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REVIEW

by Ivanka Bojkova Semerdjieva, PhD

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Regarding: A dissertation submitted in partial fulfillment of the requirements for the award of the educational and scientific degree of **Doctor (PhD)** in the field of higher education **4. Natural Sciences, Mathematics and Informatics**, professional field **4.3. Biological Sciences**, PhD Program; **Botany (01.06.03)**.

Author of the dissertation: Rumen Velinov Denev, part-time PhD candidate at the Department of Plant and Fungal Diversity and Resources, IBER-BAS, Applied Botany Section, Chemoecology and Natural Products Research Group.

Title of the dissertation:"GC–MS Analysis of silylated Amaryllidaceae alkaloids"

Scientific Supervisor: Prof. Strahil Hristov Berkov, PhD IBER, BAS

1. **General characteristics of the dissertation work – scope and structure**

A characteristic feature of species belonging to the family Amaryllidaceae is their ability to biosynthesize Amaryllis alkaloids (AAs). These alkaloids exhibit a broad spectrum of biological activities, such as antitumor, antiviral, and anti-inflammatory effects, as well as acetylcholinesterase inhibitory activity. Quantitative determination of AAs constitutes the basis for chemotaxonomic and pharmacological investigations and represents an important approach for selecting appropriate source material in biotechnological applications and for quality control. Various analytical techniques have been employed for the separation and analysis of alkaloid mixtures, including thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), and gas chromatography (GC). Each of these methods possesses specific advantages and limitations that determine its suitability for particular analytical applications. Gas chromatography coupled with electron ionization mass spectrometry (GC–EI-MS) is among the most widely used techniques for the analysis of Amaryllis alkaloids because it provides highly reproducible mass spectra suitable

for compound identification through comparison with reference spectral libraries. The principal limitation of this technique is its inability to directly analyze thermolabile, non-volatile, and highly polar compounds, as well as their salts. This limitation can be overcome through prior derivatization of the samples, most commonly by silylation.

Within this context, the doctoral dissertation submitted for review addresses a highly relevant research topic with substantial scientific and practical significance. The study contributes to expanding the analytical capabilities of GC–EI-MS through the establishment of a library of EI mass spectra of both underivatized and silylated AAs based on parallel GC–MS analysis. The obtained results have the potential to improve the accuracy of AAs identification and to broaden the applicability of the method for their quantitative determination.

Generally, the dissertation comprises 142 pages, fully complying with the requirements of Chapter 4 (9) of the Regulations governing the acquisition of academic degrees and the appointment to academic positions at the IBER, BAS (adopted by the Scientific Council and amended on 25 November 2022). The Denevs work contains all mandatory sections, which are logically organized and well balanced. The introduction spans two pages, while the literature review accounts for approximately 12% of the total dissertation volume. The research objective is clearly formulated, and three specific research tasks have been defined and successfully accomplished. The chapters entitled "Materials and Methods," "Results," and "Discussion" have been replaced by three attached scientific publications, which is fully consistent with point 9 of the Regulations governing the acquisition of academic degrees and academic appointments at IBER, BAS. The doctoral candidate is the first author of one publication and the second or third author of the remaining two. All three papers have been published in international peer-reviewed journals indexed in Web of Science or Scopus and ranked within the appropriate journal quartiles. The reported findings possess both scientific and practical value, while their publication in prestigious international journals provides additional evidence of the significance and quality of the conducted research. A list of the scientific publications, including complete bibliographic information, the principal conclusions, and the scientific contributions, is presented over five pages of the dissertation. The dissertation is illustrated with eight schemes, eighteen figures, and ten tables. Supplementary materials (Appendices) occupy 40 pages. The submitted extended abstract (25 pages) has been prepared in accordance with the institutional requirements and accurately summarizes the research included in the dissertation. An English-language summary of the dissertation (one page) is also provided. According to the plagiarism report dated 20 April 2026, no evidence of plagiarism has been identified.

2. **Literature awareness and theoretical preparation of the candidate**

The literature review comprises 73 references related to the botanical description, classification, and phylogeny of the family Amaryllidaceae, as well as the biosynthesis and alkaloid composition of amaryllis alkaloids. A substantial proportion of the cited literature has been published within the last decade and consists predominantly of international publications in English. The literature review is organized into several thematic sections that convincingly establish the scientific rationale for the present investigation. It provides a comprehensive overview of the biosynthesis of AAs and the factors influencing this process. Particular attention is devoted to the chemotaxonomic significance of the diversity in alkaloid biosynthesis and structural types within the family Amaryllidaceae. Furthermore, the review critically discusses the advantages and limitations of the principal analytical methods used for AAs analysis, while emphasizing the benefits of alkaloid derivatization, particularly silylation.

The doctoral candidate demonstrates an excellent command of the specialized terminology used in phytochemistry and possesses an in-depth understanding of the scientific issues addressed by the dissertation. The presented material reflects a high level of technical competence, comprehensive theoretical preparation, and familiarity with the current state of research in this field.

3. Methodological approach

The methodological approach adopted in the dissertation is well justified and fully consistent with the stated research objective and tasks, thereby ensuring the reliability of the obtained results and the successful implementation of the research programme. The study was conducted using three plant species belonging to Amaryllidaceae family, subfamily Amaryllidoideae - *Narcissus poeticus* L., *Galanthus nivalis* L., and *Hippeastrum papilio* Van Scheepen as well as one hybrid, *Narcissus* cv. Hawera. The biosynthesis of structurally diverse AAs is characterized in all investigated plants. Lycorine and narciclasine-type alkaloids predominate in *N. poeticus*, whereas galanthamine-type alkaloids are characterized by *G. nivalis*. Homolycorine-type alkaloids and structurally related compounds are the most specific for *H. papilio*. Particular attention was devoted to sample preparation and the extraction of AAs, which represent critical steps in ensuring reliable and scientifically sound analytical results. Alkaloid extraction from the plant material of the target species was performed using a standard acid–base extraction protocol that is well established in analytical practice. Derivatization (silylation) was carried out according to validated and widely

accepted procedures. GC–MS analyses were performed using modern instrumental platforms, namely a Thermo Scientific Focus GC/DSQ II system (electron ionization, 70 eV; ion source temperature 230°C) and an Agilent 6890+/5975 MSD, both equipped with a DB-5MS capillary column. The analytical instrumentation employed is appropriate and fully meets the requirements for reliable analysis of AAs. Method validation included the assessment of specificity and selectivity, linearity, limits of detection (LLOD) and quantification (LLOQ) under selected ion monitoring (SIM) mode, as well as precision expressed as relative standard deviation (RSD). Accuracy was evaluated through recovery experiments at fortification levels of 10%, 50%, and 100%. In addition, analyte stability and matrix effects (MF) were investigated. The validation strategy applied is comprehensive and complies with current international requirements for GC–MS analytical methods. The limits of detection for the investigated alkaloids were established, and the relationships governing their thermal stability were elucidated. The comparison of the GC–MS characteristics of silylated and underivatized AAs, as well as the evaluation of their suitability for qualitative and quantitative analyses in plant samples from the selected species, are important aspects of this study. These approaches have the potential to overcome several inherent limitations of conventional GC–MS analysis.

Furthermore, a library of EI mass spectra of both underivatized and silylated AAs obtained from the investigated plant species was established. A comparative evaluation between GC–MS analyses of underivatized and silylated reference standards was also performed. The experimental results were subjected to appropriate statistical analysis, ensuring their correct interpretation and supporting the validity of the conclusions drawn.

4. Significance and reliability of the results, their interpretation, and the conclusions

The doctoral dissertation of Rumen Denev constitutes a comprehensive investigation aimed at improving the analytical capabilities of GC–EI-MS through the development of a library of EI mass spectra of underivatized and silylated AAs.

The obtained results are thoroughly described, critically analysed, and comprehensively discussed in the accompanying scientific publications. Based on the analytical and statistical evaluation of the experimental data, the following principal findings have been established:

- ✓ Silylation of alkaloid fractions and plant extracts represents an effective approach for investigating the biosynthesis of Amaryllis alkaloids;
- ✓ For alkaloids containing two or more hydroxyl groups, silylation increases volatility, improves chromatographic separation, and lowers the limits of detection, as

exemplified by lycorine, which exhibits poor detectability at low concentrations in its underivatized form;

- ✓ For haemanthamine/crinine-type alkaloids possessing a C11 hydroxyl group, silylation enhances thermal stability and improves chromatographic performance. Haemanthidine provides a characteristic example, as it is readily detected after silylation, including the successful chromatographic separation of its epimers;
- ✓ Parallel analysis of silylated fractions and total alkaloid extracts enables discrimination between naturally occurring compounds and analytical artefacts, thereby increasing the reliability of alkaloid profiling;
- ✓ Silylation enhances analytical sensitivity, enabling the detection of minor alkaloids that remain undetectable in underivatized samples;
- ✓ Derivatization provides valuable information regarding the number of hydroxyl groups present in the molecule and facilitates structural identification;
- ✓ Epimeric haemanthamine- and crinine-type alkaloids can be differentiated on the basis of their mass spectral characteristics and retention indices;
- ✓ The configuration of the 5,10b-ethano bridge cannot be unequivocally determined by GC–MS alone and requires complementary spectroscopic techniques, such as circular dichroism (CD) spectroscopy;
- ✓ Hydroxybutyryl ester alkaloids undergo thermal degradation during GC–MS analysis to form 2-butenoyl derivatives, an observation that is essential for the correct interpretation of analytical results;
- ✓ Electron ionization fragmentation enables localization of substituent groups and supports the structural elucidation of unknown and newly identified alkaloids;

The originality, scientific relevance, and practical importance of the dissertation are unquestionable. Silylations of AAs and plant extracts represents an important analytical approach. This improvement facilitates their reliable detection and chromatographic characterization, thus contributing to both fundamental research and practical applications..

The conclusions are clearly formulated, logically derived from the experimental findings, and adequately supported by the presented results.

5. Critical remarks on the dissertation

The doctoral dissertation of Rumen Velinov Denev has been prepared in accordance with the requirements of the Regulations governing the acquisition of academic degrees and the

appointment to academic positions at the IBER, BAS. The critical remarks concerning the dissertation are not substantial and do not diminish the overall merits of the work. The identified shortcomings may be grouped into two main categories:
(1) grammatical and stylistic errors; (2) technical inaccuracies and issues related to formatting and presentation.
I would like to recommend that, in future scientific work, the doctoral candidate avoids a poetic style of expression, as it is not compatible with scientific botanical and phytochemical research. In several parts of the literature review on the family Amaryllidaceae (subfamily Amaryllidoideae), expressions of a literary or poetic nature are used, such as: “beautiful appearance,” “small plant,” "miniature bulb", “beautiful white coloration,” “symbol of spring and renewal,” “striking colours, a true spectacle of nature with its characteristic ‘butterfly’ appearance,” etc. In the botanical description of species, it is correct to use the editions of the Flora of Bulgaria, Europe or other floras, where the terms are defined and accepted.
For future publications, greater precision is recommended with respect to both grammar and technical formatting. In the dissertation, “EI-MS” is sometimes written as a single term, whereas contemporary scientific practice prefers a hyphenated format. After first mention in the text, genus names should be abbreviated in subsequent occurrences.

In the section “Aim and Objectives” (p. 21), the plant species intended for extraction and alkaloid analysis are listed (*N. poeticus*, *Narcissus* cv. *Hawera*, *G. nivalis*, and *H. papilio*), whereas in the abstract (Section 5.2, p. 13), additional species (*Crinum moorei* L. and *Amaryllis belladonna* L.) are mentioned, which are not included in the main dissertation text. These inconsistencies may lead to confusion.
It is considered inappropriate that the conclusion sections of both the dissertation and the abstract reiterate the research aim; instead, emphasis should be placed on the main findings and results. The above critical remarks do not reduce the overall scientific value of the dissertation.

Questions to the Doctoral Candidate

1. What was the purpose of including the phylogenetic scheme of the family Amaryllidaceae according to Meerow (Figure 1, p. 6) in the dissertation?
2. To what extent can silylation alter the profile of the observed alkaloids, and how is the possible formation of artefacts controlled?

3. What are the main limitations of EI-MS in the structural characterization of Amaryllidaceae alkaloids?
4. What new practical opportunities does silylation provide for the detection of minor alkaloids?

6. Nature of the Scientific Contributions

I accept the author's statement regarding the scientific contributions. They are clearly formulated and accurately reflect the results obtained by the doctoral candidate. All contributions are original and derive directly from the achievement of the stated research objective. For the first time, a library of EI mass spectra of both underivatized and silylated AAs has been created through parallel GC–MS analysis of standard compounds and alkaloid fractions from various Amaryllidaceae species. New AAs have been identified in *Narcissus* cv. *Hawera*, *G. nivalis*, and *N. poeticus*.

A GC–MS method for the quantitative determination of galanthamine in *H. papilio* has been developed and validated. In addition, a "green" GC–MS method for the quantitative analysis of AAs in plant samples has been developed and validated, using direct injection of an acidified aqueous solution, thereby avoiding the use of toxic organic solvents.

The results presented in the dissertation provide new scientific information and significantly expand current knowledge of the alkaloid composition of species within the family Amaryllidaceae. Furthermore, they are of applied scientific importance, as improvements in GC–MS methodology contribute to enhanced quality control of pharmaceutical raw materials containing Amaryllis alkaloids.

7. Evaluation of the quality of the scientific publications reflecting the dissertation research and the Doctoral Candidate's Personal Contribution

The research presented in the dissertation has been published in three scientific articles, all of which are indexed in Web of Science and Scopus. Part of the results has been published in two papers in *Rapid Communications in Mass Spectrometry* (Q2, Impact Factor 2.394), while the remaining results have been published in *Journal of Chromatography A* (Q1, Impact Factor 4.0). All publications have undergone peer review.

These publications fully meet the minimum requirements set for doctoral candidates at the Bulgarian Academy of Sciences. With a required minimum of 30 credits, the candidate has obtained 60 credits under this criterion. In addition to the publications, the doctoral candidate

has presented the results at three scientific conferences. The relevance and significance of the research are further supported by five citations.

It is my opinion that the experimental work, the obtained results, their presentation, and interpretation are largely the personal contribution of the doctoral candidate. According to the declaration of originality and authenticity submitted by the candidate (Section 5.2, p. 103), the provided information, references, and publications are accurate and reflect objective reality.

8. Conclusion

The doctoral dissertation of Rumen Velinov Denev represents a substantial contribution to the identification, improvement, and validation of GC–MS analysis of standard compounds and alkaloid fractions from various Amaryllidaceae species.

The results constitute an original scientific contribution and meet all requirements of the Act on the Development of the Academic Staff in the Republic of Bulgaria (ADASRB), the Regulations for its implementation, and the internal regulations of the IBER, BAS. The submitted materials and results fully comply with the specific requirements of the IBER, BAS regulations for the implementation of ADASRB.

The dissertation demonstrates that the doctoral candidate possesses in-depth theoretical knowledge and technical skills in the scientific field of Botany. The candidate shows the ability to independently carry out scientific research.

In view of the above, I express my positive evaluation of the conducted research and strongly recommend that the distinguished Scientific Jury award the educational and scientific degree of **Doctor** (PhD) to Rumen Velinov Denev in the field of higher education: 4. Natural Sciences, Mathematics and Informatics, professional field 4.3 Biological Sciences, doctoral programme Botany.

08.07.2026

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