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ENHANCEMENT OF THE CAPABILITIES OF GAS CHROMATOGRAPHY - MASS SPECTROMETRY BY COMBINATION WITH "GREEN" APPROACHES FOR EXTRACTION AND MODELING

ABSTRACT

OF A DISSERTATION FOR THE AWARD EDUCATIONAL AND SCIENTIFIC DEGREE OF

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The dissertation contains 165 pages (A4 format) and includes 25 figures, 38 tables, 4 appendices (contained 1 table and 8 figures) and 306 sources are cited.

The defence materials are available for review in the "Development of Academic Staff and Doctoral Studies" at the University of Plovdiv “Paisii Hilendarski”, the “National Center for Information and Documentation” at the Ministry of Education, Youth and Science, and the Central Library of the University of Plovdiv “Paisii Hilendarski”.

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Abbreviations and symbols used

CCD	Central composite design
CPE	Cloud point extraction
DLLME	Dispersive liquid-liquid microextraction
ER	Analytical yield
EF	Enrichment factor
Full scan	Scanning within a specified mass range
GC-MS	Gas chromatography-mass spectrometry
GC-MS/MS	Gas chromatography-tandem mass spectrometry
LRI	Linear retention index
NADES-DLLME	Dispersive liquid-liquid microextraction based on a natural deep-eutectic solvent
MLR	Multiple linear regression
MNT:DA	Menthol : decanoic acid
MW-CPE	Microwave assisted-cloud point extraction
QSRR	Quantitative structure retention relationship
RI	Retention index
RMSE	Root mean square error
RTIC	The reconstructed total ion current chromatograms
SD	Standard deviation
SIM	Selected Ion Monitoring
SRM	Selected Reaction Monitoring
USABE	Ultrasound-assisted re-extraction
VABE	Vortex-assisted re-extraction
VIF	Variance inflation factor
R^2	Coefficient of determination
q^2_{F1}, q^2_{F2}	Prediction power

I. INTRODUCTION

Gas chromatography with mass spectrometry detection (GC/MS) is the commonly used analytical technique for the volatile and semi-volatile organic compounds identification and quantification, in complex matrices. It is a combination of a gas chromatograph and a mass spectrometer, in which, after evaporation of the sample in the GC injector and separation of the components of the mixture in a temperature-controlled column, in addition to information about the retention times, qualitative spectral information for each compound can be obtained by the mass spectrometer. This information can be used for both qualitative structural identification of the studied components and quantitative analysis [1].

The samples with a relatively complex composition and subject to analysis by gas chromatography are essential oils. They are a mixture of terpene or phenylpropane derivatives characterised by similar mass spectra. The correct identification of such compounds is very complicated and sometimes impossible. To increase the reliability of analytical results for assessing the composition of essential oil components, a combination of criteria and different identification approaches can be used, including separation and concentration methods, comparison with spectra from databases, and calculation of retention indices (RI) [2,3]. In all cases, however, the experimental determination of retention indices, which should be compared with available reference values, is a long, complex and slow process. For this reason, and following the principles of "green" chemistry, much effort has been directed towards the development of mathematical models for predicting RI in GC-MS to facilitate and reduce the time of the identification process [4,5].

Gas chromatography-mass spectrometry is routinely used for pesticide analysis in food samples and environmental objects. The weak control and widespread use of pesticides in the past led to negative consequences of environmental pollution and danger to human health. Some pesticides are resistant to degradation in nature for a long period and can accumulate in soils and sediments, bioaccumulate and have the potential to cause harm for years. Such pesticides are classified by the Stockholm Convention in 2001 as Persistent Organic Pollutants (POPs) [6]. In 2001, an international agreement was adopted to limit or eliminate the production and use of POPs. Initially, a blacklist was compiled, including 12 POPs banned for use, in 2009, 9 compounds were added to the list, and in 2011, their number reached 22 compounds.

Organochlorine pesticides are one of the most commonly used types of POPs in the past, which have been banned or restricted for use due to their persistence and neurotoxicity [7]. Due to their lower persistence compared to organochlorine pesticides, some of the most widely used pesticides worldwide are organophosphorus pesticides [8]. To achieve sustainable use of pesticides by reducing risks and impacts on human health and the environment, Directive 2009/128/EC was introduced in the European Union and Bulgaria in 2012, a National Action Plan for Reducing the Risks and Impacts of Pesticide Use was prepared.

II. GOALS AND SCIENTIFIC RESEARCH TASK

The dissertation aims to study the capabilities of GC-MS/MS for combining with "green" approaches for qualitative and quantitative analysis.

The following tasks were set for conducting ***a qualitative analysis*** of volatile components in essential oils:

Task 1: To optimise the instrumental conditions of a GC-MS/MS system for the identification of volatile components in rose, lavender and peppermint essential oils, both during injection of liquid samples and after preliminary preparation by headspace solid-phase microextraction (HS-SPME);

Task 2: To calculate the linear retention indices of the identified compounds using the obtained experimental data and to compile a list of their molecular descriptors;

Task 3: To develop and validate a multiple linear regression algorithm for predicting linear retention indices for gas chromatographic analysis of volatile components from essential oils for a nonpolar stationary phase.

The following tasks were set for conducting ***quantitative analysis*** of pesticides:

Task 1: To study the possibility of combining the cloud point extraction (CPE) with GC-MS/MS;

Task 2: To study the possibility of combining the dispersive liquid-liquid microextraction (DLLME), based on natural deep-eutectic solvents (NADES), with GC-MS/MS;

Task 3: To assess the selectivity at mass detection of target analytes in Full Scan and SRM mode;

Task 4: To study the non-spectral matrix effects of the used extractants (surfactant and NADES) on the GC-MS/MS system;

Task 5: To assess the ecological evaluation of the developed analytical methods through quantitative metrics;

Task 6: The developed methods should be applied to organochlorine and organophosphorus pesticide analysis in water and food samples.

III. Experimental part

III.1. Apparatus and instrumental conditions

III.1.1. Gas chromatograph with tandem mass spectrometer - GC-MS/MS

The studies were conducted using a GC-MS/MS system TSQ 9000 (Thermo Scientific, USA) with an EI (70 eV) ion source equipped with a triple quadrupole mass filter, a programmable temperature vaporisation injector (PTV) and an AI1300 autosampler with a 10 µl glass syringe. In all GC-MS/MS analyses, Helium (99.999%) was used as the carrier gas and Argon (99.996%) as the collision gas. The system was controlled by Excalibur 4.1 software. The studies were performed using a glass liner (PTV Liner with Three Baffles, 1 mm ID, 2.75 mm OD, 120 mm Length, Thermo Fisher Scientific, USA) or a metal liner (PTV Siltek Metal Liner, 2 mm ID, 2.75 mm OD, 120 mm Length, Thermo Fisher Scientific, USA). A TG SQC MS column (15 m x 0.25 mm x 0.25 µm, Thermo Fisher Scientific, USA) was used for chromatographic separation. The transfer line temperature was 250 °C, and the EI was 230 °C.

In the analysis of essential oils, by injection of liquid samples and HS-SPME, a mobile phase speed of 1.0 mL min^{-1}, injection volume of 1 µl, split ratio of 10:1, PTV injector temperature of 280 °C and a column oven temperature program as follows: 40 °C for 2 min, 1 °C min^{-1} to 200 °C and 30 °C min^{-1} to 280 °C for 3 min were used. A scan mode (Full scan) in mass range 35-500 amu, dwell time 0.2 sec, was used.

The analysis of pesticides by MW-CPE-GC-MS/MS and NADES-based-DLLME-GC-MS/MS was performed under the following operating conditions: mobile phase flow rate 1.2 mL min^{-1}, injection volume 1 µl, split ratio 5:1 (MW-CPE-GC-MS/MS) and 50:1 (NADES-based-DLLME-GC-MS/MS), PTV gradient from 65 °C at 14.5 °C sec^{-1} to 260 °C and column oven temperature program as follows: 120 °C for 1 min, 40 °C min^{-1} to 155 °C, 4 °C min^{-1} to 187 °C, 1 °C min^{-1} to 194 °C and 12 °C min^{-1} to 260 °C, held for 5 min. Full scan mode was used: 20-700 amu for MW-CPE-GC-MS/MS and 45-500 amu for NADES-based-DLLME-GC-MS/MS. The solvent cut time was 5 min, and the analysis was 28 min. For each pesticide in SRM mode, one Quant transition and one Qual transition were selected with the corresponding collision energies.

III.1.2. Gas chromatograph-mass spectrometer

As an alternative instrumental technique for pesticide analysis, a GC-MS Shimadzu 2010 SE equipped with a single quadrupole mass filter, an EI ion source at 70 eV and an injector allowing operation under high pressure (PSS) was used. Injection was performed using an L-PAL3 GC autosampler (LECO) equipped with a 10 µl glass syringe. Helium (99.999%) was used as the carrier gas. The system was controlled by LabSolution software. A glass liner (Glass GC Liner, 5 mm OD, 3.4 mm ID, 95 mm Length, Trajan SGE, Australia) was used, and a TG-5MS column (30 m x 0.25 mm x 0.25 µm film thickness, Thermo Fisher Scientific, USA) was used for chromatographic separation. The transfer line temperature was 250 °C, and the EI temperature was 220 °C. The mobile phase flow rate was 0.9 mL min^{-1}; the injection volume was 2 µl; the split ratio was 5:1 at PSS 56.9 kPa and 280 °C. The column oven temperature program was as follows: 80 °C for 2 min, 8 °C min^{-1} to 280 °C and 50 °C min^{-1} to 350 °C. The solvent cut time was 5 min, and the total analysis time was 28.4 min. In SIM mode, three characteristic ions were monitored for each pesticide.

III.1.3. Other equipment

An ONDA UV 30 Scan spectrophotometer (Giorgio-Bormac, Italy) was used to study the solubility of Triton X-100 and Triton X-114 in organic solvents. The UV-VIS spectra in the UV range of 200–400 nm, with a 2 nm scan step, were recorded.

A microwave system MDS-81D (CEM corp.) with a maximum power of 600 W was used as an alternative source of energy input to conventional heating on a hot plate when conducting CPE. Heating was performed at maximum power for 4 min, followed by six heating cycles of 5 min each, with 1 min breaks between them.

III.2. Sample preparation procedures

III.2.1. Essential oils

In the study of volatile compounds in essential oils, by GC-MS/MS, the following samples were analysed: Rose oil (Rosa damascena) (Bulgaria); Lavender oil (Lavandula angustifolia) (Bulgaria); Peppermint oil (Mentha piperita L.) (Bulgaria). The samples were analysed in triplicate after appropriate dilution in dichloromethane (lavender oil DF=100, rose oil DF=200 and peppermint oil DF=400). GC-MS/MS measurements were performed according to the conditions in section III.1.1.

Based on the chemical properties of the target compounds and the ability to extract a wide range of volatile compounds, a carboxene (CAR)/polydimethylsiloxane (PDMS) fiber with a coating thickness of 85 μm (Supelco, Bellefonte, PA, USA) was used to perform the headspace solid-phase microextraction (HS-SPME). A piece of filter paper with dimensions 0.5 cm x 0.5 cm was placed at the bottom of a 10 mL glass vial, onto which a microliter volume of the analysed essential oil was pipetted (5 μl peppermint oil, 10 μl lavender oil or rose oil), the vial was closed with an aluminium cap with a polytetrafluoroethylene (PTFE) stopper. All studies were performed at a room temperature of 24 ± 2ºC, without stirring. The vials were left for the specified conditioning time: rose oil and lavender oil 45 min; peppermint oil 30 min. After the specified time, the SPME fiber was introduced into the gas space of the vial for a sorption time of 10 min for lavender oil, 5 min for rose and peppermint oil, and then it was injected for 3 min desorption.

III.2.2. Organochlorine and organophosphorus pesticides in water and food samples

Organochlorine and organophosphorus pesticides were selected as analytes: Pentachlorobenzene, Hexachlorobenzene, αlpha-HCH, β-HCH, gamma-HCH, Chlorpyrifos, Chlorpyrifos-methyl, Aldrin, Endrin, Dieldrin, α-Endosulfan, Heptachlor, Heptachlor-endo-epoxide A, o,p-DDD, p,p-DDD, o,p-DDE, p,p-DDE, o,p-DDT and p,p-DDT (GC-MS/MS) and Dichlorvos, Ethoprophos, Disulfoton, Methyl-Parathion, Fenchlorphos and Protheofos (GC-MS). Some target pesticides were investigated in lemon and red apple juice, applying cloud point extraction and a subsequent re-extraction in an organic solvent (MW-CPE-GC-MS/MS). Also, the pesticide content was determined in three brands of bottled spring water, with different physicochemical characteristics (pH 7.6-9.42 and electrical conductivity 62-263 $\mu S\ cm^{-1}$), using dispersive liquid-liquid microextraction based on hydrophobic NADES.

III.2.2.1. Procedure for microwave-assisted cloud point extraction in combination with re-extraction in an organic solvent for the pesticide analysis in fruit juices.

The following procedure was developed (Figure 1): 8 mL of apple juice or 2 mL of lemon juice diluted with 6 mL of water were pipetted into a 12 mL glass tube. 2 mL of 10% (m m^{-1}) aqueous solution of Triton X-100 was added to the samples (0.2 g of $MgSO_4$ was also added to the diluted lemon juice). The extraction systems were placed in a water bath and heated in a microwave system (section III.1.3.) above the cloud point temperature for 30 min (alternatively, tempering can be performed on a hot plate) until two phases were formed: a surfactant-rich phase (lower layer) and an aqueous phase (upper layer). The tubes were left at room temperature, 24 °C ± 2 °C, until cooled (~10 min) and placed in a refrigerator (~30 min) to increase the viscosity of the surfactant-rich phase. The supernatant was removed with a Pasteur pipette. The surfactant-rich phase was placed in a water bath at 55 °C for 1 min to reduce viscosity, then 0.25 mL of hexane was

added, and re-extraction was performed by vortexing for 1 min. 0.1 g of $MgSO_4$ was added to the resulting emulsion, heated in a water bath for 1 min at 55 °C, and vortexed for 1 min. The samples were centrifuged for 1 min at 900 xg, and then an aliquot volume of the supernatant (hexane phase) was pipetted for analysis.

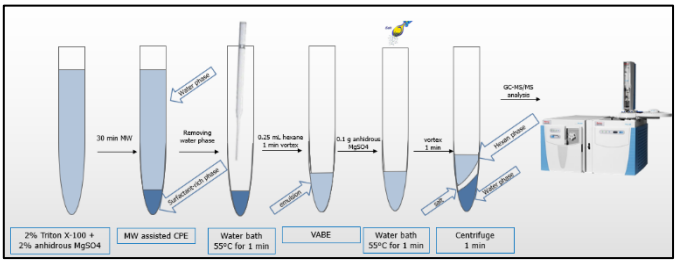


Figure 1: *MW-CPE-GC-MS/MS procedure for pesticide analysis.*

III. 2.2.2. Procedure for dispersive liquid-liquid microextraction based on a hydrophobic natural deep-eutectic solvent for the pesticide analysis in bottled spring waters.

The following procedure was developed (Figure 2): A hydrophobic deep eutectic solvent (NADES) was prepared by mixing compounds of natural origin: 31.26 g DL-menthol (2 mol) and 17.23 g decanoic acid (1 mol). The mixture was heated at 60 °C under an argon atmosphere and stirred, using a magnetic stirrer at 500 rpm, until a clear and homogeneous liquid was formed (about 30 min). 10 mL of the analysed spring water sample was transferred to a 12 mL glass tube, then 45 µL of NADES extractant was added and vortexed for 3 min. The samples were centrifuged at 900 x g for 5 min to separate the organic and aqueous phases. 1 µl of the NADES extract (upper phase) was pipetted using a micro syringe and injected into the GC-MS/MS without further dilution.

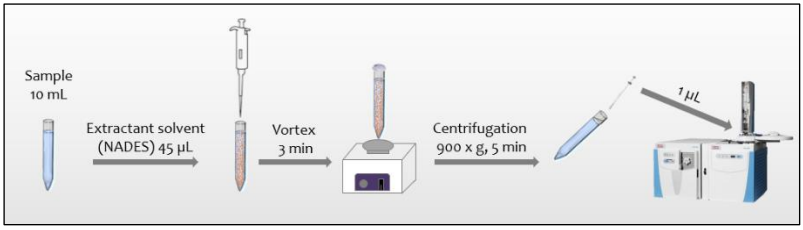


Figure 2: *NADES-based-DLLME-GC-MS/MS procedure for pesticide analysis.*

IV. RESULTS AND DISCUSSION

IV.1. QSRR model for predicting linear retention indices

The present study aimed to create a “green” approach for predicting linear retention indices of volatile compounds in essential oils (for a non-polar stationary phase) by developing a regression model that is fast and easy to apply. We used two techniques for essential oils analysis: i) by injection of liquid samples after dilution with an organic solvent, and ii) by HS-SPME.

IV.1.1. Analysis of diluted samples and calculation of linear retention indices (LRI)

We analysed the diluted essential oils (n=3) by GC- MS/MS, as described in III.1.1. When identifying the compounds, we used the NIST Mass Spectral library (NIST MS Search 2.3) and the following acceptance criteria – a match interval of ± 20 units for RI and values of Match and RMatch factors > 800. We identified 49 components in lavender oil, 51 compounds in rose oil and 32 compounds in peppermint oil, from which we selected 103 unique compounds to create our database.

Under instrumental conditions in section III.1.1., we injected a standard solution of n-alkanes C8-C20 (n=3). To calculate the LRI of the 103 identified compounds, according to the equation of van Den Dool and Kratz (Eq. 1), we used their experimentally obtained retention times (t_R) from the samples (n=3) and the t_R of the n-alkanes from the standard solution C8-C20 (n=3):

$$\mathrm{LRI} = 100\left[n + \frac{t_x - t_n}{t_{n+1} - t_n}\right] \quad \text{(Eq. 1).}$$

IV.1.2. Multiple linear regression for linear retention indices prediction.

We applied a multiple linear regression (MLR) algorithm (SPSS, IBM, USA (trial version)) to establish a statistically significant quantitative relationship between certain physicochemical properties of the molecules - molecular descriptors (independent variables - x) and the chromatographic retention parameter LRI (dependent variable y) to create a mathematical model (QSRR - Quantitative Structure Retention Relationship).

IV.1.2.1. Selection of significant independent variables (descriptors)

We created a database containing 2325 molecular descriptors (1D, 2D and PubChem Fingerprint) for the identified 103 compounds, using the open-source software product PaDEL Descriptor 2.21 and SMILES notations of the compounds. For the initial selection of significant and independent molecular descriptors, we applied stepwise multiple linear regression (Stepwise), applying a criterion for 95% statistical confidence. We found that only 16 molecular descriptors showed a linear relationship (R^2 = 0.9960, Adj. R^2 =0.9951) when modelling the linear retention indices: MLFER_L, MLFER_S, ATSC5c, ATS5i, n 3HeteroRing, n4Ring, n11Ring, GATS3c, PubChem Fingerprint: PubchemFP143, 147, 553, 582, 639, 672, 688 and TIC3. To prove the lack of collinearity of a given variable, we applied the variance inflation factor (VIF). From the obtained VIF values of 1.08-11.37, which were less than 15, we concluded that no significant collinearity was found between the listed variables. It is important to emphasise that the number of significant independent variables was relatively small – only 16 molecular descriptors, which was encouraging given the development of a regression model with a limited number of included variables.

IV.1.2.2. Development and validation of an MLR model

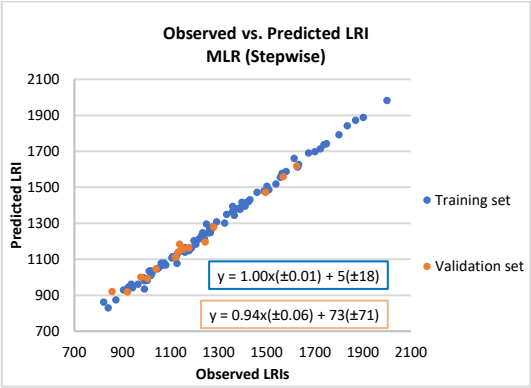
To develop and validate a regression model with the selected descriptors, we randomly divided the set of 103 compounds into a training set - 85% of the compounds (n=87) and a validation set - the remaining 15% of the compounds (n=16). We applied a Stepwise MLR algorithm to the training set. The coefficients of the developed regression model and their statistical evaluation are presented in Table 1, respectively.

We found that building a regression model using Stepwise regression using only the training set of compounds reduced the number of significant variables in the equation from 16 to 14 due to the exclusion of the descriptors PubChemFP 553 and TIC3 (p-value > 0.05). We assessed the collinearity between the molecular descriptors by calculating the corresponding variance inflation factors. Since, in all cases, they occupied values less than 15, we concluded that no collinearity was observed. We defined a range of LRI predictions from 833 to 1993. To assess the adequacy of the regression model, we calculated R^2 = 0.9958, Adj. R^2 = 0.9949 and RMSE = 17. The obtained values of R^2 and Adj. R^2, as well as the calculated low value of RMSE, gave us a reason to believe that the developed regression model is adequate.

We applied the mathematical model to the validation set (16 compounds) and assessed the predictive ability by calculating $RMSE_{val}$ = 26 and q^2_{F1} = 0.9886. Taking into account the acceptably low values of $RMSE_{val}$, in combination with values of q^2_{F1} close to unity, we considered the developed MLR model to be correct in predicting the LRI of compounds that were not used to build the regression function.

Table 1: *Regression model coefficients and their statistical estimation obtained by Stepwise regression (training set, n=87).*

Descriptor	Coefficient	SD	p-value	VIF
Intercept	226	19	<0.001	
MLFER_L	179	3	<0.001	3.73
MLFER_S	126	12	<0.001	2.27
PubchemFP639	-276	29	<0.001	2.21
ATSC5c	-205	67	0.003	1.60
PubchemFP672	52	9	<0.001	1.61
PubchemFP582	-53	12	<0.001	1.10
n4Ring	-58	9	<0.001	1.29
n3HeteroRing	101	21	<0.001	2.41
GATS3c	-40	8	<0.001	1.28
PubchemFP147	-58	20	0.006	1.10
ATS5i	0.004	0.001	<0.001	5.06
PubchemFP143	-29	7	<0.001	1.53
PubchemFP688	-19	5	0.01	1.45
n11Ring	-70	20	0.001	1.09



We further confirmed this fact by analysing the graphs representing the dependence between the experimentally determined (observed) versus predicted linear retention indices (Figure 3). We found that the resulting slope is statistically identical to unity, and the intercept is practically equal to zero, both for the training sample and for the validation set of compounds.

Figure 3*: Distribution plot of the observed versus predicted values of the dependent variable LRI using stepwise regression (uncertainty of slope and intercept is presented as a 95% confidence interval).*

IV.1.2.3. Testing an LRI prediction model with an external set

As a next step, we decided to test the validated MLR model using an external set of compounds. We analysed the essential oils using the experimentally established optimal conditions for HS-SPME (Section III.2.1.). As a result, we identified a sum of 19 additional compounds, probably due to this analysis approach's lower methodological detection limits. For these 19 compounds, we determined the molecular descriptor values using PaDEL Descriptor 2.21. We selected only 12 of the 19 identified compounds as the test set since their molecular descriptors fell within the range of descriptor values determined by the training set. We applied the model equation for predicting LRI (Eq. 2) to the test set, using the values of their molecular descriptors.

$LRI_{predicted}$ *= 226 + (179 x MLFER_L) + (126 x MLFER_S) + (-276 * PubchemFP639) + (-205 x ATSC5c) + (52 x PubchemFP672) + (-53 x PubchemFP582) + (-58 x n4Ring) + (101 x n3HeteroRing) + (-40 x GATS3c) + (-58 x PubchemFP147) + (0.004 x ATS5i) + (-29 x PubchemFP143) + (-19 x PubchemFP688) + (-70 x n11Ring) (Eq. 2).*

From the differences between the observed and predicted LRI values, we found that for 8 of the compounds, the deviations were within ± 20 of the experimentally determined LRI, while for only 4 of the compounds, they were outside this range. Additionally, we assessed the ability of the model to predict LRI for external compounds using the following metrics: RMSE = 40, q^2_{F2} = 0.9521. The obtained RMSE value for the test set of compounds was larger than that determined at the validation stage, but remained relatively low and can be considered acceptable. Despite using a relatively simple linear regression model, including only 14 molecular descriptors, the calculated value for q^2_{F2} was close to 1, which indicates the adequacy of the proposed regression model.

IV.2. Methods for quantitative analysis of pesticides

To achieve the second goal of developing "green" methods for quantitative GC-MS analysis of pesticides, we focused efforts on both replacing or minimising the amounts of organic solvents used and shortening the time for preliminary sample preparation. Taking these two factors into account, we decided to study the application of two approaches for preliminary separation and concentration, namely cloud point extraction (CPE) and dispersive liquid-liquid microextraction DLLME. Considering that the extraction procedures will be combined with GC-MS/MS, the first step in our study was to develop a chromatographic method and to define the parameters of the MS/MS system.

IV.2.1. Optimization of instrumental parameters for organochlorine and organophosphorus pesticides analysis by GC-MS/MS

When developing a GC-MS/MS method for analysis, we selected representatives of organochlorine and organophosphorus pesticides, some of which are classified as persistent organic pollutants: alpha-HCH, beta-HCH, gamma-HCH, Pentachlorobenzene, Hexachlorobenzene, Chlorpyrifos, Chlorpyrifos-methyl, Aldrin, Endrin, Dieldrin, alpha-Endosulfan, Heptachlor, Heptachlor-endo-epoxide A, o,p-DDD, p,p-DDD, o,p-DDE, p,p-DDE, o,p-DDT and p,p-DDT to optimise the chromatographic conditions. As the scan mode of the mass spectrometer, we chose selected reaction monitoring (SRM) since registration using this approach allows for achieving a high S/N ratio. We selected two transitions for each analyte, one for qualitative identification (Qual transition) and one necessary for quantitative determination (Quant transition). We optimised the parameters of the PTV injector, aiming to create a temperature gradient program that would allow rapid and consistent evaporation of the analytes after that, we varied the column oven temperature increments to obtain adequate chromatographic separation and the shortest analysis time. After optimising the temperature programs of the injector and column oven, we evaluated the influence of the carrier gas flow rate on the chromatographic separation. We varied the carrier gas flow rate at 1.0, 1.2 and 1.4 mL min^{-1}. We found that changing the flow rate did not significantly affect the retention times of the analytes and the total chromatography time. We tried to group the SRM transitions of the pesticides into different scan time ranges, considering the pesticide retention times, the width of the chromatographic peaks and the scan time. The performed optimisation allowed us to propose the instrumental conditions specified in section III.1.1.

IV.2.2. Microwave-assisted cloud point extraction in combination with re-extraction in an organic solvent

The present study investigates the possibility of adapting CPE to GC-MS or GC-MS/MS analysis of pesticides.

IV.2.2.1. Selection of surfactant for CPE pre-concentration and organic solvent for re-extraction

Due to the expected negative effect of surfactants on the GC system, we performed a target analytes re-extraction in a small volume of organic solvent compatible with the GC system. We considered it appropriate to select a surfactant (for CPE) and an organic solvent (for re-extraction) that have a low affinity for each other, as a result of which the final phase injected into the GC would contain the lowest possible amount of surfactant.

In our study, we evaluated the solubility of the most commonly used surfactants for cloud point extraction (Triton X-100 and Triton X-114) in organic solvents - hexane, isooctane, cyclohexane and ethyl acetate. We prepared 2% (m m ^{1}) model aqueous solutions of Triton X-100 and Triton X-114 with a volume of 10 mL in glass tubes. The solutions were placed in a water bath until reaching a temperature (90 ºC) above the cloud point temperature of Triton X-100 (64 ºC) and Triton X-114 (23 ºC), then heated for 30 min on a hot plate until two phases were formed: a surfactant-rich phase (lower layer) and an aqueous phase (upper layer). We cooled the tubes to room temperature, 24 °C ± 2 °C for ~10 min, then placed them in a refrigerator (4 ºC) for ~30 min to increase the viscosity of the surfactant-rich phase and removed the supernatant with a Pasteur pipette. To the surfactant-rich phase (~1mL), we added 2 mL of water to reduce the viscosity and obtain a pseudo-homogeneous solution, then added 2 mL of organic solvent (one of hexane, isooctane, cyclohexane or ethyl acetate) for re-extraction. Re-extraction was performed using vortex agitation for 10 min, centrifuged for 15 min at 900 x g, and the extraction systems were placed in a freezer at -22 ºC for 12 h to facilitate separation of the two phases. An aliquot volume of the upper organic phase was diluted (DF=12.5) and subjected to UV-VIS analysis under the conditions specified in section III.1.3. Using standard surfactant solutions (Triton X-114 or Triton X-100) with a concentration of 0.025 and 0.05%, we found that the maximum absorption in aqueous medium of both surfactants is at λ_{max}=276 nm. Therefore, the quantitative analysis of the co-extracted Triton X-100 or Triton X-114 in the studied solvents was performed by measuring the absorption at 276 nm relative to the corresponding organic solvents as a blank.

From the results obtained, we found that the content of co-extracted Triton X-100 in each organic solvent was significantly lower than the corresponding concentration of Triton X-114. Meanwhile, the level of Triton X-100 in hexane and isooctane was statistically identical and the lowest compared to the content in the other organic solvents 0.09% m m^{-1}. For this reason, we decided to use the combination of Triton X-100 and hexane or isooctane for the following studies.

IV.2.2.2 Evaluation of the compatibility of GC-MS/MS to solutions containing Triton X-100

We conducted step-by-step studies to evaluate the compatibility of the instrumentation with Triton X-100 as a matrix component and its effects on the chromatographic system.

As a first step, we evaluated the selectivity of the GC-MS/MS method by injecting solvent hexane and a matrix-matched blank (0.09% m m^{-1} Triton X-100 in hexane) in Full scan mode in a mass range of 20-700 amu (point III.1.1.). The comparison of the total ion current (TIC) chromatograms, shows that the two chromatograms were identical and when injecting the matrix-matched blank, no peaks due to Triton X-100 were detected under the chromatographic conditions used. Therefore, it can be assumed that i) due to the used PTV temperature program, Triton X-100 remains in the liner, or ii) the amount of surfactant passing through the chromatographic column is too low due to the split ratio used during injection.

We conducted an additional study of the selectivity in the SRM mode (section III.1.1.) by analysing a solution of a matrix-matched and spiked with 100 µg L^{-1} of the target analytes. When comparing the reconstructed total ion current (RTIC) chromatograms of the two samples, we found that for each of the transitions of the target analytes, no isobaric interference caused by Triton X-100 was observed. This fact indicates that even if we assume that a certain amount of surfactant enters the GC column, it does not deteriorate the selectivity of the measurements in the SRM mode. It should be noted that both beta-HCH and gamma-HCH, p,p-DDD and o,p-DDT, are coeluted since they have very close retention times, and their SRM transitions are identical. For these reasons, beta-HCH and gamma-HCH, and p,p-DDD and o,p-DDT, respectively, were analysed as the sum of their signals.

For the first time, in the present study, we investigated the stability of the GC-MS/MS system when injecting the final organic phase without further purification of the co-extracted Triton X-100. Despite the absence of isobaric interference, we aimed to identify any negative effect caused by the prolonged injection of the surfactant as a matrix component of the sample. We performed the study by conditioning the GC system

by pre-injecting three replicates of the matrix-matched blank, followed by 20 consecutive injections of a solution containing 100 μg L^{-1} pesticides in a matrix-matched blank. We assessed the precision of the recorded retention times and signals of the target analytes by measuring in SRM mode (section III.1.1.), using a glass liner and alternatively using a metal liner. From the analysis of the obtained retention times (t_R) and their standard deviations (t_R SD), we found that repeatable retention times were observed (all standard deviations (t_R SD) were less than 0.01 min). We also found that the retention times did not shift compared to those recorded when standard solutions were injected into pure hexane. The latter can be explained by the assumption that no or only a negligible amount of the surfactant passes to the GC column, the stationary phase of which remains unchanged in properties.

Regarding the precision of the recorded signals, we found that when using a glass liner for 20 consecutive injections, most of the calculated RSD% were below 2.5%, with the highest value being 5%. When a metal liner was used, the RSD% values for 20 consecutive injections were relatively higher (6 – 9%), but if the RSD% is calculated on replicates 11 to 20, we observed a significant improvement in precision. This can also be seen visually from the graphs in Figure 5.

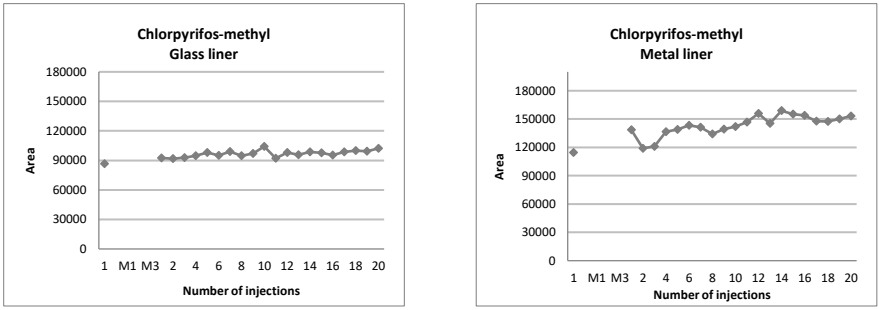


Figure 5: *Chlorpyrifos-methyl signals in sequential injections of a standard solution into a matrix-matched (n=20) using a glass or metal liner.*

It can be summarised that during sequential injection of samples containing 0.09% Triton X-100 in hexane, the introduced amount of surfactant is vented during injection by the split flow and/or is retained on the inner surface of the liner, practically not passing through the GC column. We found that three replicate injections of a blank were sufficient to condition the GC system when using a glass liner, while when using a metal liner, a higher number of injections were required to achieve satisfactory precision. This would not be necessary when analysing solutions containing Triton X-100 over a long period.

IV.2.2.3. Assessment of the non-spectral matrix effect of Triton X-100 - GC-MS/MS and GC-MS

To our knowledge, no prior study has investigated the non-spectral matrix effects in GC-MS or GC-MS/MS analysis caused by Triton X-100 as a co-extracted compound after CPE. To assess the influence of Triton X-100 on the instrumental sensitivity in GC-MS/MS analysis, we compared the slopes of the calibration curves (Eq. 3) obtained by measuring in triplicate a series of 5 standard solutions (with concentrations up to 15 μg L^{-1}) prepared in hexane as well as in a matrix-matched medium. The weighted regression method was used to derive the calibration equation, using $1/c^2$ as the weighting factor, where "c" is the concentration of the respective standard. The measurements were performed alternatively using a glass and a metal liner.

$$Matrix\ factor\ (slope\ ratio) = \frac{Area\ or\ slope\ (matrix)}{Area\ or\ slope\ (solvent)} \qquad \text{(Eq.3)}$$

From the slope ratio results obtained and presented in Table 2, we found that the achieved sensitivity for most of the target analytes using a glass liner was statistically identical in both investigated media – pure

hexane and matrix-matched. For some compounds (Chlorpyrifos, Endrin, p,p-DDE, o,p-DDD, p,p-DDD and o,p-DDT, p,p-DDT), we observed a slight increase (~10%) in the recorded sensitivity, and only for Dieldrin we found a 10% decrease in the slope ratio. When using a metal liner for all target analytes, we found different results, the slopes of the calibration curves increased 1.26 – 2.30 times in matrix-matched media compared to solutions in pure hexane (Table 2). We further estimated the standard deviations of the slopes and the propagated standard deviation of the corresponding slope ratios (Eq. 3), noting that the standard deviations of the slope ratios were larger when a metal liner was used. We hypothesised that the observed increase in analyte signals in the presence of Triton X-100 was due to processes localised in the liner. It is likely that during the evaporation step in the injector, Triton X-100 is deposited on the inner surface of the liner, reducing thermal stress and exerting a passivating effect while the target analytes pass through the GC liner. The effect as an analytes "protectant" is especially enhanced when polar analytes and those with relatively high boiling points pass through the metal liner.

Table 2: *Comparison of the slope ratios of the calibration curves (matrix-matched/hexane) obtained by weighted regression, measured by GC-MS/MS using a glass or metal liner.*

	Glass liner	***Metal liner***
Analyte	***Slope ratio ± combined uncertainty[a]***	***Slope ratio ± combined uncertainty[a]***
Pentachlorobenzene	0.97 ± 0.02	1.27 ± 0.07
alpha-HCH	0.96 ± 0.01	1.26 ± 0.06
Hexachlorobenzene	0.96 ± 0.03	1.43 ± 0.08
beta and gamma-HCH	0.95 ± 0.01	1.31 ± 0.07
Chlorpyrifos methyl	1.02 ± 0.06	2.03 ± 0.13
Heptachlor	1.03 ± 0.02	1.66 ± 0.14
Aldrin	0.96 ± 0.02	1.61 ± 0.11
Chlorpyrifos	1.12 ± 0.02	2.11 ± 0.15
Heptachlor-endo-epoxide A	0.97 ± 0.02	1.65 ± 0.16
o,p-DDE	0.98 ± 0.02	1.67 ± 0.11
alpha-Endosulfan	0.99 ± 0.02	1.64 ± 0.12
Dieldrin	0.90 ± 0.04	1.78 ± 0.13
p,p-DDE	1.14 ± 0.04	1.86 ± 0.12
o,p-DDD	1.14 ± 0.03	1.92 ± 0.14
Endrin	1.08 ± 0.03	1.95 ± 0.31
p,p-DDD and o,p-DDT	1.12 ± 0.04	2.30 ± 0.17
p,p-DDT	1.10 ± 0.03	1.64 ± 0.21

[a] The combined uncertainty was estimated by the propagation law using Eq. 3.

From Tables 3 and 4, summarising the analytical figures of merit by weighted regression for both types of dissolution media, it is striking that the obtained values for the coefficient of determination (R^2) do not deteriorate in the presence of 0.09% (m m^{-1}) Triton X-100, which is valid for both liners used. The presented limits of quantification (LOQs) were calculated based on the 10s criterion, as a standard deviation, we took the value of the standard deviation of the intercept of the regression line.

Table 3 shows that the performance of the GC-MS/MS system using a glass liner is not significantly affected by the presence of Triton X-100. In contrast, the performance with a metal liner (Table 4), the LOQs for part of the analytes decrease in the presence of surfactant using a metal liner.

Table 3: *Analytical figures of merit of GC-MS/MS (glass liner) obtained by weighted regression using hexane standard solutions or matrix-matched standard solutions.*

	Hexane			Matrix-matched		
Analyte	Slope (Area L μg^{-1})	R^2	LOQ ($\mu g\ L^{-1}$)	Slope (Area L μg^{-1})	R^2	LOQ ($\mu g\ L^{-1}$)
Pentachlorobenzene	4847	1.00	0.06	4685	1.00	0.15
alpha-HCH	6177	1.00	0.11	5937	1.00	0.19
Hexachlorobenzene	8055	0.99	0.12	7703	1.00	0.17
beta and gamma-HCH	5665	1.00	0.06	5399	1.00	0.10
Chlorpyrifos methyl	1258	0.98	3.09	1288	0.96	0.88
Heptachlor	2707	1.00	0.18	2785	1.00	0.20
Aldrin	1413	1.00	0.27	1354	1.00	0.24
Chlorpyrifos	4340	1.00	0.83	4846	1.00	0.33
Heptachlor-endo-epoxide A	632	0.99	0.31	616	1.00	0.23
o,p-DDE	9260	0.99	0.41	9095	1.00	0.15
alpha-Endosulfan	1269	1.00	0.25	1256	1.00	0.26
Dieldrin	606	0.98	0.68	548	0.99	0.36
p,p-DDE	7620	0.99	0.32	8684	1.00	0.25
o,p-DDD	17928	1.00	0.16	20430	0.99	0.13
Endrin	731	0.99	0.36	788	0.99	0.30
p,p-DDD and o,p-DDT	22054	0.99	0.52	24606	1.00	0.65
p,p-DDT	2924	1.00	0.25	3221	1.00	0.33

Conditions: 5-point calibration in the range of up to 15 µg L^{-1}. Each calibration level was injected in triplicate.

Table 4*: Analytical figures of merit of GC-MS/MS (metal liner) obtained by weighted regression for hexane or matrix-matched calibration.*

	Hexane			Matrix-matched		
Analyte	Slope (Area L μg^{-1})	R^2	LOQ ($\mu g\ L^{-1}$)	Slope (Area L μg^{-1})	R^2	LOQ ($\mu g\ L^{-1}$)
Pentachlorobenzene	2826	0.96	0.26	3578	0.98	0.18
alpha-HCH	3064	0.99	0.64	3857	0.98	0.17
Hexachlorobenzene	4470	0.96	0.28	6385	0.99	0.15
beta and gamma-HCH	5066	0.98	0.18	6615	0.98	0.20
Chlorpyrifos methyl	621	0.95	1.18	1261	0.98	0.68
Heptachlor	1456	0.85	0.35	2414	0.96	0.28
Aldrin	719	0.97	2.82	1154	0.98	0.71
Chlorpyrifos	1826	0.96	1.79	3856	0.98	0.19
Heptachlor-endo-epoxide A	269	0.96	0.77	444	0.97	1.41
o,p-DDE	4391	0.95	0.32	7340	0.99	0.14
alpha-Endosulfan	603	0.94	0.34	991	0.99	0.62
Dieldrin	298	0.98	5.23	529	0.98	5.39
p,p-DDE	3811	0.95	0.31	7098	0.99	0.58
o,p-DDD	7829	0.95	0.66	15035	0.99	0.14
Endrin	422	0.87	9.37	825	0.98	4.08
p,p-DDD and o,p-DDT	8990	0.95	1.99	20635	0.98	0.19
p,p-DDT	1316	0.91	6.82	2156	0.98	0.41

Conditions: 5-point calibration in the range of up to 15 µg L^{-1}. Each calibration level was injected in triplicate.

Despite the significant increase in sensitivity achieved with a metal liner, when comparing the slopes of the matrix-matched calibration curves, it is striking that the sensitivity with the glass liner is higher. When comparing the LOQ, it is also evident that when using a metal liner (Table 4) for one part of the analytes, the values in the matrix deteriorated, while for another, they remained unchanged. All this led us to the conclusion that the glass liner is preferable for usage.

We investigated the transferability of the positive matrix effect by a comparative study in SIM mode using a GC-MS system (Shimadzu 2010 SE), with the instrumental parameters specified in section III.1.2. For the studies, we selected a set of 7 target analytes, a glass liner and an alternative organic solvent isooctane. We compared the slopes of the calibration curves (Eq. 3) and their propagated standard deviations, obtained by measuring in a triplicate series of 5 standard solutions (with concentrations up to 1000 μg L^{-1}) prepared in pure isooctane, as well as in a matrix-matched medium (0.09% m m^{-1} Triton X-100 in isooctane). We derived the regression models by applying weighted regression, again using $1/c^2$ as a weighting factor. We found that the sensitivity increased 1.26–1.57 times when calibrated in a matrix, and the analytical performance of the GC-MS system is not significantly affected by the Triton X-100. The obtained results further demonstrate that CPE with Triton X-100 and re-extraction in organic solvents, such as hexane or isooctane, can be combined with GC-MS or GC-MS/MS analysis by injection without an additional clean-up or dilution step. The latter motivates us to continue our research in this direction.

IV.2.2.4. Optimisation of cloud point extraction in combination with re-extraction in organic solvent

In the development of a new analytical method applying CPE for preconcentration of organochlorine and organophosphorus pesticides, as a next step, we conducted a series of experiments to optimise the procedure. Both a one-factor-at-a-time and a factorial (central composite design – CCD) optimisation approach were used in the optimisation study. We were inspired to use the second approach because it allows us to reduce the number of experiments and the consumption of samples, reagents and energy.

IV.2.2.4.1 Optimization of the extraction step

At the beginning of the studies, we used a hot plate to introduce energy into the extraction systems, according to the procedure described in point IV.2.2.1. We studied the influence of the initial concentration of Triton X-100 in the aqueous phase and the influence of pH on the extraction efficiency of the target analytes. In the procedure (point IV.2.2.1.), to the surfactant-rich phase (~1 mL), we added 2 mL of water to reduce the viscosity and obtain a pseudo-homogeneous solution, after which we added 2 mL of hexane. Re-extraction was performed using vortex agitation for 10 min. Then, the extraction systems were centrifuged for 15 min at 900 x g and placed in a freezer for phase separation.

Regarding the influence of the initial concentration of Triton X-100 on the migration of pesticides in the surfactant-rich phase, it can be concluded that the registered signals for the studied analytes increase steeply with an increase in the surfactant content to 1.5% m m^{-1}. For most pesticides, a smooth increase in the generated signals follows with an increase in the Triton X-100 concentration to 3% m m^{-1}. Since obtaining a more voluminous surfactant-rich phase led to problems in the re-extraction step, as a compromise solution, in the following experiments, we chose to work with an initial Triton X-100 concentration of 2% (m m^{-1}). Part of the hexane was residually included in the form of an emulsion in the surfactant-rich phase, and neither the extension of the centrifugation time (up to 30 min) nor the increase in the centrifugal force (up to 6000 xg) destroyed the resulting emulsion. For this reason, it was necessary to place the resulting emulsion systems in a freezer at -22 ºC for 12 hours for complete phase separation. We found that the factors favouring the formation of a stable emulsion were increasing the amount of Triton X-100 in the surfactant-rich phase and reducing the volume of hexane used as a re-extractant. Due to our desire to increase the "green" nature of the procedure we developed by miniaturising the volume of the organic solvent used, we considered that using an initial concentration of Triton X-100 above 2% (m m^{-1}) would prevent the achievement of this goal in subsequent experiments.

We found that for most of the tested pesticides, the variation of the pH of the aqueous phase, in the range from 3 to 11, did not affect the extraction efficiency. Only for alpha-HCH, beta and gamma-HCH, Chlorpyrifos-methyl and p,p-DDT, a decrease in the extraction rate was observed at pH above 9, but the extracted amount of pesticides in the surfactant-rich phase was practically identical in the pH range 3 – 9. Therefore, from the point of view of the group analysis of all target analytes, we concluded that it is not necessary to adjust the pH of the medium when the native pH of the tested sample is greater than 3 and less than 9.

According to Equation 4, we calculated the achieved analytical yields, applying the CPE procedure and re-extraction in hexane, described in point IV.2.2.1.

$$ER_i\ \% = \frac{Q_{final,i}}{Q_{initial,i}}\ x\ 100, \qquad \text{(Eq.4)}$$

Q initial,i - the amount of i-th target analyte in the initial aqueous solution;
Q final,i - the amount of i-th analyte that passed into the final organic phase.

It can be concluded that the achieved analytical yields for all target analytes are relatively high in the range of 66 to 105% and are characterised by good repeatability of 3 - 12% (SD). However, significant disadvantages of the procedure include the use of a low-efficiency source of energy (hot plate) to conduct the CPE, the long time for re-extraction (over 12 hours), and the relatively large volume (2 mL) of hexane used for re-extraction. This gave us a reason to conduct further experiments to improve the mentioned characteristics of CPE.

IV.2.2.4.2. Microwave-assisted cloud point extraction (MW-CPE).

To increase the efficiency of energy transfer to the extraction systems, we studied the possibility of replacing the hot plate with irradiation by microwave radiation. We studied the influence of microwave incubation time through a one-factor experiment at microwave treatment times of 10, 20 and 30 min. For this purpose, we used a microwave system with a carousel, in which six external vessels filled with ballast water (~80 mL) were placed so that the glass tubes containing the model aqueous solutions were immersed in the water bath. We tempered the microwave system according to a previously optimised and described in point III.1.3. temperature program. After the separation of the surfactant-rich phase, we proceeded to re-extraction in hexane and subsequent analysis by GC-MS/MS. From the review of the data presented in Figure 6, we found that for most of the analytes, the analytical yields obtained were above 80% using MW treatment times of 20 to 30 min. Only for alpha-HCH and beta and gamma-HCH, the analytical yields were between 60 and 80%.

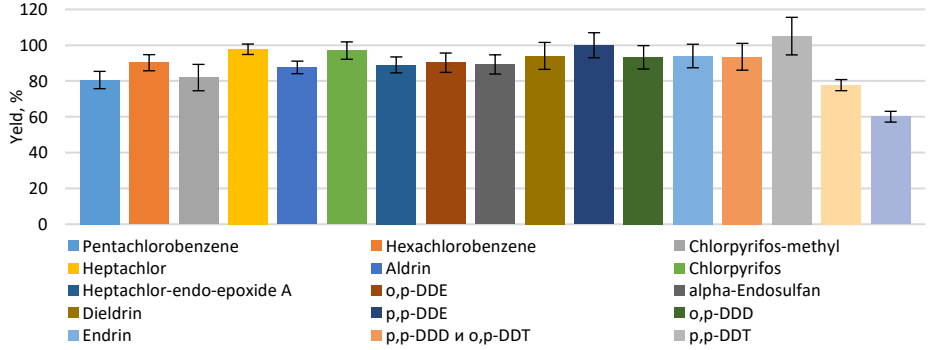


Figure 6: *Analytical yields at MW time of 30 min, accompanied by the corresponding SD (%).*
Conditions*: CPE – concentration 2% (m m^{-1}) Triton X-100, initial pesticide concentration 50 µg L^{-1}; re-extraction with added 2 mL water and 2 mL hexane, 10 min vortex agitation, 15 min centrifugation.*

The analytical yields obtained were comparable to or higher than those obtained with heating on a hot plate, but the energy consumption was reduced by more than quadruple (17 W/sample with MW-CPE versus 70 W/sample with conventional CPE). The procedure time was significantly shortened, as only 4 min were required to heat a 12 x 80 ml water bath. For this reason, the following CPE experiments we performed using microwave heating.

IV.2.2.4.3. Salting out effect

In the present study, we investigated the effect of added salts on the efficiency of CPE by carrying out the procedure in the presence of $MgSO_4$ at levels of 1%, 2%, 3%, 4%, 5% or 10% (m v^{-1}). From the obtained results, it can be concluded that for all analytes, the signals increase sharply with an increase in the salt concentration to 2% (m v^{-1}), after which only for some pesticides, a very slight increase in the signals is observed when the $MgSO_4$ content is graded to 5% (m v^{-1}). The gravitational sedimentation of the surfactant-rich phase is strongly hindered at a content of 10% $MgSO_4$ (m v^{-1}), as a phase with a flocculent structure and diffuse boundaries separates at the bottom of the reaction tube. In subsequent experiments, we decided to add $MgSO_4$ to the extraction systems at a level of 2% (m v^{-1}) for three reasons: i) at higher concentrations of $MgSO_4$, a diffuse boundary was observed between the separated surfactant-rich phase and the aqueous medium, most likely due to an increase in the density of the aqueous medium, ii) no or a relatively small increase in the analyte signals was observed when working with concentrations above 2% (m v^{-1}) and iii) to reduce the number of reagents used when conducting CPE.

IV.2.2.4.4. Optimization of the re-extraction step.

We found that the isolated surfactant-rich phase obtained after gravitational sedimentation and pipetting of the aqueous phase was still of considerable viscosity and was difficult to flow, which led to problems in mass transfer during the re-extraction step. For this reason, we decided to test two approaches to reduce the viscosity of the surfactant-rich phase: i) dilution with water in combination with different agitation approaches for dispersing the organic solvent in the surfactant-rich phase – ultrasonic treatment (ultrasound-assisted back-extraction - USABE) or vortex agitation (vortex-assisted back-extraction - VABE) or ii) heating the surfactant-rich phase before re-extraction.

To investigate the effect of adding water to the surfactant-rich phase in combination with ultrasonic or vortex agitation, we created two central composite designs using the statistical software Statistica 14.1.0.8 (TIBCO Statistica, USA), one for each agitation method. We varied the volume of added water and the re-extraction time, with the volume of hexane for re-extraction fixed at 2 mL. When conducting CPE, we used hot plate tempering, and the remaining experimental parameters were set as specified in point III.2.2.1. After re-extraction, 1µL of the organic phase was analysed by GC-MS/MS according to the optimised instrumental conditions (point III.1.1.). For all analytes studied, we observed similar behaviour both when using ultrasonic treatment and vortex agitation, and Figure 7 presents the results obtained for Pentachlorobenzene.

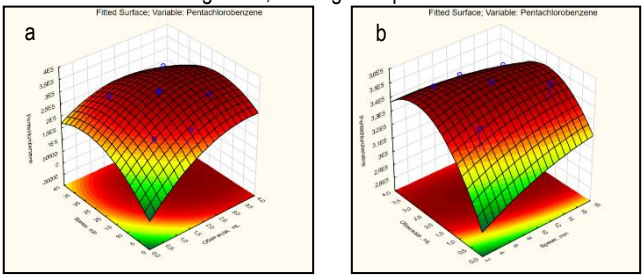


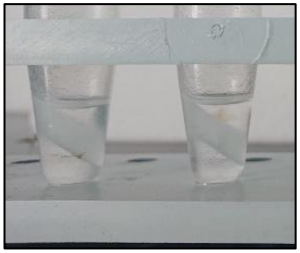
Figure 7: *The signals for Pentachlorobenzene depending on the amount of water added to the surfactant-rich phase and the re-extraction time by a) USABE and b) VABE.*
Conditions*: CPE – 2% (m m^{-1}) Triton X-100, initial pesticide concentration 25 µg L^{-1}, incubation time 30 min; re-extraction with added 2 mL hexane, 15 min centrifugation.*

Dilution of the surfactant-rich phase leads to an increase in the efficiency of re-extraction in both approaches for dispersing the organic solvent, the effect becoming significant when adding at least 1 mL of water. Regarding the time required for dispersing the organic solvent in the surfactant-rich phase, it can be concluded that with USABE, a minimum of 20 min is needed, while with VABE, this time is reduced by a factor of two to 10 min. From the point of view of reducing the extraction time and increasing the "green" character of CPE, in the following experiments at the re-extraction stage, we applied agitation by vortex.

Despite the beneficial effect of the added water on the mass transfer efficiency, prolonged cooling (~12 hours) in a freezer was still required to physically separate the hexane from the surfactant-rich phase. Therefore, our plan to minimise the volume of the organic solvent would further complicate the phase separation after re-extraction. For this reason, we decided to investigate the possibility of reducing the viscosity of the surfactant-rich phase by another approach without the need for dilution, namely by heating. We adjusted the heating temperature so that it did not exceed the coagulation temperature of Triton X-100 while at the same time being as high as possible. We found that tempering for 1 min at 55 °C, immediately before the introduction of hexane, led to a decrease in the viscosity of the surfactant-rich phase, as a result of which the results obtained were comparable to those obtained when diluting the surfactant-rich phase with 2 mL of water. Additionally, due to the reduced viscosity of the surfactant-rich phase, we achieved a reduction in the time for re-extraction by vortexing to 1 min. In subsequent experiments, in the re-extraction step, the surfactant-rich phase was tempered for 1 min at 55 °C and subjected to re-extraction with 2 mL of hexane by vortexing for 1 min.

IV.2.2.4.5. Minimisation of the organic solvent volume for re-extraction.

Adhering to the principles of "green" analytical chemistry, we investigated the possibilities of minimising the volume of the organic solvent used in the re-extraction step. To achieve this goal, we conducted a one-factor experiment in which we varied the volume of hexane used at levels of 0.25 mL, 0.5 mL, 1.0 mL and 2.0 mL. We found that despite the reduced viscosity of the surfactant-rich phase, at volumes smaller than 1.0 mL, a stable emulsion is formed, which can be destroyed only by prolonged cooling in a freezer (over 12 hours). This motivated us to look for an alternative approach to break the emulsion. For this purpose, we studied the influence of the salting-out effect upon the addition of 0.05 g, 0.1 g, and 0.2 g anhydrous $MgSO_4$, and to assist the migration of $MgSO_4$ into the emulsified extraction system, we again applied tempering for 1 min at 55 °C, followed by 1 min vortex agitation. Then, the extraction systems were centrifuged at 900 xg for 1 min. From the experiments conducted, we found that the addition of 0.1 g of the salt led to the formation of a separated upper phase of hexane, even when the volume of the organic solvent was only 0.25 mL (Figure 8). A probable reason for this was that the introduction of an excess of solid salt ($MgSO_4$) to the emulsified extraction system led to the formation of a saturated solution of the salt in the surfactant-rich phase, as a result of which the polarity of the water included in it increases. It can be assumed that from a thermodynamic point of view, this leads to enlargement and aggregation of the dispersed microdroplets of hexane into a separate phase.



The formation of an intermediate layer of solid undissolved $MgSO_4$ (Figure 8) further facilitated the pipetting of an aliquot volume from the hexane phase for subsequent analysis. It is important to note that as a result of the optimisation carried out, the step of cooling the samples in a freezer was not necessary.

Figure 8: *Surfactant-rich phase of model aqueous solutions of 2% Triton X-100, with added 0.1 g anhydrous $MgSO_4$ and 0.25 mL hexane for re-extraction: after tempering at 55 °C for 1 min, vortexing for 1 min and after centrifugation of the samples.*

To assess the repeatability of the procedure, we developed model solutions according to the optimised CPE and re-extraction parameters, using 0.5 mL and 0.25 mL hexane for re-extraction in parallel. From the analytical yield results presented in Table 5, it can be seen that when using 0.25 mL hexane, the analytical yields achieved decrease, and their repeatability significantly deteriorates compared to those when using 0.5 mL hexane. For 0.5 mL hexane, most of the determined relative standard deviations fell in the range of 2–5%, while the corresponding values for 0.25 mL hexane were in the range of 9 – 14%. Despite the twofold higher concentration factor when using 0.25 mL hexane, the LOQ did not decrease by a factor of two due to the lower analytical yields achieved. In the case where the analysed concentrations are low (e.g. close to or below the limits of determination), despite the reduced precision, it is more appropriate to work with a higher concentration factor due to the chance of improving the detection capabilities of the method. Given the expected relatively low concentrations of pesticides in most analysed samples (e.g. water, food, beverages, etc.), we recommend using 0.25 mL of hexane at the re-extraction stage.

Table 5: *Analytical figures of merit of the developed MW-CPE-GC-MS/MS method.*

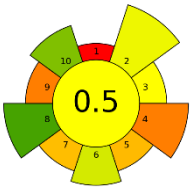
	0.5 mL hexane			***0.25 mL hexane***		
Analyte	***ER (%)***	***RSD (%)***	***LOQ (µg L^{-1})***	***ER (%)***	***RSD (%)***	***LOQ (µg L^{-1})***
Pentachlorobenzene	87	9	0.009	81	9	0.005
alpha-HCH	56	4	0.017	41	8	0.012
Hexachlorobenzene	92	5	0.009	82	13	0.005
beta-HCH and gamma-HCH	46	2	0.011	38	7	0.007
Chlorpyrifos-methyl	66	2	0.067	47	11	0.047
Heptachlor	89	2	0.011	74	14	0.007
Aldrin	94	2	0.013	83	14	0.007
Chlorpyrifos	86	2	0.019	67	13	0.012
Heptachlor-endo-epoxide A	66	5	0.018	47	10	0.012
o,p-DDE	81	3	0.009	64	13	0.006
alpha-Endosulfan	83	2	0.016	66	13	0.010
Dieldrin	74	3	0.024	52	12	0.017
p,p-DDE	92	2	0.014	74	14	0.008
o,p-DDD	59	5	0.011	40	10	0.008
Endrin	78	6	0.019	58	12	0.013
p,p-DDD and o,p-DDT	62	4	0.052	42	11	0.039
p,p-DDT	80	4	0.021	62	13	0.013

Conditions*: CPE - pesticide concentration 10 µg L^{-1}, 2% (m m^{-1}) Triton X-100, 2% (m v^{-1}) $MgSO_4$ in the aqueous phase, microwave incubation for 30 min; 10 min re-extraction by vortex and addition of 0.1g $MgSO_4$, (n=3).*

The reduction of the re-extraction time (to 5 min) and the possibility of reducing the organic solvent volume to 0.25 mL were the final steps in achieving the goal of creating a sample preparation procedure based on CPE. The analytical yields achieved under these conditions are in the ranges of 46–94% (for 0.5 mL hexane) and 38–83 (for 0.25 mL hexane), respectively (Table 5).

IV.2.2.5. Assessment of the MW-CPE-GC-MS/MS method "greenness" – AGREEprep

We assessed the "greenness" of the developed method using the AGREE prep software, and Figure 9 presents a pictogram of the distribution according to the relevant assessment criteria.



Legend :
1. Sample preparation placement;
2. Hazardous materials;
3. Sustainability, renewability and reusability of materials;
4. Waste;
5. Size economy of the sample;
6. Sample throughput;
7. Automation / Integration and automation;
8. Energy consumption;
9. Instrumental technique / Post-sample preparation configuration for analysis;
10. Operator's safety.

Figure 9: *Assessment of the MW-CPE-GC-MS/MS method "greenness" using AGREEprep.*

We concluded that the resulting score of 0.50 suggests that the method has a moderate environmental impact, with the main factors negatively affecting the calculated metric being the need for sample development in the laboratory, the volume of final waste generated (including the volume of the initial sample), and the analysis technique used.

IV. 2.2.6. Analysis of food samples using the developed MW-CPE-GC-MS/MS method

Maximum residue levels of pesticides (MRL) in or on food and feed of plant and animal origin and amending are defined in Regulation (EC) No 396/2005. For pesticides not explicitly mentioned in the regulatory documents, an MRL of 0.01 mg kg^{-1} is applicable [9]. The achieved low LOQ for all studied pesticides (Table 5) was the reason for applying the developed MW-CPE-GC-MS/MS method for the analysis of fruit juices. In the present work, we studied concentrated lemon juice with pulp content and pasteurised red apple juice with pigment content.

IV.2.2.6.1. Analysis of lemon juice (concentrate)

The lemon juice (concentrate) had a high pulp content (fruit pieces/vesicles from the fruit endocarp). We performed the CPE (point III.2.2.1) at different dilution factors (DF= 1.25, 1.67, 2.5 and 5), and in the re-extraction step, we used 0.25 mL hexane. We found that when analysing lemon juice with DF = 1.25, the formation of the surfactant-rich phase was not observed, probably due to the high density of the aqueous phase. When higher dilution factors were used, sedimentation of the surfactant-rich phase was observed. Since the pesticide content in the analysed juices was below the limit of quantification, we used the spiked recovery method to assess the accuracy of the MW-CPE-GC-MS/MS method. We spiked the target analytes to each sample at a concentration of 10 µg L^{-1}. The highest analytical yields were obtained when diluting lemon juice with DF = 5. Their values, calculated using matrix-matched calibration standards, were in the range of 72-114% (Table 6).

Notably, for most of the analytes, the achieved analytical yields are statistically identical to those obtained for model solutions (Table 5). For other pesticides such as Pentachlorobenzene, alpha-HCH, beta and gamma-HCH and Chlorpyrifos-methyl, an increase in analytical yields is observed when extracted from the lemon juice matrix. For a part of the target analytes (Pentachlorobenzene, Hexachlorobenzene, Chlorpyrifos, p,p-DDE, Endrin and p,p-DDT), the analytical yields had values close to 100%. Lower yields than 70% were obtained only for beta and gamma-HCH and Heptachlor-endo-epoxide A, but the concentration of these pesticides can be easily calculated in real samples by using a correction factor for partial degree of extraction. This gives us grounds to conclude that the proposed MW-CPE-GC-MS/MS method can be applied for the control of pesticides in lemon juice.

Table 6: *Analytical yields of 10 µg L^{-1} pesticide addition to lemon juice (DF=5) obtained by the MW-CPE-GC-MS/MS, (n=3).*

Analyte	*ER (%)*	*RSD (%)*	*Methodological LOQs (µg L^{-1})*	*MRL[a] (µg L^{-1})*
Pentachlorobenzene	114	5	0.025	10
alpha-HCH	72	11	0.060	10
Hexachlorobenzene	91	15	0.025	10
beta and gamma-HCH	62	10	0.035	10 each
Chlorpyrifos-methyl	74	14	0.235	10
Heptachlor	76	22	0.035	10 (in citrus)
Aldrin	78	22	0.035	10 (sum of Aldrin and Dieldrin)
Chlorpyrifos	88	14	0.060	10
Heptachlor-endo-epoxide A	65	12	0.060	See Heptachlor
o,p-DDE	77	16	0.030	See p,p-DDT
alpha-Endosulfan	87	13	0.050	50 (in citrus)
Dieldrin	78	15	0.085	See Aldrin
p,p-DDE	87	20	0.040	See p,p-DDT
o,p-DDD	76	26	0.040	See p,p-DDT
Endrin	86	17	0.065	10
p,p-DDD and o,p-DDT	78	26	0.195	See p,p-DDT
p,p-DDT	95	26	0.065	50

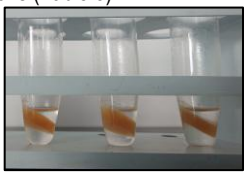
[a]MRLs for pesticides [9].

IV.2.2.6.2. Analysis of red apple juice

To evaluate the capabilities of the developed method for the analysis of samples containing pigments, we chose red apple juice. To investigate the influence of the matrix, we prepared diluted juice samples at dilution factors DF = 1.25, 1.67, 2.5 and 5, using water as a solvent. We analysed the samples by the MW-CPE-GC-MS/MS procedure (section III.2.2.1), using 0.25 mL of hexane for re-extraction. When analysing samples with DF = 1.67, 2.5 and 5, we obtained comparable results for the analytical yields for each target analyte, while at DF = 1.25, we did not observe the formation of a surfactant-rich phase. A possible reason for this was the increased density of the aqueous phase. To test our hypothesis, we performed CPE without adding 2% (m v^{-1}) $MgSO_4$. We found that under these conditions, the formation of a surfactant-rich phase was not hindered (Figure 10 a), and the obtained results for the analytical yields were comparable to those when working with more diluted samples and statistically identical to those obtained for model solutions (Table 5).

a) Surfactant-rich phase at 8 mL juice without added $MgSO_4$

b) after vortex re-extraction for 1 min at 55 °C



c) after centrifugation

Figure 10: *Sample preparation steps of a red apple juice at DF = 1.25 a) surfactant-rich phase obtained without the addition of $MgSO_4$ at the CPE stage, b) at the re-extraction stage with 0.25 mL hexane and c) after centrifugation.*

Additionally, we found that the method we developed combines a pre-concentration step and a sample clean-up step. From Figure 10 b), it can be seen that the pigments are extracted into the non-polar core of the micelles during the extraction step, and during the re-extraction step, they are deposited in the intermediate layer of $MgSO_4$, with the hexane phase remaining colourless (Figure 10 c).

The achieved analytical yields, calculated using calibration standards in a simulated matrix, are presented in Table 7.

Table 7: *Analytical yields of 10 µg L^{-1} pesticide addition to the red apple juice samples (DF=1.25) obtained by the MW-CPE-GC-MS/MS, (n=3)*

Analyte	*ER (%)*	*RSD (%)*	*Methodological LOQs (µg L^{-1})*
Pentachlorobenzene	88	8	0.006
alpha-HCH	51	8	0.015
Hexachlorobenzene	83	6	0.006
beta and gamma-HCH	41	9	0.009
Chlorpyrifos-methyl	60	6	0.059
Heptachlor	79	9	0.009
Aldrin	87	5	0.009
Chlorpyrifos	82	9	0.015
Heptachlor-endo-epoxide A	64	10	0.015
o,p-DDE	73	9	0.008
alpha-Endosulfan	76	7	0.013
Dieldrin	71	8	0.021
p,p-DDE	83	8	0.010
o,p-DDD	57	7	0.010
Endrin	74	9	0.016
p,p-DDD and o,p-DDT	58	8	0.049
p,p-DDT	66	4	0.016

*MRLs for pesticides are listed in Table 6.

From the studies conducted with lemon and apple juice, it can be concluded that the developed combined MW-CPE-GC-MS/MS method can be used to control the content of pesticides in fruit juices. An important emphasis is that the preliminary sample preparation procedure simultaneously concentrates the target analytes and separates them from the pigments in the samples. The latter is essential for the successful implementation of subsequent GC-MS/MS analysis.

IV.2.3. Dispersive liquid-liquid microextraction based on the natural hydrophobic deep eutectic solvent (NADES-based-DLLME) for extraction of organochlorine and organophosphorus pesticides

An alternative procedure for preconcentration of organochlorine and organophosphorus pesticides that we investigated was dispersive liquid-liquid microextraction (DLLME). From a "green" analytical chemistry perspective, we desired to completely replace the use of organic solvents in the sample preparation process by using a natural deep eutectic solvent (NADES) as the extractant (point III.2.2.2.). Additionally, to achieve the set goal, we decided to replace the dispersing solvent with an alternative method for dispersing the extractant, namely by applying vortex agitation.

IV.2.3.1. Evaluation of the selectivity of a GC-MS/MS method

We conducted step-by-step studies to evaluate the compatibility of GC-MS/MS with NADES as a matrix component and the effects it has on the chromatographic system. As a first step, we injected NADES extractant (without dilution) in Full scan mode, according to the conditions specified in point III.1.1. The

comparison of the recorded TIC chromatogram showed the presence of two peaks eluting at 9.54 and 10.77 min, which we identified as Carbonic acid, menthyl hexyl ester and 3-cyclopentylpropionic acid, tetradecyl ester. Despite the presence of these two peaks, we found that at the retention times of the target analytes, no peaks due to NADES were eluted.

To determine whether isobaric interference caused by NADES (MNT:DA) was observed, we conducted an additional study to evaluate the selectivity in the SRM mode under the conditions described in section III.1.1. We prepared two model aqueous solutions, a blank of purified water and a second containing pesticides at a level of 10 μg L^{-1} (in purified water), which developed according to point III.2.2.2 and injected an aliquot of the extractant phase into GC-MS/MS without further dilution. The comparison of RTIC chromatograms of the NADES blank with those of the NADES containing the studied pesticides showed that NADES causes no isobaric interference at the transitions of the target analytes.

IV.2.3.2. Assessment of non-spectral matrix effects in GC-MS/MS

To our knowledge, matrix effects in GC-MS/MS analysis caused by NADES (MNT:DA) as a matrix medium have not been studied to date. For this reason, we investigated the effect of NADES (MNT:DA) on the instrumental sensitivity by comparing the slopes of the calibration curves (Eq. 3) obtained by measuring a series of 3 standard solutions (with concentrations up to 2000 μg L^{-1} in the final solution) prepared in acetonitrile and NADES medium. We additionally evaluated the standard deviations of the slopes and the propagated standard deviation of the corresponding slope ratios.

From the slope ratios results obtained, we found that the sensitivity for all target analytes increased in the tested MNT:DA medium compared to acetonitrile in the range of 1.27 – 1.74 times (Table 8).

Table 8: *Slope ratios of calibration curves in NADES environment and acetonitrile for each target analyte measured by GC-MS/MS.*

Analyte	*Slope ratio ± combined uncertainty*[a]	*Analyte*	*Slope ratio ± combined uncertainty*[a]
Pentachlorobenzene	1.36 ± 0.10	o,p-DDE	1.58 ± 0.21
alpha-HCH	1.33 ± 0.14	alpha-Endosulfan	1.48 ± 0.20
Hexachlorobenzene	1.27 ± 0.12	Dieldrin	1.34 ± 0.19
beta and gamma-HCH	1.32 ± 0.15	p,p-DDE	1.58 ± 0.24
Chlorpyrifos-methyl	1.74 ± 0.24	o,p-DDD	1.56 ± 0.23
Heptachlor	1.46 ± 0.17	Endrin	1.46 ± 0.27
Aldrin	1.51 ± 0.23	p,p-DDD and o,p-DDT	1.72 ± 0.31
Chlorpyrifos	1.69 ± 0.25	p,p-DDT	1.69 ± 0.18
Heptachlor-endo-epoxide A	1.41 ± 0.15		

[a] The combined uncertainty was estimated according to the propagation law using Eq.3.

The general trend of the beneficial effect of MNT:DA media was that the longer the retention time of the target analyte (higher boiling point) and the higher its polarity, the higher the sensitivity achieved compared to the use of acetonitrile. A possible explanation for this "matrix-induced signal enhancement effect" is analogous to what we found in the analysis of pesticides by CPE, namely the reduction of thermal stress when the target analytes pass through the GC liner and/or the deactivation of active sites on the inner surface of the liner, which affects more polar analytes.

IV.2.3.3. The chromatographic system stability study in presence of NADES

It was important to study the stability of the signals and retention times (t_R) of the target pesticides in the presence of a NADES matrix (MNT:DA), which exhibits non-spectral matrix effects. We measured three replicates of standard solutions in NADES containing the tested pesticides at a level of 10 μg L^{-1} and 1000

μg L^{-1} in SRM mode (section III.1.1.). As a measure of repeatability, we calculated the relative standard deviations of the retention times (t_R RSD (%)) and the signals of the target analytes (area RSD (%)). From the analysis of the obtained RSD values (%), we found that despite the positive matrix effect in the extractant medium and the relative viscosity of NADES, the retention times and signals of the analytes were repeatable at both studied concentrations. The area RSD (%) at 10 μg L^{-1} were in the range of 5 - 17%, and t_R RSD (%) of 0.04 - 0.35%, the results at 1000 μg L^{-1} for area RSD (%) were in the range of 3 - 22%, and <0.01 - 0.16% for t_R RSD (%).

IV. 2.3.4. Optimization of dispersive liquid-liquid microextraction

In developing a new analytical method for the simultaneous determination of a large number of analytes, it was important to conduct a series of experiments to optimise the efficiency of the extraction procedure. From the perspective of "green" analytical chemistry, when optimising the main experimental factors affecting the DLLME procedure, we applied a two-step multivariate optimisation approach. In the first step, as a screening approach to identify significant factors, we used a Plackett-Burman design. In the optimisation step, to establish the optimal values for the significant factors, we applied a central composite design (CCD). The application of the experimental design allowed us to significantly reduce the number of experiments, samples, reagents and energy consumption.

IV.2.3.4.1. Factor screening experiment – Plackett-Burman design

In the screening stage, we chose to conduct a Plackett-Burman design, including 11 factors and 12 experiments. We set seven factors with a potential influence on DLLME, and for their low (-) and high (+) levels we chose respectively: i) NADES volume (50 and 100 μL); ii) sample volume (6 and 10 mL); iii) extraction time (1 and 3 min); iv) centrifugation time (5 and 10 min); v) centrifugation speed (900 xg and 1300 xg); vi) ionic strength, NaCl concentration (0 and 3% m v^{-1}) and vii) sample pH (5 and 7). We used the remaining four factors as dummy factors, with the help of which we could estimate the uncertainty of the measurement.

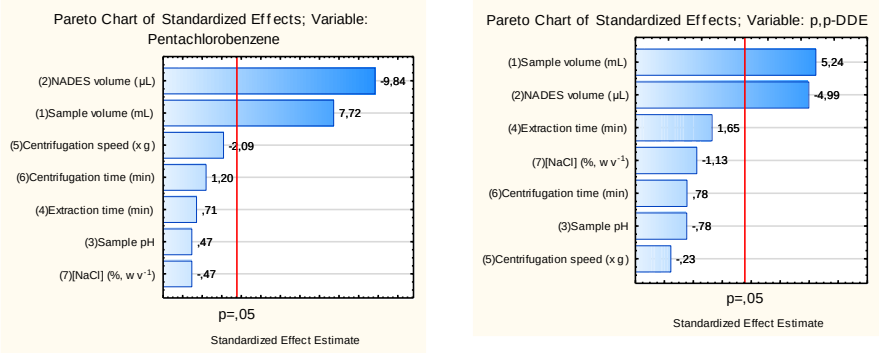


Figure 11: *Pareto charts of standardised effects from Plackett-Burman design for the studied target analytes.*

To conduct the experiments, we developed 12 model aqueous solutions containing the studied pesticides at a concentration of 10 μg L^{-1}, according to the experimental matrix and the procedure described in point III.2.2.2. The obtained NADES extractant phases (upper layer) were analysed by injecting aliquot volumes into GC-MS/MS (point III.1.1.). From the analysis of the Pareto charts presented in Figure 11, we found that the factors that have a significant effect on the signals of all target analytes are the volume of NADES and the volume of the sample. The remaining five factors do not have a significant effect on the DLLME of all the studied pesticides. We took into account the effects of the Pareto diagrams when determining the values of three of the insignificant factors for further experiments: no addition of NaCl, centrifugation speed at 900 xg

and extraction time of 3 min. For practical reasons, we set the values of the centrifugation time and pH factors to 5 min (due to temperature increase during extended centrifugation time) and pH = 7 for model solutions or natural pH of the analysed bottled spring water, respectively.

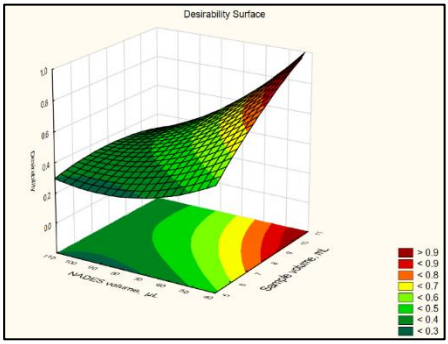
IV. 2.3.4.2 Determining the optimum values of the significant factors - Central composite design and Desirability function

To optimise the values of the two significant factors, we created a central composite design (CCD), choosing 8 mL sample volume and 75 µL NADES extractant volume as central points, respectively. We prepared model aqueous solutions containing 10 µg L^{-1} of the studied pesticides, following the CCD design matrix and the NADES-based-DLLME procedure described in section III.2.2.2.. An aliquot of the extractant phase after DLLME were analysed by GC-MS/MS (section III.1.1.). When analysing the obtained response surface plots we found that the model functions for all studied pesticides have similar shapes - the recorded peak area increases when the NADES volume decreases and/or the sample volume increases.

Due to the group extraction of a large number of pesticides, it was necessary to find compromise values for the sample volume and NADES volume at which to maximise the signals for all target analytes. For this purpose, we applied a Desirability function, which we calculated according to Equation 5 and presented the response surface plot in Figure 12.

$$\text{Desirability} = \frac{\sum_{i=1}^{n} \frac{(Signal_{i,\,run})}{(Signal_{i,max})}}{n} \qquad \text{(Eq. 5)}$$

Signal $_{i,run}$ - registered signal of the i - th pesticide under the conditions of a given experiment from the CCD matrix;
Signal $_{i,max}$ - the maximum registered signal of the i-th pesticide from all experiments on the CCD matrix;
n - the number of pesticides analysed.



In the analysis of the results, we reported a high value of the coefficient of determination (R^2 = 0.9501) of the constructed regression model, which showed a good fit of the regression equation with the experimental data. Based on the obtained response surface plot (Figure 12), we concluded that the best results can be expected at 10.8 mL sample volume and 40 µL NADES extractant volume.

Figure 12: *Response surface plot of the Desirability function.*

From a "green" analytical chemistry perspective, however, our goal was to reduce sample consumption, and from a practical perspective, it was necessary to more easily pipette a volume of 1 µL of extractant located on the surface of the aqueous phase. For these reasons, we decided to compare the signals recorded at 10.8 mL of aqueous phase volume and 40 µL of extractant volume with those at 10 mL of aqueous phase volume and 45 µL of NADES. We found that the signals were statistically identical, which is why, in subsequent experiments, we chose 10 mL as the aqueous sample volume and 45 µL of NADES as the extractant volume.

We additionally tested the adequacy of the model by comparing the observed signals for the target analytes (under optimal conditions) with the predicted signals by the Desirability function. The obtained ratios between predicted and observed values for most of the analytes were in the range of 71% - 87%, which allowed us to consider the constructed regression model as appropriate. Based on the results of the multivariate optimisation approach, we determined the optimal values of the studied factors for conducting the NADES-based-DLLME-GC-MS/MS procedure, presented in point III.2.2.2.

IV.2.3.5. Analytical figures of merit

To evaluate the calibration characteristics of the developed NADES-based-DLLME-GC-MS/MS method, we prepared a series of standard solutions with increasing concentrations of the studied pesticides in an aqueous medium at the following concentration levels: 0.001, 0.005, 0.01, 0.05, 0.1, 1.0, 2.5, 5.0, 7.5, 10.0, 12.5 μg L^{-1}. We subjected the aqueous standard solutions to the NADES-based-DLLME procedure (section III.2.2.2.). The values of the limits of quantification (LOQ) were calculated based on a signal-to-noise ratio of 10. The obtained results and the corresponding maximum residue levels (MRL) for each pesticide in water intended for human consumption are presented in Table 9. It can be seen that for most of the analytes, the LOQ values are significantly below the MRL for pesticide residues. The working range of the method was determined in the concentration range from LOQ to 12.5 μg L^{-1} of the initial aqueous solution or 2.78 mg L^{-1} after pre-concentration by NADES-based-DLLME.

Table 9: *Analytical figures of merit of the NADES-DLLME-GC-MS/MS method.*

Analyte	*Slope[a], (Area L μg^{-1})*	*Intercept[a] (Area)*	*R^2*	*LOQ (ng L^{-1})*	*MRL[b], (ng L^{-1})*
Pentachlorobenzene	62863 ± 1649	802 ± 9233	0.9938	0.7	100
alpha-HCH	100414 ± 1978	9477 ± 11074	0.9965	1.5	100
Hexachlorobenzene	97057 ± 1266	5181 ± 7087	0.9985	0.2	100
beta and gamma-HCH	171363 ± 3357	10659 ± 18795	0.9966	0.7	100
Chlorpyrifos-methyl	35395 ± 779	4802 ± 4574	0.9961	2.8	100
Heptachlor	57499 ± 578	722 ± 3235	0.9990	0.6	30
Aldrin	20510 ± 277	443 ± 1714	0.9987	10	30
Chlorpyrifos	98911 ± 1480	4001 ± 9161	0.9984	18	100
Heptachlor-endo-epoxide-A	10680 ± 172	1674 ± 1064	0.9982	9.8	30
o,p-DDE	170473 ± 2199	-4640 ± 12310	0.9984	4.1	100
alpha-Endosulfan	23437 ± 290	1342 ± 1621	0.9985	2.4	100
Dieldrin	8907 ± 156	261 ± 966	0.9979	13	30
p,p-DDE	138221 ± 2011	-5951 ± 11809	0.9983	4.1	100
o,p-DDD	323412 ± 4117	-13164 ± 24176	0.9987	8.4	100
Endrin	14144 ± 302	91 ± 1981	0.9976	69	100
p,p-DDD and o,p-DDT	460714 ± 7446	-3301 ± 43719	0.9979	5.1	100
p,p-DDT	164178 ± 6248	-37452 ± 41019	0.9914	78	100

[a] Standard deviation calculated using the least squares method.

[b] Maximum permissible levels (MRL) [10]

The method was further evaluated for repeatability by RSD (%) at two of the calibration levels, 0.05 and 5.0 μg L^{-1}. The analytical yields were calculated according to Equation 4, where in this case, Q final,i is the amount of analyte in the final phase of NADES (estimated by matrix-matched calibration in NADES) and subjected to instrumental analysis. The analytical yields for the pesticides at a concentration of 5 μg L^{-1} are

presented in Table 10, in which we also added enrichment factors. To account for the influence of the positive matrix effect (point IV.2.3.2.), we calculated the real increase in the signals - GAIN (Table 10), which represents how many times the signal for each pesticide is amplified by the NADES-DLLME-GC-MS/MS combination. The calculation of the GAIN for a given pesticide was performed by multiplying enrichment factor (EF) and the slope ratio of the calibration curves (Table 8).

Table 10: *Analytical yields, enrichment factors and signal enhancement GAIN of pesticides obtained by the developed NADES-DLLME-GC-MS/MS method (5 µg L^{-1} in aqueous solution).*

Analyte	*ER (%) ± SD[a]*	*EF ± u_c[b]*	*GAIN[c] ± u_c[d]*
Pentachlorobenzene	94 ± 9	209 ± 9	284 ± 23
alpha-HCH	117 ± 3	222 ± 3	347 ± 37
Hexachlorobenzene	116 ± 10	222 ± 10	327 ± 34
beta and gamma-HCH	112 ± 3	222 ± 3	327 ± 37
Chlorpyrifos-methyl	121 ± 8	222 ± 8	465 ± 67
Heptachlor	118 ± 6	222 ± 6	381 ± 44
Aldrin	114 ± 7	222 ± 7	382 ± 59
Chlorpyrifos	118 ± 5	222 ± 5	443 ± 65
Heptachlor-endo-epoxide A	108 ± 6	222 ± 6	339 ± 36
o,p-DDE	109 ± 9	222 ± 9	383 ± 52
alpha-Endosulfan	121 ± 6	222 ± 6	397 ± 55
Dieldrin	118 ± 6	222 ± 6	350 ± 49
p,p-DDE	110 ± 10	222 ± 10	386 ± 61
o,p-DDD	111 ± 8	222 ± 8	385 ± 57
Endrin	113 ± 5	222 ± 5	366 ± 67
p,p-DDD and o,p-DDT	111 ± 8	222 ± 8	425 ± 64
p,p-DDT	106 ± 6	222 ± 6	398 ± 44

[a] Uncertainty is estimated as the standard deviation at n=3.

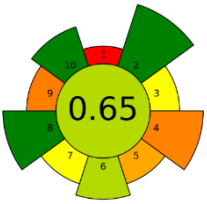
[b] Combined uncertainty was calculated according to the EF calculation.

[c] Factor indicating how many times the signal (area) for each pesticide is increased after the NADES-DLLME-GC-MS/MS compared to GC-MS/MS.

[d] Combined uncertainty was calculated according to the model equation for estimating GAIN.

From the obtained results, we found that analytical yields for some of the target analytes are close to 100% and are accompanied by relatively low SDs. This is undoubtedly an important positive characteristic of the developed method.

IV.2.3.6. Assessment of the NADES-based-DLLME-GC-MS/MS method "greenness" using AGREEprep



To assess the "greenness" of the method, we applied the analytical greenness metric AGREEprep. The final score for the "greenness" of the method was 0.65, which shows that the method is relatively green and has a moderate to good level of environmental friendliness. From the analysis of the resulting pictogram, presented in Figure 13, we found that the highest-rated criteria in our proposed method are criterion 2, criterion 8 and criterion 10. The equipment used in the extraction process (8-position centrifuge and vortex), in combination with the short extraction time, leads to a small amount of energy consumption for the development of one sample (2.9 W/sample).

Figure 13: *Assessment of the "greenness" of the NADES-DLLME-GC-MS/MS method using AGREEprep software. (Legend is presented in Figure 9).*

IV. 2.3.7. Analysis of real samples using the developed method

The achieved methodological LOQs for all tested pesticides (Table 9) are below the respective MRL specified in the EU Directive on the quality of water intended for human consumption [10]. This gave us the reason to apply the developed NADES-based-DLLME-GC-MS/MS method for the analysis of bottled spring waters. We selected three brands of bottled spring water with different conductivity and pH.

During the water analysis, we found that the pesticide content in the analysed samples was below the LOQ. For this reason, we assessed the accuracy of the method, the robustness and the precision of the obtained analytical yields using the spike recovery method. Each sample of bottled spring water was analysed in triplicate by the addition of pesticides at three concentration levels (0.1, 1 and 5 μg L^{-1}). The achieved results for the analytical yields (calculated using the calibration standards subjected to the NADES-DLLME procedure), depending on the spring water matrices, are presented in Table 11. Most of the presented analytical yields had values statistically identical to 100%. The latter was evidence that no significant matrix effects were observed when applying the NADES-based-DLLME procedure for the extraction of these pesticides from spring water. Only for p,p -DDT we obtain lower yields, probably due to the different total content of dissolved substances (estimated by conductivity). The trend that can be observed with p,p -DDT is that the higher the conductivity, the lower the analytical yields.

Table 11: *Analytical yields (ER(%)) of the target pesticides in bottled spring waters.*

	ER (%) ± SD [a]					
	Sample 1, spike (μg L^{-1})		***Sample 2, spike (μg L^{-1})***		***Sample 3, spike (μg L^{-1})***	
Analyte	***0.1***	***1***	***0.1***	***1***	***0.1***	***1***
Pentachlorobenzene	113 ± 11	90 ± 8	97 ± 3	99 ± 6	110 ± 15	100 ± 5
alpha-HCH	100 ± 4	91 ± 1	102 ± 9	100 ± 2	124 ± 11	101 ± 1
Hexachlorobenzene	100 ± 6	91 ± 9	96 ± 1	99 ± 5	98 ± 3	102 ± 6
beta & gamma-HCH	95 ± 2	98 ± 2	98 ± 3	100 ± 1	97 ± 1	99 ± 2
Chlorpyrifos-methyl	84 ± 3	77 ± 4	86 ± 10	80 ± 1	85 ± 5	75 ± 2
Heptachlor	112 ± 7	97 ± 3	105 ± 5	99 ± 3	109 ± 12	94 ± 3
Aldrin	100 ± 5	101 ± 4	100 ± 2	102 ± 4	103 ± 5	96 ± 6
Chlorpyrifos	98 ± 5	94 ± 7	92 ± 4	98 ± 1	101 ± 5	95 ± 2
Heptachlor-endo-epoxide A	72 ± 14	93 ± 6	90 ± 2	92 ± 2	66 ± 8	97 ± 1
o,p-DDE	113 ± 1	111 ± 6	110 ± 12	108 ± 5	109 ± 8	99 ± 4
alpha-Endosulfan	100 ± 4	105 ± 7	100 ± 2	99 ± 1	100 ± 4	98 ± 1
Dieldrin	121 ± 20	90 ± 7	95 ± 5	93 ± 2	95 ± 6	89 ± 3
p,p-DDE	105 ± 6	104 ± 2	118 ± 11	107 ± 7	100 ± 7	100 ± 7
o,p-DDD	99 ± 4	101 ± 10	110 ± 9	103 ± 7	106 ± 6	99 ± 3
Endrin	108 ± 8	91 ± 9	101 ± 5	96 ± 5	106 ± 10	94 ± 6
p,p-DDD & o,p-DDT	97 ± 4	101 ± 11	104 ± 1	110 ± 2	93 ± 0	96 ± 4
p,p-DDT	77 ± 13	64 ± 12	100 ± 16	93 ± 5	72 ± 2	68 ± 8

[a] Standard deviation calculated on three individually prepared samples according to the optimised procedure.

V. Conclusions

V.1. An approach for qualitative analysis of volatile components in essential oils based on modelling of linear retention indices has been developed and validated.

- **i.** Two approaches have been successfully developed for the qualitative analysis of volatile compounds in rose, lavender and peppermint oil by liquid sample injection and pre-concentration by HS-SPME in combination with gas chromatography with mass spectrometry;
- **ii.** A QSRR model for predicting linear retention indices (LRIs) via multiple linear regression (MLR) was successfully developed, including only 14 independent variables (molecular descriptors) based on experimental data;

iii. The proposed QSRR model is validated (q^2_{F1} = 0.9886, RMSE = 26) and successfully tested using an external set of compounds (q^2_{F2} = 0.9521, RMSE = 40);

iv. The model provides a valuable tool for green chemistry purposes in identifying components in essential oils for a nonpolar stationary phase by predicting LRI.

V.2. Two new procedures with a pronounced "greenness" have been developed for the group separation and concentration of a total of 19 organochlorine and organophosphorus pesticides: MW-CPE-GC-MS/MS and NADES-DLLME-GC-MS/MS, which have been successfully combined with GC-MS/MS analysis.

i. For the first time, microwave-assisted CPE using Triton X-100 as a surfactant, in combination with re-extraction in hexane or isooctane for group separation and concentration of pesticides (organochlorine and organophosphorus), has been implemented in combination with GC-MS/MS. For MW-CPE-GC-MS/MS, matrix-matched calibration has been successfully applied.

ii. For the first time, a vortex-assisted DLLME procedure for group separation and concentration of pesticides (organochlorine and organophosphorus) has been developed, in which the extractant is a hydrophobic deep-eutectic solvent prepared from components of natural origin. For NADES-DLLME-GC-MS/MS, calibration using standard solutions prepared through the extraction procedure has been successfully applied;

iii. For the first time, the extractant phases used, containing 0.09% m m^{-1} Triton X-100 or NADES – menthol/decanoic acid, were injected into the GC-MS/MS without an additional cleanup step;

iv. For the first time, selective determination of pesticides in a matrix containing 0.09% m m^{-1} Triton X-100 or NADES – menthol/decanoic acid, using SRM mode, has been achieved. It has been demonstrated that the introduction of 0.09% m m^{-1} Triton X-100 and NADES (menthol:decanoic acid) into the gas chromatography system does not deteriorate the instrumental analysis;

v. For the first time, the stability of the chromatographic system in the presence of Triton X-100 or NADES – menthol/decanoic acid in GC-MS/MS analysis has been investigated;

vi. For the first time, the matrix effects of the used extractants (Triton X-100 / NADES – menthol/decanoic acid) in GC-MS/MS analysis have been investigated, and for the first time, the dependence of the matrix effects on the used liners has been investigated and discussed. The established positive effects lead to an increase in the instrumental sensitivity in MW-CPE-GC-MS/MS (metal liner: 1.26 – 2.30 times; glass liner: up to 1.14 times) and NADES-DLLME-GC-MS/MS (glass liner: 1.27 – 1.74 times);

vii. The achieved analytical yields for most of the tested pesticides in both procedures are in the range of 70 - 120%, characterised by satisfactory precision;

viii. It has been proven that the developed methods have a strongly pronounced "greenness";
- MW-CPE-GC-MS/MS: AGREEprep score 0.50;
- NADES-DLLME-GC-MS/MS: AGREEprep score 0.65;

ix. The proposed analytical methods have significantly lower methodological limits of quantification (for some pesticides) than the MRL values for food samples and drinking water;

x. The developed combined methods were successfully applied to the analysis of real samples:
- fruit juices by MW-CPE-GC-MS/MS;
- spring waters by NADES-DLLME-GC-MS/MS.

In conclusion, it can be summarised that the development of gas chromatographic qualitative and quantitative analysis has been achieved through the application of mathematical modelling and the successful combination of instrumental techniques with "green" procedures for preliminary sample preparation.

Contributions

i. A database of experimentally determined linear retention indices on a non-polar gas chromatography column for 122 compounds included in the composition of essential oils has been created;
ii. A new easily applicable regression model for predicting linear retention indices has been proposed, which can be used in the identification of volatile components in essential oils by GC-MS and GC-MS/MS;
iii. A protocol has been created for performing GC-MS/MS analysis of 19 organochlorine and organophosphorus pesticides - optimization of both the parameters affecting the gas chromatographic separation and the mass spectrometric registration of the analyzed substances has been performed.
iv. The application of cloud point extraction for preliminary separation and concentration of organochlorine and organophosphorus pesticides has been expanded. A procedure is proposed, the "greenness" of which is enhanced by the use of microwave radiation. The procedure has been applied to the analysis of real samples - fruit juices.
v. Knowledge of combining cloud point extraction with gas chromatographic analysis has been enriched. Evidence has been provided that the introduction of a surfactant into the gas chromatographic system not only does not have a negative impact on instrumental analysis, but conversely can lead to an increase in sensitivity and a decrease in detection limits. An important advantage in implementing the combination of cloud point extraction and gas chromatographic analysis is the possibility of performing using matrix-matched calibration;
vi. An innovative approach to dispersive liquid-liquid extraction is proposed, based on the use of a deep eutectic solvent of natural origin as a method for preliminary sample preparation in the analysis of organochlorine and organophosphorus pesticides. Operating conditions were established, ensuring simultaneous extraction of 19 target analytes, while completely eliminating the use of toxic organic solvents. The procedure was applied to the analysis of real samples – bottled spring waters.
vii. The possibilities of combining dispersive liquid-liquid microextraction using a deep eutectic solvent as an extractant with gas chromatography analysis were studied. It was found that the final phase obtained after extraction can be injected directly into GC-MS/MS. In the analysis of organochlorine and organophosphorus pesticides, it was observed that the deep eutectic solvent (menthol: decanoic acid) acts as a protectant of the analytes, which leads to an increase in the sensitivity of instrumental detection and a decrease in the detection limits.

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Publications in scientific journals:

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

Participation in scientific conferences:

III. A. Hristozova, K. Simitchiev, V. Kmetov, E. Rosenberg, “Optimization of GC - MS/MS method for analysis of organochlorine and organophosphorus pesticides after extraction at coagulation temperature and re-extraction in hexane”, “Days of Science 2021” of SUB - Plovdiv, 25 - 27 November 2021, PLOVDIV, BULGARIA. (poster);

IV. A. Hristozova, K. Simitchiev, V. Kmetov, E. Rosenberg, “Adaptation of extraction at coagulation temperature to GC - MS/MS analyses”, Seminar “Instrumental techniques for chemical analysis”, 02 June 2022, PLOVDIV, BULGARIA. (oral presentation);

V. A. Hristozova, K. Simitchiev, V. Kmetov, E. Rosenberg, “Studying the effect of Triton X-100 as a matrix component on the GC-MS/MS analysis of the pesticides HCB and o,p-DDD”, ANAKON2023, April 10-14, 2023, VIENNA, AUSTRIA. (poster);

VI. A. Hristozova, Lorena Vidal, Miguel Ángel Aguirre, Kiril Simitchiev, Antonio Canals, "Natural deep eutectic solvent-based dispersive liquid-liquid microextraction of pesticides in water samples", 12th Chemistry Conference, October 13-14, 2023, PLOVDIV, BULGARIA. (poster) ;

VII. A. Hristozova, M. Batmazian, K. Simitchiev, E. Rosenberg, "Development and validation of regression models for predicting linear retention indices of volatile components from essential oils analysed by gas chromatography with tandem mass spectrometry", 12th Chemistry Conference, October 13-14, 2023, PLOVDIV, BULGARIA. (poster);

VIII. A. Hristozova, K. Simitchiev, V. Kmetov, E. Rosenberg, "Effect of co-extracted TRITON X-100 on the instrumental measurements of pesticide analysis by GC-MS/MS with cloud point extraction as sample preparation technique", 18th HTS, 28-31 May 2024 - LEUVEN, BELGIUM. (poster);

IX. A. Hristozova, M. Batmazian, S. Tsoneva, K. Simitchiev, E. Rosenberg, "Multiple linear regression models for predicting linear retention indices of volatile compounds in essential oils analysed by gas chromatography with tandem mass spectrometry - development and validation", 18-th HTS, 28-31 May 2024 - LUVEN, BELGIUM. (poster);

X. A. Hristozova, L. Vidal, M. Ángel Aguirre, K. Simitchiev, A. Canals, "DISPERSIVE LIQID-LIQUID MICROEXTRACTION BASED ON NATURAL DEEP EUTECTIC SOLVENT COMBINED WITH GC-MS/MS FOR ANALYSIS OF PESTICIDES IN BOTTLED SPRING DRINKING WATER", "Instrumental Techniques and Methods for Chemical Analysis - CHALLENGES AND NEW SOLUTIONS", 05 June 2024, PLOVDIV, BULGARIA. (poster);

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