Volume 18, Issue 1, January 2025
ISSN 1791-3691 (Print)
ISSN 2732-656X (OnLine)

Hellenic Plant Protection Journal

A semiannual scientific publication of the
BENAKI PHYTOPATHOLOGICAL INSTITUTE

Editorial Policy

The *Hellenic Plant Protection Journal (*HPPJ) (ISSN 1791-3691) is the scientific publication of the Benaki Phytopathological Institute (BPI) replacing the *Annals of the Benaki Phytopathological Institute* (ISSN 1790-1480) which had been published since 1935. Starting from January 2008, the *Hellenic Plant Protection Journal* is published semiannually, in January and July each year.

HPPJ publishes scientific work on all aspects of plant health and plant protection referring to plant pathogens, pests, weeds, pesticides and relevant environmental and safety issues. In addition, the topics of the journal extend to aspects related to pests of public health in agricultural and urban areas.

Papers submitted for publication can be either in the form of a complete research article or in the form of a sufficiently documented short communication (including new records). Only original articles which have not been published or submitted for publication elsewhere are considered for publication in the journal. Review articles in related topics, either submitted or invited by the Editorial Board, are also published, normally one article per issue. Upon publication all articles are copyrighted by the BPI.

Manuscripts should be prepared according to instructions available to authors and submitted in electronic form on line at http://www.hppj.gr. All submitted manuscripts are considered and published after successful completion of a review procedure by two competent referees.

The content of the articles published in HPPJ reflects the view and the official position of the authors. The information and opinions contained herein have not been adopted or approved by the HPPJ Editorial Board. The HPPJ Editorial Board neither guarantees the accuracy of the information included in the published articles nor may be held responsible for the use to which information contained herein may be put.

For all parties involved in the act of publishing (the author(s), the journal editor(s), the peer reviewers, and the publisher) it is necessary to agree upon standards of expected ethical behavior. HPPJ follows the ethics statements of De Gruyter journals, which are based on the Committee on Publication Ethics (COPE) Code of Conduct guidelines available at www.publicationethics.org.

For back issues, exchange agreements and other publications of the Institute contact the Library, Benaki Phytopathological Institute, 8 St. Delta Str., GR-145 61 Kifissia, Attica, Greece, e-mail: m.kitsiou@bpi.gr.

This Journal is indexed by: *AGRICOLA, CAB Abstracts*-Plant Protection Database, *INIST* (Institute for Scientific and Technical Information) and *SCOPUS*.

The olive tree of Plato in Athens is the emblem of the Benaki Phytopathological Institute

sciendo

Hellenic Plant Protection Journal **18:** 1-9, 2025
DOI 10.2478/hppj-2025-0001

Effect of abiotic factors in seed germination of the medicinal aromatic plant *Ziziphora hispanica* L.

M. Djebbouri[1,2], M. Zouidi[3*] and M. Terras[1]

Summary *Ziziphora hispanica* L. (Lamiaceae) is a medicinal aromatic plant which is native to Algeria in semi-arid regions, Morocco and southern Spain. Despite the medicinal and economic interests as well as the uncertain future that this species may encounter in its natural habitats, various aspects of the biology of its seeds have not been recognized up to date. To fulfill these gaps, seed germination has been studied under the effect of different incubation temperatures, and under the influence of deferential concentrations of water stress and saline stress. The seeds were first incubated at different temperatures (15, 20 and 25°C). Then, at 20°C, germination studies were carried out at various sodium chloride concentrations (0, 25, 50, 75 and 100 Mmol /l) and various solutions of polyethylene glycol (PEG_{6000}) (0, -0.2, -0.4, and-0.6 Mpa). The results showed that *Z. hispanica* seeds were not dormant, and the optimal temperature of germination was 20°C, at which the maximum final germination percentage occurred (94%). A high negative correlation was obtained between the germination percentage and the various concentrations of NaCl. In comparison to other plant species that share its habitats, the results of the water stress impact tests indicate that *Z. hispanica* is very sensitive to water stress, indicating difficulties in germinating in semi-arid and arid regions and that climate change may restrict its natural habitats. The results of this study can serve as guidelines for propagation protocols of this species to support its conservation and cultivation.

Additional keywords: abiotic factors, conservation, germination, *Ziziphora hispanica*

Introduction

Lamiaceae is the fifth largest family of flora in Algeria after Asteraceae, Fabaceae, Brassicaceae and Caryophyllaceae. It is represented by 36 genera in the Algerian flora and 183 taxa (Dobignard and Chatelain, 2010-2013). The genus *Ziziphora* of the Lamiaceae is represented by three species (*Z. hispanica* L., *Z. capitata* L. and *Z. tenuior* L.) in Algeria (Dobignard and Chatelain, 2010-2013).

Ziziphora hispanica is an annual herbaceous plant (Quézel and Santa, 1961-1962) which is native to Morocco, Algeria and southern Spain (Dobignard and Chatelain, 2010-2013, Euro+Med, 2015). In Algeria, it is found in the highlands and the regions of the Saharan Atlas (Quézel and Santa, 1961-1962). It is medicinal and aromatic (Bekhechi *et al*., 2007; Benlamdini *et al*., 2014). Several studies have shown its various pharmaceutical activities such as antibacterial (Bekhechi *et al*., 2007; Rabah *et al*., 2013; Zenati *et al*., 2014) anti-fungal (Meratate *et al*., 2015) or antioxidant (Meratate *et al*., 2015).

As medicinal plants and their cultivation is becoming popular due to the increased demand for natural products in the world (Tanga *et al*., 2018), in Algeria, many species of medicinal plants are collected by the local population and herbalists in an anarchic and abusive manner. The combined effect of this overexploitation with climate change makes these species increasingly rare, or threatened with extinction (Bouasla and Bouasla, 2017; Nasrallah *et al*., 2020). In the arid natural environment which characterizes many regions of Algeria, certain environmental constraints (drought and salinity of the soils) are considered to be the main factors limiting the es-

[1] Laboratory of Water Resources and Environment, Department of Biology, Faculty of Science, University of Saida - Dr Tahar Moulay, Saida, 20000, Algeria.
[2] Conservation of forests in the wilaya of Saida -DGF-Algeria.
[3] Centre de Recherche en Aménagement du Territoire (CRAT), Campus Zouaghi Slimane, Route de Ain el Bey, 25000 Constantine, Algérie.
* Corresponding author: zouidibiologie20@gmail.com
mohamed.zouidi@crat.dz

tablishment and cultivation of plants (Zouidi *et al*., 2020). Moreover, Mediterranean plant communities have exhibited adaptation failures to climate change (Cochrane *et al*., 2015; Dadach and Mehdadi, 2021).

The establishment of medicinal plant cultivation procedures is an alternative measure to ensure the preservation of floristic diversity on the one hand, and the availability of these medicinal plants for commercialization on the other (Rebbas *et al*., 2012; Tanga *et al*., 2018). Multiplication of plant species by seed for commercial propagation, including several medicinal plants, is a simple method (Laghmouchi *et al*., 2017). However, many abiotic factors, such as salt stress, temperature, and water stress affect the germination of medicinal plant seeds (Gorai *et al*., 2011; Said-Al Ahl and Omer, 2011; Laghmouchi *et al*., 2017). Indeed germination is one of plant growth stages more sensitive to salt, severely inhibited by increased salinity (Sosa *et al*., 2005). According to several authors (Botía *et al*., 1998; Jamil *et al*., 2006, Medjebeur *et al*., 2018), salinity affects seed germination by reducing osmotic potential to such an extent that it delays or prevents the absorption of water necessary for the mobilization of nutrients necessary for germination and/or the toxic effects of Na^+ and Cl^- which can be toxic to the embryo. Therefore, information regarding seed germination could be of immense help in the domestication of wild medicinal plants (Sharma and Sharma, 2017).

To our knowledge, no published research has investigated the influence of abiotic factors on the germination of seeds of *Z. hispanica*. In this study, and in order to provide useful information for conservation programs and to promote its cultivation and reintroduction, we assessed the effect of temperature, saline stress and water stress on the germination of *Z. hispanica* seeds.

Materials and methods

Seed collection area

In this study, ripe seeds of *Ziziphora hispanica* L. (Fig. 1) were collected in the Saida region in northwest Algeria in the summer of 2020 (Fig. 2). The climate of this region is semi-arid with typical Mediterranean climatic characteristics, i.e., low precipitation (between 300 and 370 mm/year), a wet and cold winter, and a dry and hot summer (Djebbouri and Terras, 2019).

Collection and preparation of seeds

The largest number of *Z. hispanica* plants were found in the Saida region (more than 200 individuals distributed in 13 locations) where from the seeds were collected (Fig. 2). In the laboratory, the seeds were immediately peeled manually and then sterilized with 5% bleach (sodium hypochlorite) for 1 min, followed by an abundant rinse with distilled and air-dried water before being used in the germination experiments.

Experimental design

To determine the optimum temperature for germination and to test seed dormancy and viability, seed germination of *Z. hispanica* was studied under different incubation temperatures of 15°C, 20°C and 25°C. For water stress four concentrations of PEG_{6000} were used, corresponding to osmotic pressures of -0, -0.2, -0.4 and -0.6 Mpa (Michel and Kaufmann, 1973). To examine the impact of salinity stress, the germination of the seeds was studied under solutions of increasing NaCl concentrations (0, 25, 50, 75 and 100 mmol L^{-1}). For water stress, four concentrations of PEG_{6000} were used, corresponding to osmotic pressures of -0, -0.2, -0.4 and -0.6 Mpa (Michel and Kaufmann, 1973). The germination tests were carried out in Petri dishes, each containing 5 ml of test solutions at 20°C because the temperature experiment revealed that the optimum temperature of germination was 20°C. Each experiment consisted of 90 seeds divided into 3 replicates with 30 seeds per Petri dish.

Expressions of germination results and statistical analyses

Two germination parameters were calculated after 30 days of germination (germination was stabilized on day 21st):

Figure 1. *Ziziphora hispanica* L. (full bloom and seeds).

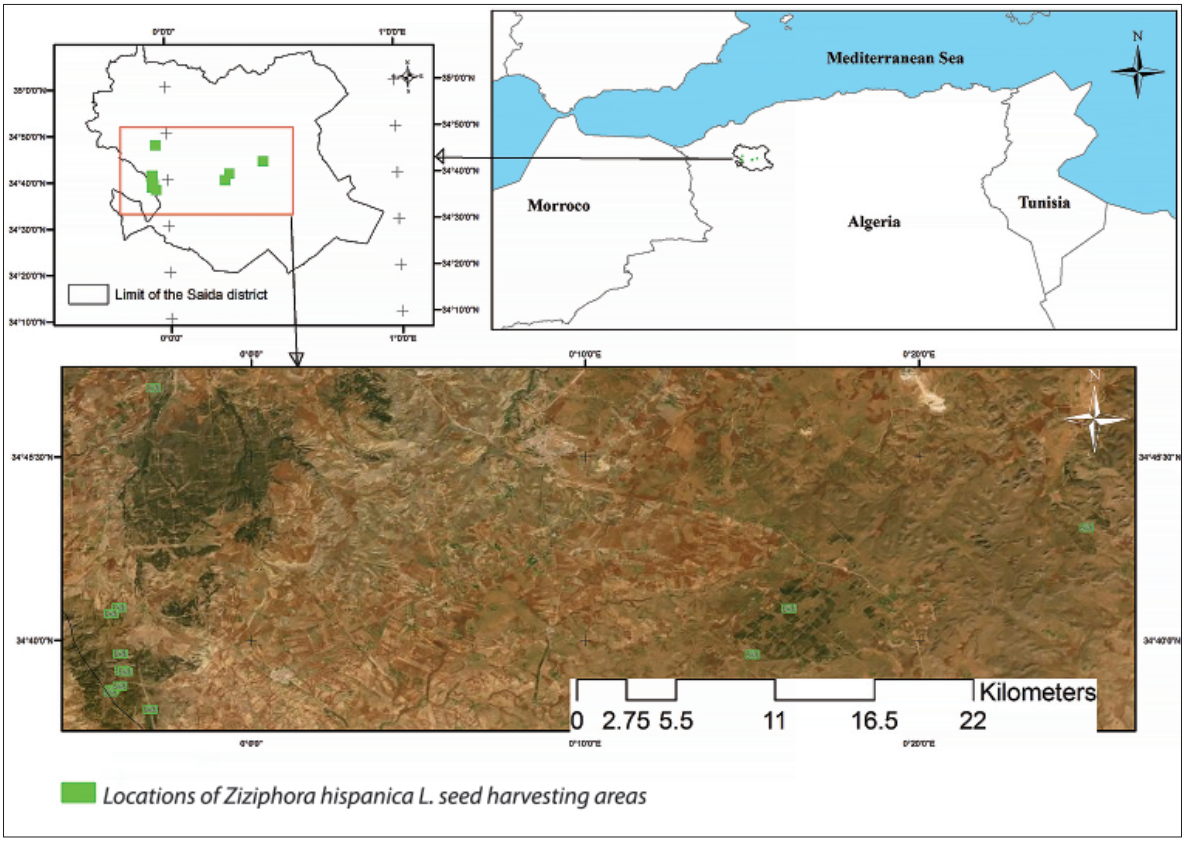

Figure 2. Locations of seed harvesting areas *Ziziphora hispanica* L. in Algeria.

Percentage of germination (GP%) = (number of germinated seeds / total number of seeds) x 100

Mean time of germination (MTG) according to the formula given by Ellis and Roberts (1981):

$MTG = \sum n \times D / \sum n$

Where n: number of newly germinated seeds at time,

D: number of days since the start of the germination test,

Σn: number of seeds that germinated during the germination test.

The lower the MTG value, the faster is the germination (Melendo and Giménez, 2019). Germination percentages and average germination time were calculated with GerminaQuant software (Marques *et al*., 2015). The values for each treatment were calculated as averages of three replicates and were subjected to an analysis of variance (ANOVA). Significant differences between the mean values were tested using the Duncan test. The SPSS version 24 software was used in all analyses and the significance level was set to 0.05.

Results

Effect of temperature on seed germination

The germination rate as a function of the tested temperatures varied significantly ($p < 0.001$). At temperatures of 15 and 25°C, the GP% ranged between 23.33% and 74.44% respectively (Fig. 3). The highest GP% was observed at 20°C (94.44%). The mean MTG at the different test temperatures are shown in Figure 3; it was lower at 20°C compared to 25°C (Fig. 3).

Effect of water stress on seed germination

The highest GP% was recorded in control seeds (96.66%). At the osmotic pressure of -0.2 MPA, the GP% was reduced to 43.33% corresponding to a 55.17% drop compared to the control. The GP% decreased with the increase in the osmotic pressure of the imbibition medium. At the pressure of -0.4 MPA, the reduction in germination reached 96.55%. The germination was completely inhibited at -0.6 MPA (Fig. 4).

Like GP%, the MTG required for germination of *Ziziphora hispanica* L. seeds increased with osmotic pressure. The control seeds germinated faster than those treated with different PEG concentrations (Fig. 4). They required an average of 5.96 days to germinate, whereas, at higher osmotic pressures (-0.2 Mpa and -0.4 Mpa), they germinated after a longer time, 6.99 and 11.33 days, respectively (Fig. 4).

Effect of saline stress on seed germination

The effect of NaCl concentrations on GP% is statistically significant ($p < 0.001$). Seed GP% decreased in response to increased concentration of NaCl in the imbi-

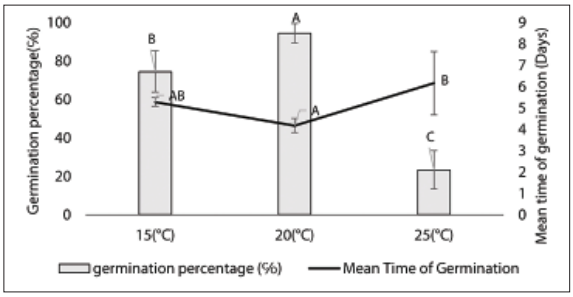

Figure 3. Effect of temperature on percentage and mean time of germination of seeds of *Ziziphora hispanica* L. Bars represent the mean ± S.E. (n = 3). Values with different letters are significantly different at $p < 0.05$.

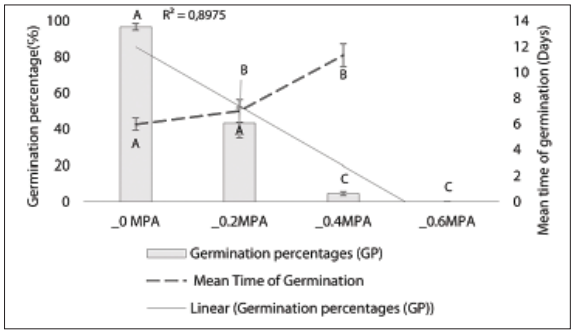

Figure 4. Effect of different water stress on the percentage and the mean time of germination of seeds of *Ziziphora hispanica* L. Bars represent the mean ± S.E. (n = 3). Values with different letters are significantly different at $p < 0.05$.

bition medium (Fig. 5). The higher the osmotic pressure, the higher was the recorded rate of reduction of germinated capacity (Fig. 5). Indeed, from 25 Mmol /l the influence of salinity was significantly perceptible ($p < 0.001$) with a germination percentage of 66.66% corresponding to a reduction of 26.41%. The germination percentage decreased to a minimum value of 3.33% in seeds treated with 100 Mmol/Na Cl (Fig. 5).

The MTG of Z. *hispanica* seeds increased significantly ($p < 0.001$) with the rise of the salinity of the medium (Fig. 5). In controls, the MTG was 5.58 days whereas germination took 17.66 days at 100 Mmol /l of NaCl (Fig. 5).

Discussion

Effect of temperature

Z. hispanica seeds were not dormant in the context of Baskin and Baskin (2004), since they germinated at high percentages (>74%) over a wide temperature range (15–20°C), with no pre-treatment. This is similar to many other species of the Lamiaceae family in the Mediterranean regions (Laghmouchi *et al*., 2017). Although germination of *Z. hispanica* seeds was possible at temperatures between 15°C and 25°C, a peak of germination percentage was recorded at 20°C.

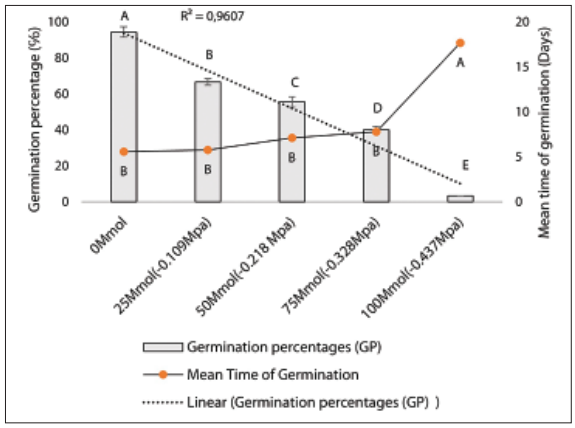

Figure 5. Effect of different saline stress on the percentage and the mean time of germination of seeds of *Ziziphora hispanica* L. Values with different letters are significantly different at $p < 0.05$.

This thermal optimum germination is comparable to some Mediterranean Lamiaceae in arid and semi-arid regions such as *Ballota hirtusa* (Dadach and Mehdadi, 2018), *Marrubium vulgare*, *Sideritis incana* and *Stachys ocymastrum* (Dadach *et al*., 2018), *Thymus maroccanus* Ball. and *Thymus broussonetii* Boiss (Abbad *et al*., 2011). The lowest germination percentage at 25°C indicates that most *Z. hispanica* seeds will probably germinate in late fall and throughout the winter, when temperatures are below 25°C.

Effect of water stress

Seed germination decreased with increased stress and stopped completely at -0.6 MPa which is considered value of tolerance for germination thresholds. Similar results for seed germination affected by the decline in water potential and high germination rate in the absence of stress, have been reported for several species of the family Lamiaceae in arid regions, such as *Sideritis incana, Stachys ocymastrum,* and *Thymus fontanesii* (Dadach and Mehdadi, 2021) *Thymus maroccanus, T. broussonetii* and *T. serpyllum* (Abbad *et al*., 2011; Dadach *et al*., 2015). Similarly, germination of *Salvia verbenaca*, flourishing in semi-arid Mediterranean climate zones, was also very sensitive to a range of water potential from 0 to -0.6 Mpa: at an osmotic potential of -0.4 Mpa, the germination percentage was 5% and it was completely inhibited at an osmotic potential of -0.6 Mpa (Javaid *et al*., 2018).

Drought tolerance during seed germination is an essential criterion for identifying varieties of species capable of resisting water deficit in the early stages of plant growth (Hamdini *et al*., 2021). In this regard, our results revealed that although *Z. hispanica* is native to the Mediterranean, it will have a great difficulty germinating in arid and semi-arid regions because it would not tolerate a water potential greater than -0.4 Mpa (3.33 %GP). Conversely, *Plantago lanceolata* L. and *Ballota hirsute* Benth, species sharing the same habitats where *Z. hispanica*, are more tolerant to water stress and could germinate at -1 Mpa (Dadach *et al*., 2015: Dadach and

Mehdadi, 2018; Mira *et al*., 2018).

As the climate change in the Mediterranean Basin results in widespread increases in average temperatures and longer and more frequent drought periods, and decreased rainfall (Pachauri *et al*., 2014), the germination of *Z. hispanica* has been severely disturbed by water stress. This indicates that climate change restricts its natural habitats and possibly poses a threat to this species.

Effect of salt stress

In arid and semi-arid regions of Algeria, the long-standing drought has clearly led to the salinization of soils. The combination of these two natural stresses is becoming increasingly stressful for the germination and growth of plants in their natural environment (Zouidi *et al*., 2019). Since the reduction of the germination percentage was significant from the concentration of 25 mmol /L of NaCl and the germination was almost inhibited beyond 100 mmol/l of NaCl, this concentration seems to be the threshold of tolerance to the salinity of this species. The negative response to salinity stress at the germination stage has also been reported for other Lamiaceae species (Laghmouchi *et al*., 2017; Dadach and Mehdadi, 2018; Dadach *et al*., 2018; Nedjimi *et al*., 2020). In addition, similar influence of saline stress at a concentration of 100 Mmol/l of NaCl has been recorded on the germination percentage of semi-arid climate Lamiaceae species growing in Mediterranean climate such as *Marrubium vulgare, Sideritis incana* and *Stachys ocymastrum* (Dadach *et al*., 2018).

The effect of PEG and NaCl solutions on seed germination under these conditions can be compared with similar osmotic potential. The -0.2 Mpa and -0.4 Mpa PEG solutions had similar osmotic potential with the 50 and 100 Mmol/l NaCl solutions, respectively. Seeds showed similar germination percentages under similar osmotic potentials and this was consistent with what has been reported in other species (Mira *et al*., 2018, Song *et al*., 2021).

Conclusion

The results of this study showed that based on its seed germination capacity, Z. *hispanica* seems to be more sensitive under water stress compared to other species that share the same habitats. Thus, future projections of climate change, combined with the abusive collection of this species as a medicinal plant will have a negative impact on the population dynamics of this species in Algeria. However, to extrapolate our results, the effects of temperature, salinity and water stress should be assessed in the laboratory and in the field at later growth stages of this species.

The authors thank the members of Laboratory of Water Resources and Environment, Department of Biology, Faculty of Science, University of Saida and the Forestry Department for this work.

Literature Cited

Abbad, A., Belaqziz, R., Bekkouche, K. and Markouk, M. 2011. Influence of temperature and water potential on laboratory germination of two Moroccan endemic thymes: *Thymus maroccanus* Ball. and *Thymus broussonetii* Boiss. *African Journal of Agricultural Research*, 6(20): 4740-4745.

Baskin, J.M. and Baskin, C.C. 2004. A classification system for seed dormancy. *Seed Science Research,* 14(1): 1-16.

Bekhechi, C., Bekkara, F.A., Abdelouahid, D.E., Liu, K., Casanova, J. and Tomi, F. 2007. Composition and antibacterial activity of the essential oil of *Ziziphora hispanica* (L.) from Algeria. *Journal of Essential Oil Bearing Plants*, 10(4): 318-323.

Benlamdini, N., Elhafian, M., Rochdi, A. and Zidane, L. 2014. Etude floristique et ethnobotanique de la flore médicinale du Haut Atlas oriental (Haute Moulouya). *Journal of Applied Biosciences,* 78: 6771-6787.

Botía, P., Carvajal, M., Cerdá, A. and Martínez, V. 1998. Response of eight *Cucumis melo* cultivars to salinity during germination and early vegetative growth. *Agronomie*, 18(8-9): 503-513.

Bouahmed, A., Vessella, F., Schirone, B., Krouchi, F., and Derridj, A. 2019. Modeling *Cedrus atlantica* potential distribution in North Africa across

time: new putative glacial refugia and future range shifts under climate change. *Regional Environmental Change,* 19(6): 1667-1682.

Bouasla, A. and Bouasla, I. 2017. Ethnobotanical survey of medicinal plants in northeastern of Algeria. *Phytomedicine,* 36: 68-81. DOI: 10.1016/j.phymed.2017.09.007.

Cochrane, J.A., Hoyle, G.L., Yates, C.J., Wood, J. and Nicotra, A.B. 2015. Climate warming delays and decreases seedling emergence in a Mediterranean ecosystem. *Oikos,* 124(2): 150-160.

Dadach, M. and Mehdadi, Z. 2018. Germination responses of *Ballota hirsuta* seeds under conditions of temperature, salinity and water stress. *Hellenic Plant Protection Journal*, 11: 34-39.

Dadach, M. and Mehdadi, Z. 2021. Drought tolerance of three ethnomedicinal shrubs evaluated based on their seed germination rates at different drought levels induced by using polyethylene glycol, PEG. *Folia Oecologica,* 48(1).

Dadach, M., Mehdadi, Z. and Adjouj, A.L. 2018. Evaluation of the tolerance to temperature and salinity on seed germination of three Lamiacea from Algerian scrubland. *Advanced Research in Life Sciences,* 2(1): 11-15.

Dadach, M., Mehdadi, Z. and Latreche, A. 2015. Effect of water stress on seed germination of *Thymus serpyllum* L. from Tessala mount. *Journal of Plant Sciences*, 10(4): 151.

Dadach, M., Mehdadi, Z. and Latreche, A. 2015. Germination Responses of *Marrubium vulgare* L. under various water stress conditions. *Journal of Applied Environmental and Biological Sciences*, 5(9): 28-33.

Djebbouri, M. and Terras, M. 2019. Floristic diversity with particular reference to endemic, rare orendangered flora in forest formations of Saïda (Algeria). *International Journal of Environmental Studies,* 76(6): 990-1003.

Dobignard, A. and Chatelain, C. 2010-2013. Index synonymique de la flore d'Afrique du Nord.

Ellis, R. and Roberts, E. 1981. The quantification of ageing and survival in orthodox seeds. *Seed Science and Technology* (Netherlands), 9(2): 373-409.

Euro+Med. 2015. The Euro+Med Plantbase Project.

Gorai, M., Gasmi, H. and Neffati, M. 2011. Factors influencing seed germination of medicinal plant *Salvia aegyptiaca* L. (Lamiaceae). *Saudi Journal of Biological Sciences*, 18(3): 255-260.

Hamdini, A., Mehdadi, Z., Dadach, M. and Latreche, A. 2021. Effect of abiotic stresses on germination behaviour of *Ruta chalepensis*. *Botanica*, 27(1): 23–33.

Jamil, M., Deog Bae, L., Kwang Yong, J., Ashraf, M., Sheong Chun, L. and Eui Shik, R. 2006. Effect of salt (NaCl) stress on germination and early seedling growth of four vegetables species. *Journal of Central European Agriculture,* 7(2): 273–282, DOI: https://doi.org/10.5513/jcea.v7i2.370.

Javaid, M.M., Florentine, S., Ali, H.H. and Weller, S. 2018. Effect of environmental factors on the germination and emergence of *Salvia verbenaca* L. cultivars (verbenaca and vernalis): An invasive species in semi-arid and arid rangeland regions. *PLoS One*, 13(3), e0194319.

Laghmouchi, Y., Belmehdi, O., Bouyahya, A., Senhaji, N.S. and Abrini, J. 2017. Effect of temperature, salt stress and pH on seed germination of medicinal plant *Origanum compactum*. *Biocatalysis and Agricultural Biotechnology,* 10: 156-160.

Marques, F.R.F., Meiado, M.V., Castro, N.M.C.R.D., Campos, M.L.D.O., Mendes, K.R., Santos, O.D.O. D., and Pompelli, M.F. 2015. GerminaQuant: A new tool for germination measurements. *Journal of Seed Science*, 37: 248-255.

Medjebeur, D., Hannachi, L., Ali-Ahmed, S., Metna, B. and Abdelguerfi, A. 2018. Effets de la salinité et du stress hydrique sur la germination des graines de *Hedysarum flexuosum* (Fabaceae). *Revue d'écologie*, 73(3): 318-329.

Melendo, M. and Giménez, E. 2019. Seed germination responses to salinity and temperature in *Limonium supinum* (Plumbaginaceae), an endemic halophyte from Iberian Peninsula. *Plant Biosystems-An International Journal Dealing with all Aspects of Plant Biology,* 153(2): 257-263.

Meratate, F., Lalaoui, A., Rebbas, K., Benmakhbi, L., Bouhadda, A., Souhila, B., Akkal, S., Ouali, E. and Laouer, H. 2015. Antioxidant and antibacterial activities of essential oil and extract of *Ziziphora hispanica* L. of M'sila. *Advances in Environmental Biology*, 9(14): 90-96.

Michel, B.E. and Kaufmann, M.R. 1973. The osmotic potential of polyethylene glycol 6000. *Plant physiology,* 51(5): 914-916.

Mira, S., Veiga-Barbosa, L., González-Benito, M.E. and Pérez-García, F. 2018. Inter-population variation in germination characteristics of *Plantago lanceolata* seeds: effects of temperature, osmotic stress and salinity. *Mediterranean Botany*, 39(2): 89-96.

Nasrallah, Y., Aouadj, S.A. and Khatir, H. 2020. Impact of the exploitation of medicinal plants on biodiversity conservation in Saida and El Bayadh regions, Algeria. *Biodiversity: Research and Conservation*, (60): 11-22.

Nedjimi, B., Souissi, Z.E., Guit B. and Daoud, Y. 2020. Differential effects of soluble salts on seed germination of *Marrubium vulgare* L. *Journal of Applied Research on Medicinal and Aromatic Plants*, 17: 100-250.

Pachauri, R.K., Allen, M.R., Barros, V.R., Broome, J., Cramer, W., Christ, R., Church J.A., Clarke, L., Dahe, Q. and Dasgupta, P. 2014. *Climate change 2014*: synthesis report. *Contribution of Working Groups I, II and III to the fifth assessment report of the Intergovernmental Panel on Climate Change*, Ipcc.

Quézel, P. and Santa, S. 1961-1962. *Nouvelle flore de l'Algérie et des régions désertiques*. Paris.

Rabah, B., Lograda, T., Ramdani, M., Chalard, P. and Feguiredo, G. 2013. Chemical composition and antibacterial activity of essential oil of *Ziziphora hispanica* L. *Global Journal of Research on Medicinal Plants and Indigenous Medicine*, 2(2): 73.

Rebbas, K., Bounar, R. Gharzouli, R., Ramdani, M., Djellouli, Y. and Alatou, D. 2012. Plantes d'intérêt médicinale et écologique dans la région d'Ouanougha (M'sila, Algérie). *Phytothérapie,* 10(2): 131-142.

Said-Al Ahl, H. and Omer, E. 2011. Medicinal and aromatic plants production under salt stress. A review. *Herba Polonica*, 57(1): 72-87.

Sharma, R.K. and Sharma, S. 2017. Seed longevity, germination and seedling vigour of *Rheum australe* D. Don: A step towards conservation and cultivation. *Journal of Applied Research on Medicinal and Aromatic Plants,* 5: 47-52.

Sharma, S., Arunachalam, K., Bhavsar, D. and Kala, R. 2018. Modeling habitat suitability of *Perilla frutescens* with MaxEnt in Uttarakhand—A conservation approach. *Journal of Applied Research on Medicinal and Aromatic Plants*, 10: 99-105.

Song, X., Zhou, G., Shi, L., Ahmad, I., Shi, X., Zhu, G. and Jiao, X. 2021. Comparative effects of salinity and drought on seed germination, seedling growth, photosynthetic productivity, pigments content and antioxidant enzymes of castor bean (*Ricinus communis*). *Crop and Pasture Science*, 72(7): 541-550.

Sosa, L., Llanes, A., Reinoso, H., Reginato, M. and Luna, V. 2005. Osmotic and specific ion effects on the germination of *Prosopis strombulifera*. *Annals of Botany,* 96(2): 261-267.

Tanga, M., Lewu, F., Oyedeji, O. and Oyedeji, O. 2018. Cultivation of medicinal plants in South Africa: A solution to quality assurance and consistent availability of medicinal plant materials for commercialization. *Academia Journal of Medicinal Plants,* 6(7): 168-177.

Zenati, F., Benbelaid, F., Khadir, A., Bellahsene, C. and Bendahou, M. 2014. Antimicrobial effects of three essential oils on multidrug resistant bacteria responsible for urinary infections. *Journal of Applied Pharmaceutical Science*, 4(11): 15-18.

Zouidi, M. Borsali, A.H., Hachem, K., Ayoub, A., Naimi, A. and Hakm, I. 2019. Assessing of the tolerance of *Pinus halepensis* Mill. seeds to water and saline stress at the germination stage. *Forestry Ideas,* 25(1): 160–170.

Zouidi, M., Borsali, A.H., Allam, A., Gros, R., Rebufa, C. and Da Silva, A.M.F. 2020. Comparative Local Case Study of Coniferous Forest Litter of the "*Pinus halepensis* Mill" in Arid and Semi-arid Areas of Western Algeria. *Acta Silvatica et Lignaria Hungarica*, 16(1): 39-50.

Received: 31 October 2021; Accepted: 28 November 2024

Επίδραση αβιοτικών παραγόντων στη βλάστηση των σπόρων του φαρμακευτικού αρωματικού φυτού *Ziziphora hispanica* L.

M. Djebbouri, M. Zouidi και M. Terras

Περίληψη Το *Ziziphora hispanica* L. (Lamiaceae) είναι ένα φαρμακευτικό αρωματικό φυτό, ιθαγενές στην Αλγερία σε ημιξηρικές περιοχές, στο Μαρόκο και στη νότια Ισπανία. Παρά το φαρμακευτικό και οικονομικό ενδιαφέρον καθώς και το αβέβαιο μέλλον που μπορεί να έχει αυτό το είδος στους φυσικούς του βιότοπους, διάφορα θέματα σχετικά με τη βιολογία των σπόρων του δεν είναι γνωστά μέχρι σήμερα. Για να καλυφθούν αυτά τα κενά γνώσης, μελετήθηκε η βλάστηση των σπόρων υπό την επίδραση διαφορετικών θερμοκρασιών επώασης, διαφορετικών συγκεντρώσεων υδατικής καταπόνησης και αλατότητας. Οι σπόροι επωάστηκαν αρχικά σε διαφορετικές θερμοκρασίες (15, 20 και 25°C). Στη συνέχεια, στους 20°C, πραγματοποιήθηκαν μελέτες βλαστικότητας σε διάφορες συγκεντρώσεις χλωριούχου νατρίου (0, 25, 50, 75 και 100 Mmol/l) και σε διάφορα διαλύματα πολυαιθυλενογλυκόλης (PEG6000) (0, -0.2, -0.4, και -0,6 Mpa). Τα αποτελέσματα έδειξαν ότι οι σπόροι του *Z. hispanica* δεν έχουν λήθαργο και η βέλτιστη θερμοκρασία βλάστησης ήταν 20°C, όπου σημειώθηκε το μέγιστο τελικό ποσοστό βλάστησης (94%). Παρατηρήθηκε υψηλή αρνητική συσχέτιση μεταξύ του ποσοστού βλάστησης και των

διαφόρων συγκεντρώσεων NaCl. Σε σύγκριση με άλλα φυτικά είδη που μοιράζονται τους οικοτόπους του, τα αποτελέσματα των δοκιμών επίδρασης υδατικής καταπόνησης δείχνουν ότι το *Z. hispanica* είναι πολύ ευαίσθητο στην υδατική καταπόνηση, υποδεικνύοντας δυσκολία στη βλάστηση σε ημιξηρικές και ξηρικές (άνυδρες) περιοχές και ότι η κλιματική αλλαγή μπορεί να περιορίσει τους φυσικούς του βιότοπους. Τα αποτελέσματα αυτής της μελέτης μπορούν να χρησιμεύσουν ως κατευθυντήριες γραμμές για τα πρωτόκολλα πολλαπλασιασμού αυτού του είδους για την υποστήριξη της διατήρησης και της καλλιέργειάς του.

Hellenic Plant Protection Journal **18:** 1-9, 2025

sciendo

Hellenic Plant Protection Journal **18:** 10-12, 2025
DOI 10.2478/hppj-2025-0002

SHORT COMMUNICATION

First record of the scale insect *Parlatoria camelliae* Comstock (Hemiptera: Coccomorpha: Diaspididae) in Greece

G.J. Stathas[1*], E.D. Kartsonas[2], P. Skouras[1] and M. Dervišević[3]

Summary The scale insect *Parlatoria camelliae* Comstock (Hemiptera: Coccomorpha: Diaspididae) is recorded for the first time in Greece on 12 August 2024. It was found on *Camellia japonica* L. (Theaceae: Ericales) in Chalkidiki. From preliminary studies it was found that *P. camelliae* is an oviparous, biparental species.

Additional keywords: *Camellia japonica,* Greece, *Parlatoria camelliae*

Parlatoria camelliae Comstock (Hemiptera: Coccomorpha: Diaspididae) is recorded for the first time in Greece on *Camellia japonica* L. (Theaceae: Ericales) from northwestern area of Karyes, Holy Mount Athos (40°18′ N, 24°12′ E), on 12 August 2024, in Halkidiki. The confirmation of the species *P. camelliae* was made by Professor Giuseppina Pellizzari (Dipartimento di Agronomia, Animali, Alimenti), University of Padua, Italy. Vouchers of permanent slides of the scale insect are kept in the Laboratory of Agricultural Entomology and Zoology, Department of Agriculture, School of Agriculture and Food, University of the Peloponnese.

Genus *Parlatoria* Targioni Tozzetti, includes 80 species (García Morales *et al.,* 2016). Four species of *Parlatoria* are recorded in Greece: *P. oleae* (Colvée) (Koroneos, 1934), *P. pergandii* Comstock (Koroneos, 1964), *P. theae* Cockerell (García Morales *et al.,* 2016) and *P. ziziphi* (Lucas) (Koroneos, 1934; Normark *et al.,* 2019).

Parlatoria camelliae has been recorded in several countries of north and south America, Africa, Asia and Australia (García Morales *et al.,* 2016). In Europe it has been recorded in France (Foldi, 2001), Georgia (on *Camellia japonica, C. sasanqua* (Theaceae) and *Prunus laurocerasus* (Rosaceae)) (Miller and Davidson, 2005; Batsankalashvili *et al.*, 2017), Italy (Longo *et al.,* 1995), Portugal (Nakahara, 1982) and Spain (Morrison, 1939).

In Chalkidiki, *P. camelliae* was found in high infestation density on a twenty year old *C. japonica* plant (Fig. 1). The scale was mainly settled on the upper surface of the leaves (Fig. 2). The examination of samples of the infested leaves in the laboratory showed that the species is biparental and oviparous. The majority of the live individuals on the samples were ovipositing female adults (Fig. 3). However, preovipositing female adults and crawlers were also recorded. The female adults of the scale were used for the identification of the species (Figs 4 and 5). In addition, scales with an exit hole of unidentified endoparasite individuals were recorded.

Little information is provided in bibliography on the biology and economic importance of *P. camelliae*. Miller and Davidson (2005) referred that males and females were found on the top and underside of camellia leaves, close to the veins. McKenzie (1956) reported *P. camelliae* as the most important scale insect pest of camellias in California whereas Miller and Davidson (1990) considered this species to be an occasional pest.

[1] Laboratory of Agricultural Entomology and Zoology,
[2] Laboratory of Floriculture and Landscape Architecture, Department of Agriculture, School of Agriculture and Food, University of the Peloponnese, GR-241 00 Kalamata, Greece.
[3] Institute of Pesticides and Environmental Protection, SE-11080 Belgrade, Serbia.
* Corresponding author: g.stathas@uop.gr

Figure 1. *Camellia japonica* infested by *Parlatoria camelliae*.

Figure 3. Eggs laid by ovipositing female of *Parlatoria camelliae* (↓).

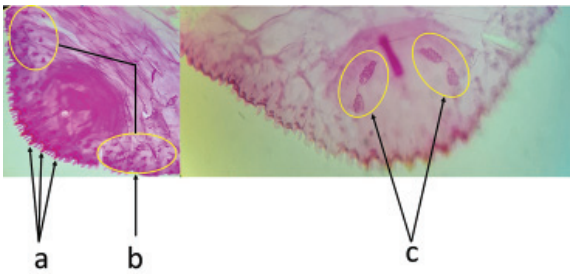

Figure 5. Pygidiun of *Parlatoria camelliae* female adult: (a): median (L1), second (L2) and third lobe (L3), (b): microducts, (c): perivulval pores.

The senior author expresses his gratitude to Professor Giuseppina Pellizzari (Dipartimento di Agronomia, Animali, Alimenti), University of Padua, Italy, for the confirmation of Parlatoria camelliae.

Figure 2. Colony of *Parlatoria camelliae* on leaf of *Camellia japonica*.

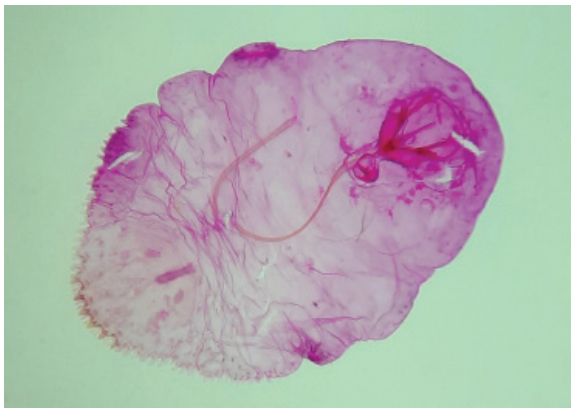

Figure 4. Body of female adult of *Parlatoria camelliae*.

Literature cited

Batsankalashvili, M., Kaydan, M.B., Kirkitadze, G. and Japoshvili, G.O. 2017. Updated checklist of scale insects (Hemiptera: Coccomorpha) in Sakartvelo (Georgia). *Annals of Agrarian Science,* 15: 252-268.

Foldi, I. 2001. Liste des cochenilles de France (Hemiptera, Coccoidea). [List of scale insects of France (Hemiptera, Coccoidea).]. *Bulletin de la Société Entomologique de France,* 106(3): 303-308.

García Morales, M., Denno, B.D., Miller, D.R., Miller, G.L., Ben-Dov, Y. and Hardy, N.B. 2016. *ScaleNet: A literature-based model of scale insect biology and systematics*. Retrieved September 27, 2024, from http://scalenet.info.

Koroneos, J. 1934. *Les Coccidae de la Grèce surtout du Pélion (Thessalie). I. Diaspinae.* Athens, 95 pp, 77 tables.

Longo, S., Marotta, S., Pellizzari, G., Russo, A. and Tranfaglia, A. 1995. An annotated list of the

scale insects (Homoptera: Coccoidea) of Italy. *Israel Journal of Entomology,* 29: 113-130.

McKenzie, H.L. 1956. The armored scale insects of California. *Bulletin of the California Insect Survey,* 5: 1-209.

Miller, D.R. and Davidson, J.A. 2005. *Armored Scale Insect Pests of Trees and Shrubs*. Cornell Univ. Press Ithaca, NY 442 pp.

Miller, D.R. and Davidson, J.A. 1990. A List of the Armored Scale Insect Pests. *Armored Scale Insects, Their Biology, Natural Enemies and Control* [Series title: World Crop Pests, Vol. 4B]. Elsevier Amsterdam, the Netherlands, 688 pp. 299-306.

Morrison, H. 1939. Taxonomy of some scale insects of the genus *Parlatoria* encountered in plant quarantine inspection work. United States Department of Agriculture, *Miscellaneous Publications*, 344: 1-34.

Nakahara, S. 1982. Checklist of the armored scales (Homoptera: Diaspididae) of the conterminous United States. United States Department of Agriculture, *Animal and Plant Health Inspection Service,* 110 pp.

Received: 28 September 2024; Accepted: 8 December 2024

ΣΥΝΤΟΜΗ ΑΝΑΚΟΙΝΩΣΗ

Πρώτη καταγραφή του κοκκοειδούς εντόμου *Parlatoria camelliae* Comstock (Hemiptera: Coccomorpha: Diaspididae) στην Ελλάδα

Γ.Ι. Σταθάς, Ε.Δ. Κάρτσωνας, Π. Σκούρας και M. Dervišević

Περίληψη Το κοκκοειδές έντομο *Parlatoria camelliae* Comstock (Hemiptera: Coccomorpha: Diaspididae) καταγράφεται για πρώτη φορά στην Ελλάδα στις 12 Αυγούστου 2024. Το έντομο βρέθηκε επί του φυτού *Camellia japonica* L. (Theaceae: Ericales) στην Χαλκιδική. Από προκαταρκτικές μελέτες διαπιστώθηκε ότι το είδος *P. camelliae* είναι ωοτόκο και αμφιγονικό.

Hellenic Plant Protection Journal **18:** 10-12, 2025

sciendo

Hellenic Plant Protection Journal **18:** 13-16, 2025
DOI 10.2478/hppj-2025-0003

SHORT COMMUNICATION

First record of *Maconellicoccus hirsutus* (Green) (Hemiptera: Pseudococcidae) on *Albizia julibrissin* - Honeybee foraging on mealybug honeydew

A.E. Tsagkarakis[1], M.B. Kaydan[2,3], G.J. Stathas[4] and G. Gastouniotis[1]

Summary In August 2024, the pink hibiscus mealybug, *Maconellicoccus hirsutus* (Green) (Hemiptera: Coccomorpha: Pseudococcidae), was found in heavy infestations on *Albizia julibrissin* Durazz. (Fabaceae) and *Grevillea robusta* A. Cunn. (Proteaceae) in Athens, Greece. This is the first global record of *A. julibrissin* and the first record on *G. robusta* in Greece, as hosts of *M. hirsutus*. All developmental stages: eggs, nymphs, and adult males and females, were present. Honeybees (*Apis mellifera* L.) were observed actively foraging on the abundant honeydew secreted by the mealybugs. Further research is necessary to assess the potential impact of this invasive pest on host plants in Greece, and the significance of its honeydew as an alternative food resource for honeybees.

Additional keywords: Albizia julibrissin, *Apis mellifera,* climate change, *Grevillea robusta,* honeydew, *Maconellicoccus hirsutus*

Maconellicoccus hirsutus (Green) (Hemiptera: Coccomorpha: Pseudococcidae) was first recorded in Greece on *Hibiscus rosa-sinensis* (Linnaeus) (Malvaceae) on the island of Rhodes in 2014. Subsequent records in 2016 included *Hibiscus* sp., *Ceratonia siliqua*, *Erythrina* sp. and *Bauhinia* sp. (Fabaceae) (Milonas and Partsinevelos, 2017).

The present study reports the first global record of the ornamental plant *Albizia julibrissin* Durazz. (Fabaceae) and the first Greek record of *Grevillea robusta* A. Cunn. (Proteaceae) as host plants for *M. hirsutus*. Both records were documented in Alimos, Attiki (37°55'36 N, 23°44'37 E) in August 2024 (Figs 1 and 2). The plants were heavily infested. Samples of the infested shoots were transferred to the laboratory for examination. All developmental stages of the scale were recorded: eggs covered by waxy ovisac, nymphs and male and female adults. Female adults were collected and microscopic permanent slides were made according to the method of Kosztarab and Kozár (1988). The identification of the species *M. hirsutus* was made by Professor Mehmet Bora Kaydan (Biotechnology Development and Research Centre, Adana), Çukurova University, Turkey. The vouchers of the permanent slides are kept in the Biotechnology Development and Research Centre of Çukurova University and in the Laboratory of Agricultural Entomology and Zoology, Department of Agriculture, University of the Peloponnese.

The genus *Maconellicoccus* Ezzat (Hemiptera: Coccomorpha: Pseudococcidae includes nine species. Only the species *M. hirsutus,* which is native to southern Asia, is recorded in Greece. *Maconellicoccus hirsutus* is a highly polyphagous species infesting plants of 258 Genera, belonging to 84 Families. It is distributed in 110 countries worldwide (García Morales *et al.,* 2016) while in Europe it is recorded in Greece and Cyprus

[1] Laboratory of Sericulture and Apiculture, Agricultural University of Athens, GR-118 55 Athens, Greece.
[2] Biotechnology Application and Research Centre, Çukurova University, 01250, Adana, Türkiye.
[3] Çukurova University, Imamoğlu Vocational School, 01330, Balcalı, Adana, Türkiye.
[4] Department of Agriculture, University of the Peloponnese, GR-241 00 Kalamata, Greece.
* Corresponding author: atsagarakis@aua.gr

Figure 1. Infestation of *Albizia julibrissin* by *Maconellicoccus hirsutus.*

Figure 2. Infestation of *Grevillea robusta* by *Maconellicoccus hirsutus.*

(Ülgentürk and Hocaali, 2019). Moreover, other records from the Mediterranean basin include the countries Algeria (EFSA PLH Panel, 2022), Egypt (Williams, 1986), Israel (Spodek *et al*., 2018), Tunisia (Halima-Kamel *et al*., 2015) and Turkey (EFSA, 2021).

The first global record of *M. hirsutus* on *A. julibrissin* and the first Greek record on *Grevillea robusta* expand the pest's known host range and geographic distribution. Georgopoulou *et al.* (2025) highlights the pest's wide host range and its ability to thrive in diverse climates; the climate change scenario SSP5-8.5 projects further range expansion of *M. hirsutus* into areas of southern Europe, including Spain, Italy, Greece, and the Mediterranean coastline, with increased climatic suitability for persistent populations. This aligns with our current findings, and suggests that urban and peri-urban landscapes, with their mix of ornamental and agricultural plants, could serve as critical zones for further establishment and spread. According to EFSA PLH Panel (2022), *M. hirsutus* meets the criteria that are within the remit of EFSA to assess for it to be regarded as a potential Union quarantine pest.

Al-Fwaeer *et al.* (2014) reported that *M. hirsutus* completes one generation in warm period of the year in Jordan. Regarding the host plants, damage and economic importance of *M. hirsutus,* Charlet (1997) referred that the mealybug invaded the US Virgin Islands, Puerto Rico, St. Kitts, Trinidad, Tobago, Grenada and several other Caribbean islands, where it was damaging coffee, cotton, soybean, citrus and some other crops. According to the same study, the mealybug feeds on soft plant tissues and injects a toxic saliva that distorts leaves and buds, causing curling, twisting, and a bushy appearance at shoot tips. Infestations also lead to shortened internodes, and heavy honeydew excretion, which fosters black sooty mold and can stunt, particularly seedlings and weakened trees. The pest occurs on stems, leaves, buds, fruit, and even underground parts (e.g., roots, pods, and pegs in peanuts), reducing yields and compromising plant vigor. In cotton, it infests growing parts, producing bunchy growth and fewer, smaller bolls, while in grapevines, severely infested clusters may shrivel and drop (Williams, 1996).

Natural enemies of *M. hirsutus* include 72 species belonging to 22 Insect Families of parasitoids and predators (mainly Hymenoptera, Coleoptera, Neuroptera, Diptera, Lepidoptera, Hemiptera, Thysanoptera), the entomopathogenic fungi *Beauveria bassiana* and the nematode *Heterorhabditis amazonensis*. (García Morales *et al.*, 2016). The coccinellid predator *Cryptolaemus montrouzieri* Mulsant and the parasitoid wasp *Anagyrus kamali* Moursi are recommended for biological control of the mealybug (Etienne *et al.*, 1998).

A noteworthy observation on *A. julibrissin* and *G. robusta* was the presence of abundant honeydew secreted by *M. hirsutus*. This honeydew attracted honeybees, offering an alternative food source during periods of scarce floral resources (Fig. 3). In the insight of climatic crisis, the potential impact of the

Figure 3. Honeybees feeding on honeydew secretions of *Maconellicoccus hirsutus.*

pest on host crops in Greece should be investigated, as well as the role of the mealybug's honeydew in apiculture.

Literature Cited

Al-Fwaeer, M., Abu-Obaid, I., Al-Zyoud, F., Abo-Alosh, A. and Halaybeh, M. 2014. Population Dynamics of the Hibiscus Mealybug *Maconellicoccus hirsutus* Green (Hom., Pseudococcidae) and Its Parasitoid on Guava Trees in Madaba-Jordan. *International Journal of Agriculture and Forestry*, 4(3): 171-177.

Charlet, L. 1997. Pink Hibiscus mealybug. *IOBC Newsletter,* 66: 6-7.

EFSA PLH Panel (EFSA Panel on Plant Health), Bragard, C., Baptista, P., Chatzivassiliou, E., Di Serio, F., Gonthier, P., Jaques Miret, J.A., Justesen, A.F., Magnusson, C.S., Milonas, P., Navas-Cortes, J.A., Parnell, S., Potting, R., Reignault, P.L., Stefani, E., Thulke, H.-H., Van der Werf, W., Vicent Civera, A., Yuen, J., Zappalà, L., Gregoire, J.-C., Malumphy, C., Antonatos, S., Kertesz, V., Maiorano, A., Papachristos, D. and MacLeod, A. 2022. Scientific Opinion on the pest categorisation of *Maconellicoccus hirsutus*. *EFSA Journal,* 20(1): 7024, 45 pp. https://doi.org/10.2903/j.efsa.2022.7024.

Étienne, J., Matile-Ferrero, D., Leblanc, F. and Marival, D. 1998 Premier signalment de la cochenille *Maconellicoccus hirsutus* (Green) en Guadeloupe; situation actuelle de ce ravageur des cultures dans les Antilles françaises (Hem., Pseudococcidae). *Bulletin de la Société Entomologique de France,* 103: 173-174.

García Morales, M., Denno, B.D., Miller, D.R., Miller, G.L., Ben-Dov, Y. and Hardy, N.B. 2016. *ScaleNet: A literature-based model of scale insect biology and systematics*. Retrieved November 28, 2024, from http://scalenet.info.

Georgopoulou, I.A., Papachristos, D.P., Milonas, P.G., Alkafy Baldi, S. and Kriticos, D.J. 2025. Climate change impacts on the potential global distribution of *Maconellicoccus hirsutus*. *Biological Invasions,* 27: 21. https://doi.org/10.1007/s10530-024-03460-w.

Halima-Kamel, M.B., Germain, J.-F. and Mdellel, L. 2015. First records of two mealybugs, *Maconellicoccus hirsutus* (Green) and *Phenacoccus peruvianus* Granara de Willink, in Tunisia and the North of Africa. *Bulletin OEPP/EPPO,* 45(1): 139–143.

Kosztarab, M. and Kozár, F. 1988. *Scale insects of central Europe. Boletin del Museo de Entomologia de la Universidad del Valle Akademiai Kiado*. Budapest. 456 p.

Milonas, P.G. and Partsinevelos, G.K. 2017. The pink hibiscus mealybug *Maconellicoccus hirsutus* (Green) (Hemiptera: Pseudococcidae) in Greece. *Hellenic Plant Protection Journal,* 10: 80-83.

Spodek, M., Ben-Dov, Y., Mondaca, L., Protasov, A., Erel, E. and Mendel, Z. 2018. The cotton mealybug, *Phenacoccus solenopsis* Tinsley (Hemiptera: Pseudococcidae) in Israel: pest status, host plants and natural enemies. *Phytoparasitica*. https://doi.org/10.1007/s12600-018-0642-1.

Ülgentürk, S. and Hocaali, S.Ş. 2019 Pest Status of *Dactylopius opuntiae* (Cockerell) (Hemiptera: Dactylopiidae) and New Records of Scale Insects from Northern Turkish Republic of Cyprus. *Munis Entomology and Zoology*, 14(1): 294-300.

Williams, D.J. 1986. The identity and distribution of the genus *Maconellicoccus* Ezzat (Hemiptera: Pseudococcidae) in Africa. *Bulletin of Entomological Research,* 76: 351-357.

Williams, D.J. 1996. A brief account of the hibiscus mealybug *Maconellicoccus hirsutus* (Hemiptera: Pseudococcidae), a pest of agriculture and horticulture, with descriptions of two related species from southern Asia. *Bulletin of Entomological Research,* 86: 617-628.

Received: 14 December 2024; Accepted: 5 January 2025

ΣΥΝΤΟΜΗ ΑΝΑΚΟΙΝΩΣΗ

Πρώτη καταγραφή του ψευδόκοκκου *Maconellicoccus hirsutus* (Green) (Hemiptera: Coccomorpha: Pseudococcidae) στο καλλωπιστικό φυτό *Albizia julibrissin* - Διατροφή μελισσών στις μελιτώδεις εκκρίσεις

Α.Ε. Τσαγκαράκης, M.B. Kaydan, Γ.Ι. Σταθάς και Γ. Γαστουνιώτης

Περίληψη Ο ψευδόκοκκος *Maconellicoccus hirsutus* (Green) (Hemiptera: Coccomorpha: Pseudococcidae) βρέθηκε τον Αύγουστο του 2024 σε μεγάλους πληθυσμούς στα καλλωπιστικά φυτά *Albizia julibrissin* και *Grevillea robusta* στην Αθήνα. Αυτή είναι η πρώτη παγκόσμια καταγραφή του *A. julibrissin* και η πρώτη καταγραφή του *G. robusta* στην Ελλάδα, ως ξενιστές του *M. hirsutus*. Κατά τις δειγματοληψίες, βρέθηκαν όλα τα στάδια ανάπτυξης του εντόμου: ωά, νύμφες και άρρενα και θήλεα ακμαία. Επί των αποικιών του εντόμου παρατηρήθηκε παρουσία μελισσών (*Apis mellifera* L.) που διατρέφονταν στις άφθονες μελιτώδεις εκκρίσεις του ψευδόκοκκου. Περαιτέρω έρευνα απαιτείται για την αξιολόγηση της πιθανής επίδρασης αυτού του χωροκατακτητικού εντόμου στα φυτά ξενιστές της χώρας μας και της σημασίας του μελιτώματός του ως εναλλακτικού πόρου τροφής για τις μέλισσες.

Hellenic Plant Protection Journal **18:** 13-16, 2025

sciendo

Hellenic Plant Protection Journal **18:** 17-39, 2025
DOI 10.2478/hppj-2025-0004

Determination of nitroimidazole and fumagillin residues in honey employing Liquid Chromatography-Tandem Mass Spectrometry: An insight in the 2023-2024 Greek honey production

E. Manea-Karga[1], F. Kanteli[1], E.A. Papadopoulou[1,2]*, K. Machera[1] and K.M. Kasiotis[1]*

Summary Honey bees (*Apis* sp.) are vital to ecosystems, enhancing agricultural productivity and preserving biodiversity. However, the observed decline of their populations, caused by, among other, Nosema infections has led to the use of the antibiotics fumagillin and nitroimidazoles. Nonetheless, due to increasing concerns over their genotoxic, mutagenic, and carcinogenic properties, the presence of fumagillin residues in honey is prohibited, while similar is the case for the parent nitroimidazoles and their metabolites in animal-derived food. Within this context and considering the limited availability of pertinent data on Greek honey, we have developed and applied robust analytical methods for the detection of fumagillin and nitroimidazole residues in honey, employing liquid chromatography-tandem mass spectrometry (LC-MS/MS). The developed protocols, based on solid phase extraction, proved fit for the purpose of detecting fumagillin and nitroimidazoles in honey samples with substantial sensitivity (detection capability, CCβ not exceeding 0.78 µg/kg), comparable to recent literature, and could be applied in routine analyses to ensure consumers' safety. Application of the methods in 30 Greek honey samples did not unveil residues above the CCβ of the analytes, and the developed pipeline can be further exploited in future large-scale monitoring studies to investigate Greek apiculture and its adherence to regulatory obligations.

Additional keywords: Dimetridazole, LC-MS/MS, Metronidazole, Ronidazole, Solid Phase Extraction, Veterinary antibiotics

Introduction

Honey bees is a subset of bees in the genus *Apis* that are indispensable to ecosystems and agriculture, mainly due to their exceptional pollination abilities, which not only enhance agricultural productivity and yield, but also safeguard biodiversity conservation (Galajda *et al.*, 2021). Nonetheless, over the last two decades, a decline in honey bee populations has been observed worldwide, a multifaceted issue driven by several biotic and abiotic factors, including, among others, the reduction of their natural habitats, climate change, exposure to pesticides, as well as various diseases caused by pests and pathogens (Goulson *et al.*, 2015).

Microsporidian parasites of the genus *Nosema*, and more specifically the species *Nosema apis*, *Nosema ceranae*, and the recently detected *Nosema neumanni*, cause one of the most devastating honey bee diseases, nosemosis (Mazur and Gajda, 2022). These parasites infect the gut epithelial cells of adult bees causing symptoms such as dysentery, reduced lifespan, and decreased foraging efficiency. Infected bees often show signs of lethargy, poor coordination, and inability to return to their hive. The disease can result in colony collapse, diminished honey production, and increased winter mortality (Grupe and Quandt, 2020; Hristov *et al.*, 2020). Prevention and treatment of nosemosis include regular monitoring and a combination of hive management practices

[1] Laboratory of Pesticides' Toxicology, Scientific Directorate of Pesticides' Control & Phytopharmacy, Benaki Phytopathological Institute, GR-145 61 Kifissia, Greece.
[2] Laboratory of Pesticide Science, Department of Crop Science, Agricultural University of Athens, GR-118 55 Athens, Greece.
* Corresponding authors: k.kasiotis@bpi.gr
a.papadopoulou@bpi.gr

and chemical treatment, with the most used antibiotics being fumagillin and nitroimidazoles (NIs) (Formato *et al*., 2022).

Fumagillin is a mycotoxin initially reported in 1949 by Hanson and Eble, who isolated it from the filamentous fungus *Aspergillus fumigatus* (Hanson and Eble, 1949). It is a meroterpenoid of low molecular weight (458.54 g·mol^{-1}), with poor water solubility, whereas it is soluble in organic solvents (Guruceaga *et al*., 2019). In the early 1950s, not long after its discovery, fumagillin was recognized as a bioactive compound against *Nosema* infections and has been used extensively since (Peirson and Pernal, 2024; Stanimirovic *et al*., 2007; van den Heever *et al*., 2015a). Its mode-of-action involves its binding to the active site of the methionine aminopeptidase type 2 (MetAP-2) protein through the epoxide located on its cyclohexane moiety (Van den Heever et al., 2014). Since the MetAP-2 enzyme plays a key role in protein synthesis, this inhibition disrupts essential cellular processes, leading to impaired growth and replication of the *Nosema* spores within the honey bees' guts (Guruceaga *et al*., 2019).

On the other hand, NIs is a group of antibiotics discovered in the early 1950s when researchers isolated azomycin, a 2-nitroimidazole compound, from a *Streptomyces* extract (Nakamura, 1955). This discovery spurred the synthesis of an array of derivatives with remarkable bioactivity, mainly against anaerobic bacteria, protozoa, and parasites (Ang *et al.,* 2017). Their chemical structure is characterized by the presence of an imidazole ring with a nitro group attached to it in different positions (Guo *et al*., 2017). Synthetic 5-nitroimidazole (5-NI) derivatives, such as dimetridazole, metronidazole, ronidazole, and ipronidazole, have been extensively exploited in the prevention and management of nosemosis in honey bees (Zhou *et al*., 2007; Mitrowska *et al*., 2014). These metabolites seem to exert their antimicrobial properties due to the presence of the nitro group in their molecule, which generates reactive nitro radical anions, inhibiting microbial nucleic acid synthesis and leading to cell death (Ang *et al*., 2017).

Despite the effectiveness of fumagillin and NIs in combating Nosema disease in honey bees, the potential presence of their residues in honey can pose a potential risk to consumer health. Fumagillin is believed to be genotoxic, which can be a threat to both beekeepers and consumers of honey-containing residues (Stanimirovic *et al*., 2007), and therefore, it is no longer authorized in most European Union (EU) member states (European Commission 2009; Kunat-Budzyńska *et al*., 2022). Since no maximum residue limit (MRL) has been established for honey, any detectable fumagillin residue levels are prohibited, adhering to a zero-tolerance policy (Nozal *et al*., 2008). Likewise, NIs are considered mutagenic and carcinogenic to humans and thus, they are classified at the group A6 of prohibited substances (European Commission 1996). Consequently, their use in food-producing animals is not allowed in the EU under Regulation 2377/90, depicted also for dimetridazole, metronidazole, ronidazole and other NIs in EU Regulation 37/2010 (European Commission 2010). Also, metabolites formed by hydroxylation of the main NIs (e.g., hydroxy metronidazole and hydroxy ipronidazole) could display similar mutagenic properties and, as a result, EU Reference Laboratory's recommended concentration for analytical methods to determine parent NIs and their metabolites in animal-derived food is suggested at 3 μg·kg-1 (CRL 2007).

Based on the abovementioned, there is an increasing need for the generation of analytical data on contaminants in honey due to its broad consumption and acknowledgment of health benefits. In this context, the development and application of sensitive analytical methods for the detection of a wide range of levels of NIs and fumagillin in honey, for the discovery of any illegal uses that may occur, is of high importance. Such a task requires the development and validation of sophisticated sample preparation and analytical protocols. Within this context, and focusing on consumers' safe-

ty, here, enhanced experimental protocols and robust analytical methods were developed for the detection of fumagillin and NI residues in honey based on Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) and Solid Phase Extraction (SPE). The methods were applied to Greek honey samples collected during the period 2023-2024. Greek honey has exceptional quality with pronounced biological properties (Patouna *et al*., 2023), which is attributed to the unique plant diversity of the country and respective chemical constituents (Karabagias *et al*., 2014; Kasiotis *et al*., 2022). Therefore, the prevalence of these compounds in this valuable food commodity should be contemplated. To our knowledge, there is a shortage of data on monitoring the presence of such chemicals in Greek apicultural products.

Materials and methods

1. Chemicals and Reagents

All chemicals and reagents used in the sample preparation and analysis were of the highest commercially available grade. More specifically, the organic solvents, methanol (MeOH) and acetonitrile (ACN) (purity ≥ 99.9%) were purchased from Fisher Chemical (Thermo Fisher Scientific, Waltham, MA, USA). Ultrapure water was obtained by an Ultra-Clear® TWF UV ultra-clear water system (Evoqua Water Technologies, Pittsburgh, PA, USA). Acetic acid (purity ≥99.9%) was purchased from Merck (Merck KGaA, Darmstadt, Germany), and formic acid (purity ≥99.9%) from Honeywell Research Chemicals (Morris Plains, NJ, USA). Magnesium sulfate and sodium chloride were obtained from Fisher Chemical (Thermo Fisher Scientific, Waltham, MA, USA). The ammonium hydroxide solution that was used in the sample preparation for nitroimidazole analysis was prepared by diluting the desired amount of ammonia (Merck KGaA, Darmstadt, Germany) in ultrapure water.

For method development and analyses, the analytical standards fumagillin (purity 93%, Toronto Research Chemicals, North York, ON, Canada), metronidazole (MNZ) and dimetridazole (DMZ) (purity ≥ 98.0%, Sigma-Aldrich Solutions, St. Louis, MO, USA), ronidazole (RNZ) (purity ≥ 95%, Sigma-Aldrich Solutions, St. Louis, MO, USA), ipronidazole (IPZ) (purity 99.5%, Dr. Ehrenstorfer Reference Materials, LGC Standards, Teddington, Middlesex, UK), hydroxymetronidazole (MNZ-OH) (purity ≥ 99.0 %, Sigma-Aldrich Solutions, St. Louis, MO, USA), hydroxydimetridazole (DMZ-OH) (purity 98%), hydroxyipronidazole (IPZ-OH) (purity 99.14%), and ternidazole hydrochloride (TRZ) (purity 86.0%) (Dr. Ehrenstorfer Reference Materials, LGC Standards, Teddington, Middlesex, UK), were used. Ipronidazole D3 (IPZ-D3) (purity 99.47%) (Dr. Ehrenstorfer Reference Materials, LGC Standards) served as the internal standard for the LC-MS/MS analysis of NIs.

2. Preparation of standard solutions

Stock solutions were prepared using the analytical standards listed in section 2.2, in ACN for fumagillin and in MeOH for NIs, which were stored at –20° C until further use. Appropriate dilutions of the stock solutions of the analytical standards were performed to obtain the corresponding working solutions for experimentation. For the construction of calibration curves, solutions of the analytical standards were prepared to final concentrations ranging from 0.5 to 250 ng/mL in ACN for fumagillin, and MeOH for NIs.

3. Honey sample preparation

The developed experimental protocols for the detection of fumagillin and NIs in honey samples were based on Solid Phase Extraction (SPE), applying minor modifications to previously published protocols (Dmitrovic and Durden, 2013; Mitrowska and Antczak, 2017; Li *et al.,* 2018), such as the use of mixed-mode SPE (but not in dispersion), the incorporation of salts in the extraction step for NIs, and the Biotage VacMaster 20 device (Biotage, Uppsala, Sweden).

For fumagillin, initially, a portion of hon-

ey (5 g) was weighed in 50-mL falcon tubes and 10 mL of ultrapure water were added. Samples were then extracted using an automatic axial extractor (Agytax®, AgytaxLab, Madrid, Spain) for 10 min, taking advantage of the magnetic-axial movement under controlled conditions, and centrifuged (Hermle Z446K, Hermle Labortechnik GmbH, Wehingen, Germany) for 15 min at 4500 rpm, at ambient temperature. SPE was performed employing Phenomenex Strata-X cartridges (60 mg, 3 mL) (Phenomenex, Torrance, CA, USA), which were conditioned with 5 mL MeOH and 5 mL ultrapure water prior to sample loading. Afterwards, the cartridges were washed with 10 mL of a water-MeOH mixture (60:40, v/v), and were dried under vacuum. The elution step was conducted using 2 portions of 2 mL of ACN, and the solutions were evaporated to dryness in a rotary evaporator (Heidolph WB 2001, Schwbach, Germany). Dried samples were reconstituted in 1 mL ACN and filtered (13 mm, 0.22 μm, Membrane Solutions, Seattle, Shanghai) into 2 mL amber glass autosampler vials (Macherey-Nagel, Düren, Germany). All handling was performed under a low light regime to avoid potential fumagillin degradation.

For NIs, 2 g of honey were weighed in 50-mL falcon tubes and the internal standard (IS) ipronidazole D3 was added. Samples were then extracted with 15 mL of a 2% (v/v) acetic acid solution in an ultrasonic bath (Isolab Laborgeräte GmbH, Eschau, Germany) for 10 min. Afterwards, 0.5 g of magnesium sulfate and sodium chloride (4:1 w/w) were added to the sample, the mixture was hand-shaken for 1 min, placed again for 2 min in an ultrasonic bath (Grant Instruments, Shepreth, Cambridgeshire, UK), and the resulting extract was centrifuged for 5 min at 4000 rpm (Hettich Universal 32 R Centrifuge, Tuttlingen, Germany) with constant temperature at 10°C. Then, the supernatant was passed through Oasis MCX (Mixed Mode Cation Exchange, for interaction with MNZ see Figure 1, and for similar interaction Dugheri *et al*., 2017) cartridges (6 mL) (Waters Corporation, Milford, MA, USA). The cartridges had been previously conditioned with 5 mL MeOH followed by 5 mL of the 2% acetic acid solution. The same solvent system in reverse order was used for cartridge washing. Elution was performed with 5 mL of an ACN-ammonium hydroxide (25% v/v) mixture in a 95:5 v/v ratio, and evaporation followed in a SpeedVac vacuum concentrator (HyperVac VC2124, Gyrozen, Gimpo, South Korea). Reconstitution was performed with 0.5 mL of the following system: 0.1% (v/v) aqueous solution of formic acid: 0.1% (v/v) ACN solution of formic acid. The reconstituted solutions were filtered into inserts (Macherey-Nagel, Düren, Germany) and placed into glass autosampler vials (2 mL).

4. Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) analysis

For method development and honey sample analysis for the detection of fumagillin and NIs, two instruments were employed, with the key parameters for their LC-MS/MS analyses being demonstrated in Table 1. Regarding fumagillin analysis, elution was performed in a mobile phase (A):(B) 30:70 (v/v) ratio, whereas for NIs, the following gradient elution was applied: 5% of the mobile phase B was held for 1 min, increased to 40% at 1 min and held for 4 min. Then, linearly decreased within 0.5 min to initial conditions and held for 10 min for equilibration of pump pressure. The precursor and productions of the analytes are presented in Ta-

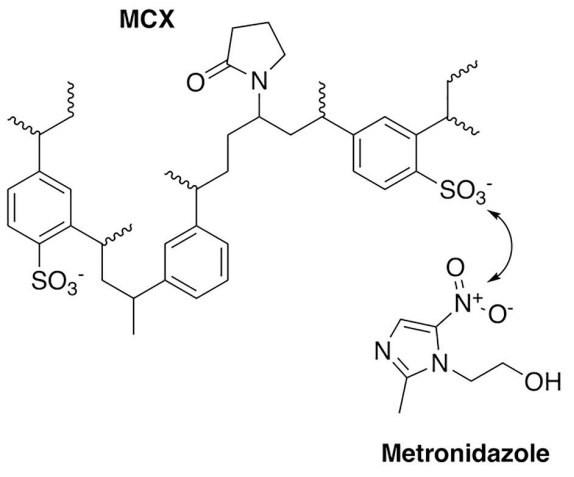

Figure 1. Metronidazole interacting with MCX sorbent.

Table 1. LC-MS/MS equipment and parameters employed for fumagillin and nitroimidazole analysis (AF: Ammonium formate, FA: Formic acid, MRM: Multiple Reaction Monitoring, ESI^+: positive Electron Spray Ionization).

Instrument & Parameters \ Analyte	Fumagillin	Nintroimidazoles
Instrument	Agilent Technologies 6410 Triple Quad LC/MS system	Shimadzu 80/60NX Triple Quad LC-MS/MS system
Column Type	Agilent reversed phase Zorbax Eclipse XDB-C18	reversed phase Kinetex
Column Dimension	150 mm x 2.7 mm, 3.5 μm	150 mm x 2.1 mm, 2.6 μm
Column Temperature	30°C	35°C
Injection volume	10 μL	10 μL
Flow rate	0.3 $mL{\cdot}min^{-1}$	0.25 $mL{\cdot}min^{-1}$
Mobile phase composition	(A) H2O 2mM AF (B) ACN: H2O 90:10 (v/v) 2mM AF	(A) H2O 0.1% FA (B) ACN0.1% FA
Elution mode	Isocratic	Gradient
MS/MS parameters	MRM mode ESI^+	MRM mode ESI+
Nebulizer and collision gas	Nitrogen	Nitrogen
Data acquisition	Agilent Mass Hunter data acquisition Triple Quad B.01.04	LabSolutions LCMS software v. 4.52
Data processing	Agilent MassHunter Workstation Qualitative Analysis B.01.04	LabSolutions LCMS software v. 4.52

bles 2 and 3, for NIs and fumagillin, respectively. In Figure 2, MRM chromatograms of fumagillin in control and fortified control samples, along with fumagillin standards, that were analyzed for method validation purposes, are displayed. In Figure 3, the elution order of NIs is displayed (in Figure S1, indicative MRM chromatograms for DMZ and IPZ are presented).

5. Analytical Method Validation

Analytical methods' validation is a key element that determines their robustness and validity. Here, the two chromatographic analytical methods were validated according to Commission Decision 2002/657/EC (Commission-Decision-2002/657/EC, 2002) and based on the guidelines for screening methods for veterinary medicines (CRLs-20/1/2010, 2010), which complements the aforementioned EC Decision. A previous work of our group dealing with the determination of antibiotics in honey was also advised for the establishment and determination of respective validation criteria (Kasiotis *et al.,* 2023). The latter were linearity, specificity, matrix effect (ME), decision limits (CCα), detection capabilities (CCβ), limit of quantitation (LOQ), precision, trueness and uncertainty. CCα and CCβ were calculated using the calibration plots metrics in agreement with suggestions by ISO 11843-2 (ISO-11843-2, 2000), considering also the work by Van Loco and colleagues (Van Loco *et al.*, 2007). Linearity was assessed in an overall range of 0.5-250 μg/L, incorporating eight calibration levels using standard solutions of all analytes and corresponding matrix-matched extracts prepared from blank honey. Trueness was studied by performing recovery experiments for all substances at three concentration levels utilizing twenty replicates at CCβ, and six replicates for two additional levels (3×CCβ, 10×CCβ). Precision was determined by evaluating repeatability

Table 2. Retention time and mass spectrometer parameters for nitroimidazoles (MNZ; Metronidazole, RNZ; Ronidazole, DMZ; Dimetridazole, IPZ; Ipronidazole, IPZ-D3; Ipronidazole D3; MNZ-OH; Hydroxymetronidazole, DMZ-OH; Hydroxydimetridazole, IPZ-OH; Hydroxyipronidazole, TRZ; Ternidazole).

Analyte	Retention time (min)	Precursor Ion (m/z)	Product Ion (m/z)*	Q1 Pre Bias (V)	CE (eV)	Q3 Pre Bias (V)	Ratio**
MNZ	5.92	171.9	**82.2**	22	24	15	0.95
			128.2	19	15	13	
RNZ	6.54	200.8	**140.1**	22	12	24	0.24
			98.05	10	17	21	
DMZ	6.04	141.9	81.1	16	27	14	0.51
			96.1	16	17	17	
IPZ-D3	8.48	169.9	124.2	21	20	23	0.59
			109.2	20	24	21	
IPZ	8.43	172.9	**127.3**	20	21	24	
MNZ-OH	4.46	187.9	126.2	20	25	23	0.63
			123.2	22	17	29	
DMZ-OH	6.56	157.9	**140.2**	17	13	12	0.07
			55.3	19	16	27	
IPZ-OH	7.3	186	**168.2**	17	19	22	0.67
			122.2	12	14	17	
TRZ	5.87	185.9	**128.2**	12	21	22	0.58
			82.2	14	15	23	
				10	26	14	

*in bold quantitation ion, **(area qualification ion/area quantitation ion)

Table 3. Retention time and mass spectrometer parameters for fumagillin.

Analyte	Retention time (min)	Precursor Ion (m/z)	Product Ion (m/z)*	Fragmentor voltage (V)	CE (eV)	Collision Cell Accelerator Voltage (V)	Ratio**
Fumagillin	1.82	459.2	**177**	50	-12	4	0.54
			131	50	-33	4	

*in bold quantitation ion, **(area qualification ion/area quantitation ion)

(intra-day) and reproducibility (inter-day) at the same concentration levels (with an identical number of replicates) of the trueness study and expressed through the % relative standard deviation (RSD). ME was extrapolated by juxtaposing the slope of standard and matrix-matched calibration curves. Methods' expanded measurement uncertainty (U') was determined following the approach and equations described in the SANTE document (SANTE, 2021) and in the recently published work of our group (Kasiotis *et al.*, 2023), encompassing recovery data as the main driver of uncertainty.

6. Honey samples

The developed experimental and analytical protocols for the determination of

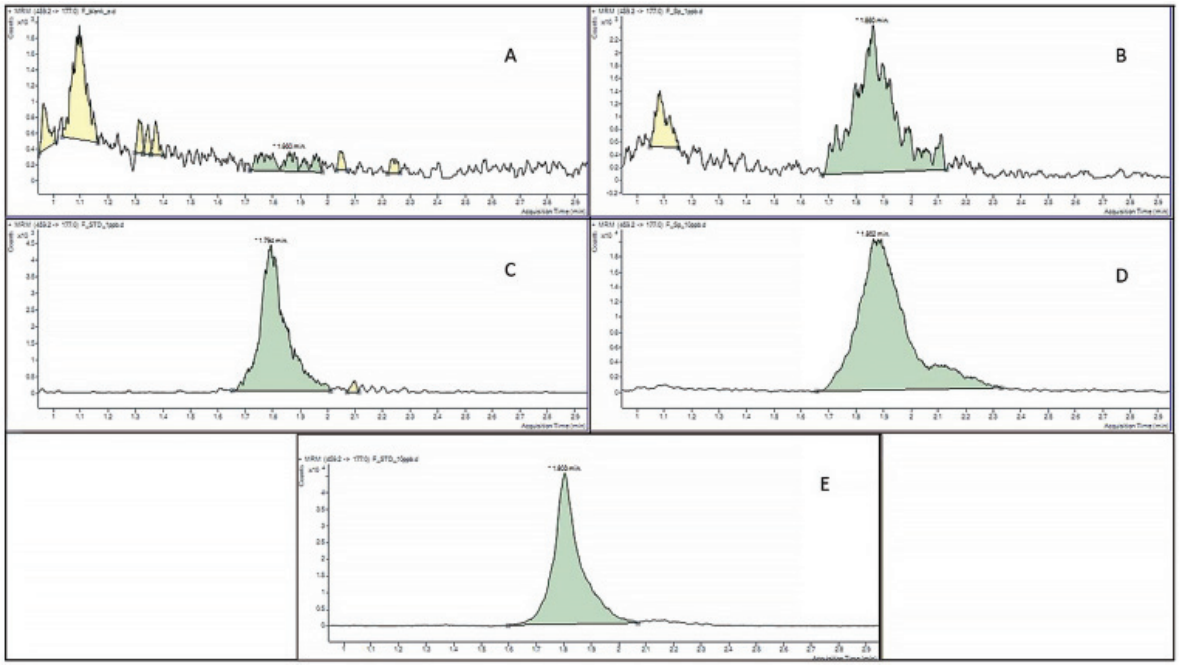

Figure 2. Multiple Reaction Monitoring (MRM) chromatograms (quantitation transition) of Fumagillin in A: control sample, B: Fortified control sample at 1 µg/kg, C: Standard solution (STD) 1 µg/L, D: Fortified sample at 10 µg/kg and E: STD 10 µg/L.

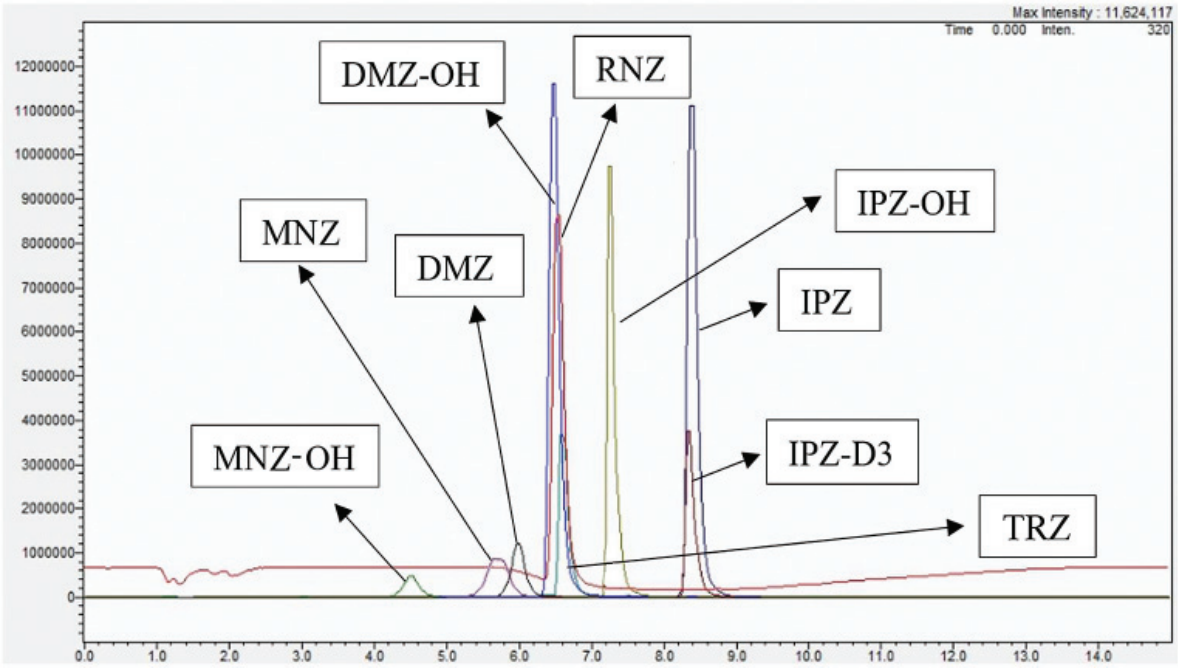

Figure 3. TIC MRM chromatogram of a standard mix solution at 100 µg/L showing the elution order of NIs (MNZ; Metronidazole, RNZ; Ronidazole, DMZ; Dimetridazole, IPZ; Ipronidazole, IPZ-D3; Ipronidazole D3; MNZ-OH; Hydroxymetronidazole, DMZ-OH; Hydroxydimetridazole, IPZ-OH; Hydroxyipronidazole, TRZ; Ternidazole).

fumagillin and NI residues in honey were applied, as a proof-of-concept, to honey samples, collected and sent to Benaki Phytopathological Institute, Laboratory of Pesticides' Toxicology by public authorities and individual beekeepers. In total, thirty honey samples of different floral origins, consistencies, and colors, were collected from

different beehives from various Greek prefectures (Attica, Evia and Aegean islands, Macedonia, Central Greece, Crete, and Peloponnese) (Table 4) during the period 2023-2024, to include as much variability as possible in terms of geographical distribution (yet, it was out of the scope of the work to proceed to melissopalynological analysis for exact pollen identification and botanical source attribution). The samples were stored in boxes following the completion of sampling to avoid light-induced fumagillin degradation, and they were immediately cooled at 0-4°C using ice packs. When received at the laboratory, the samples were stored at –20°C until further processing. For the validation of the analytical methods, blank monofloral honey samples from the repository of the laboratory (routine samples) were used.

Results and Discussion

The introduction of salts in the extraction (partitioning) step and mixed-mode SPE (two modes of interaction, see also discussion below) in the sample preparation step proved efficient in recovering NIs from honey (recoveries depicted in Table 4) exploiting, in this way, the amphiphilic properties of NIs, and leading to slightly cleaner extracts (preliminary tests not shown, demonstrated this behavior compared to the sole use of MCX sorbent). The decision was also based on the intention to expand the scope of the method to other veterinary substances with different physicochemical properties than NIs that would benefit also from this combinatorial sample preparation. Calibration curves of both standards and matrix-matched solutions demonstrated very good linearity for each analyte in honey (0.5-250 μg/L) with a correlation coefficient above 0.9996. The analytical methods' specificity was evaluated by analysing a series (more than 20) of commercially available honey samples. The absence of major interferences in the retention time of the target compounds corroborated the high specificity of the method. The recoveries (as a measure of precision) were found to vary between 76.5% and 113.1% for all the analytes. The reproducibility and repeatability RSD% values of both methods were investigated at three concentration levels (CCβ, 3 × CCβ, 10 × CCβ) and were, in all cases, acceptable (≤20%). For NIs and ME, a negligible to slight suppression of the overall signal intensity was corroborated only when the IS was regarded, fluctuating from -0.8 to -7.8%, which is in line with the findings of Li et al. (2018). For fumagillin, a slight enhancement of ME was evidenced (ME, 8.2%). Lastly, the U' of the analytical methods was evaluated mainly from data of the precision (recovery) investigation (Table 5). For the three concentrations tested U' did not exceed 27.2% (for fumagillin was <16.9%).

Table 4. Geographical origin of honey samples.

No.	Origin	Number of samples
1	Attica	3
2	Macedonia	10
3	Crete	5
4	Peloponnese	4
5	Evia and Aegean islands	2
6	Central Greece	6

With regard to the sensitivity of the herein-developed methods, it was fit for purpose, considering both the pertinent literature (in terms of analytical method performance and actual findings) and the recommended concentration for NIs at 3 μg/kg (CRL 2007). Nevertheless, as mentioned in previous work of our group, particular attention should be given to the way of calculating these metrics (Kasiotis *et al.,* 2023). The values of CCα for NIs following the implementation of our protocol ranged between 0.17 and 0.54 μg/kg and those of CCβ were calculated in the range of 0.30 to 0.78 μg/kg for all NIs being analyzed (Table 5). In the same context, the LOQs for all NIs and fumagillin were established at 1 μg/kg, after spiking blank honey samples (n=5) at this concentration, considering the accept-

Table 5. Validation results for nitroimidazoles (MNZ; Metronidazole, RNZ; Ronidazole, DMZ; Dimetridazole, IPZ; Ipronidazole, MNZ-OH; Hydroxymetronidazole, DMZ-OH; Hydroxydimetridazole, IPZ-OH; Hydroxyipronidazole, TRZ; Ternidazole) and fumagillin determined in honey samples – decision limit (CCα) and detection capability (CCβ) values, recoveries (%), intra and inter-day (RSD%).

Analyte	CC*α* (µg/kg)	CC*β* (µg/kg)	Linear range (µg/L)	Recovery (%)			Intra-day RSD (%)			Inter-day RSD (%)		
				CC*β*	3 × CC*β*	10 × CC*β*	CC*β*	3 × CC*β*	10 × CC*β*	CC*β*	3 × CC*β*	10 × CC*β*
MNZ	0.44	0.63	0.5-250	89.2	93.7	99.2	8	3	6	10	8	7
RNZ	0.18	0.39		76.5	82.1	87.5	13	9	7	12	13	10
DMZ	0.37	0.56		88.4	93.7	101.9	6	7	2	10	8	5
IPZ	0.17	0.30		94.9	102.4	113.1	9	9	5	9	12	7
IPZ-OH	0.18	0.32		79.3	83.3	85.8	13	11	7	10	11	11
MNZ-OH	0.54	0.78		84.3	92.5	103.8	12	7	4	15	10	8
DMZ-OH	0.16	0.30		80.7	85.4	97.3	7	5	5	6	7	10
TRZ	0.34	0.51		97.6	95.9	108.4	11	9	6	13	10	9
Fumagillin	0.45	0.71		86.2	93.3	97.4	8	7	4	8	7	6

able precision (recovery in the range of 85.2 to 100.2%) and trueness of the method (RSD%<12.3). The latter was deemed necessary to avoid any confusion between CC*β* and LOQ values, since based on definition CC*β* is the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of *β*. Following thorough research of the literature on the NI analysis in honey, these values lay between previously reported ones employing various sample preparation protocols and analytical platforms (Table 6) (Zhou *et al*., 2007; Cronly *et al*., 2010; Huang *et al*., 2011; Sakamoto *et al*., 2011; Kanda *et al*., 2012; Tölgyesi *et al*., 2012; Mitrowska *et al.,* 2014; Galarini *et al*., 2015; Shendy *et al*., 2016; Guo *et al*., 2017; Mitrowska and Antczak, 2017; Lei *et al*., 2018; Guo *et al*., 2021; Liu *et al*., 2022; Melekhin *et al*., 2024). Overall, the herein-developed method was improved in terms of the abovementioned values compared to the ones published by Cronly (Cronly *et al*., 2010), Guo (Guo *et al*., 2021), Huang (Huang *et al*., 2011), Lei (Lei *et al.,* 2018), Liu (Liu *et al*., 2022), Shendy (Shendy *et al.,* 2016), and Zhou (Zhou *et al.,* 2007) and their associates. Similarly, the performance of the presented method was similar to those of Galarini (Galarini *et al.,* 2015), Guo (Guo *et al*., 2017), Melekhin (Melekhin *et al*., 2024), and Mitrowska (Mitrowska *et al*., 2014; Mitrowska and Antczak, 2017) and their teammates. The only exceptions were the methods of Li and coworkers (Li *et al.,* 2018), Kanda (Kanda *et al*., 2012), Sakamoto (Sakamoto *et al.,* 2011), and Tölgyesi (Tölgyesi *et al.,* 2012), which displayed superior performance.

More specifically, the comparison with the literature disclosed comparable performance with the work by Melekhin and colleagues, who applied an ultrasonic-assisted derivatization – magnetic solid-phase extraction protocol for the determination of NIs and other antibiotics using LC-MS/MS (Melekhin *et al.,* 2024). The achieved LOQs were in the range of 0.3 to 1 µg/kg, therefore, comparable to the ones presented here. In another multiresidue LC-MS/MS-based method, developed by Galarini and fellows (Galarini *et al*., 2015) for the determination of 27 antibiotics in honey, the range in which CC*α* and CC*β* values fluctuated was relatively broader and, while detection of most NIs reached similar to our work levels, for some NIs those values were higher (Tables S1-S8). In other key works the LOQs for

Table 6. Comparison of the developed method with other analytical methods for the determination of nitroimidazoles in honey published in the literature.

Sample preparation	Analyzer	Linear range (µg/L)	Recovery (%)	RSD (%)	CCα (µg/kg)	CCβ (µg/kg)*	LOQ (µg/kg)	Reference
ACN extraction	LC-MS/MS	0-20	94.7-108.9	3.5-12.4	0.38-1.16	0.66-1.98	ND	(Cronly *et al.*, 2010)
acidic hydrolysis & SPE	LC-MS/MS	0.1-10	84.8-122	6.2-23	0.15-1.70	0.21-2.50	ND	(Galarini *et al.*, 2015)
SPE (MIPs)[a]	HPLC-MS/MS	1–500	79.7–110	8.2-13.2	ND	0.10-0.50	1.00	(Guo *et al.*, 2017)
SPE (T-POPs)[b]	HPLC-UV	1.5-1200	81.2-117	1-10	ND	0.50-1.50	1.50-4.50	(Guo *et al.*, 2021)
SBSE[c]	HPLC-DAD	2-200	71.1–114	1.4-9.1	ND	0.47–1.52	1.54–5.00	(Huang *et al.*, 2011)
QuEChERS & SPE (alumina-N)	LC-MS/MS	0.005–5	76.1-98.5	2.8-14.2	ND	0.03-0.15	0.10-0.50	(Kanda *et al.,* 2012)
QuEChERS	LC-MS/MS	1–100	81-115.6	0.7-5.8	ND	0.64-1.58	2.13-5.27	(Lei *et al.*, 2018)
d-SPE (MCX)	LC-MS/MS	0-500	90.2-105.6	4.3-11.2	ND	ND	0.05-0.2	(Li *et al.*, 2018)
SPE (MDP-HCP)[d]	HPLC-UV	1-200	93-111.3	1.9-7.1	ND	0.30-0.60	1.00-2.00	(Liu *et al.*, 2022)
UAD–MSPE[e]	LC-MS/MS	0.3-200	97-118	4-16	ND	ND	0.30-1.00	(Melekhin *et al.*, 2024)
SPE (SCX)[f]	LC-MS/MS	0.5-3000	98-104.6	5.1-9.5	0.50	ND	ND	(Mitrowska & Antczak, 2017)
SPE (MIPs)[a]	LC-MS/MS	0-10	96-105.9	1-10.1	0.110-0.387	0.179-0.508	ND	(Mitrowska *et al.*, 2014)
SPE (silica)	LC-MS/MS	0.5-100	91.2-100.7	2.6-17.1	ND	0.05-0.20	ND	(Sakamoto *et al.,* 2011)
QuEChERS	LC-MS/MS	0.25-10	90.96-97	1.51-12.58	0.53–0.74	0.91–1.27	ND	(Shendy *et al.,* 2016)
SPE (SDB)[g]	LC-MS/MS	0-9	55.6-86.8	8.6-26.4	0.05-0.10	0.08-0.15	0.17-0.33	(Tölgyesi *et al.,* 2012)
SPE (amine)	LC-UV	10-500	71.5–101.4	2.4-17.2	ND	1.00-2.00	3.00-6.00	(Zhou *et al.* 2007)
SPE (MCX)[h]	LC-MS/MS	0.5-250	76.5-113.1	3-13	0.17-0.54	0.30-0.78	1.00	This work

a: molecularly imprinted polymers
b: triazine-based porous organic polymers
c: stir bar sorptive extraction
d: m-phenylenediamine hypercrosslinked polymer
e: ultrasonic-assisted derivatization-magnetic solid-phase extraction
f: strong cation exchange
g: styrene-divinylbenzene
h: mixed-mode cation exchange
ND: not determined
*can be equivalent to limit of detection (LOD)

NIs varied from 0.1 to 0.5 μg/kg (Kanda *et al.*, 2012), and 0.05-0.2 μg/kg (Li *et al.*, 2018).

Interestingly, the work of Mitrowska and Antczak (2017), on which our experimental protocol was largely based, revealed similar to this study CC*α* values (0.5 μg/kg for all NIs), although the respective values for some of the NIs of our study were somewhat lower (Table 6). Minor modifications in Mitrowska and Antczak sample preparation protocol included the replacement of SCX (Strong Cation Exchange) SPE cartridges with the mixed-mode MCX ones, used effectively by Li and coworkers (2018). In our attempts, the clogging phenomena (Li *et al.,* 2018) were not so extensive to direct us to work in dispersive mode, yet this observation might be attributed to the type of honey analyzed in this work (both blank and actual samples). MCX cartridges combine both cation exchange and hydrophobic interactions, providing a comprehensive retention mechanism for NIs, which exhibit both hydrophilic and hydrophobic properties (see also Figure 1). This dual interaction mode could enhance selectivity, ensuring that NIs are retained, while minimizing the co-extraction of unwanted matrix components, such as sugars and proteins commonly found in honey. In this way, it is plausible to assume that MCX cartridges could handle the complex matrix of honey more effectively, resulting in cleaner extracts and higher recovery rates.

For fumagillin, the comparison of the presented findings with the work by Dmitrovic and Durden (2013) showed an analogous performance of CC*α*, CC*β* and LOQ. Nevertheless, when compared to the LOQ established in the work by Kanda and co-workers (Kanda *et al.*, 2011) the herein LOQ is one order of magnitude higher, yet not computed the same way. An elaborate stability study was not conducted since it is described in the literature for both fumagillin (van den Heever *et al.*, 2015b) and NIs (Mitrowksa *et al.*, 2014) and conditions contemplated in the presented study. Lastly, the runtime of both methods is relatively short, ensuring their applicability, especially for routine laboratories.

Overall, combining the results of our study with those of the literature, we can think about the use of SPE in sample preparation, together with the employment of an LC-MS/MS system, as the method of choice for the analysis of NIs and fumagillin in honey. Nonetheless, further experimentation can be considered to additionally improve the CC*α* and CC*β* values for some of the analytes. Moreover, after validating the analytical methods, analysis of thirty Greek honey samples of different floral and geographical origins, consistencies, and colors for NIs and metabolites and fumagillin showed that they were devoid of NIs or fumagillin residues. Considering the substantial sensitivity of the methods and the diversity of the analyzed samples, the study provides a first insight into the potential use of these chemicals in association with beekeeping practices in Greece, which seem to comply with the zero tolerance regulation regime regarding the application of such substances. In the same context, considering the approval of metronidazole for use in companion animals (EMA, 2019) the absence of residues in this work might also imply that improper use of veterinary medicines is not embraced by Greek beekeepers. The impact of such findings is particularly significant, especially considering similar studies conducted in other countries, such as those applied in Albanian (Pasho *et al.*, 2024) and Egyptian (Rabea *et al.*, 2024) honey, which have reported NI residues above EU standards. While the current evidence may not be sufficient to draw definite conclusions for the whole Greek apiculture sector, it provides a valuable first overview, which could be considered in future large-scale monitoring studies.

Conclusions

In this work, the first attempt for the determination of eight nitroimidazoles and fumagillin in Greek honey samples is reported, which, regardless of the banning of these chemicals in some countries, are still used by beekeepers on a global basis to control

honey bee diseases. Both analysis methods and protocols were optimized, validated, and successfully applied to a variety of honey samples (different flower origins, different geographical areas, different consistencies, and colors). The validation results and the relatively short analysis time propose that these methods are suitable for routine analysis. The actual samples analyzed were devoid of residues of all compounds investigated herein. Next steps, regard the incorporation of additional veterinary substances in the scope of the methods, and the consideration of analyzing honey samples with high-resolution mass spectrometry instrumentation to unveil additional related molecules.

Supplementary Material

Figure S1. MRM chromatograms of standard solutions of DMZ (A) and IPZ (B) at 100 μg/L.

Table S1. Comparison of the developed method with other analytical methods for the determination of metronidazole in honey published in the literature.

Table S2. Comparison of the developed method with other analytical methods for the determination of ronidazole in honey published in the literature.

Table S3. Comparison of the developed method with other analytical methods for the determination of dimetridazole in honey published in the literature.

Table S4. Comparison of the developed method with other analytical methods for the determination of ipronidazole in honey published in the literature.

Table S5. Comparison of the developed method with other analytical methods for the determination of hydroxymetronidazole in honey published in the literature.

Table S6. Comparison of the developed method with other analytical methods for the determination of hydroxydimetridazole in honey published in the literature.

Table S7. Comparison of the developed method with other analytical methods for the determination of hydroxyipronidazole in honey published in the literature.

Table S8. Comparison of the developed method with other analytical methods for the determination of ternidazole in honey published in the literature.

Conflicts of Interest

The authors declare no conflict of interest.

The authors express their gratitude to the veterinary authorities of Greece for providing the honey samples and to the National Residue Control Program (ΕΠΕΚ, honey sampling was conducted within its frame) funded by the Greek Ministry of Rural Development and Food.

Literature cited

Ang, C.W., Jarrad, A.M., Cooper, M.A. and Blaskovich, M.A. 2017. Nitroimidazoles: molecular fireworks that combat a broad spectrum of infectious diseases. *Journal of Medicinal Chemistry*, 60(18): 7636-7657.

Commission-Decision-2002/657/EC. 2002. Implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Official Journal of the European Union*, L221/8: 8-36.

CRL. 2007. CRLs view on state-of-the-art analytical methods for the national residues plans for control of residues. Sanco. p. 3228. [cited 2016 Aug 27]. Available from: http://www.rivm.nl/bibliotheek/digitaaldepot/crlguidance2007.pdf.

CRLs-20/1/2010. 2010. Community Reference Laboratories Residues: Guidelines For The Validation Of Screening Methods For Residues Of Veterinary Medicines (Initial Validation And Transfer).

Cronly, M., Behan, P., Foley, B., Malone, E., Martin, S., Doyle, M. and Regan, L. 2010. Rapid multi-class multi-residue method for the confirmation of chloramphenicol and eleven nitroimidazoles in milk and honey by liquid chromatography-tandem mass spectrometry (LC-MS). *Food Additives and Contaminants*, 27(9): 1233-1246.

Dmitrovic, J. and Durden, D.A. 2013. Analysis of fumagillin in honey by LC-MS/MS. *Journal of AOAC International*, 96(3): 687-695.

Dugheri, S., Bonari, A., Pompilio, I., Colpo, M., Mucci, N., Montalti, M. and Arvangeli, G. 2017. Development of an Innovative Gas Chromatography–Mass Spectrometry Method for Assessment of Formaldehyde in the Workplace Atmosphere. *Acta Chromatographica*, 29(4):1-4.

EMA. 2019. EMA/CVMP/CHMP/682198/2017. European Medicines Agency, Categorisation of antibiotics in the European Union.

European Commission. 1996. Council directive

96/23/EC. *Official Journal of the European Union,* L125:10–32.

European Commission. 2009. Commission Regulation No 470/2009 of the European Parliament and of the Council of 6 May 2009 laying down Community procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin, repealing Council Regulation (EEC) No. 2377/90 and amending Directive 2001/82/EC of the European Parliament and of the Council and Regulation (EC) No. 726/2004 of the European Parliament and of the Council. *Official Journal of the European Union,* L152: 11–22.

European Commission. 2010. Commission regulation EU No 37/2010. *Official Journal of the European Union,* L15:1–72.

Formato, G., Rivera-Gomis, J., Bubnic, J., Martín-Hernández, R., Milito, M., Croppi, S. and Higes, M. 2022. Nosemosis prevention and control. *Applied Sciences*, 12(2): 783.

Galajda, R., Valenčáková, A., Sučik, M. and Kandráčová, P. 2021. Nosema disease of European honey bees. *Journal of Fungi*, 7(9): 714.

Galarini, R., Saluti, G., Giusepponi, D., Rossi, R. and Moretti, S. 2015. Multiclass determination of 27 antibiotics in honey. *Food Control*, 48: 12-24.

Goulson, D., Nicholls, E., Botías, C. and Rotheray, E.L. 2015. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science*, 347(6229): 1255957.

Grupe, A.C. and Quandt, C.A. 2020. A growing pandemic: A review of *Nosema* parasites in globally distributed domesticated and native bees. *PLoS pathogens*, 16(6): e1008580.

Guo, X.-C., Xia, Z.-Y., Wang, H.-H., Kang, W.-Y., Lin, L.-M., Cao, W.-Q., Zhang, H.-W. and Zhou, W.-H. 2017. Molecularly imprinted solid phase extraction method for simultaneous determination of seven nitroimidazoles from honey by HPLC-MS/MS. *Talanta*, 166: 101-108.

Guo, Y., Wang, J., Hao, L., Wu, Q., Wang, C. and Wang, Z. 2021. Triazine-triphenylphosphine based porous organic polymer as sorbent for solid phase extraction of nitroimidazoles from honey and water. *Journal of Chromatography A*, 1649: 462238.

Guruceaga, X., Perez-Cuesta, U., Abad-Diaz de Cerio, A., Gonzalez, O., Alonso, R.M., Hernando, F. L., Ramirez-Garcia, A. and Rementeria, A. 2019. Fumagillin, a mycotoxin of *Aspergillus fumigatus*: biosynthesis, biological activities, detection, and applications. *Toxins*, 12(1): 7.

Hanson, F.R. and Eble, T.E. 1949. An antiphage agent isolated from *Aspergillus* sp. *Journal of Bacteriology*, 58(4): 527-529.

Hristov, P., Shumkova, R., Palova, N. and Neov, B. 2020. Factors associated with honey bee colony losses: A mini-review. *Veterinary Sciences*, 7(4): 166.

Huang, X., Lin, J. and Yuan, D. 2011. Simple and sensitive determination of nitroimidazole residues in honey using stir bar sorptive extraction with mixed mode monolith followed by liquid chromatography. *Journal of Separation Science*, 34(16-17): 2138-2144.

ISO-11843-2, Capability of detection—Part 2: Methodology in the linear calibration case, (2000).

Kanda, M., Sasamoto, T., Takeba, K., Hayashi, H., Kusano, T., Matsushima, Y., Nakajima, T., Kanai, S. and Takano, I. 2011. Rapid determination of fumagillin residues in honey by liquid chromatography/tandem mass spectrometry using the QuEChERS method. *Journal of AOAC International*, 94(3): 878-885.

Kanda, M., Sasamoto, T., Takeba, K., Hayashi, H., Kusano, T., Matsushima, Y., Nakajima, T., Kanai, S. and Takano, I. 2012. Rapid determination of nitroimidazole residues in honey by liquid chromatography/tandem mass spectrometry. *Journal of AOAC International*, 95(3): 923-931.

Karabagias, I.K., Badeka, A.V., Kontakos, S., Karabournioti, S. and Kontominas, M.G. 2014. Botanical discrimination of Greek unifloral honeys with physico-chemical and chemometric analyses. *Food Chemistry*, 165: 181-190.

Kasiotis, K.M., Baira, E., Iosifidou, S., Bergele, K., Manea-Karga, E., Theologidis, I., Barmpouni, T., Tsipi, D. and Machera, K. 2022. Characterization of Ikaria heather honey by Untargeted Ultrahigh-Performance Liquid Chromatography-High Resolution Mass Spectrometry Metabolomics and melissopalynological analysis. *Frontiers in Chemistry*, 10:924881.

Kasiotis, K.M., Manea-Karga, E., Tzanetou, E.N., Barmpouni, T., Liapatas, G. and Machera, K. 2023. Introducing direct probe electrospray ionization tandem mass spectrometry in apiculture: Comparison with other mass spectrometric methods for the determination of antibiotics in bees and honey. *International Journal of Mass Spectrometry*, 489: 117064.

Kunat-Budzyńska, M., Budzyński, M., Schulz, M., Strachecka, A., Gancarz, M., Rusinek, R. and Ptaszyńska, A.A. 2022. Natural substances, probiotics, and synthetic agents in the treatment and prevention of honeybee nosemosis. *Pathogens*, 11(11): 1269.

Lei, H., Guo, J., Lv, Z., Zhu, X., Xue, X., Wu, L. and Cao, W. 2018. Simultaneous determination of nitroimidazoles and quinolones in honey by modified QuEChERS and LC-MS/MS analysis. *International Journal of Analytical Chemistry*, 2018(1): 4271385.

Li, X., Ke, Y., Wang, Y., Wang, C., Ye, D., Hu, X., Zhou, L., Xia, X. 2018. Confirmatory analysis of nitroimidazoles and hydroxy metabolites in honey by Dispersive-Solid Phase Extraction and Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry. *Molecules*, 23:3350.

Liu, Z., Wang, J., Guo, Y., Liu, J., Wang, J., Wang, C., Wu, Q. and Wang, Z. 2022. Amino-functionalized hypercrosslinked polymer as sorbent for effective extraction of nitroimidazoles from water, drink and honey samples. *Journal of Chromatography A*, 1676: 463206.

Mazur, E.D. and Gajda, A.M. 2022. Nosemosis in honeybees: a review guide on biology and diagnostic methods. *Applied Sciences*, 12(12): 5890.

Melekhin, A., Tolmacheva, V., Goncharov, N., Apyari, V., Parfenov, M. Y., Bulkatov, D., Dmitrienko, S. and Zolotov, Y.A. 2024. Rapid multi-residue LC-MS/MS determination of nitrofuran metabolites, nitroimidazoles, amphenicols, and quinolones in honey with ultrasonic-assisted derivatization–magnetic solid-phase extraction. *Journal of Pharmaceutical and Biomedical Analysis*, 237: 115764.

Mitrowska, K. and Antczak, M. 2017. Transfer of nitroimidazoles from contaminated beeswax to honey. *Food Additives & Contaminants: Part A*, 34(4): 573-581.

Mitrowska, K., Posyniak, A. and Zmudzki, J. 2014. Selective determination of fourteen nitroimidazoles in honey by high-performance liquid chromatography–tandem mass spectrometry. *Analytical Letters*, 47(10): 1634-1649.

Nakamura, S. 1955. Structure of azomycin, a new antibiotic. *Pharmaceutical Bulletin*, 3(5): 379-383.

Nozal, M.J., Bernal, J. L., Martín, M.T., Bernal, J., Alvaro, A., Martín, R. and Higes, M. 2008. Trace analysis of fumagillin in honey by liquid chromatography-diode array–electrospray ionization mass spectrometry. *Journal of Chromatography A*, 1190(1-2): 224-231.

Pasho, I., Vaso, K., Dardha, L., Marku, E. and Hoxha, V. 2024. Detection and analysis of nitroimidazole residues in Albanian honey: Implications for food safety and regulatory oversight. In *Proceedings of the 4th International Conference on Innovative Academic Studies (ICIAS)*, Konya, Turkey 12-13 March 2024, p. 1391-1401.

Patouna, A., Vardakas, P., Skaperda, Z., Spandidos, D.A. and Kouretas, D. 2023. Evaluation of the antioxidant potency of Greek honey from the Taygetos and Pindos mountains using a combination of cellular and molecular methods. *Molecular Medicine Reports*, *27*, 54. https://doi.org/10.3892/mmr.2023.12941.

Peirson, M. and Pernal, S.F. 2024. A Systematic review of fumagillin field trials for the treatment of Nosema disease in honeybee colonies. *Insects,* 15(1): 29.

Rabea, D., Ryad, L., Shehata, M.R. and Khalaf-Alla, P.A. 2024. Nitroimidazole residues in Egyptian honey using UPLC-Orbitrap-HRMS. *Food Additives and Contaminants: Part B*, 1-13.

Sakamoto, M., Takeba, K., Sasamoto, T., Kusano, T., Hayashi, H., Kanai, S., Kanda, M. and Nagayama, T. 2011. Determination of dimetridazole, metronidazole and ronidazole in salmon and honey by liquid chromatography coupled with tandem mass spectrometry. *Journal of the Food Hygienic Society of Japan,* 52(1): 51-58.

SANTE/11312/2021. 2021. Guidance document on analytical quality control and method validation procedures for pesticides residues analysis in food and feed.

Shendy, A.H., Al-Ghobashy, M.A., Alla, S.A.G. and Lotfy, H.M. 2016. Development and validation of a modified QuEChERS protocol coupled to LC–MS/MS for simultaneous determination of multi-class antibiotic residues in honey. *Food Chemistry,* 190: 982-989.

Stanimirovic, Z., Stevanovic, J., Bajic, V. and Radovic, I. 2007. Evaluation of genotoxic effects of fumagillin by cytogenetic tests *in vivo. Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 628(1): 1-10.

Tölgyesi, Á., Sharma, V.K., Fekete, S., Fekete, J., Simon, A. and Farkas, S. 2012. Development of a rapid method for the determination and confirmation of nitroimidazoles in six matrices by fast liquid chromatography–tandem mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis,* 64: 40-48.

Van den Heever, J.P., Thompson, T.S., Curtis, J.M., Ibrahim, A. and Pernal, S.F. 2014. Fumagillin: an overview of recent scientific advances and their significance for apiculture. *Journal of Agricultural and Food chemistry,* 62(13): 2728-2737.

Van den Heever, J.P., Thompson, T.S., Curtis, J.M. and Pernal, S.F. 2015a. Determination of dicyclohexylamine and fumagillin in honey by LC-MS/MS. *Food Analytical Methods,* 8: 767-777.

Van den Heever, J.P., Thompson, T.S., Curtis, J.M. and Pernal, S.F. 2015b. Stability of dicyclohexylamine and fumagillin in honey. *Food Chemistry,* 179: 152-158.

Van Loco, J., Jànosi, A., Impens, S., Fraselle, S., Cornet, V. and Degroodt, J.M. 2007. Calculation of the decision limit (CC*α*) and the detection capability (CC*β*) for banned substances: The imperfect marriage between the quantitative and the qualitative criteria. *Analytica Chimica Acta,* 586: 8-12.

Zhou, J., Shen, J., Xue, X., Zhao, J., Li, Y., Zhang, J. and Zhang, S. 2007. Simultaneous determination of nitroimidazole residues in honey samples by high-performance liquid chromatography with ultraviolet detection. *Journal of AOAC International,* 90(3): 872-878.

Received: 24 July 2024; Accepted: 15 January 2025

Προσδιορισμός υπολειμμάτων νιτροϊμιδαζολών και φουμαγγιλίνης στο μέλι με χρήση υγρής χρωματογραφίας συζευγμένης με φασματομετρία μάζας τριπλού τετράπολου (LC-MS/MS): Εφαρμογή σε δείγματα ελληνικού μελιού παραγωγής 2023-2024

Η. Μανέα-Κάργα, Φ. Καντέλη, Ε.Α. Παπαδοπούλου, Κ. Μαχαίρα και Κ.Μ. Κασιώτης

Περίληψη Οι μέλισσες (*Apis* sp.) αποτελούν αναπόσπαστο κομμάτι του οικοσυστήματος, ενισχύοντας τη γεωργική παραγωγή και διασφαλίζοντας τη διατήρηση της βιοποικιλότητας. Παρόλα αυτά, η παρατηρούμενη μείωση του πληθυσμού τους, η οποία οφείλεται, μεταξύ άλλων, σε λοιμώξεις από μικροσπορίδια του γένους *Nosema*, οδήγησε στη χρήση αντιβιοτικών όπως της φουμαγγιλίνης και των νιτροϊμιδαζολών. Ωστόσο, εξαιτίας των ολοένα αυξανόμενων στοιχείων για τις γονοτοξικές, μεταλλαξιογόνες και καρκινογόνες ιδιότητές τους, η παρουσία υπολειμμάτων φουμαγγιλίνης στο μέλι απαγορεύεται, ενώ παρόμοια είναι η κατάσταση και για τα μητρικά μόρια νιτροϊμιδαζολών αλλά και για τους μεταβολίτες τους σε τρόφιμα ζωικής προέλευσης. Στο πλαίσιο αυτό, και λαμβάνοντας υπόψη την περιορισμένη διαθεσιμότητα των σχετικών δεδομένων για το ελληνικό μέλι, αναπτύχθηκαν και εφαρμόστηκαν προηγμένες αναλυτικές μέθοδοι για την ανίχνευση υπολειμμάτων φουμαγγιλίνης και νιτροϊμιδαζολών στο μέλι, με εφαρμογή υγρής χρωματογραφίας συζευγμένης με φασματομετρία μάζας τριπλού τετράπολου (LC-MS/MS). Τα πρωτόκολλα βασίστηκαν στην εκχύλιση στερεής φάσης και αποδείχθηκαν κατάλληλα για την ανίχνευση φουμαγγιλίνης και νιτροϊμιδαζολών σε δείγματα μελιού, επιδεικνύοντας υψηλή ευαισθησία (ικανότητα ανίχνευσης, CCβ που δεν υπερέβη τα 0.78 μg/kg), συγκρίσιμη με την αντίστοιχη πρόσφατων μελετών της διεθνούς βιβλιογραφίας, και θα μπορούσαν να εφαρμοστούν σε αναλύσεις ρουτίνας για τη διασφάλιση της ασφάλειας των καταναλωτών. Οι μέθοδοι που αναπτύχθηκαν εφαρμόστηκαν σε 30 δείγματα ελληνικού μελιού, στα οποία δεν ανιχνεύθηκαν υπολείμματα που υπερβαίνουν την τιμή CCβ των αναλυτών, και η μεθοδολογία που αναπτύχθηκε μπορεί να αξιοποιηθεί περαιτέρω σε μελλοντικές μελέτες παρακολούθησης μεγάλης κλίμακας, για τη διερεύνηση του ελληνικού μελισσοκομικού τομέα σχετικά με την τήρηση των κανονιστικών ρυθμίσεων.

Hellenic Plant Protection Journal **18:** 17-39, 2025

Supplementary material

Figure S1. MRM chromatograms of standard solutions of DMZ (A) and IPZ (B) at 100 µg/L.

Table S1. Comparison of the developed method with other analytical methods for the determination of metronidazole in honey published in the literature.

Sample preparation	Analyzer	Linear range (µg/L)	Recovery (%)	RSD (%)	CC*α* (µg/kg)	CC*β* (µg/kg)*	LOQ (µg/kg)	Reference
ACN extraction	LC-MS/MS	0-20	108.9	6.7	0.72	1.22	ND	(Cronly *et al.,* 2010)
acidic hydrolysis & SPE	LC-MS/MS	0.1-10	84.8-105	6.9-16	0.43	0.52	ND	(Galarini *et al.,* 2015)
SPE (MIPs)[a]	HPLC-MS/MS	1–500	90.2-99.4	8.60-9	ND	0.50	1.00	(Guo *et al.,* 2017)
SPE (T-POPs)[b]	HPLC-UV	1.5-1200	90.8-115	1-8.3	ND	0.50-0.60	1.50-1.80	(Guo *et al.*, 2021)
SBSE[c]	HPLC-DAD	5-200	87.2-114	1.4-9.1	ND	1.23	4.06	(Huang *et al.,* 2011)
QuEChERS & SPE (alumina-N)	LC-MS/MS	0.005–0.5	83.7-94.1	3.0-10.6	ND	0.03	0.10	(Kanda *et al.,* 2012)
QuEChERS	LC-MS/MS	1–100	81-100.7	2.0-5.8	ND	0.64-0.83	2.13-2.77	(Lei *et al.*, 2018)
d-SPE (MCX)	LC-MS/MS	0-500	90.8-105.6	4.3-8.9	ND	ND	0.05	(Li *et al.,* 2018)
SPE (MDP-HCP)[d]	HPLC-UV	1-200	99.2-111.3	1-6	ND	0.30	1.00	(Liu *et al.*, 2022)
UAD–MSPE[e]	LC-MS/MS	1-200	115-118	6-10	ND	ND	1.00	(Melekhin *et al.,* 2024)
SPE (SCX)[f]	LC-MS/MS	0.5-3000	98.1-101.7	5.1-6.6	0.50	ND	ND	(Mitrowska & Antczak, 2017)
SPE (MIPs)[a]	LC-MS/MS	0-10	97.8-103.2	1.4-2.2	0.15	0.20	ND	(Mitrowska *et al.*, 2014)
SPE (silica)	LC-MS/MS	0.5-100	96.5-100.7	2.6-8.7	ND	0.05	ND	(Sakamoto *et al.*, 2011)
SPE (SDB)[g]	LC-MS/MS	0-9	61.1–69.7	15.3–26.4	0.10	0.13	0.33	(Tölgyesi *et al.*, 2012)
SPE (amine)	LC-UV	10-500	90.2-101.4	5.5-14.9	ND	1.00	3.00	(Zhou *et al.* 2007)
SPE (MCX)[h]	LC-MS/MS	0.5-250	89.2-99.2	3-8	0.44	0.63	1.00	This work

a: molecularly imprinted polymers
b: triazine-based porous organic polymers
c: stir bar sorptive extraction
d: m-phenylenediamine hypercrosslinked polymer
e: ultrasonic-assisted derivatization-magnetic solid-phase extraction
f: strong cation exchange
g: styrene-divinylbenzene
h: mixed-mode cation exchange
ND: not determined
*can be equivalent to limit of detection (LOD)

Table S2. Comparison of the developed method with other analytical methods for the determination of ronidazole in honey published in the literature.

Sample preparation	Analyzer	Linear range (µg/L)	Recovery (%)	RSD (%)	CC*α* (µg/kg)	CC*β* (µg/kg)*	LOQ (µg/kg)	Reference
ACN extraction	LC-MS/MS	0-20	102.4	3.5	0.38	0.66	ND	(Cronly *et al.*, 2010)
acidic hydrolysis & SPE	LC-MS/MS	0.1-10	90.4-101	12-26	1.60	2.30	ND	(Galarini *et al.*, 2015)
SPE (MIPs)[a]	HPLC-MS/MS	1–500	86.7-90.3	10.3-13.2	ND	0.50	1.00	(Guo *et al.*, 2017)
SPE (T-POPs)[b]	HPLC-UV	3-1200	89.1-116	1.9-10	ND	1.00-1.50	3.00-4.50	(Guo *et al.*, 2021)
SBSE[c]	HPLC-DAD	5-200	89-114	1.7-8.9	ND	1.52	5.00	(Huang *et al.*, 2011)
QuEChERS & SPE (alumina-N)	LC-MS/MS	0.01–1	76.1-85.4	5.7-9.5	ND	0.06	0.20	(Kanda *et al.*, 2012)
d-SPE (MCX)	LC-MS/MS	0-500	93.8-98.2	7.9-11.2	ND	ND	0.05	(Li *et al.*, 2018)
SPE (MDP-HCP)[d]	HPLC-UV	1.7-200	96-99.4	2.7-6.8	ND	0.50	1.70	(Liu *et al.*, 2022)
UAD–MSPE[e]	LC-MS/MS	1-200	97-100	5-10	ND	ND	1.00	(Melekhin *et al.*, 2024)
SPE (SCX)[f]	LC-MS/MS	0.5-3000	98.6-101.1	6.7-8	0.50	ND	ND	(Mitrowska & Antczak, 2017)
SPE (MIPs)[a]	LC-MS/MS	0-10	96.7-104.9	1.4-4.9	0.16	0.21	ND	(Mitrowska *et al.*, 2014)
SPE (silica)	LC-MS/MS	0.5-100	95.8-96.9	4.1-6.8	ND	0.20	ND	(Sakamoto *et al.*, 2011)
QuEChERS	LC-MS/MS	0.25-10	94.03-96.2	1.51-6.81	0.53	0.91	ND	(Shendy *et al.*, 2016)
SPE (SDB)[g]	LC-MS/MS	0-9	55.6–60.9	15.5–24	0.05	0.08	0.17	(Tölgyesi *et al.*, 2012)
SPE (amine)	LC-UV	10-500	83.4-99.2	2.4-12.3	ND	1.00	3.00	(Zhou *et al.* 2007)
SPE (MCX)[h]	LC-MS/MS	0.5-250	76.5-87.5	7-13	0.18	0.39	1.00	This work

a: molecularly imprinted polymers
b: triazine-based porous organic polymers
c: stir bar sorptive extraction
d: m-phenylenediamine hypercrosslinked polymer
e: ultrasonic-assisted derivatization-magnetic solid-phase extraction
f: strong cation exchange
g: styrene-divinylbenzene
h: mixed-mode cation exchange
ND: not determined
*can be equivalent to limit of detection (LOD)

Table S3. Comparison of the developed method with other analytical methods for the determination of dimetridazole in honey published in the literature.

Sample preparation	Analyzer	Linear range (µg/L)	Recovery (%)	RSD (%)	CC*α* (µg/kg)	CC*β* (µg/kg)*	LOQ (µg/kg)	Reference
ACN extraction	LC-MS/MS	0-20	94.7	9.4	0.73	1.24	ND	(Cronly *et al.*, 2010)
acidic hydrolysis & SPE	LC-MS/MS	0.1-10	92.1-105	12-20	0.48	0.64	ND	(Galarini *et al.*, 2015)
SPE (MIPs)[a]	HPLC-MS/MS	1–500	89-90.8	8.9-12.5	ND	1.00	1.00	(Guo *et al.*, 2017)
SPE (T-POPs)[b]	HPLC-UV	4.5-1200	81.2-114	1.5-8.3	ND	1.00-1.50	4.50	(Guo *et al.*, 2021)
SBSE[c]	HPLC-DAD	2-200	71.1-87.3	2.3-9	ND	0.47	1.54	(Huang *et al.*, 2011)
QuEChERS & SPE (alumina-N)	LC-MS/MS	0.01–1	86.8-95.1	3-8.1	ND	0.06	0.20	(Kanda *et al.*, 2012)
d-SPE (MCX)	LC-MS/MS	0-500	93.4-101.8	5.7-10.1	ND	ND	0.1	(Li *et al.*, 2018)
SPE (MDP-HCP)[d]	HPLC-UV	1-200	95.7-104.2	1.9-5.9	ND	0.30	1.00	(Liu *et al.*, 2022)
UAD–MSPE[e]	LC-MS/MS	0.3-200	99-101	5-15	ND	ND	0.30	(Melekhin *et al.*, 2024)
SPE (SCX)[f]	LC-MS/MS	0.5-3000	98.9-101.4	5.8-6.2	0.50	ND	ND	(Mitrowska & Antczak, 2017)
SPE (MIPs)[a]	LC-MS/MS	0-10	98.8-101.9	2-4.2	0.13	0.23	ND	(Mitrowska *et al.*, 2014)
SPE (silica)	LC-MS/MS	0.5-100	91.2-100.7	2.6-17.1	ND	0.20	ND	(Sakamoto *et al.*, 2011)
QuEChERS	LC-MS/MS	0.25-10	90.96-97	1.81-12.58	0.74	1.27	ND	(Shendy *et al.*, 2016)
SPE (SDB)[g]	LC-MS/MS	0-9	64.4–86.8	8.6–23.3	0.10	0.12	0.33	(Tölgyesi *et al.*, 2012)
SPE (amine)	LC-UV	10-500	71.5-89.7	6.4-15.8	ND	2.00	6.00	(Zhou *et al.* 2007)
SPE (MCX)[h]	LC-MS/MS	0.5-250	88.4-101.9	2-7	0.37	0.56	1.00	This work

a: molecularly imprinted polymers
b: triazine-based porous organic polymers
c: stir bar sorptive extraction
d: m-phenylenediamine hypercrosslinked polymer
e: ultrasonic-assisted derivatization-magnetic solid-phase extraction
f: strong cation exchange
g: styrene-divinylbenzene
h: mixed-mode cation exchange
ND: not determined
*can be equivalent to limit of detection (LOD)

Table S4. Comparison of the developed method with other analytical methods for the determination of ipronidazole in honey published in the literature.

Sample preparation	Analyzer	Linear range (µg/L)	Recovery (%)	RSD (%)	CC*α* (µg/kg)	CC*β* (µg/kg)*	LOQ (µg/kg)	Reference
ACN extraction	LC-MS/MS	0-20	97.8	4.3	0.40	0.68	ND	(Cronly *et al.*, 2010)
acidic hydrolysis & SPE	LC-MS/MS	0.1-10	84.9-108	8.8-21	0.44	0.54	ND	(Galarini *et al.*, 2015)
SPE (MIPs)[a]	HPLC-MS/MS	1–500	90.4-102	8.6-9.9	ND	0.10	1.00	(Guo *et al.*, 2017)
QuEChERS & SPE (alumina-N)	LC-MS/MS	0.005–0.5	88.5-98.5	2.9-8.7	ND	0.03	0.10	(Kanda *et al.*, 2012)
d-SPE (MCX)	LC-MS/MS	0-500	90.2-96.9	5.8-9.9	ND	ND	0.1	(Li *et al.*, 2018)
UAD–MSPE[b]	LC-MS/MS	0.3-200	98-102	4-10	ND	ND	0.30	(Melekhin *et al.*, 2024)
SPE (SCX)[c]	LC-MS/MS	0.5-3000	98-102.3	6.6-8.6	0.50	ND	ND	(Mitrowska & Antczak, 2017)
SPE (MIPs)[a]	LC-MS/MS	0-10	99.4-100.9	2.2-7.3	0.11	0.19	ND	(Mitrowska *et al.*, 2014)
SPE (MCX)[d]	LC-MS/MS	0.5-250	94.9-113.1	5-9	0.17	0.30	1.00	This work

a: molecularly imprinted polymers
b: ultrasonic-assisted derivatization-magnetic solid-phase extraction
c: strong cation exchange
d: mixed-mode cation exchange
ND: not determined
*can be equivalent to limit of detection (LOD)

Table S5. Comparison of the developed method with other analytical methods for the determination of hydroxymetronidazole in honey published in the literature.

Sample preparation	Analyzer	Linear range (µg/L)	Recovery (%)	RSD (%)	CC*α* (µg/kg)	*CC*β (µg/kg)*	LOQ (µg/kg)	Reference
ACN extraction	LC-MS/MS	0-20	103.8	4.1	0.39	0.67	ND	(Cronly *et al.*, 2010)
acidic hydrolysis & SPE	LC-MS/MS	0.1-10	91.6-119	13-22	1.70	2.50	ND	(Galarini *et al.*, 2015)
QuEChERS & SPE (alumina-N)	LC-MS/MS	0.05–5	79.8-86.5	3.9-14.2	ND	0.15	0.50	(Kanda *et al.*, 2012)
d-SPE (MCX)	LC-MS/MS	0-500	92.3-95.0	4.7-10.3	ND	ND	0.2	(Li *et al.*, 2018)
UAD–MSPE[a]	LC-MS/MS	1-200	98-101	7-15	ND	ND	1.00	(Melekhin *et al.*, 2024)
SPE (SCX)[b]	LC-MS/MS	0.5-3000	100.2-102.5	7-8.1	0.50	ND	ND	(Mitrowska & Antczak, 2017)
SPE (MIPs)[c]	LC-MS/MS	0-10	97.6-103.6	1-1.7	0.16	0.21	ND	(Mitrowska *et al.*, 2014)
SPE (SDB)[d]	LC-MS/MS	0-9	<35	ND	0.10	0.13	0.33	(Tölgyesi *et al.*, 2012)
SPE (MCX)[e]	LC-MS/MS	0.5-250	84.3-103.8	4-12	0.54	0.78	1.00	This work

a: ultrasonic-assisted derivatization-magnetic solid-phase extraction
b: strong cation exchange
c: molecularly imprinted polymers
d: styrene-divinylbenzene
e: mixed-mode cation exchange
ND: not determined
*can be equivalent to limit of detection (LOD)

Table S6. Comparison of the developed method with other analytical methods for the determination of hydroxydimetridazole in honey published in the literature.

Sample preparation	Analyzer	Linear range (µg/L)	Recovery (%)	RSD (%)	CC*α* (µg/kg)	CC*β* (µg/kg)*	LOQ (µg/kg)	Reference
ACN extraction	LC-MS/MS	0-20	104.2	4.6	0.42	0.71	ND	(Cronly *et a*l., 2010)
acidic hydrolysis & SPE	LC-MS/MS	0.1-10	86.6-115	15-20	1.50	2.00	ND	(Galarini *et al.*, 2015)
QuEChERS & SPE (alumina-N)	LC-MS/MS	0.05–5	80.4-88.5	2.8-9.4	ND	0.15	0.50	(Kanda *et al.*, 2012)
SPE (SCX)[a]	LC-MS/MS	0.5-3000	98.2-102.1	7.6-8.3	0.50	ND	ND	(Mitrowska & Antczak, 2017)
SPE (MIPs)[b]	LC-MS/MS	0-10	98.7-102	1.9-4.6	0.14	0.18	ND	(Mitrowska *et al.,* 2014)
SPE (SDB)[c]	LC-MS/MS	0-9	64.3–68.8	14.5–24.2	0.10	0.15	0.33	(Tölgyesi *et al.*, 2012)
SPE (amine)	LC-UV	10-500	82.9-99.5	2.5-15.4	ND	1.00	3.00	(Zhou *et al.,* 2007)
SPE (MCX)[d]	LC-MS/MS	0.5-250	80.7-97.3	5-7	0.16	0.30	1.00	This work

a: strong cation exchange
b: molecularly imprinted polymers
c: styrene-divinylbenzene
d: mixed-mode cation exchange
ND: not determined
*can be equivalent to limit of detection (LOD)

Table S7. Comparison of the developed method with other analytical methods for the determination of hydroxyipronidazole in honey published in the literature.

Sample preparation	Analyzer	Linear range (μg/L)	Recovery (%)	RSD (%)	CC*α* (μg/kg)	CC*β* (μg/kg)*	LOQ (μg/kg)	Reference
ACN extraction	LC-MS/MS	0-20	100.8	6.2	0.64	1.10	ND	(Cronly *et al.*, 2010)
acidic hydrolysis & SPE	LC-MS/MS	0.1-10	90-115	8.6-23	0.15	0.21	ND	(Galarini *et al.*, 2015)
QuEChERS & SPE (alumina-N)	LC-MS/MS	0.05–5	82.4-95	2.8-10	ND	0.15	0.50	(Kanda *et al.*, 2012)
d-SPE (MCX)	LC-MS/MS	0-500	92.7-104.2	4.8-7.9	ND	ND	0.2	(Li *et al.*, 2018)
UAD–MSPE[a]	LC-MS/MS	1-200	100-101	4-9	ND	ND	1.00	(Melekhin *et al.*, 2024)
SPE (SCX)[b]	LC-MS/MS	0.5-3000	98.9-104.6	7.8-9.5	0.50	ND	ND	(Mitrowska & Antczak, 2017)
SPE (MIPs)[c]	LC-MS/MS	0-10	96.9-104.6	2.3-4.5	0.16	0.21	ND	(Mitrowska *et al.*, 2014)
SPE (MCX)[d]	LC-MS/MS	0.5-250	79.3-85.8	7-13	0.18	0.32	1.00	This work

a: ultrasonic-assisted derivatization-magnetic solid-phase extraction
b: strong cation exchange
c: molecularly imprinted polymers
d: mixed-mode cation exchange
ND: not determined
*can be equivalent to limit of detection (LOD)

Table S8. Comparison of the developed method with other analytical methods for the determination of ternidazole in honey published in the literature.

Sample preparation	Analyzer	Linear range (μg/L)	Recovery (%)	RSD (%)	CC*α* (μg/kg)	CC*β* (μg/kg)*	LOQ (μg/kg)	Reference
ACN extraction	LC-MS/MS	0-20	102	9	0.78	1.34	ND	(Cronly *et al.*, 2010)
acidic hydrolysis & SPE	LC-MS/MS	0.1-10	87.3-106	6.2-12	0.38	0.43	ND	(Galarini *et al.*, 2015)
UAD–MSPE[a]	LC-MS/MS	1-200	108-111	4-9	ND	ND	1.00	(Melekhin *et al.*, 2024)
SPE (MIPs)[b]	LC-MS/MS	0-10	98.3-102.7	1.9-5	0.19	0.33	ND	(Mitrowska *et al.*, 2014)
SPE (MCX)[c]	LC-MS/MS	0.5-250	95.9-108.4	6-11	0.34	0.51	1.00	This work

a: ultrasonic-assisted derivatization-magnetic solid-phase extraction
b: molecularly imprinted polymers
c: mixed-mode cation exchange
ND: not determined
*can be equivalent to limit of detection (LOD)

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

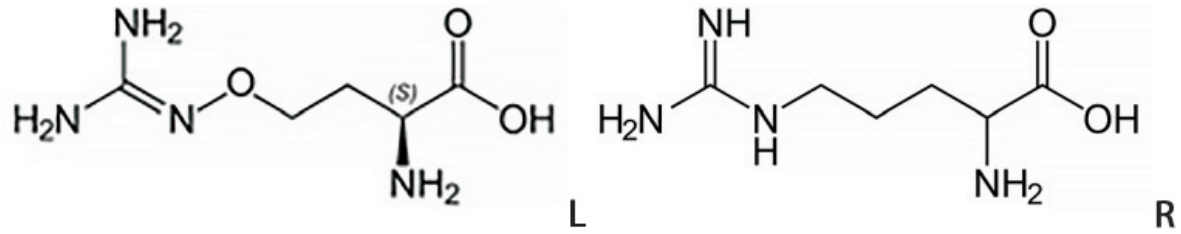

Figure 1. Chemical structure of L-Canavanine (Left) and L-Arginine (Right)

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.

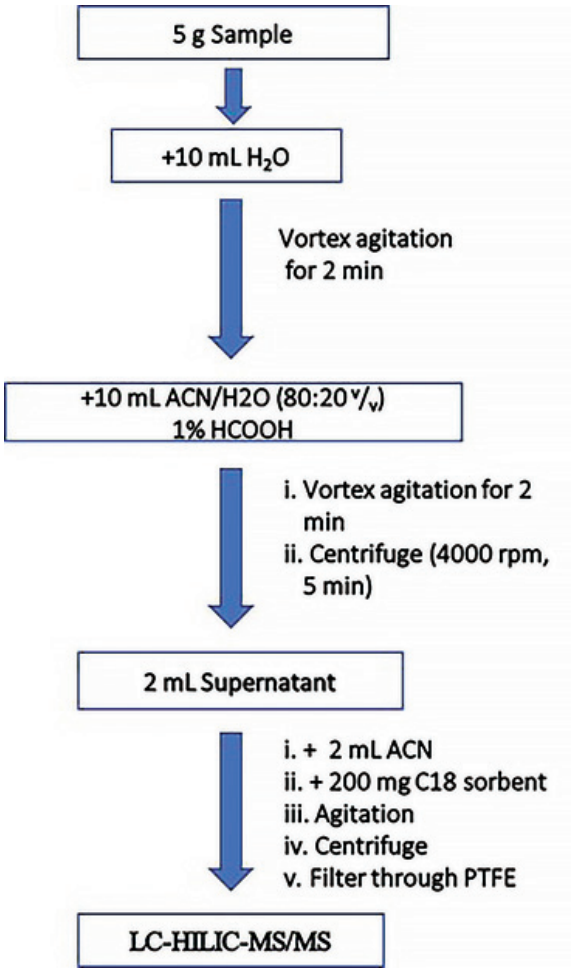

Figure 2. Abbreviated flow diagram describing extraction procedure.

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

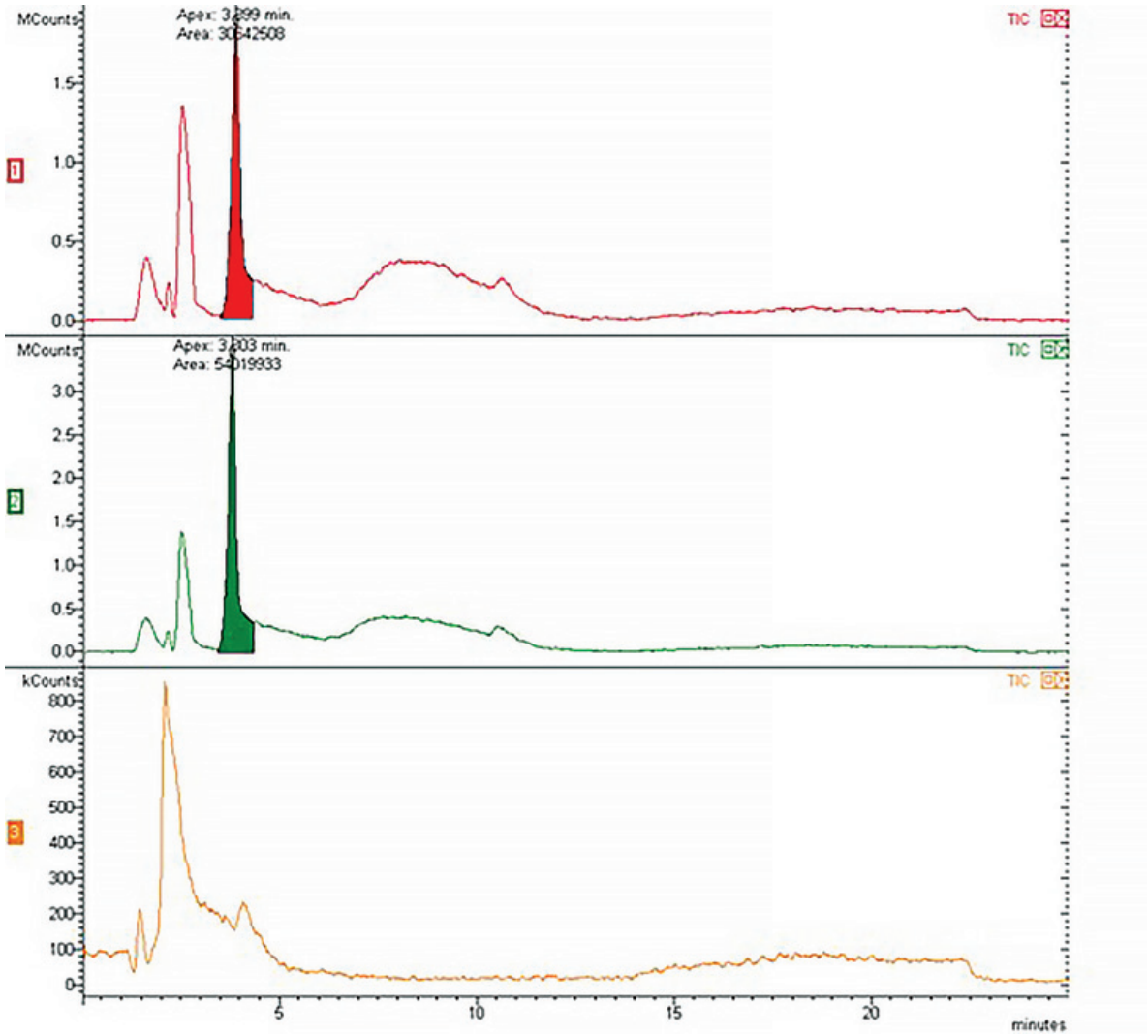

Figure3. TIC chromatogram for matrix matched calibration standards of Canavanine (at 0.25 μg mL^{-1} (1^{st} row) and 0.5 μg mL^{-1} (2^{nd} row) and for blank sample.

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

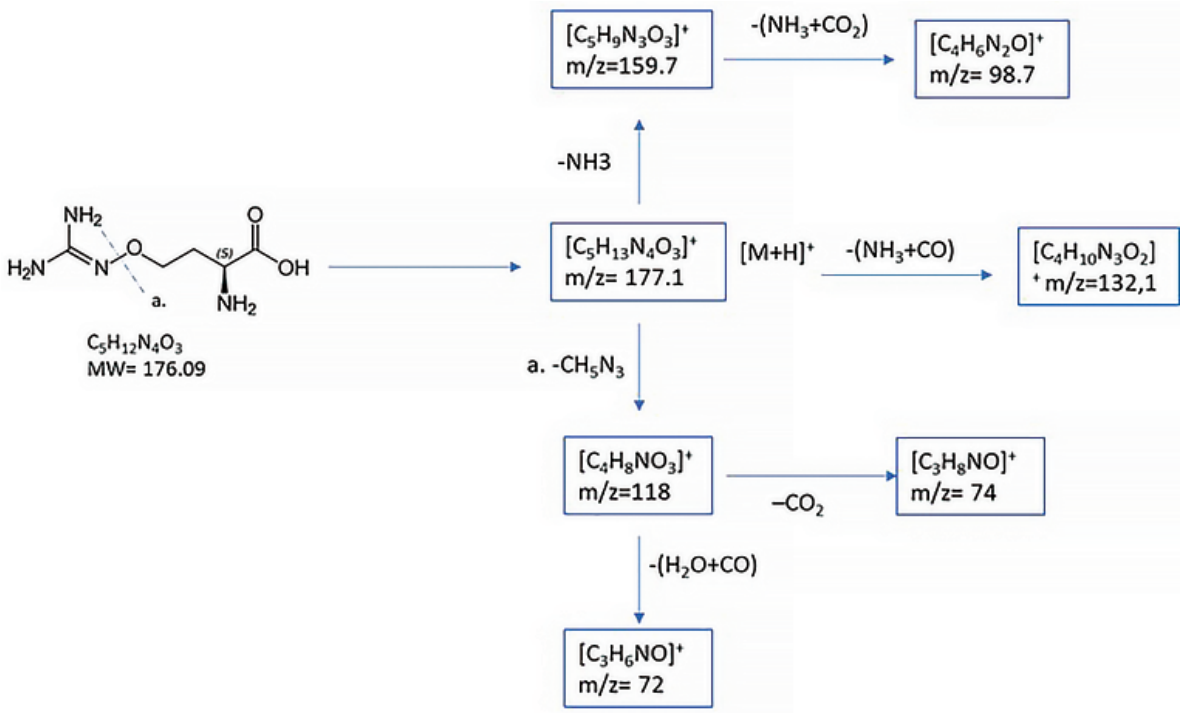

Figure S1. Theoretical fragmentation pattern of L-Canavanine under L-ESI(+)-MS/MS conditions.

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

Τόμος 18, Τεύχος 1, Ιανουάριος 2025
ISSN 1791-3691 (Print)
ISSN 2732-656X (OnLine)

Περιεχόμενα

Volume 18, Issue 1, January 2025
ISSN 1791-3691 (Print)
ISSN 2732-656X (OnLine)

Hellenic Plant Protection Journal

Contents

www.hppj.gr