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Analysis of antioxidants in foods and food raw materials using modern analytical methods

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List of published articles related to presented dissertation

1. Kateřina Pravcová, Nikola Macháčková, Lenka Česlová. Determination of selected pesticides in honey and mead by HPLC. Scientific Papers of the University of Pardubice, Series A. 26 (2020) 125-137.
2. Kateřina Pravcová, Tomáš Mikysek, Lenka Česlová. Comparison of HPLC and electrochemical determination of 5-hydroxymethylfurfural in honey and mead samples Scientific Papers of the University of Pardubice, Series A. 26 (2020) 139-146.
3. Mariola Brycht, Anna Łukawska, Michaela Frühbauerová, Kateřina Pravcová, Radovan Metelka, Sławomira Skrzypek, Milan Sýs. Rapid monitoring of fungicide fenhexamid residues in selected berries and wine grapes by square-wave voltammetry at carbon-based electrodes. Food Chemistry. 338 (2021) 127975.
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5. Lenka Česlová, Kateřina Pravcová, Miroslava Juričová, Jan Fischer. Rapid HPLC/MS/MS analysis of phenolic content and profile for mead quality assessment. Food Control. 134 (2022) 108737.

Abstract

The thesis focuses on developing analytical methods for the determination of selected biologically active substances in foods. The phenolic profile in mead, pseudocereals was elucidated by liquid chromatography coupled with tandem mass spectrometry. In addition, the content of pesticides was determined in samples of mead and honey. Further, the pesticide profile was monitored in environmental waters and fruit rinsing.

Abstrakt

Disertační práce se věnuje vývoji analytických metod pro stanovení vybraných biologicky aktivních látek v potravinách a potravinových surovinách. Pomocí kapalinové chromatografie ve spojení s hmotnostní spektrometrií byl objasněn fenolický profil v medovinách a pseudoobilovinách. Ve vzorcích medovin a medu byl navíc stanoven obsah pesticidů. Profil pesticidů byl dále stanoven i v enviromentálních vodách a oplachu ovoce.

Keywords

Phenolic compounds, meads, honey, pseudocereals, pesticides, liquid chromatography, mass spectrometry, extraction

Klíčová slova

Fenolické látky, medoviny, med, pseudoobiloviny, pesticidy, kapalinová chromatografie, hmotnostní spektrometrie, extrakce

Table of Contents

1 Antioxidants

Antioxidants play an important role in the food industry. Their main advantages are their affordability and convenient use, and therefore, they are part of virtually all foods. The main purpose of all antioxidants in food is to preserve the food for as long as possible without changing it, thus obtaining taste or color [1]. Antioxidants prevent free radical formation and can reduce the incidence of various diseases such as cancer, aging, cardiovascular disease, cataracts, immune system decline, and brain dysfunction. One of the most important groups of antioxidants is phenolic compounds [2].

Phenolic compounds are heterogeneous substances that are classified as secondary metabolites of plants and can react with free radicals. They occur mainly in plants [3]. They have at least one aromatic ring to which one or more hydroxyl groups are attached to aliphatic or aromatic skeletons. The most important classes include flavonoids and phenolic acids [4,5]. Phenolic acids and flavonoids could be present in free or bound form. In food, they are often glycosylated with various sugars, especially glucose, or they can be bound to the cell wall. They are important for the quality of food of plant origin and play an important role in shaping the organoleptic properties of food and beverages [6]. Phenolic compounds have different biological activities, but the most important is their antioxidant activity, which is caused by the reduction or elimination of oxygen and nitrogen free radicals in the human body. The antioxidant reacts with a reactive free radical to form a non-reactive antioxidant radical. This reaction will reduce the body's oxidative stress. In the case of phenols, the hydrogen radical reacts to form a stable quinone. The antioxidant efficiency in food depends not only on the number and location of hydroxyl groups, but also on factors such as interactions with other food ingredients, and environmental conditions (e.g. pH) [7-9].

2 Extraction processes used for the determination of phenolic compounds

The basis for the determination of individual phenolic compounds is their isolation from the sample matrix, separation, identification, and quantification [10,11]. The extraction is the most common isolation method of phenolic compounds with antioxidant effects from materials of plant origin [12]. Extraction efficiency can be affected by many factors such as solvent composition, extraction time, extraction temperature, or solvent to solid ratio. The extraction of phenolic compounds in plant materials is influenced by their chemical nature, the extraction method used, the particle size of the sample, the storage time and conditions, as well as the presence of interferences [13]. Before the extraction step, it is necessary to adjust the material by grinding, drying, lyophilisation, or simply dipping [14]. The most widely used extraction techniques include liquid-liquid extraction (LLE) [13], solid-phase extraction (SPE) [15], microwave-assisted extraction (MAE) and ultrasonic-assisted extraction (UE) [10], supercritical fluid extraction (SFE) [16], or pressurized liquid extraction (PLE) techniques [10].

In the case of pseudocereals, the sample must be homogenized prior to analysis. First, it is necessary to remove fats and lipids from the cereals using n-hexane. Further, soluble phenolic compounds are extracted into aqueous-organic solvents. These are mainly free, unconjugated phenolic compounds and soluble conjugated compounds with carbohydrates by ester or ether bond. The remaining solid phase is generally

subjected to acidic or alkaline hydrolysis to release phenolic compounds from the ester or ether bond [10,11,13].

3 Methods for determination of phenolic compounds in food and food raw materials matrices

The most common method for the determination of phenolic compounds in foods is high-performance liquid chromatography (HPLC). The analysis is carried out primarily using gradient elution with the mobile phase consisting of acidified redistilled water (with formic acid) and an organic solvent (acetonitrile or methanol) and with C18 columns as a stationary phase. The most common detector in HPLC is the spectrophotometric detector [17,18]. However, for the analysis of phenolic compounds, HPLC coupled with tandem mass spectrometry (HPLC/MS/MS) is the most important in terms of identification and sensitivity [19].

4 Mead quality evaluation based on the phenolic profile determination using rapid HPLC/MS/MS

4.1 Introduction

Mead is classified as a traditional alcoholic beverage. Mead production is based on alcoholic fermentation of a honey solution [20]. In general, it can be produced in two different ways: in a cold or hot process. The hot process includes boiling the honey solution before fermentation. The disadvantage is the possibility of a decrease in the concentration of valuable substances due to thermal degradation. For this reason, a gentle but more technologically demanding cold process of mead production is preferred. Mead contains many biologically active substances that come from honey. This research is focused on phenolic compounds that affect the taste of mead [21,22]. The type of honey, the technological process of production, and the addition of fruit juices to the honey solution significantly affect the profile of phenolic compounds of mead [23,24]. Matrix compounds cause the suppression of ionization, so the extraction of phenolic compounds from mead is important [25]. The most commonly used methods are LLE or SPE [25]. The analysis of phenolic compounds is usually performed by RP-HPLC on C18 [26] or C8 columns [25]. Aqueous acetonitrile [25] or methanol [25,26] are used as the mobile phase. HPLC is often used in combination with spectrophotometric [25,27], coulometric [21,28] or fluorescence detection [25]. In terms of sensitivity and selectivity, it is best to use MS detection [21]. GC [25,29] or MEKC [30] can also be used for the analysis of phenolic compounds in honey.

Due to frequent adulteration, it is important to control the quality of the mead. This product is most often adulterated by replacing expensive honey with cheaper molasses or glucose-fructose syrup or mixing honey with ethanol without a fermentation process. The phenolic profile and content can serve as a parameter of mead quality. If there are no phenolic compounds in the mead, it can be stated that the mead was not made from honey or low-quality honey was used, and the producer did not follow the good technological practice. Therefore, the aim of this research was to optimize the rapid HPLC/MS/MS separation for the determination of phenolic compounds in different samples of mead and to evaluate the monitored samples of mead by the method of principal component analysis.

4.2 Result and discussion

The concentration and profile of phenolic compounds in selected mead were influenced by the type of honey and by the technological process of mead production. The example can be seen in Figure 1, where the HPLC/MS/MS separation of three different samples of mead is shown.

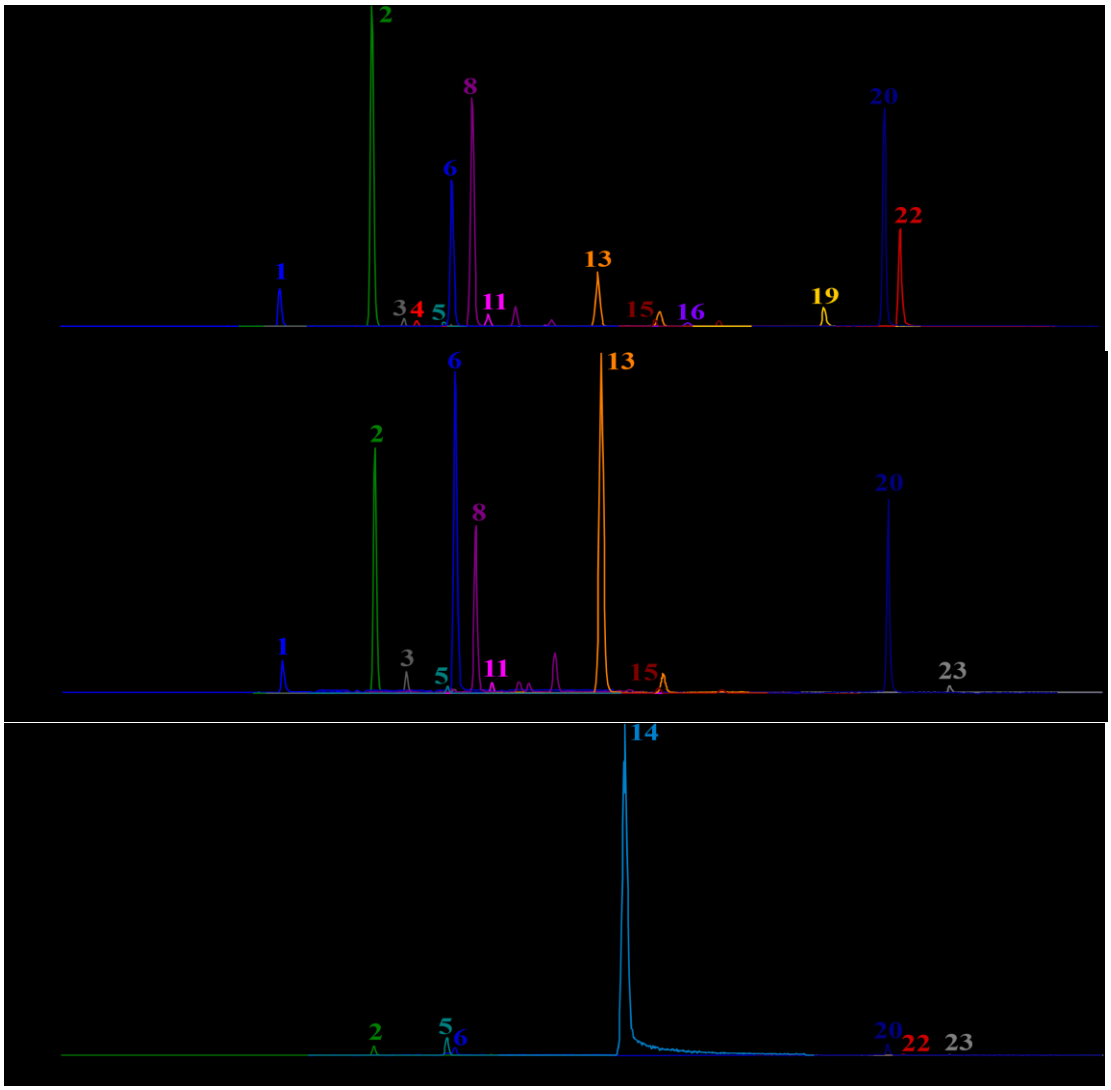

Figure 1: The examples of HPLC/MS/MS separation of selected mead samples.
A/ mead sample No. 9, B/ mead sample No. 16, C/ mead sample No. 15. Ascentis Express C18 column (150 mm, 3.0 mm, 2.7 µm particles), MF A: 0.3 % (v/v) formic acid in deionized water, MF B: acetonitrile, gradient 0 min - 10% B, 0.1 min - 23% B, 3 min - 24% B, 4 min - 50% B, 5 min - 60% B, 6 min - 10% B, flow rate 0.6 ml/min, temperature 30 °C, MS/ESI$^-$, MRM mode.
Notes: 1 = gallic acid; 2 = 3,4-dihydroxybenzoic acid; 3 = chlorogenic acid; 4 = catechin; 5 = dihydroxybenzaldehyde; 6 = 4-hydroxybenzoic acid; 8 = caffeic acid; 11 = gentisic acid; 13 = *p*-coumaric acid; 14 = vanillin; 15 = isomers of ferulic acid (15a: trans- 15b: cis-), 16 = taxifolin; 19 = myricetin; 20 = salicylic acid; 22 = quercetin; 23 = apigenin.

Figure 1A shows the separation of sample No. 9 (honeydew wine), which is a product of the beekeeper prepared by the cold process without further sweetening. Compared to sample No. 16 (Figure 1B), purchased at a local market, the intensity of phenolic compounds is one order of magnitude higher. In addition, the phenolic profile of the two samples is slightly different. Moreover, in samples Nos. 7 and 9, there are present compounds that have entered the mead from the fruit with which it has been flavored. Figure 1 shows a higher concentration of chlorogenic acid (sample No. 7) and quercetin (samples Nos. 7 and 9).

Mead made from forest (honeydew) honey (Nos. 1, 5, 6, 8, 9, and 10) showed a higher concentration of salicylic acid than samples prepared from nectar honey. In general, mead from beekeepers had a higher total concentration of phenolic compounds compared to mass-produced mead. The reason for the low concentration of the phenolic profile may be the use of insufficient honey, low-quality honey, or high-temperature processing. Figure 1C shows the chromatogram of sample No. 15, where one order of magnitude higher concentration of vanillin was found than in the other samples. Vanillin is often used as a flavouring, and it has also been found in other samples (Nos. 13, 14, 18, 21, and 22) purchased from local shops. Samples of mead received from beekeepers did not contain any aromatic substances, only natural aromas derived from fruits or herbs.

4.3 Conclusion

Rapid RP-HPLC/MS/MS separation was developed and applied to 22 mead samples obtained by beekeepers or purchased from local markets. Gradient elution of acetonitrile in acidified water on core-shell C18 stationary phase was used, and the whole separation took only 6 minutes. For its sensitivity and selectivity, mass spectrometry in the multi-reaction monitoring mode was used. The type of honey and the technological process affect the phenolic profile of the mead and therefore can be used to verify the quality of the mead. For most samples, the concentration of all selected phenolic compounds was in the range of 10-40 mg/l. The exceptions were two honeydew wines produced in a "cold" way, where the concentration was about 60 mg/l. Sample No. 15 contained a high concentration of vanillin and a low total concentration of phenolics. It can be said that low-quality honey was used in this sample or that the mead was made from insufficient honey. Another reason may be the long-term cooking of honey before inoculation, which reduces the concentration of thermally unstable compounds.

5 Determination of phenolic profile in pseudocereals

5.1 Introduction

Pseudocereals are dicotyledonous plants that are an important source of macronutrients. Compared to conventional cereals, they contain more protein, minerals, and fiber, so they are also important for the prevention of cardiovascular disease, cancer, obesity, diabetes or high blood pressure [31-34]. Their main advantages are the absence of gluten, which is a mixture of proteins contained in grains, so they are especially suitable for people with celiac disease and are becoming a modern trend in the field of healthy nutrition [31-37]. However, this lack of gluten in pseudocereals makes their processing difficult or requires at least some specific adjustments [38]. The main representatives include buckwheat and amaranth. They are often used in the diet as an alternative to rice due to their low allergicity [39].

In conventional cereals, phenolic compounds are bound to the cell wall, while in buckwheat, they occur throughout the grain. The most important flavonoids in buckwheat include rutin, quercetin, orientin, vitexin, isoorientin, and phenolic acids: 3,4-dihydroxybenzoic, caffeic, and ferulic acids [31,40]. Rutin is the most important present antioxidant. Its concentration is higher compared to other pseudocereals. Its content is influenced by ambient conditions and variety (buckwheat has a demonstrably higher content than amaranth and therefore has a bitter taste). It follows that phenolic compounds and their antioxidant activity can be optimized by several factors, such as growth or selection of a specific species, which is especially important for manufacturers [41-43]. In the food industry, there are three main species: *Amaranthus cruentus*, *Amaranthus cauatus,* and *Amaranthus hypochondriacus*. Amaranth grains are a relevant source of flavonoids and phenolic acids. The highest concentration of phenolic compounds can be found in the outer layers of the grain. Amaranth grain contains mainly free phenolic acids, where the main representatives are gallic, vanillic, 3,4-dihydroxybenzoic, *p*-coumaric, caffeic, and ferulic acids. Amaranth leaves and stems have the highest content of flavonoids and their derivatives [33,36,44,45].

Due to the degradation of the phenolic compounds analyzed, it is significant to adjust the sample before the actual analysis. Already prepared mechanically hulled grains [33,35,46], leaves [47,48] or sprouts [33,35,46] can be the form of the sample used for the analysis. There are several options for modifying grains through technological processes such as drying, freeze-drying or freezing in order to preserve all biological active compounds present in the grain [49]. Lyophilization is especially important for the preservation of all nutrients, where water is sublimated under low pressure and low temperature and the grains are ground into flour [50], which is homogenized using a sieve [48]. The next step is extraction with *n*-hexane to eliminate lipophilic compounds [47] and SPE extraction, which eliminates interference and increases the analyte concentration [40]. Subsequently, the pseudocereal extract is dried with nitrogen [51] or in an oven at 40-45 °C for 4 hours [40,48]. Samples are filtered and stored without access to light at 4-5 °C [51].

For the extraction of polar phenolic compounds, aqueous solvent solutions of ethanol [46,47], methanol [35,46,48,51], acetone [46] or aqueous ethyl acetate solution [52] are used. Higher extraction efficiency can be achieved by acidification. The liquid part of the pseudocereal extract is separated from the solid part by

centrifugation [46]. The solvent is then evaporated with nitrogen [53] or in a vacuum oven [52]. The extracts are stored in the cold, most often at -20 °C [35,46,52]. Alkaline [51] or acidic hydrolysis [53] is necessary for the analysis of bound phenolic compounds due to disruption of cell wall-binding. One hydrolysis or their combination can be used. Alkaline hydrolysis takes place at room temperature in the presence of variably concentrated NaOH [54-56]. Ascorbic acid and EDTA are added due to the highly alkaline environment that would cause the degradation of phenolic compounds [51]. Compared to alkaline hydrolysis, acid hydrolysis takes place at high temperatures (85 °C). In the case of acid hydrolysis bound phenolic compounds are released with hydrochloric acid [51,57]. After both alkaline and acid hydrolysis, extraction with ethyl acetate [58] or diethyl ether [51] is necessary. To eliminate the solvent, the final product is finally dried under nitrogen [58] or argon [53]. The extracts are stored at -20 °C until final analysis [35,46,52,58].

RP-HPLC [59] with C18 [59] or C8 [29] columns is usually used for the analysis of phenolic compounds. The mobile phase is most often aqueous solutions of methanol [60] or acetonitrile [35,45,47,59,60]. To eliminate dissociation of phenolic acids [61], the aqueous portion of the mobile phase is acidified with formic acid [62], acetic acid [35,62], phosphoric acid [59] or trifluoroacetic acid [47,60]. The separation temperature is between 25-40 °C [45,54,63-65]. Spectrophotometric detection [63,64], NMR [64,65] and MS [65] are standardly used for the determination of phenolic compounds in pseudocereals. Mass spectrometry is currently the most widely used [66-68]. However, a combination of spectrophotometric detection and MS is also frequently encountered [66-68].

5.2 Result and discussion

An external standard calibration method was used for quantitative analysis. Two different types of buckwheat samples were analyzed, namely Kroupa and Lámanka. Chlorogenic, *p*-coumaric, benzoic, 3,4-dihydroxybenzoic acid were mainly found in both buckwheat samples. The highest content of rutin was recorded [42,43]. In contrast, hyperoside, quercitrin, isoquercitrin, quercetin, 7-hydroxyflavone and caffeic acid were present in low concentrations. Higher amounts of phenolic compounds were found in the Lámanka sample, which may be due to the technological process of production. Buckwheat germination was also studied. The germination time has almost no effect on the release of phenolic compounds from the binding to the cell wall. Their concentrations are almost the same during the individual days of germination, so it is sufficient to germinate buckwheat for one day only. Compared to amaranth, buckwheat contains more phenolic compounds. However, the amount of phenolic compounds can be influenced by selecting a suitable variety. Among the monitored compounds, glycosides of ferulic, vanillic, and 4-hydroxybenzoic acids were mainly present in samples of amaranth. In contrast, salicylic and vanillic acids, quercetin, and kaempferol did not occur at concentrations above the LOQ. Alkaline and acid hydrolyses (Figure 2) are used to release bound phenolic compounds from the cell wall of pseudocereals.

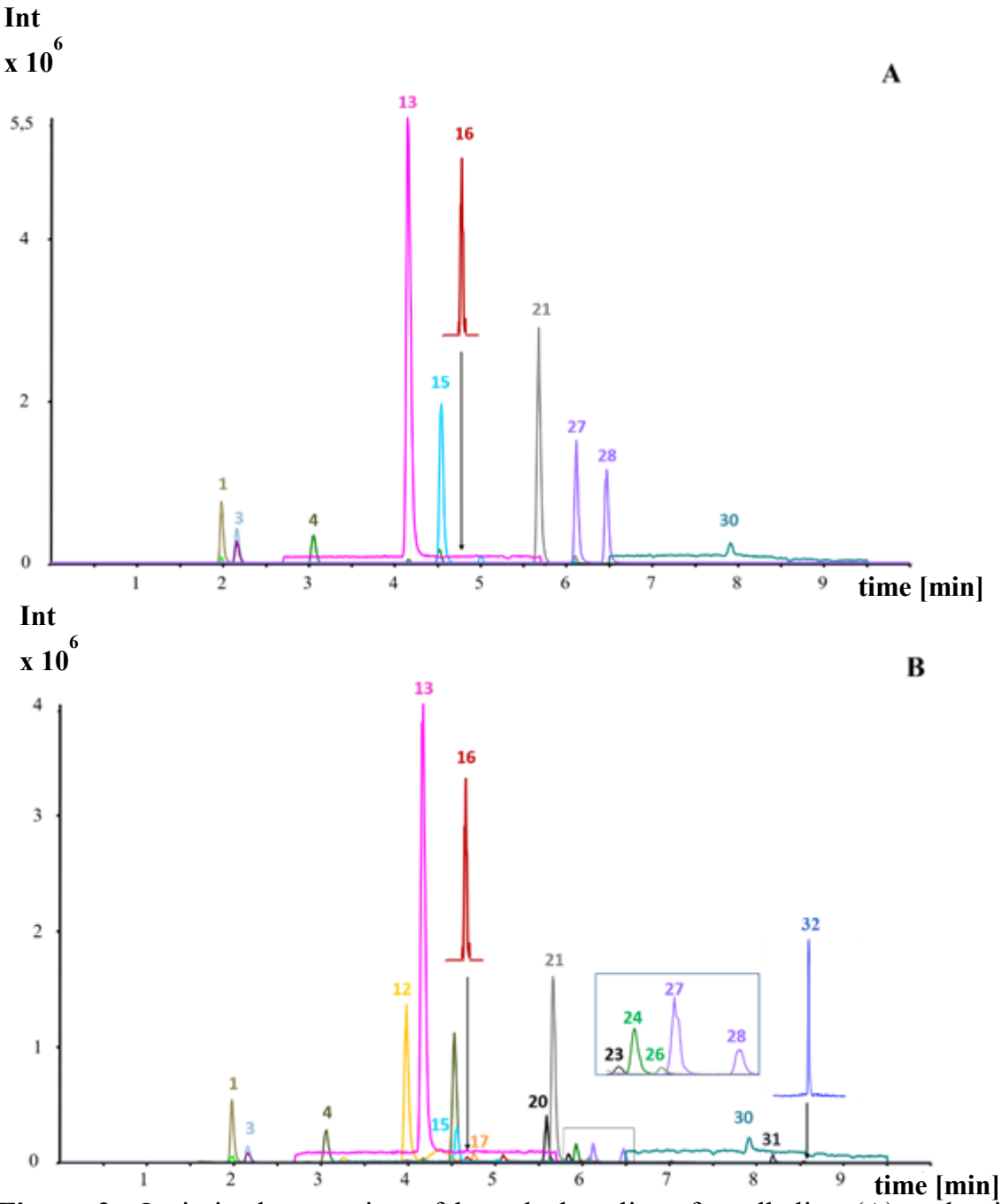

Figure 2: Optimized separation of bound phenolics after alkaline (A) and acid (B) hydrolysis.
Column: Ascentis Express C18 (150 mm, 3.0 mm, 2.7 µm); mobile phase: acetonitrile/water + formic acid (pH 2.43); gradient: 0 minutes - 10%, 6 minutes - 37%, 7 minutes - 100% acetonitrile; flow rate: 0.4 ml/min; dosage: 2 µl; column temperature: 40 °C; detection: ESI^- / MS; MRM transitions see Table 10. 1 = 4-hydroxybenzoic acid glycoside; 3 = vanillic acid glycoside; 4 = 3,4-dihydroxybenzoic acid; 12 = vanillic acid glycoside; 13 = 4-hydroxybenzoic acid; 15 = caffeic acid; 16 = vanillic acid; 17 = caffeoylquinic acid; 20,23 = coumaroylquinic acid; 21 = *p*-coumaric acid; 24,26 = feruoylquinine acid; 27,28 = ferulic acid; 30 = salicylic acid; 31 = quercetin; 32 = kaempferol.

If we compare the chromatograms of both hydrolyzed samples (Figure 2), a higher amount of phenolic compounds released was found in the sample after alkaline hydrolysis (Figure 2A) than after acid hydrolysis (Figure 2B), and mainly caffeic acid (15), *p*-coumaric acid (21), and ferulic acid (27 and 28) were found in the extracts. In contrast, a greater diversity of the phenolic profile was observed after acid hydrolysis. Two peaks of ferulic acid were observed due to its *cis* and *trans* configuration. The intensity of 4-hydroxybenzoic acid increased compared to the original extract. The reason was the large amount of glycosides present in the extracts.

5.3 Conclusion

The qualitative and quantitative profile of phenolic compounds in pseudocereals was determined using HPLC/MS/MS system after their previous isolation by a LLE or liquid-solid extraction. In pseudocereals, phenolic compounds are present in the free and bound form. A hydrolysis step was used to release the bound phenolic acids. In the case of amaranth, several types of extractions were compared. Furthermore, the effect of pH on extraction efficiency was tested, where it was shown that the extraction efficiency is higher under acidic conditions. In buckwheat, the effect of germination on the content of phenolic compounds was investigated. During the first day of germination, the content of phenolic compounds increased significantly. The following days of germination, the values were almost the same, so it is sufficient to germinate buckwheat for only one day.

All analyzes were performed on an Ascentis Express C18 column with gradient elution. The organic part of the mobile phase was methanol (buckwheat) or acetonitrile (amaranth). The aqueous part of the mobile phase was deionized water acidified with formic acid to suppress the dissociation of phenolic acids. The analysis time of the phenolic compounds ranged from 6 to 14 minutes and depended on the specific extract (buckwheat or amaranth) and thus the number of compounds that needed to be separated. The most time consuming (14 minutes) was the separation of 37 phenolic compounds in buckwheat extract. For the identification of phenolic compounds, HPLC/MS analysis in positive and negative ion mode was used. Tandem mass spectrometry was used to obtain more structural information. Moreover, the MRM transition was optimized for each substance. Quantitative analysis was performed using an external standard calibration method. The accuracy and precision of the method were determined from the validation parameters.

Furthermore, the antioxidant activity was determined using the ABTS, DPPH, and FCM methods. In the case of amaranth, extracts with the addition of formic acid had the highest values of antioxidant activity and total phenolic compounds, while the lowest values have multiple extracts, where extraction was performed first with methanol followed by extraction with diethyl ether and ethyl acetate. To compare these two pseudocereals, it can be stated that amaranth grains have lower antioxidant activity. In the case of buckwheat, the effect of germination time on the content of phenolic compounds was tested. Germination was important only on the first day, in the following days this content was comparable to the first day. Lámanka grains showed lower antioxidant activity than grains of sample Kroupa.

6 Zeolitic imidazolate framework-8 decorated with gold nanoparticles for solid-phase extraction of neonicotinoids in agricultural samples

6.1 Introduction

Neonicotinoids belong to a group of synthetic insecticides that are important pest control in agricultural plants, vegetables, and fruits [70]. The widespread use of neonicotinoids in agriculture and their properties (hydrophilicity and persistence) result in contamination and transfer of their residues to the environment. Originally, seven neonicotinoid insecticides, imidacloprid, acetamiprid, dinotefuran, thiacloprid, thiamethoxam, nitenpyram and clothianidin, were commercially available [71]. In 2018, the use of clothianidin, imidacloprid and thiamethoxam was banned, and in 2020, thiacloprid was also banned. Today, only acetamiprid can be legally used [72-74]. Because these pollutants are potentially dangerous for ecosystems and human health at trace levels, their application must be controlled. Therefore, the development of sensitive analytical methods for monitoring these compounds is important [75]. In general, neonicotinoids are determined in the environment and food matrices by HPLC/MS [76]. The main disadvantage of this technique is use of several pretreatment steps in complex matrices to increase the signal-to-noise ratio and reduce potential interference [77,78]. For this reason, SPE using conventional phases (such as C18 and HLB) has mainly been used as a sample preparation technique. The disadvantages of these sorbents are the high amount of phase (≥ 200 mg), low selectivity, and non-reusability. Therefore, the development of new materials with improved retention properties is important to improve the analytical performance of SPE sorbents [79].

Metal-organic frameworks (MOFs) are microporous materials synthesized by the coordination of inorganic centers (metals or metal clusters) with bridging organic ligands. Their main advantages are large surface area, porosity, tunable topology, and simple functionalization [80]. The main goal today is to obtain specific and more selective sorbents. Therefore, the development of MOF composites (Figure 3) prepared by integration with functional nanomaterials, such as carbon nanostructures, magnetic and metal nanoparticles (NPs), has improved adsorption capabilities and provides new properties [81]. The combination of MOF and magnetic nanoparticles is globally used for the extraction of organic pollutants from complex samples [82]. In the analytical field, the combination of MOF with gold nanoparticles (AuNP) has not been studied yet. So far there is no research on the direct applications of AuNP@MOF to neonicotinoid extraction and the relevant discussion of the mechanism of interaction [83].

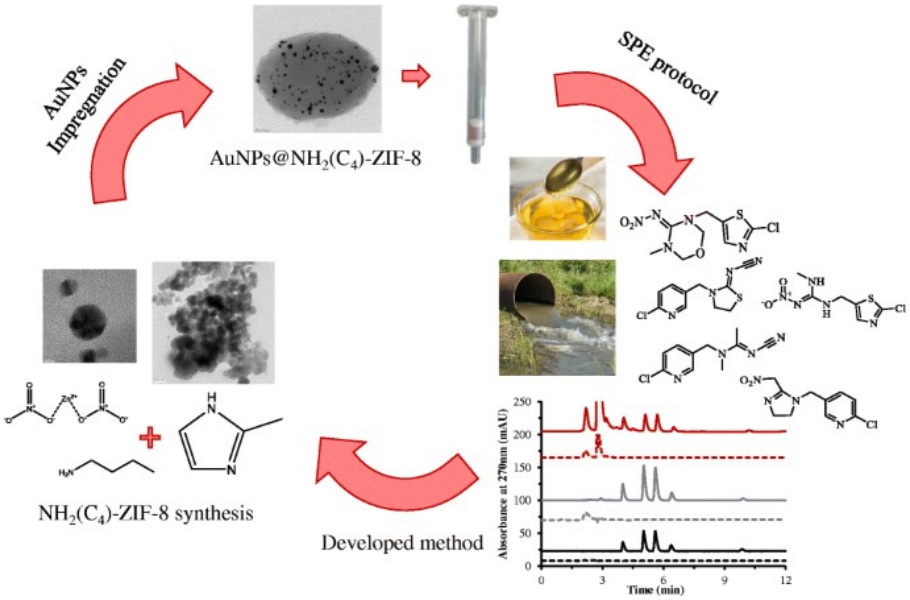

Figure 3: Graphical abstract of the experimental part.

This research describes the hybridization of the zeolitic imidazole framework-8 (ZIF-8) MOF with AuNP, where a composite with improved properties is formed. The resulting material is used for extraction and preconcentration of neonicotinoid pesticides from agricultural samples (Figure 3). First, ZIF-8 with amine groups (specifically $NH_2(C_4)$-ZIF-8) was synthesized and subsequently, AuNPs were immobilized on amino-functionalized MOF based on the high affinity between the amino group and AuNPs. The resulting material (AuNP@$NH_2(C_4)$-ZIF-8) was correctly characterized and its extraction efficiency as an SPE sorbent was evaluated (including several parameters such as sample volume, composition, and elution solvent volume). The resulting optimized method was successfully used to monitor five selected neonicotinoids in real samples (environmental water, soil, and syrup) before determination by HPLC-DAD [84,85].

6.2 Result and discussion

The ability to extract AuNP@$NH_2(C_4)$-ZIF-8 as an SPE sorbent for neonicotinoids was investigated. To achieve optimal SPE performance, key parameters such as feed solution pH, ionic strength, elution solvent composition and volume, and sampling volume were optimized. Furthermore, the reuse of sorbents, which has an important aspect from an economic and environmental point of view, was investigated. The extraction units were regenerated by washing with 2 ml of methanol and 1 ml of water. The results show that the extraction performance did not change significantly (> 85 %) even after 10 reuses. Subsequently, to illustrate the extraction capacity of AuNP@NH_2(C4)-ZIF-8 for insecticides, a comparative study was performed with another commercial sorbent (phase C18) and also with bare MOF (using the same amount of sorbent, 20 mg) under the same extraction conditions. Figure 4 shows the low yields of neonicotinoids (<40 %) obtained in C18, which can be explained by its hydrophobicity in contrast to the polar behavior of these analytes. On the other hand, bare MOF provided better extraction efficiency than the C18 phase, which may be due to interactions. The extraction capacity increased after the incorporation of AuNP. This fact demonstrates an important aspect of AuNP in the final composite

by introducing additional interactions based on the affinity of the amino and cyano groups present in the target compounds, which enhances the interaction between the sorbent and the analytes. To evaluate the applicability of this method, the synthesized composite was used for trace analysis of neonicotinoids in real water, soil, and syrup samples. None of the target pollutants were found in the samples and therefore five neonicotinoids were enriched. The yields of neonicotinoids ranged from 80-110 %.

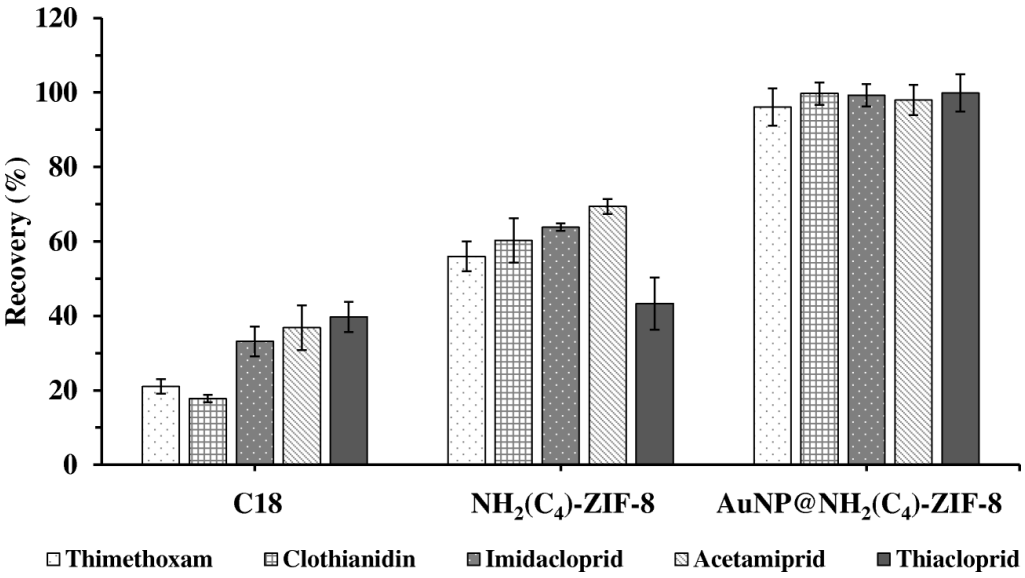

Figure 4: Extraction efficiency of neonicotinoides using different sorbents (commercial C18 phase; bare MOF and composite). Conditions: concentration, 250 μg/ml of neonicotinoides in 0.25 wt.% NaCl at pH 6.0; sample volume, 15 ml; eluent volume, 0.25 × 2 ml methanol:water (60:40, v:v)

6.3 Conclusion

A composite based on $NH_2(C_4)$-ZIF-8 and AuNP was synthesized and used as an SPE sorbent to extract five neonicotinoids for the first time. This material was easily made by the direct assembly of MOF with AuNP without any post-functionalization step. A combination of the benefits of MOF and AuNP has been demonstrated. The retention mechanism of this composite was mainly due to π-interactions, hydrogen bonding, and the affinity of amino or cyano groups for AuNP surfaces. The combination of $NH_2(C_4)$-ZIF-8 and AuNP as an SPE sorbent for neonicotinoid extraction with available HPLC-DAD equipment provided high recoveries, low LOD, and good reusability of the extraction units. Compared to other conventional SPE methods, this method used a small amount of eluent in the pretreatment step. The proposed protocol is cost-effective, simple, feasible, and can be used for the determination of neonicotinoids in water samples in the environment. All these results show that this composite is a potential sorbent in sample preparation and the assembly method can be extended to other nanostructures, thus expanding the field of research on composite nanoparticles.

7 Determination of selected pesticides in honey and mead by HPLC

7.1 Introduction

Pesticides are among the chemicals used in agriculture to protect crops from pests [86]. The use of pesticides poses a risk of contamination of soil, water, and food, so monitoring them in food and the environment is important to protect consumer safety [87]. Maximum residue limits (MRLs) are set for the pesticide content of individual raw materials [88]. Honey is a product that contains a mixture of sugars and other complex carbohydrates, enzymes, amino acids, organic acids, proteins, vitamins, volatile compounds, pigments, phenolic compounds and minerals [20,89]. Mead is an alcoholic beverage prepared by fermentation of a honey solution, therefore, all honey compounds are also contained in mead and the quality of mead is mainly influenced by the quality of honey [89]. Although it contains compounds that have a positive effect on health, both honey and mead can contain harmful compounds such as pesticides. The source of contamination may be beekeeping itself [90] or the application of pesticides in agriculture [91].

The first step during the determination of pesticides in honey and mead is the isolation and enrichment of the monitored pesticides. The most common extraction techniques are liquid-liquid extraction (LLE) [92], solid-phase extraction (SPE) [93], solid-supported liquid-liquid extraction (SLE) [94] and QuEChERS [95]. The choice of separation technique depends on the properties of the pesticides. Due to polarity and thermal instability of pesticides, HPLC is preferred for their analysis [94]. The most commonly used combination is HPLC/MS [96].

7.2 Result and discussion

The monitored pesticides were determined in twenty honey samples and twelve mead samples. Each extraction was performed twice and each extract was measured three times. The content of selected pesticides in honey (Table 1) and mead samples (Table 2) was determined from the calibration curves. The results show that the origin of the honey/mead (market or beekeeper) does not affect the amount of pesticides, because their content, which enters the honey from the environment, cannot be influenced.

More or less similar pesticides were found in all samples studied, although the samples came not only from the Czech Republic, but also from Italy, Croatia and Slovakia. Higher concentrations of thiacloprid were found in honey samples Nos. 5 and 7 from the same area (Náchodsko). Sample No. 7 also contained a high concentration of T-fluvalinate. Thiacloprid was present in similar concentrations in the samples from the Central Bohemian Region and its most common presence was recorded in the Hradec Králové region. T-fluvalinate was also found in the vicinity of Nové Město nad Metují (honey samples Nos. 6 and 7). Carbendazim was found only in the village of Doubravice (Hradec Králové Region, honey sample No. 5). The most frequent presence of thiacloprid was found in the Pardubice Region and in some cases (honey samples Nos. 10, 11 and 13) the presence of prochloraz was also found. Pesticides have also been shown to be present in BIO organic products, although in the case of BIO organic products, bees must collect pollen in a place that has not been chemically treated.

The pesticide content was lower in the mead samples than in the honey samples. Carbendazim has been found in some samples.

Table 1: Quantitative representation of pesticides in honey samples.

NO.	Carbendazim [mg/kg]	Acetamiprid [mg/kg]	Thiacloprid [mg/kg]	Prochloraz [mg/kg]	T-fluvalinate [mg/kg]
1	–	–	1.44 ± 0.19	–	–
2	–	2.78 ± 0.03	2.04 ± 0.03	–	–
3	–	–	1.67 ± 0.02	–	–
4	–	0.24 ± 0.02	1.25 ± 0.07	0.20 ± 0.01	–
5	5.9 ± 0.47	–	16.4 ± 0.42	–	–
6	–	0.22 ± 0.91	1.9 ± 0.32	–	0.02 ± 0.01
7	–	–	21.7± 0.33	–	76 ± 0.53
8	–	–	1.25 ± 0.07	0.50 ± 0.01	–
9	–	–	1.34 ± 0.05	–	–
10	–	–	–	0.63 ± 0.01	–
11	–	0.41 ± 0.06	0.85 ± 0.07	1.12 ± 0.01	–
12	–	16.9 ± 0.71	0.92± 0.08	–	–
13	–	–	1.91 ± 0.06	0.59 ± 0.01	–
14	–	–	–	0.42 ± 0.06	–
15	–	5.32 ± 0.02	–	–	–
16	–	1.3 ± 0.65	1.2 ± 0.84	–	–
17	–	–	0.90 ± 0.04	–	–
18	–	–	–	–	–
19	–	–	0.88 ± 0.03	–	5.46 ± 0.02
20	–	–	2.25 ± 0.04	–	3.22 ± 0.06

Table 2: Quantitative representation of pesticides in mead samples.

NO.	Carbendazim [mg/l]	Acetamiprid [mg/l]	Thiacloprid [mg/l]
1	–	1.64 ± 0.01	–
2	–	2.39 ± 0.07	1.92 ± 0.03
3	–	–	–
4	–	–	–
5	–	–	–
6	–	–	–
7	–	–	–
8	–	–	–
9	–	–	–
10	–	0.676 ± 0.02	–
11	–	8.33 ± 0.51	–
12	0.193 ± 0.02	79.16 ± 0.35	17.79 ± 0.92

7.3 Conclusion

Pesticides can serve as a parameter for environmental impact assessment. For this reason, the aim of the research was to determine 8 selected pesticides in honey and mead using optimized HPLC with QuEChERS extraction. The separation took only 10 minutes and was applied to 20 samples of honey and 12 samples of mead, which were obtained from beekeepers or purchased at local markets. It can be seen from the results that it does not matter where the honey comes from. Similar pesticides were present in all samples. Samples 5 and 7 contained a high concentration of thiacloprid and additionally sample 7 had also a high concentration of T-fluvalinate. Both of these samples came from the beekeepers in Náchod region. Although BIO organic products should not contain any pesticides, they have also been found there. The concentration of pesticides in mead samples is lower than in honey samples, which may be caused due to dilution during mead production. The results show the presence of carbendazim in some samples, although it has been banned since 2013. This pesticide is probably still in the environment. In the case of mead, there is a risk of contamination of the product with pesticides from other material that alters the taste of the mead.

8 Rapid monitoring of fungicide fenhexamid residues in selected berries and wine grapes by square-wave voltammetry at carbon-based electrodes

8.1 Introduction

Fenhexamide is a fungicide and is the main active substance in the commercially available fungicide TELDOR 500 SC. According to Directive 2006/53/EC, the maximum residue level for fenhexamide is influenced by the type of crop. The MRL for fenhexamide in or on fruits and vegetables is around 0.05 - 30 mg/kg, leaf vegetables 30 mg/kg, kiwi 10 mg/kg, berries and small fruits 5 mg/kg, tomatoes and aubergines 1 mg/kg, root and onion vegetables 0.05 mg/kg [97]. Monitoring of pesticide residues in food is based on two protocols ČSN 56 0253 and ČSN EN 15662 (560680) based on European Union regulations. The first protocol sets out sampling guidelines for the determination of pesticides in and on foods and raw materials of plant and animal origin. The second protocol includes two instrumental analytical methods for the determination of pesticide residues by gas chromatography with mass spectrometric detection and/or high-performance liquid chromatography with tandem mass spectrometric detection. The main goal of this research was to develop a new and fast direct voltammetric method for the determination of fenhexamide residues in selected foods [98].

8.2 Results and discussion – reference RP- HPLC method

The aim of this work was rapid monitoring of fenhexamide fungicide residues in selected berries and wine grapes by square-wave voltammetry at carbon-based electrodes. As the reference method, the RP-HPLC was used. The optimal separation of fenhexamid from matrix compounds was achieved with gradient elution 60 % to 100 % of pure methanol in 0.1% formic acid in 10 minutes. The precision of the reference method was determined using 20 μmol/l fenhexamid (RSD = 1.1%) based on five repeated measurements (n = 5). Further, satisfactory accuracy with recovery value of 94.8 % has been achieved. From Table 3 is evident that the blueberry and grape samples tested did not contain a statistically significant amount of fenhexamide

residues. Strawberries and red grapes did not contain this fungicide at all, as might be expected. To obtain more information on the matrix effect, fenhexamide-free samples were intentionally enriched with a known amount of analyte, and satisfactory recovery was observed. In the case of the maximum permitted amount of fenhexamid residues, all analyzed samples can be considered harmless.

Table 3: Analysis of fenhexamide content in commercially available berries and wine grapes obtained by square wave voltammetry (SWV) and RP-HPLC.

Berries	**SWV**	**RP-HPLC**	**Maximum residue limit (spiked content)**
	Fenhexamid (mg per kg)	**Fenhexamid (mg per kg)**	**Fenhexamid (mg per kg)**
Blueberries	0.8 ± 0.2	0.6 ± 0.1	5
Blueberries spiked	4.4 ± 0.4	4.8 ± 0.3	5
Strawberries	—	—	5
Strawberries spiked	17 ± 1.2	16 ± 0.4	5
Red grapes	5.4 ± 0.4	5.3 ± 0.4	5
White grapes	—	—	5

8.3 Conclusion

During this experiment, a new, simple and fast voltammetric method was developed, which was successfully used to determine fenhexamide residues in blueberries, strawberries, and grapes. Research shows that various carbon-based electrode materials tested as working electrodes for fenhexamide analysis are potentially useful, but GCPE is the optimal choice due to its high sensitivity, mechanical stability in aqueous/methanol mixtures, and its ability to analyze food samples using standard methods. The developed square wave voltammetry method was also compared with the reference method RP-HPLC with spectrophotometric detection and there was no significant difference between the results obtained between these two methods. It can therefore be concluded that both methods can be used to determine fenhexamide in food samples (fruit and vegetables). However, the voltammetric method could be used for routine purposes as a method of rapid screening for food safety inspections due to its simplicity, cheapness, and less time-consuming nature.

9 References

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10 List of Students' Published Works

Presentations

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