

## REVIEW

by prof. d-r Pavletta Shestakova, Institute of Organic Chemistry with Centre of Phytochemistry,  
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of a dissertation for awarding the educational and scientific degree 'doctor'  
in the field of higher education 4. 'Natural sciences, mathematics and informatics',  
professional direction 4.2. 'Chemical Sciences', PhD program 'Organic Chemistry'

**Author:** assistant Dessislava Plamenova Gerginova

**Topic:** NMR metabolomics of honey and wine

**Supervisors:** prof. DSc Svetlana Simova (IOCCP-BAS) and Assistant prof. Dr. Yavor Mitrev (IOCCP-BAS)

### General presentation of the procedure

The set of materials presented by assistant Dessislava Gerginova for the defense of a PhD thesis for awarding the educational and scientific degree "doctor" is in accordance with the Regulations for the Development of the Academic Staff of the IOCCP-BAS, and meets the criteria of the IOCCP-BAS for the awarding of the educational and scientific degree "doctor".

The PhD candidate has presented 4 publications on the topic of the dissertation, a list of citations of the papers included in the dissertation, a list of participation in scientific events, a list of participation in the implementation of scientific projects, awards and the relevant supporting material. The documents were submitted on time and are prepared carefully and precisely.

### Brief biographical data of the PhD student

Dessislava Gerginova completed her secondary education in 2011 at the Professional High School of Ecology and Biotechnology "Prof. Asen Zlatarov, Sofia, with major in "Ecology and Environmental Protection". In 2016, she graduated from the University of Chemical Technology and Metallurgy in Sofia as a chemical engineer with a Bachelor degree in "Chemical Inorganic Technologies". During the period November 2013 - January 2014, Dessislava Gerginova was an intern at the Bulgarian NMR Centre (BulNMR) under the "Student Practices" program of the Ministry of Education and Culture, under the supervision of Prof. Svetlana Simova. The practice at the BulNMR marked the beginning of her interests in the field of NMR spectroscopy, and in 2016 she continued her studies at the Faculty of Chemistry and Pharmacy, Sofia University "St. Kliment Ohridski", where she completed her Master degree in "Modern Spectral and Chromatographic Analysis Methods" in 2017. In 2018, she was enrolled as a full-time doctoral student at the BULNMR at the IOCCP-BAS, with scientific supervisors Prof. Svetlana Simova and Assistant Professor Yavor Mitrev. Since January 2021, she has been dismissed with the right of defense and has been appointed as an assistant at the BulNMR at IOCCP-BAS.

### Relevance of the topic

The topic of the PhD thesis is highly relevant and important, both in a scientific and applied-practical aspect, in view of the growing number of frauds related to both the quality of food products and beverages, as well as their true origin, which lead to economic and socially significant problems. Regardless of the actions taken at the level of the European Union, there is still a lack of effective generally recognized legislative and regulatory documents and measures that would allow strict control of the content, quality and origin of food and beverages. The problems are largely related to the limited number of established methods for the analysis of their qualitative and quantitative composition, which would allow fast, reliable and economically relevant detection of the substances used for counterfeiting. In recent years, NMR spectroscopy has been increasingly established as an important, reliable and fast method for controlling the quality and origin of food and beverages. In

connection with this, the main goal of the dissertation was formulated - development of new NMR-based approaches for fast and reliable analysis of the quality and authenticity of honey, wine and other products with a high sugar content. The specific tasks to achieve this goal are appropriately defined, methodically and logically connected with each other, which already at the beginning of the dissertation gives the impression that the dissertation work has been developed on the basis of a carefully considered and well-designed concept.

### **Knowing the problem**

The dissertation includes a total of 326 literature sources, some of which are NMR spectral databases, software packages for analysis of NMR spectra and chemometric data, as well as packages for metabolomic analysis. The literature review is very well systematized, covers all aspects of the research in the dissertation, it is designed very precisely, and the information is presented with beautifully designed figures, graphs and tables. The literature review is interestingly and engagingly written, comprising six parts containing a description and analytical analysis of the main experimental and statistical methods used in metabolomic studies, briefly presenting the basic principles of NMR spectroscopy and the most important aspects of the method in its application to metabolomic studies, the used chemometric methods and statistical approaches are also presented. The composition and main analytical methods used for the analysis of honey, wine, mead and pine jam are described, and their advantages and disadvantages are critically analyzed. The brief historical notes at the beginning of each part of the literature review create a pleasant impression and show the desire of the doctoral student to acquire a wider culture and complete familiarity with the methods used and the objects studied.

The presentation of the literature review convincingly shows that the doctoral student thoroughly knows the current state of research and the problems addressed in the dissertation, she is able to critically and competently analyze, interpret and summarize the existing literature. This definitely contributed to the correct formulation of the main goal and specific tasks of the dissertation, as well as to finding an appropriate scientific strategy and approaches for their successful completion, which also determines the high level of the conducted research and the obtained results.

### **Research methodology, characterization and evaluation of the dissertation work**

The PhD thesis is written on 235 pages and is richly illustrated with 84 figures, 3 schemes and 26 tables. Of the cited 326 literary sources, more than half are articles published after 2015. The dissertation consists of six main sections: Introduction (2 pages), Literature review (104 pages), Results and discussion (89 pages), Experimental part (26 pages), Conclusion (1 page), References (13 p.). Along with the main text included in the dissertation, electronic access is provided to 11 Appendices, containing numerous and excellently presented experimental data, graphs and a detailed description of the components found in honey and wine.

The results of the own scientific research are presented and discussed in three main sections, which are grouped according to the type of the studied objects: 1) bee honey, 2) wine and 3) other products with a high sugar content - mead and pine jam.

**Research on honey** covers 300 honey samples of different botanical and geographical origins. Due to the lack of an established protocol for NMR metabolomic analysis of honey, the optimal conditions for conducting the research are defined in the thesis: concentration, pH, temperature, pulse sequences for the  $^1\text{H}$  and  $^{13}\text{C}$  NMR experiments, as well as the most appropriate experimental parameters. The complex chemical composition of honey, including a large number of substances with similar structure, poses serious challenges in the analysis of  $^1\text{H}$  NMR spectra, as they contain a large number of closely spaced or overlapping signals, with complex multiplicity, which creates difficulties in

identification and quantification of the components. To solve this problem, a set of standard homo- and heterocorrelation two-dimensional NMR techniques, a selective one-dimensional TOCSY experiment, as well as a comparison with the spectra of the individual saccharides and literature data were applied in the dissertation. By this approach, 46 compounds were identified in the  $^1\text{H}$  NMR spectra of honey. A methodology was also developed for the quantification of saccharides by using  $^{13}\text{C}$  NMR spectra, and 41 compounds were identified and quantified. The obtained data on the qualitative and quantitative composition of honey samples were analyzed by chemometric techniques, as a result of which characteristic components were established and a methodology was proposed for distinguishing honey by botanical, entomological and geographical origin. A method for rapid identification of dilute honey, by a single-accumulation  $^1\text{H}$  spectrum and without water signal suppression, using the maltose/glucose and sucrose/glucose ratios is also proposed. A method was developed for calculating the optical rotation angle of honey and jam, based on the concentrations of 21 disaccharides determined from  $^{13}\text{C}$  spectra and the optical rotation angle of the individual saccharides.

**Studies on wine** cover 70 samples (of which three are diluted), including red and white wines from different grape varieties, as well as foreign and Bulgarian wines, from 4 different regions of Bulgaria. One-dimensional and two-dimensional NMR techniques were applied to determine the qualitative and quantitative composition and for identification of various organic components in the investigated wine samples, and the approach is similar to the one used in the study of honey. 31 compounds were identified and quantified, the data were analyzed with various chemometric techniques, and the possibilities of the proposed approach for distinguishing wine by grape variety, by geographical origin, as well as for determining main components, through which it is possible to distinguish between diluted from real wines, as well as to distinguish red wines from the same grape variety with and without the use of maceration in the production process. A model has been proposed that allows distinguishing wines from typical Bulgarian grape varieties and foreign varieties produced in Bulgaria.

**Studies on other high sugar products** included 2 samples of pine jam and one sample of mead. The qualitative and quantitative composition of 30 organic substances in mead was established. A comparative study of mead, honey and wine was conducted, and components in mead were found that can be used to assess its quality or for the presence of adulteration. 14 saccharides in pine jam were identified and quantified, and a comparative study of the sugar profile of pine jam and honey, as well as pine cone and pine tip jam, was conducted. Through chemometric analyses, differences in their content were found that could serve as markers for their differentiation, however to create a reliable model, it is necessary to continue the research by analyzing a larger number of samples.

All the experiments were carried out using a systematic, well-planned and consistent approach, the interpretation of the experimental data was done carefully and precisely, the analysis of the data with the proposed chemometric approaches and statistical models was convincing. I would like to note that the work performed is impressive in volume and is related to the processing and analysis of a large set of experimental data. The doctoral student coped with this task perfectly, as in addition to indisputable skills for working with a huge amount of data, she also demonstrated great skills for summarizing, describing and visualizing them in an extremely interesting and attractive way.

**In the Experimental part**, the origin of the studied samples, the sample preparation methods, the experimental parameters for recording the NMR spectra, their processing and their quantitative analysis are described in detail. The software programs, methods and parameters for performing the chemometric analyzes of the data for honey, wine, mead and pine jam are indicated. The way in which

the experimental part is presented provides an opportunity to reproduce the experimental data and conduct independent analyses, which I consider a positive aspect of the dissertation.

**My overall impression** is that the dissertation represents NMR experimental work of large volume, combined with precise chemometric and statistical data analysis, and it has been carried out with extreme precision and at a high scientific level. Important contribution for the highly relevant topic of the thesis and its scientific quality has the high competence of the scientific supervisors Prof. DSc. Svetlana Simova and assistant professor Dr. Yavor Mitrev.

### **Contributions and significance of the developments for science and practice**

The research carried out in the thesis and the results obtained have a scientific and scientific-applied nature. Essentially, they are related to the development of new NMR-based methodologies for the qualitative and quantitative characterization of honey, wine and mead by applying standard NMR experimental techniques in combination with chemometric approaches for data analysis. The conducted studies contain original scientific results and lay the foundations for a new direction "NMR metabolomics" in the research activities of the BulNMR and in the research in Bulgaria, The large volume of analyzed samples and the precision with which the experiments, processing and analysis of the obtained data were carried out, as well as the impressive layout of the dissertation could serve as a standard.

The main contributions of the dissertation can be summarized as follows:

- Through analysis of  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra recorded with different pulse sequences, parameters and samples prepared under different conditions, the optimal conditions for sample preparation and the most suitable experimental parameters for the analysis of honey by NMR metabolomics were established.
- Development and verification of  $^{13}\text{C}$  NMR spectroscopy based methodology, which was applied for semi-quantitative determination of 41 components in bee honey and pine jam.
- Original research contributions related to the study of bee honey: for the first time it was experimentally proven that honey from African stingless bees contains a high amount of the disaccharide trehalulose; for the first time, the presence of the monosaccharide quinovose in bee honey was established; two characteristic components for oak honeydew have been identified - 1-kestose and meso-2,3-butanediol; specific components have been established that allow distinguishing the Strandzha oak honey dew honey from oak honey of other geographical origins.
- Using chemometric analyses, specific components have been identified that can serve as markers for determining the origin of honey and wine, in order to establish and determine the botanical origin and country of production of honey, as well as the region in Bulgaria from which is the wine.
- It has been shown that wines from typical Bulgarian grape varieties contain a higher amount of sugars and alcohols compared to wines from the studied international varieties.
- Development of methodology based on  $^1\text{H}$  NMR spectra, allowing quick and easy identification of diluted honey and wine, which is an original contribution of the dissertation.
- A method for calculating the optical angle of rotation of honey depending on the amount of contained sugars is proposed, allowing easy identification of diluted honey.
- Through NMR metabolomics, the profiles of pine jam and mead were determined for the first time and a comparative analysis of the composition of pine jam, honey, wine and mead was performed.

### **Assessment of dissertation publications**

The results obtained in the PhD thesis are summarized in 4 scientific publications, two of which are published in journals that belong to the category Q1 (Molecules with IF 4.412 and Foods with IF 4.350), one to category Q4 (Bulgarian Chemical Communication with IF 0.242) and one publication is in a journal that is not referenced in international databases (Journal of the Bulgarian Academy of Sciences, in Bulgarian). A total of 15 citations were observed on the publications, with 14 of them being on the two publications in Molecules and Foods published in 2020 and 2021, respectively, which shows the great interest of the scientific community and is indicative for the significance of the research conducted.

The results of the scientific work of Assistant Gerginova have been presented to the scientific community through oral presentations (9 issues) and posters (9 issues) at international and national scientific forums. It is noteworthy the significant participation with oral presentations, of which 5 in national and 4 in international events, as well as the large number of awarded posters (3 pieces), which is a recognition not only for the high quality of the research, but also of the doctoral student's excellent presentation skills of the results.

### **Personal contribution of the PhD candidate**

I am fully convinced that Assistant Dessislava Gerginova has an indisputable and decisive personal involvement in every stage of the conducted research. I would especially like to highlight her important contributions for careful sample preparation, processing, analysis and interpretation of the NMR spectra, data summary and analysis through the application of chemometric and statistical methods. During her work on the dissertation, Assistant Gerginova completed with excellent results courses in NMR spectroscopy and statistical methods, acquired skills in working with various software packages for processing and analysis of NMR spectra, for metabolomic analysis, for chemometric and statistical data analysis. She is entirely responsible for writing and impressively presenting the data and results with figures, tables and diagrams. The PhD student is deservedly the first author in 3 of the 4 publications, which is recognition of her significant personal contribution to the conducted research.

I would like to point out that, in addition to the 4 publications included in the Thesis, Assistant Professor Dessislava Gerginova is the co-author of 4 more publications and one book chapter. As Gerginova participated in 18 scientific projects financed by the National Science Fund, the Ministry of Education and Science; OP-SESG, within the framework of the bilateral academic cooperation of the BAS and from other sources, and already has experience in project management under the National Program "Young scientists and postdoctoral fellows".

### **Abstract**

The presented abstract has been prepared in accordance with the requirements and objectively reflects the structure, content and results of the PhD thesis.

### **Critical remarks and recommendations**

Some inaccuracies have been noted in the description of the principles of NMR spectroscopy, which I assume are due to the PhD student's desire to explain the principles of the method at a level that is understandable and accessible to a wider audience. I would like to note that this does not affect my excellent impression of the quality of the work and the high scientific level of the PhD thesis.

I have no critical remarks to the presented research and to the dissertation materials.

### **Personal impressions**

I have known Dessislava Gerginova since 2013, when she first came to the NMR laboratory at IOCCP, BAS, as an intern under the "Student Practices" program. Over the years, she gained serious knowledge and skills for working with the NMR equipment, got involved and actively participated in the

various activities related to the maintenance of the NMR spectrometers, being responsible for summarizing and preparing the monthly reports on the service activities of the BuINMR. As a head of the BuINMR my personal impressions of Dessislava Gerginova are wonderful. I was pleased to watch her scientific development and growth as a competent, responsible and thorough young researcher who is able to successfully face new challenges, work independently and solve the problems. Impressive are the high criteria and requirements she sets for her own work, as well as her desire for new knowledge, the inspiration and strong motivation she puts into scientific work. With her precise, persistent and thorough work, Desi contributed to the introduction of NMR metabolomics and chemometric analyzes as new research line of the NMR group. Thanks to all these qualities, Desi has become an irreplaceable and highly valued member of the BuINMR team.

#### **Recommendations for future use of dissertation contributions and results**

I would recommend that the NMR-based methods for the analysis of the qualitative and quantitative composition of honey and wine developed in the dissertation work be more widely popularized and presented to the Bulgarian Agency for Food Safety or other food and beverage control institutions and bodies. It would also be good to consider including the obtained data on the composition of the studied subjects in databases for NMR metabolomic studies (internet-based or other type). This would contribute to popularizing the developed methods and increasing the visibility of the obtained results.

#### **CONCLUSION**

The PhD Thesis contains original scientific and scientific-applied developments, results and contributions, which cover and exceed the requirements of the Law on the Development of the Academic Staff in the Republic of Bulgaria (LDASRB), the Regulations for its Implementation and the Regulations for its Implementation of the Bulgarian Academy of Sciences. The presented materials and dissertation results fully comply with the specific requirements of the Regulations of IOCCP-BAS for the application of LDASRB.

The dissertation presents assistant Desislava Gerginova as a talented, serious and promising young scientist, distinguished by her own scientific profile, in-depth approach, enthusiasm and highly professional skills in the field of NMR metabolomic analysis of foods and beverages, which is an important scientific field. I wish Desi health, good luck, success and more interesting topics and exciting results in her future work. I would also like to congratulate the supervisors for the interesting scientific topic, the well-formulated research strategy and the original results obtained in the dissertation.

Based on the above, I am pleased to give my **positive assessment** of the research carried out, the results achieved and the contributions presented in the dissertation work and I propose to the Honorable Scientific Jury **to award the educational and scientific degree "doctor" to assistant Dessislava Gerginova in the field of higher education: Natural sciences, mathematics and informatics, professional direction 4.2. Chemical sciences, PhD program "Organic chemistry"**.

25.08.2022r.

Reviewer:

Prof. d-r Pavletta Shestakova