STATEMENT

By Assoc. Prof. Dr. Violeta Milenkova Stefanova

Department of Analytical Chemistry and Computer Chemistry,

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on a PhD thesis for the awarding of the educational and scientific degree "Doctor"

Area of higher education: **4. Natural sciences, mathematics and informatics**

Professional field: **4.2. Chemical sciences**

Doctoral program: **Analytical Chemistry**

Author: Asya Dimitrova Hristozova

Title: ***Enhancement of the capabilities of gas chromatography - mass spectrometry by combination with "green" approaches for extraction and modeling***

PhD Supervisor: Assoc. Prof. Dr. Kiril Kostov Simitchiev - Plovdiv University "Paisii Hilendarski", Faculty of Chemistry, Department of Analytical and Computer Chemistry

1. General overview of the procedure and the PhD student

According to Order № РД -22-486 dated 21.02.2025 by the Rector of University of Plovdiv "Paisii Hilendarski" (PU), I have been appointed as a member of the scientific jury under the procedure for defence of PhD thesis entitled ***Enhancement of the capabilities of gas chromatography - mass spectrometry by combination with "green" approaches for extraction and modeling*** for awarding the educational and scientific degree "Doctor" in higher education area **4. Natural sciences, mathematics and informatics**; professional field - **4.2. Chemical sciences**; doctoral program - **Analytical Chemistry**. The author of the thesis is **Asya Dimitrova Hristozova** - PhD student in full-time education at the Department of Analytical Chemistry and Computer Chemistry, Faculty of Chemistry, PU "Paisii Hilendarski".

The complete set of materials presented by Asya Hristozova, both in paper and electronic form, fully complies with the requirements outlined in Article 36(1) of the PU's Academic Staff Development Regulations.

Asya Hristozova is an alumna of the Faculty of Chemistry at PU. In 2005, she earned a Bachelor's degree in *Biology and Chemistry*, and in 2006, a Master's degree in *Medical Chemistry*. The part-time format of her Master's program allowed her to begin working immediately at "Tchaikapharma High-Quality Medicines" AD (2006–2020), where she started as a medical representative and later shifted toward analytical work as an analytical chemist, chromatographer, and LIMS system administrator in the Quality Control Department. Her professional path in analytical methodologies and quality control systems inspired her to successfully complete a second Master's program in *Spectrochemical Analysis* (2019).

Since March 2020, she has been appointed as a assistant professor at the Department of Analytical and Computer Chemistry at PU, where she leads practical classes in Analytical Chemistry and Instrumental Analysis, Pharmaceutical Analysis (for Chemistry and Biology undergraduates), and Combined Chromatographic Techniques (for master's students in the program Spectrochemical analysis) and courses on "Analytical capabilities of modern gas chromatography" and "Analytical capabilities of modern liquid chromatography" (for master's students program in the "Chromatographic and spectral analytical control").

2. Relevance of the topic

Modern gas chromatography techniques for separation in combination with mass spectrometric detectors (GC-MS and GC-MS/MS) have established themselves as powerful instrumental systems capable of meeting the ever-increasing demands of society for the quality of analytical data, as well as the need to identify and quantify a large number of organic compounds with relatively low boiling points, in ever-lower concentrations, present in a variety of objects (pharmaceutical, clinical, environmental, food, etc.).

The research is focused on expanding the capabilities of GC-MS for determining sub-trace levels of volatile and semi-volatile compounds contained in essential oils, as well as pesticides in waters and fruit juices through environmentally friendly approaches.

Over the past two decades, non-target analysis has gained popularity as a promising approach for the identification of a vast number of organic compounds in complex matrices. A key challenge is the development of reliable identification approaches based on mathematical modelling of chromatographic data without the need for experimental determination of retention times.

Another scientific direction addressed in the dissertation is the development of environmentally sustainable analytical methods aligned with "green chemistry" principles — using minimal quantities of relatively non-toxic reagents while conserving energy, instrumentation, and time.

3. Knowledge of the problem

A comprehensive literature review — 306 sources (over 56% of which were published since 2015) demonstrates the candidate's deep understanding of current advancements in the field. The review critically assesses GC-MS development prospects in the context of 'green chemistry', as well as the main advantages, limitations and challenges of the method with attention to pesticide and essential oil component identification and quantification.

Significant focus is given to chemometric methods for chromatographic analysis and chemical experiment modelling.

Sample preparation is a critical stage of the analytical procedure and is therefore constantly in the focus of scientific research, but the present work focuses on some of the most promising environmentally benign methods for preparing samples for gas chromatographic analysis: cloud point extraction (CPE); dispersive liquid-liquid microextraction (DLLME); solid-phase microextraction

(SPME); microextraction with deep eutectic solvents (DES) and natural deep eutectic solvents (NADES).

4. Research methodology

The entire research on the dissertation work to expand the capabilities of gas chromatography in combination with tandem mass spectrometry (GC-MS/MS) follows the paradigm of environmentally appropriate "green" approaches in analytical chemistry. A systematically planned research strategy addresses both the qualitative (identification) and quantitative (measurement) aspects of the analysis.

The entire structure of the study convincingly demonstrates the key role of the expert analyst in the intelligent selection and systematic tracking of cause-and-effect relationships, which creates the prerequisites both for the development of environmentally friendly procedures that ensure the obtaining of well-validated final analytical results, and for the effective planning of the entire research process.

Three types of essential oils were experimentally tested for the purpose of qualitative analysis. The chromatographic separation was optimized using a non-polar stationary phase. The multicomponent non-targeted analysis identified the presence of 51 compounds in two brands of rose oil, 49 compounds in lavender oil and 32 compounds in peppermint oil. The obtained experimental results are combined into a private database, based on which a multiple linear regression algorithm has been developed and validated, which allows to predict the linear retention indices (LRI) of volatile and semi-volatile compounds. The algorithm is easy to implement and is based on only 14 molecular descriptors.

A procedure for preliminary separation of the components from the matrix by head space solid-phase microextraction (HS-SPME) has also been developed.

The quantitative analysis is oriented towards the determination of organophosphorus and organochlorine pesticides, which are considered to be some of the most dangerous persistent organic pollutants with extremely low regulated concentrations in a number of sites. Two novel environmentally friendly extraction procedures have been developed for the group separation and preconcentration of 19 compounds: Microwave-assisted CPE (MW-CPE) and DLLME based on natural hydrophobic deep-eutectic solvents (NADES-DLLME).

Experimental design methods (Plackett-Burman and Central Composite Design) were used to optimize the procedures, reducing experimental workload while capturing factor interactions.

Combined methods for separation and preconcentration, coupled with subsequent GC-MS/MS analysis, have been systematically studied, both in terms of the stability of the chromatographic column in the presence of surfactant and NADES, and in terms of matrix effects and calibration strategies.

The "green" nature of the two developed methods for the determination of organochlorine and organophosphorus pesticides was assessed using a consensus approach, popular in scientific circles: *Analytical greenness metric for sample preparation* (AGREEprep). Taking into account the fact that chromatographic separation, in itself, is a slow process, streamlining the optimization procedure can also be considered as a "green" contribution to the development of a specific analytical method.

Both methods were applied to determine pesticides in real samples: MW-CPE-GC-MS/MS for fruit juices (lemon, red apple) and NADES-DLLME-GC-MS/MS for three brands of bottled spring water.

5. Characteristics and evaluation of the dissertation

The dissertation is structured into five main sections: Literature review (38 pages) Aims and objectives (1 page); Experimental section (11 pages); Results and discussions (62 pages); Conclusions and contributions summary (2 pages); References list (306 sources). The entire study is visualized through 38 tables and 25 figures. Additionally, 4 appendices are presented, which contain chemical structures of the target analytes, figures illustrating the influence of some parameters on extraction efficiency, and chromatograms.

The aim and relevant research objectives of the dissertation are clearly formulated.

The main contributions can be summarized as follows:

- An approach for the identification of volatile components in essential oils by GC-MS and GC-MS/MS has been developed and validated. A new regression model for the prediction of LRI based on multiple linear regression has been proposed. A database of experimentally determined LRI for over 120 compounds contained in essential oils (rose, mint and lavender) has been created, using a non-polar stationary phase.
- Two optimized approaches have been developed for group separation and concentration of trace amounts of organochlorine and organophosphorus pesticides through extraction techniques combined with subsequent GC-MS/MS analysis.
  - ✓ For the first time microwave-assisted CPE with Triton X100, followed by re-extraction in hexane (or isooctane) was applied for pre-concentration and subsequent gas chromatographic analysis of pesticides.
  - ✓ For the first time, an original approach for the group separation and concentration of pesticides by vortex-assisted DLLME was proposed, in which the extractant was a hydrophobic deep-eutectic solvent prepared from components of natural origin (menthol: decanoic acid) (NADES).
  - ✓ For both sample preparation procedures, adequate calibration strategies are suggested, taking into account the matrix influence in subsequent GC-MS/MS) analysis;
  - ✓ The proposed combined analytical methods for preconcentration and quantification of pesticides MW-CPE-GC-MS/MS and NADES-DLLME-GC-MS/MS have been applied to the analysis of real waters and fruit juices. The achieved method limits of quantification (MLOQ) are significantly lower than the corresponding regulatory values.

- It has been proven that the two developed and validated methods have a significantly more pronounced "green" character compared to routinely used methods for the analysis of pesticides in waters intended for human consumption and pesticide analysis in foods of plant origin.

Some of the contributions of the dissertation work go beyond the specific research and have a more general applied and fundamental nature:

- It has been shown that the appropriate use of mathematical modeling to predict linear retention indices has significant potential for revealing the multicomponent composition in untargeted panoramic analysis of real samples. This capability not only frees the analyst from the need for standards in compound identification, but also offers the possibility of discovering suitable markers, allowing the determination of the origin of various essential oils or other natural objects.
- Knowledge has been enriched regarding the possibilities of combining CPE with chromatographic analysis. It has been shown that the introduction of surfactants into the chromatographic system can lead to an increase in sensitivity, respectively a decrease in MLOQ.
- The capabilities of gas chromatography with mass spectrometric detection for pesticide determination have been extended by combining it with DLLME preconcentration using natural deep-eutectic solvents as extractants. NADES has been shown to act as a “protector” for the analytes, leading to increased sensitivity.

6. Evaluation of the scientific publications and the contribution of the PhD student

Two articles have been published on the subject of the dissertation in refereed high-impact journals where Asya Hristozova is first author:

1) Hristozova, A., Simitchiev, K., Kmetov, V., Rosenberg, E., „*Compatibility of cloud point extraction with gas chromatography: Matrix effects of Triton X-100 on GC-MS and GC-MS/MS analysis of organochlorine and organophosphorus pesticides*“, Talanta, 2024, 269, 125445. https://doi.org/10.1016/j.talanta.2023.125445, (Scopus, SJR 2023=0.956, (Q1, 5 citations)

2) Hristozova, A., Batmazyan, M., Simitchiev, K., Tsoneva, S., Kmetov, V., Rosenberg, E., „*Headspace – Solid phase microextraction vs liquid injection GC-MS analysis of essential oils: Prediction of linear retention indices by multiple linear regression*“, Acta Chromatographica, 2024, 2025, 37 (1), 76–86., https://doi.org/10.1556/1326.2024.01207 (Scopus, SJR 2023=0.344; Q2; 1 cit.)

Research results have been presented in 8 poster and 2 oral presentations at scientific conferences and seminars in Bulgaria and abroad.

Asya Hristozova is a member in the research teams of four scientific projects, two of which are with international participation.

The total scientometric score from the published articles is 45 (Q1 – 25 points and Q2 – 20 points), which exceeds the national minimum requirements for acquiring the degree of "Doctor" and the requirements of the Regulations for the Development of the Academic Staff of Plovdiv University "Paisii Hilendarski" (30 points required). Convincing evidence that the PhD student actively participated in both the experimental and scientific design of the publications and posters is that Asya Hristozova is the first author in both published articles, as well as in all poster presentations.

7. Abstract

The abstract systematically, correctly and clearly represents the main achievements and contributions of PhD thesis of **Asya Hrstozova** and meets the formal requirements of the Regulations of the Plovdiv University "Paisii Hilendarski"

8. Critical remarks and recommendations

I have no remarks concerning the PhD-Thesis, the abstract, and the materials.

CONCLUSION

The PhD-Thesis contains ***scientific and applied results which are original contributions to science*** and **meet all the requirements** of the *Law for Development of Academic Staff in the Republic of Bulgaria*, *the Regulations for implementation of the Law for the development of the academic staff in the Republic of Bulgaria and the respective Regulations of the Plovdiv University "Paisii Hilendarski"*. The presented materials and the results obtained completely correspond to the specific requirements of the Faculty of Chemistry.

Asya Hristozova has demonstrated solid theoretical knowledge and practical skills in analytical chemistry, which indicates that she has acquired capability to independently plan, conduct and report scientific research.

Based on the above, I am convinced to give my **positive assessment** to the research presented by the above reviewed PhD-Thesis, abstract, results, and contributions, and ***I propose the esteemed scientific jury to award the educational and scientific degree "Doctor"*** to **Asya Dimitrova Hristozova** in the field of higher education: **4. Natural sciences, mathematics and informatics;** professional field **4.2. Chemical sciences**; doctoral program **Analytical Chemistry**.

08. 05. 2025

Reviewer: .....

/Assoc. Prof. Dr. Violeta Stefanova/