.A. 1955. Canavanine: detection and occurrence in *Colutea arborescens*. *The Biochemical Journal,* 59(2): 221-224.

Irakli, M., Tsialtas, I.T. and A. Lazaridou, A. 2018. Determination of Free and Total Underivatized Amino Acids including L-Canavanine in bitter vetch seeds using Hydrophilic Interaction Liquid Chromatography.*Analytical Letters,* 51(16): 2602-2613.

Lu, Y., Tian, Y., Wang, R., Wu, Q., Zhang, Y. and X. Li, L. 2015. Dual fluorescent protein-based bioassay system for the detection of genotoxic chemical substances in Saccharomyces cerevisiae. *Toxicology Mechanism and Methods*, 25(9): 698-707.

Malinow, M.R., Pirofsky, E.J.B., Craig, S. and McLaughlin, P. 1982. SLE-like syndrome in monkeys fed alfalfa sprouts: role of a non-protein amino acid. *Science of the Total Environment*, 2016: 415-417.

Megías, C., Cortés-Giraldo, I., Girón-Calle, J., Vioque, J. and Alaiz, M. 2015. Determination of l-canavanine and other free amino acids in *Vicia disperma* (Fabaceae) seeds by precolumn derivatization using diethyl ethoxymethylenemalonate and reversed-phase high-performance liquid chromatography. *Talanta*, 131: 95-98.

Molognoni, L., *et al.* 2024. Determination of Polar Veterinary Drugs and Melamine in Feeds: Method Using Ion-Pair Reversed-Phase or Hydrophilic Interaction Liquid Chromatography Coupled to Tandem Mass Spectrometry. *Chemical Food Contaminants Analysis.* R. Hoff and L. Mologoni. New York, NY, Springer US**:** 221-229.

Staszek, P.L.A.W., Ciacka, K., Krasuska, U. and Gniazdowska, A. 2017. L-Canavanine: How does a simple non-protein amino acid inhibit cellular function in a diverse living system? *Phytochemistry Reviews*, 16: 1269–1282.

Prete, P.E. 1985. Effects of L-canavanine on immune function in normal and autoimmune mice: disordered B-cell function by a dietary amino acid in the immunoregulation of autoimmune disease. *Canadian Journal of Physiology and Pharmacology,* 7: 843-854.

Rosenthal, G.A. 2001 L-Canavanine: A higher plant insecticidal allelochemical. *Amino acids*, 21(3):319-330.

Scopus 2024. Scopus: Comprehensive, multidisciplinary, trusted abstract and citation database.2024, from https://www.scopus.com/.

Yang J. and Rainville, P.D. 2022. Analysis of minogly-

cosides in foods using a Zwitterionic Stationary Phase and Liquid Chromatography-Tandem Mass Spectrometry. Waters, Waters Corporation**:** 20.

Moulton, V.R., Suarez-Fueyo, A., Meidan, E,. Li, H., Mizui, M. and Tsokos, G.C. 2017. Pathogenesis of Human Systemic Lupus Erythematosus: A Cellular Perspective. *Molecular Medicine,* 7: 615–635.

Received: 13 November 2024; Accepted: 21 January 2025

Προσδιορισμός της L-Καναβανίνης σε όσπρια και ιχθυοτροφές με πρώτη ύλη τα όσπρια

Χ. Αναγνωστόπουλος, Α.Χ. Χαραλάμπους και Κ. Λιαπής

Περίληψη Η L-Καναβανίνη είναι ένα φυσικό μη πρωτεϊνικό αμινοξύ που περιέχεται σε ορισμένες κατηγορίες οσπρίων. Ο ρόλος της στην άμυνα των φυτών είναι σημαντικός, καθώς δρα ως προστατευτική ένωση έναντι της φυτοφαγίας, επηρεάζοντας τη φυσιολογία των εντόμων ή/και των ζώων που καταναλώνουν όσπρια. Στην παρούσα μελέτη αναπτύχθηκε μια εύχρηστη και αξιόπιστη αναλυτική μεθοδολογία για τον προσδιορισμό αυτής της ενδογενούς ουσίας, χρησιμοποιώντας οξινισμένο ακετονιτρίλιο ως διαλύτη εκχύλισης και χρωματογραφία υδρόφιλης αλληλεπίδρασης υγρής φάσης (HILIC) σε συνδυασμό με φασματομετρία μάζας τριπλού τετραπόλου (MS/MS). Η μέθοδος επικυρώθηκε με επιτυχία ακολουθώντας τις Ευρωπαϊκές Κατευθυντήριες Γραμμές, μελετώντας την ακρίβεια, την επαναληψιμότητα, τη γραμμικότητα της απόκρισης (κλίση της καμπύλης βαθμονόμησης) σε εύρος συγκεντρώσεων 1,25 - 25 μg L^{-1}, την επίδραση του υποστρώματος και το όριο ποσοτικοποίησης. Για την εκτίμηση της συνολικής διευρυμένης αβεβαιότητας χρησιμοποιήθηκε μονοπαραγοντική ανάλυση διακύμανσης (ANOVA), λαμβάνοντας υπόψη τα στάδια ομογενοποίησης και υποδειγματοληψίας.

Hellenic Plant Protection Journal **18:** 40-49, 2025

Supplementary material

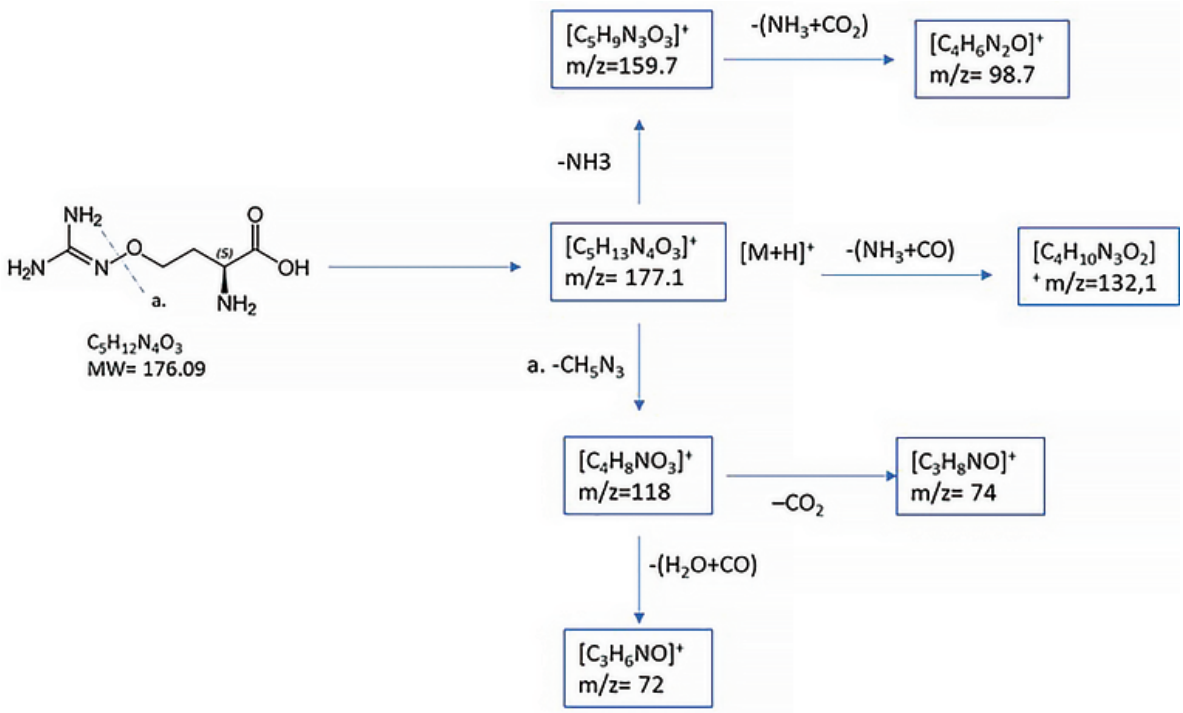

Figure S1. Theoretical fragmentation pattern of L-Canavanine under L-ESI(+)-MS/MS conditions.

Table S1. Retention time t_R and data acquisition parameters for LC-MS/MS operating in positive MRM mode.

Type of transition	t_R (min)	Precursor Ion (amu)	Product Ion (amu)	Capillary Voltage (eV)*	Collision energy (V)*
Quant	3.85	177	75.6	40	14.5
Qual 1		177	76.9	40	14
Qual 2		177	117.6	40	11
Qual 3		77	159.7	40	6

* These values are optimized for the specific instrument conditions, model, and manufacturer. A small variation may be observed between different models and instrument condition. However, they may differentiate significant between different manufacturers.

Table S2. Mean recoveries (% REC) and percentage relative standard deviation (%RSD) values for estimating accuracy and precision.

Matrix	Fortification Level (mg/kg)	REC (%)	RSD (%)
Animal Feed	0.01	76.3	5.6
	0.02*	87.2	2.3
	0.05*	74.6	1.2
	0.1	78	8
	0.2*	81.7	9.3
Lentils	0.01	90.2	1.9
	0.02*	85.3	3.5
	0.05*	95.1	7.2
	0.1	84.2	3.1
	0.2*	99.1	2.1

*n = 3 replicates

Table S3. Regression analysis data.

Matrix	n	r^2	b	Sb	a	Sa	Su
Lentils	5	0.9997	3.0E+08	2.8E+06	1.4E+07	5.9E+06	0.03
Animal Feed	5	0.9997	1.7E+08	1.6E+06	1.7E+07	1.6E+06	0.01
Solvent	5	0.9999	5.2E+08	3.4E+06	-5.0E+06	7.1E+06	0.02