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REVIEW

of a doctoral dissertation for the award of the educational and scientific degree **"Doctor"** in the field of higher education: 4. Natural Sciences, Mathematics and Informatics, professional field: 4.3. Biological Sciences, scientific speciality: Botany

Author of the doctoral dissertation: RUMEN VELINOV DENEV, full-time doctoral candidate at the Research Group "Chemoecology and Natural Products", Section "Applied Botany", Department "Plant and Fungal Diversity and Resources" at IBER-BAS

Scientific supervisor: Prof. Dr Strahil Hristov Berkov

Dissertation topic: "GC-MS Analysis of Silylated Amaryllidaceae Alkaloids"

Reviewer: Assoc. Prof. Ivayla Nedyalkova Dincheva, PhD, Agrobioinstitute – Sofia, Agricultural Academy of Bulgaria; Field of higher education 4. Natural Sciences, Mathematics and Informatics, professional field 4.3. Biological Sciences, scientific speciality "Biologically Active Substances"

1. Brief Introduction of the Candidate

Doctoral candidate Rumen Velinov Denev was born on 22.09.1983 in Zlatograd, Bulgaria. During the period 2006–2008, he studied at the Faculty of Chemistry of Sofia University "St. Kliment Ohridski", where he obtained the educational qualification degree Master in "Modern Spectral and Chromatographic Methods of Analysis". Since November 2009, he has been appointed as a research associate-chemist at the Institute of Organic Chemistry with the Centre of

Phytochemistry at the Bulgarian Academy of Sciences. In the period 2017–2024, he worked as a chemist at the Institute of Biodiversity and Ecosystem Research (IBER-BAS). Since 2024, he has been a service engineer at ACM2 Ltd., while simultaneously continuing his research activities at IBER-BAS. He completed a specialisation at the University of Pardubice (Czech Republic) under Prof. Ing. Michal Holčapek, PhD (01.05.2011 – 01.09.2011). He has 18 scientific publications in peer-reviewed and indexed in world-renowned scientific databases (Web of Science and Scopus). The doctoral dissertation was developed within the framework of Project No. ПВУ-67 of 16.12.2024 /BG-RRP-2.017-0021-C01 under the National Recovery and Resilience Plan of the Republic of Bulgaria.

2. Documents Submitted under the Procedure

The materials submitted by the doctoral candidate Rumen Denev comply with the Regulations on the Terms and Conditions for Acquiring Scientific Degrees and Holding Academic Positions at IBER-BAS. The following mandatory documents are included: doctoral dissertation; abstract; summary of contributions; list of publications on the dissertation topic and copies of the publications; list of citations of the works on the dissertation topic; scientific autobiography with a full list of publications; copy of the Master's degree diploma, certified by the Scientific Secretary of IBER-BAS; protocol from the meeting that referred the dissertation for defense; statement of the conducted educational process and earned credits; summary of the dissertation in English. The documents are presented correctly and in accordance with the requirements.

3. General Characteristics of the Doctoral Dissertation – Scope and Structure

The dissertation is presented in a total volume of 120 pages and contains the following sections: title page; acknowledgements (1 page); introduction (1 page); literature review (16 pages); aims and objectives (1 page); publications on the topic (47 pages); conclusion (1 page); main findings (2 pages); declaration of

originality and authenticity (1 page); contributions (1 page); references (6 pages); list of scientific publications with full bibliographic description (1 page); Appendix 1 – supplementary materials to the publications (39 pages). The structure of the dissertation complies with the requirements of the Regulations for the terms and procedures for acquiring academic degrees and for occupying academic positions at the Institute of Biodiversity and Ecosystem Research at the Bulgarian Academy of Sciences (IBER-BAS). According to Article 9 of the Regulations, the chapters “Materials and Methods”, “Results”, and “Discussion” are presented in the form of three scientific publications, which is fully permissible and gives the work a complete and publication-based character.

4. Relevance of the Topic

The topic of the doctoral dissertation is undoubtedly relevant, as Amaryllidaceae alkaloids represent a group of natural compounds with exceptionally high pharmacological value. Galantamine, lycorine, haemantamine, and other representatives of this family exhibit acetylcholinesterase inhibition (application in Alzheimer's disease), antitumor, antiviral, and anti-inflammatory activities. The correct identification and quantitative determination of these compounds in plant samples is of essential importance for chemotaxonomic, pharmacological, biotechnological research, and for quality control of plant raw materials. GC-MS is one of the most widely applied methods for the analysis of these alkaloids, but its limitations – low volatility of polar compounds, thermolability, and poor detection – are well known. In this context, the investigation of silylation as an approach to overcome these limitations has not only scientific but also direct practical significance. The development of reliable analytical methods for the control of plant raw materials containing Amaryllidaceae alkaloids is a key step in

creating sustainable industrial chains for the production of pharmaceutical substances from plant sources.

5. Literature Knowledge and Theoretical Preparation of the Candidate

The literature review is thoroughly developed and demonstrates the doctoral candidate's very good awareness of the current state of research in the field of Amaryllidaceae alkaloids, their chemical diversity, chemotaxonomic significance, and biological activity. Up-to-date information is presented on the structure and classification of over 600 known Amaryllidaceae alkaloids, as well as their importance in pharmacology and medicine. The review covers 78 sources from the period after 2000, with the cited works entirely in Latin script and including renowned international journals in the fields of phytochemistry, analytical chemistry, and pharmacology. Particularly thorough attention is given to analytical methods – from sample preparation to instrumental techniques (TLC, HPLC, CE, GC-MS) – with emphasis on the advantages and limitations of GC-MS and the possibilities for derivatisation. The candidate demonstrates excellent knowledge of mass spectral fragmentation and chromatographic behaviour of Amaryllidaceae alkaloids, which is of key importance for the interpretation of the obtained results. All of this testifies to a high level of theoretical preparation and ability for critical analysis of the scientific literature.

6. Correspondence of the Chosen Research Methodology to the Aim and Objectives of the Doctoral Dissertation

The methodology used in the doctoral dissertation is exceptionally well-planned and fully corresponds to the stated aim and formulated objectives. To achieve the goals, modern analytical approaches were applied:

- **GC-MS analysis** with electron impact (EI) at 70 eV and a DB-5MS capillary column – ensures high resolution and reproducibility.

- **Silylation with BSTFA in pyridine** – converts polar and thermolabile alkaloids into more volatile and stable trimethylsilyl derivatives.
- **Parallel analysis** of underivatized and silylated standard substances and plant extracts – enables comparative study of fragmentation patterns.
- **Use of retention indices (RI)** – facilitates the identification of isomers.
- **Validated quantitative method** for determination of galantamine in *Hippeastrum papilio* with direct injection of aqueous extracts – a significant contribution to green analytical chemistry.
- **Statistical processing** of data, including ANOVA and regression analysis.

The applied techniques are scientifically sound, modern, and fully suitable for achieving the objectives of the research. Particularly impressive is the developed "green" method for quantitative analysis, which eliminates the use of toxic organic solvents and allows direct injection of aqueous extracts. This is a significant step forward compared to conventional methods that require multiple extractions with chloroform or dichloromethane.

7. Significance and Persuasiveness of the Obtained Results, Interpretations, and Conclusions

The obtained results have undoubted scientific value and significantly expand the knowledge regarding the application of GC-MS analysis in the study of Amaryllidaceae alkaloids. They are published in reputable international journals with impact factor – *Rapid Communications in Mass Spectrometry* (Q2, IF 2.586 and Q3, IF 1.7) and *Journal of Chromatography A* (Q1, IF 4). The doctoral candidate is a co-author in all three publications, and in the third he is the first author, which testifies to his leading role in the research work. The publications are well-structured, with clear experimental design, application of modern methods, and

well-substantiated results and conclusions. The statistical processing is correct and reliable.

The research convincingly demonstrates that silylation leads to a substantial improvement in volatility, thermal stability, chromatographic separation, and analytical sensitivity for several alkaloids. It has been proven that silylation enables the detection of compounds that remain undetected in the direct analysis of underivatized samples. Particularly important are the results related to: improved detection of lycorine and other polyhydroxylated alkaloids; stabilisation of thermolabile compounds of the haemantamine and crinine types; the possibility of distinguishing natural compounds from artifacts; the improved structural information obtained after derivatisation.

The scientific value of the results is enhanced by the identification of new alkaloids and the development of new analytical methods for their quantitative determination. The conclusions are clearly formulated, logically coherent, and fully consistent with the experimental data obtained.

Particularly impressive is the third publication in *Journal of Chromatography A*, which presents an innovative "green" method for quantitative analysis of galantamine. This work demonstrates not only in-depth knowledge in the field of analytical chemistry but also the ability to develop methodologies consistent with the principles of green chemistry.

The results have not only fundamental significance but also serious practical applicability in phytochemistry, pharmacognosy, biotechnology, and quality control of plant raw materials.

8. Assessment of the Scientific Contributions

The scientific contributions of the doctoral dissertation can be summarised in the following directions:

A library of EIMS spectra of underivatized and silylated Amaryllidaceae alkaloids has been created – a valuable resource for phytochemical and chemotaxonomic research.

A detailed metabolic analysis has been performed on an alkaloid fraction from *Narcissus cv—Hawera* with proven antidepressant and anxiolytic activity.

Seven new Amaryllidaceae alkaloids have been identified:

Narcissus cv. Hawera: 2-oxomesembrine and 2-oxo-epi-mesembrenol;

Galanthus nivalis: 11-O-acetylhamayne, 3-(3'-O-hydroxybutanoyl)hamayne, 11-O-(3'-acetoxybutanoyl)hamayne, and 3,11-O-(2',2''-dibutanoyl)hamayne;

Narcissus poeticus: 11-O-(3-hydroxybutenoyl)haemanthamine.

A GC-MS method has been developed and validated for the quantitative determination of galantamine in *Hippeastrum papilio*, demonstrating its high content in this species – 7.44 mg/g dry weight in the bulbs.

A "green" GC-MS method has been developed and validated for the quantitative analysis of Amaryllidaceae alkaloids by direct injection of an acidified aqueous extract, eliminating the use of toxic organic solvents.

The fragmentation pathways of haemantamine- and crinine-type alkaloids have been elucidated, allowing the differentiation of epimeric forms and the identification of unknown compounds.

These contributions have both fundamental significance for understanding the chemical diversity and biosynthesis of Amaryllidaceae alkaloids and direct practical application in the fields of phytochemistry, pharmacology, and quality control of plant raw materials.

9. Critical Remarks on the Doctoral Dissertation

Despite the high scientific level of the doctoral dissertation, some critical remarks can be made which do not diminish its qualities but could serve for further refinement of the research approach:

Chronological inconsistencies and anachronisms

Mismatch in cited project: On page 2, it is stated that the dissertation was developed within project No. PVU-67 dated 16.12.2024. At the same time, the first scientific publication included in the Rapid Communications in Mass Spectrometry journal was accepted and published in April 2021. Chronologically, a paper published in 2021 can't have been conceived or carried out within a project that started at the end of 2024.

Research history: On page 19, it is stated that “GC-MS analysis of AAs began to be used more intensively at the beginning of the 20th century.” This is a serious factual inaccuracy. At the beginning of the 20th century, gas chromatography and mass spectrometry did not exist at all (their intensive combined application and introduction into phytochemistry began in the second half of the 20th century, around the 1960s and 1970s).

Gaps in the literature review and structure

Limited scope of biological activity: In the literature review (pages 13–15), the effects of galantamine, lycorine, and haemantamine are described in detail. However, since the main focus of the first included article is on Sceletium-type alkaloids, their pharmacological activity should have been addressed in greater detail in the theoretical part of the dissertation, rather than appearing only in the discussion section of the article.

Opportunities for assessing the practical applicability of the developed method

The proposed green method for the quantitative analysis of galantamine offers advantages in speed and environmental profile. Further evaluation of costs, analysis time, and comparisons with conventional approaches would strengthen the assessment of its potential for broader application.

Minor technical and stylistic issues

*In certain parts of the dissertation, repetitions and stylistic imperfections are observed. These do not affect the scientific value of the work but could be refined to improve readability.*10. Conclusion

The presented doctoral dissertation of Rumen Velinov Denev represents a complete, up-to-date, and significant scientific study in the field of botany, phytochemistry, and analytical chemistry. The stated scientific aim has been successfully achieved. Original results have been obtained, new analytical approaches have been developed, and both scientific and applied contributions of high value have been achieved. The doctoral dissertation contains new knowledge, has a clearly expressed contributory character, and demonstrates the candidate's ability to conduct independent scientific research.

The doctoral candidate is a co-author in all three publications related to the dissertation topic, and in one of them, he is the first author, which confirms his leading role in the research work.

Having thoroughly reviewed the submitted defence materials, I consider that the doctoral dissertation fully meets the requirements of the Law on the Development of the Academic Staff in the Republic of Bulgaria, the Regulations for its Implementation, as well as the internal regulatory documents of IBER-BAS. On this basis, I give a decidedly positive assessment of the doctoral dissertation and recommend to the esteemed Scientific Jury to award Rumen Velinov Denev the educational and scientific degree **"Doctor"** in the doctoral program **"Botany"**.

Date: 30.06.2026 **REVIEWER:**

Sofia (Assoc. Prof. Ivayla Dincheva, PhD)