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Development of methods for the analysis of phenolic substances in plant products using liquid chromatography and mass spectrometry

Theses of the Doctoral Dissertation

Pardubice 2024

Study program: **Analytical Chemistry**

Study field: **Analytical Chemistry**

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Year of the defence: 2024

References

KAŠPAR, Michal. Application of liquid chromatography and mass spectrometry for the analysis of phenolic compounds in plant products. Pardubice, 2024. 114 pages. Dissertation thesis (PhD.). University of Pardubice, Faculty of Chemical Technology, Department of Analytical Chemistry. Supervisor doc. Ing. Petr Česla Ph.D.

Abstract

This doctoral thesis focuses on the determination of phenolic compounds in vinegars and coffee beans using the advanced analytical technique of liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Phenolic compounds are important bioactive substances with antioxidant, anti-inflammatory, and other beneficial effects on human health. Vinegar and coffee beans are rich in these compounds, which contribute to their flavor properties and health benefits. The aim of this study was to optimize and validate the LC-MS/MS method for the simultaneous quantification of several phenolic compounds in these matrices. Detailed experiments were conducted to optimize chromatographic separation conditions, as well as to select appropriate precursor and product ions for mass spectrometric detection. The validation of the method included the evaluation of parameters such as linearity, accuracy, repeatability, and detection limit. The results of the study showed that the developed method is highly efficient and reliable for the analysis of phenolic compounds in vinegars and coffee beans. The results obtained evaluate the differences in the phenolic profile of various types of vinegar and help to understand the fate of phenolic compounds during the roasting of coffee beans. This methodology can be used for further research on bioactive components in foods and for quality control of these products.

Abstrakt

Tato disertační práce se zaměřuje na stanovení fenolických sloučenin v octech a kávových zrnech pomocí pokročilé analytické techniky kapalinové chromatografie spojené s tandemovou hmotnostní spektrometrií (LC-MS/MS). Fenolické sloučeniny jsou významné bioaktivní látky s antioxidačními, protizánětlivými a dalšími prospěšnými účinky na lidské zdraví. Ocet a kávová zrna jsou bohaté na tyto látky, které přispívají k jejich chuťovým vlastnostem a zdravotním benefitům. Cílem této studie bylo optimalizovat a validovat metodu LC-MS/MS pro simultánní kvantifikaci několika fenolických sloučenin v těchto matricích. Byly provedeny podrobné experimenty pro optimalizaci chromatografického dělení, stejně jako pro výběr vhodných prekurzorových a produktových iontů pro hmotnostně spektrometrickou detekci. Validace metody zahrnovala hodnocení parametrů jako je linearita, správnost, opakovatelnost a mez detekce. Výsledky studie ukázaly, že vyvinutá metoda je vysoce účinná a spolehlivá pro analýzu fenolických sloučenin v octech a kávových zrnech. Dosažené výsledky hodnotí odlišnosti ve fenolickém profilu různých typů octa a pomáhají pochopit osudy fenolických látek v průběhu pražení kávových zrn. Tato metodika může být použita pro další výzkum bioaktivních složek v potravinách a pro kontrolu kvality těchto produktů.

Keywords

Liquid chromatography, mass spectrometry, phenolic compounds, vinegar, coffee beans

Klíčová slova

Kapalinová chromatografie, hmotnostní spektrometrie, fenolické sloučeniny, ocet, kávová zrna

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Objectives of the doctoral thesis

The aim of the first part of this work was to evaluate the basic retention characteristics of selected phenolic compounds in a reversed-phase chromatographic system. This was intended to provide a better understanding of the behaviour of phenolic compounds in such a system, and on the basis of the obtained results, columns exhibiting high efficiency and acceptable values of other retention characteristics for selected phenolic compounds were chosen.

One of these columns was subsequently used in the second part of this work, which aimed to develop a universal, fast, and reliable LC-MS/MS method for the qualitative and quantitative determination of phenolic compounds. This method needed to be optimized to achieve the best possible performance characteristics, and then validated to verify its accuracy, repeatability, and other commonly validated parameters in the field of liquid chromatography.

The universality of the method was verified in the third and fourth parts of this work, which dealt with comparing the phenolic profiles of different types of vinegar and monitoring changes in the amount of phenolic compounds during roasting of coffee beans. Here, it was necessary to select a suitable sample preparation technique and choose the correct approach to quantifying phenolic compounds to minimise the effects of the matrix.

The achievement of these objectives was made possible, among other things, thanks to two grant projects from the Czech Science Foundation, 18-14893S and 22-09556S.

Introduction

Compounds that contain an aromatic ring with at least one hydroxyl group directly attached are generally referred to as phenolic compounds, with over 8,000 such structures identified in nature [1]. These are secondary plant metabolites that plants use primarily for protection against external factors [2][3]. Phenolic compounds can be further classified on the basis of their structure into two main categories: flavonoids and nonflavonoids. Flavonoids are the dominant group of phenolic compounds in fruits and vegetables and can constitute up to two-thirds of dietary phenol intake [2]. Consumption of plant-based foods, particularly fruits and vegetables, is a current trend in a healthy lifestyle, along with activities such as regular physical exercise, adequate hydration (mainly from water), and quality sleep. Eating fruits and vegetables is commonly associated with the intake of vitamins, minerals, fiber, and antioxidants. The last group of the compounds, specifically their antioxidant properties, can be attributed to phenolic compounds [4]. However, antioxidant activity is not the only benefit of phenols for the human body, as they also exhibit antimicrobial [5] and anti-inflammatory activities [6], thus helping the body prevent a wide range of diseases.

In the field of phenolic compound analysis, liquid chromatography in reversed-phase mode combined with spectrometric detection techniques currently dominates, with the most information provided by coupling with mass spectrometry. Plants and plant products, including vinegar and coffee beans, are of particular interest for the analysis of phenolic compounds [7].

Vinegar can be considered a traditional food product, produced through a two-step fermentation process involving yeasts and bacteria of the *Acetobacter* genus. Practically any plant material containing the appropriate carbohydrates, which can be converted into ethanol by yeasts, can serve as the raw material for the vinegar production. Traditional raw materials for vinegar production include apples, grapes, rice, starches, beer, pears, and various berries, from raspberries to blueberries. This diversity of starting

materials, as well as the different methods and approaches to vinegar production, suggests a variety of profiles of biologically active compounds, including phenols [8].

Coffee is one of the most popular beverages in the world, consumed daily by millions of people. It is valued not only for its stimulating effect due to its high caffeine content but also for its complex aroma and flavor properties that develop during the roasting of coffee beans. The roasting process is crucial for achieving the characteristic coffee profile [9]. During roasting, green coffee beans are heated to temperatures up to around 200 °C for several minutes, causing a series of chemical changes [10], including changes in the content of phenolic compounds.

1 The use of liquid chromatography to characterize the phenolic profile of vinegars and coffee

Several studies (Table 1) have been conducted to examine the content of phenolic compounds in various types of vinegar. For this purpose, the most commonly used technique is liquid chromatography combined with diode-array or mass spectrometry detectors.

Table 1: The articles using the LC technique to characterize the phenolic profile of different types of vinegar. (UV – ultraviolet detector, DAD – diode array detector, SPE – solid phase extraction)

Type of vinegar	Column	Detection	Sample preparation	Compounds	Ref.
Wine	Kromasil C18	UV	Filtration	Phenolic acids, flavonols, naringenin (8 compounds)	[11]
Wine	Eclipse XDB-C18	DAD	Not specified	Various (19 compounds)	[12]
Wine (red)	Acclaim C18	UV	Filtration	Phenolic acids	[13]

Wine and balsamic	Waters C18	MS/MS	Online - SPE	Various (56 compounds)	[14]
Wine and balsamic	BEH C18	DAD	Not specified	Phenolic acids, tyrosol (7 compounds)	[15]
Balsamic	Atlantis C18	DAD	SPE	Phenolic acids, catechin, vanillin, myricetin (10 compounds)	[16]
Wine and apple	Luna C18	DAD	Filtration	Phenolic acids, catechin (6 compounds)	[17]
Wine and apple	Hypersil Gold	UV	Filtration	Phenolic acids, catechins, myricetin, rutin (15 compounds)	[18]
Wine and apple	Luna C18	DAD-MS/MS	Filtration	Various (27 compounds)	[19]
Apple	Nucleosil 100 C18	DAD	Ultrasound	Phenolic acids, naringenin, myricetin (5 compounds)	[20]
Rice	Phenyl RP column	UV	Ex. by ethylacetate	Phenolic acids, catechin, rutin (11 compounds)	[21]
Various types (15 in total)	Luna C18	DAD	Filtration	Phenolic acids, catechin (8 compounds)	[22]

Previous works in this field are usually limited to a specific type of vinegar, and the number of analytes monitored is relatively low. The developed methods differ in terms of detection type, chromatographic column chosen, mobile phases, sample preparation methods, and, last but not least, total analysis time. In the past, the greatest attention has been paid to wine and apple vinegar, whereas there are only a limited number of studies characterizing the phenolic profile of rice and spirit vinegar.

Several studies can be found in the literature which deal with the determination of the content of phenolic compounds in coffee beans and coffee. These studies have typically focused on characterizing chlorogenic acid (and its various isomers), which is one of the main components of coffee beans and is responsible for the majority of their antioxidant capacity. However, relatively few studies have paid attention to other phenolic compounds.

Table 2: The articles using the LC technique to characterize changes in the phenolic profile during the roasting of coffee beans. (MS – mass spectrometer detector)

N. of roasting degrees	Detection	Sample preparation	Compounds	Ref.
3	UV	Ex. by boiling water	Flavonoids and non-chlorogenic acids (12 compounds)	[23]
3	MS/MS	Ex. by 70% ethanol + hydrolysis	Phenolic acids and others (23 compounds)	[24]
Green + 3	DAD	Ex. by boiling water	Chlorogenic acids (3 compounds)	[25]
Green + 3	DAD	Ex. by hot water	Chlorogenic acids	[26]
Green + 3	DAD	Ex. by hot water	Phenolic acids (10 compounds)	[27]
Green + 4	UV	Ex. by boiling water	Chlorogenic acids (7 compounds)	[28]
Green + 6	DAD - MS	Ex. by 80% methanol + hydrolysis	Phenolic acids (21 compounds)	[29]

The focus of individual works varies; they have usually investigated changes in the phenolic profile during coffee beans roasting (Table 2), the use of phenolic compounds to determine the origin of green coffee beans, and the comparison of different techniques to extract phenolic compounds from coffee beans. For all of these purposes, the most commonly used technique has been liquid chromatography in reversed-phase mode combined with spectrophotometric or mass detectors.

2 Experimental part

2.1 LC-MS/MS analysis

An Agilent 1260 liquid chromatography (LC) system (Agilent, Santa Clara, CA, USA) was used for the separation of phenolic compounds. Separations were carried out on a Luna Omega Polar C18 column (Phenomenex, Torrance, CA, USA) with dimensions of 150 × 3 mm and particle size of 3 μm. This column was selected on the basis of the results achieved in the first part of this doctoral thesis, as it provided acceptable values of retention characteristics and efficiency. A gradient elution mode was used with mobile phases A (water + 0.1% (v/v) acetic acid) and B (acetonitrile + 0.1% (v/v) acetic acid). All of the chemicals used were purchased from Sigma-Aldrich (St. Louis, MO, USA) with purity for LC-MS applications with the exception of water, which was obtained by purification in the Mili-Q system (Merck KGaA, Darmstadt, Germany). The gradient profile of the final method was as follows: 0 min – 15% B; 12 min - 51% B; 12.01 min - 100% B; 14 min – 100% B. After the end of the analysis, the conditions were stabilized to the initial values of 15% B for 4 minutes. The separations was performed at a temperature of 40 °C, a mobile phase flow rate of 0.5 ml/min and an injection volume of 3 μL.

For detection, the LC system was coupled with the QTrap 4500 mass spectrometer from AB Sciex LLC (Framingham, MA, USA). Ionization of phenolic compounds was carried out by electrospray in negative mode. Data were collected in MRM (multiple reaction monitoring) mode with optimized DP (declustering potential), CE (collision energy), and CXP (collision cell exit potential) parameters. Each MRM transition was monitored for 90 seconds (schedule mode).

Stock solutions of 48 phenolic compounds (purchased from Sigma-Aldrich at the highest possible purity) with a concentration of 1 mg/mL were prepared by weighing the appropriate amount on an analytical balance (SARTORIUS, Ústí nad Labem, Czech Republic) and dissolving in methanol.

A different method was used using a Luna Omega PS C18 column for the analysis of chlorogenic acids in the coffee bean samples. However, the description of this method is beyond the scope of this text, as well as the description of the experimental work of the first part of this doctoral thesis, which evaluated the reversed-phase columns in terms of retention, selectivity, peak symmetry, and efficiency for the analysis of phenolic compounds.

2.2 Vinegar samples preparation

Samples of six different types of vinegar (spirit, rice, apple, white wine, red wine, balsamic) were purchased in Czech stores. Samples were prepared by centrifugation and filtration and diluted with water prior to LC-MS/MS analysis. The quantification of the the phenolic compounds was carried out using the method of multiple standard addition.

2.3 Coffee beans samples preparation

The obtained coffee bean samples were ground using an ETA Magico electric coffee grinder (Prague, Czech Republic). The ground samples were further dried in the dark at room temperature. Phenolic compounds were extracted from the samples treated in this way using the extraction method in an ultrasonic bath. 0.5 g of ground coffee beans were extracted with 20 mL of solvent, which was 50% (v/v) methanol. The extraction took place at a controlled temperature of 40 °C and for 10 minutes. The extracts obtained were centrifuged and filtered through polytetrafluorethylen (PTFE) syringe filters and stored in the refrigerator until analysis. The quantification of phenolic compounds in coffee bean samples was carried out using the calibration curve method in the matrix.

3 Results and discussion

3.1 Reversed-phase liquid chromatography of phenolic compounds

Eight columns (Table 3) designed for separations in reversed-phase mode were tested, each with a length of 15 cm, an internal diameter typically 3 mm, and particle sizes ranging from 2.6 to 3 μm. The columns were evaluated in terms of retention, selectivity,

peak symmetry, and efficiency for selected phenolic compounds. All columns tested were suitable for the analysis of the selected phenolic compounds, but they exhibited different values, particularly in terms of retention factor and peak symmetry.

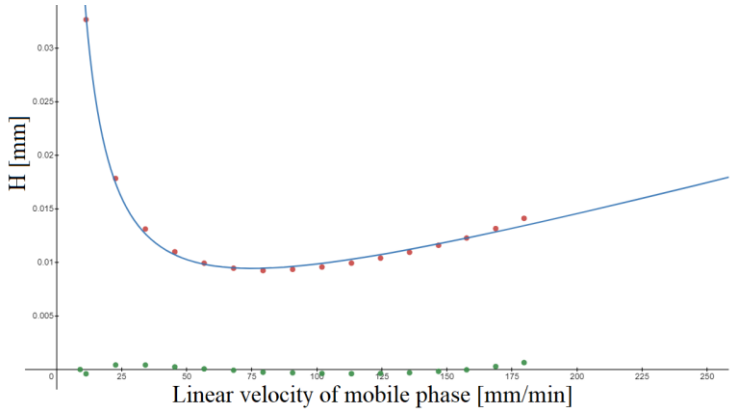

Figure 1: Van Deemter curve of the propylbenzene using ACE 3 C18-PFP column.

The efficiency of the columns was verified based on the van Deemter equation. For this purpose, the analysis of a homologous series of alkylbenzenes were perfomed at different mobile phase flow rates. On the basis of the obtained data, the contributions of the A, B, and C terms in the van Deemter equation were evaluated. The results obtained for propylbenzene can be used to present the findings (Figure 1; Table 3).

Table 3: Height equivalent to a theoretical plate (H) of propylbenzene for various columns in reversed-phase mode at a flow rate of 0.5 mL/min.

Column name	**Particle type**	***A* [µm]**	***B/u* [µm]**	***C*×*u* [µm]**	***H* [µm]**
ACE 3 C18-PFP	Fully porous	-0.353	3.257	7.417	**10.322**
Luna Omega Polar C18	Fully porous	0.089	2.892	7.700	**10.681**
Luna Omega PS C18	Fully porous	1.632	2.747	7.751	**12.130**
Ascentis Express C8	Fully porous	-1.285	2.967	10.914	**12.596**
Evosphere Diphenyl	Core-shell	1.996	2.981	9.437	**14.414**

Kinetex C18	Core-shell	0.285	2.692	13.369	**16.346**
Kinetex EVO C18	Core-shell	0.492	2.261	14.929	**17.682**
Kinetex Biphenyl	Core-shell	-11.82	3.911	38.666	**30.757**

3.2 LC-MS/MS determination of phenolic compounds

Based on the results of the first part, the Luna Omega Polar C18 column was chosen, as it exhibited acceptable retention characteristics for the selected phenolic compounds and good efficiency for alkylbenzenes. Using this column, a method was developed (Figure 2) for the simultaneous analysis of 48 phenolic compounds, including flavonoids, phenolic acids, aldehydes, alkylphenols, esters, benzentriols, coumarins, methoxybenzenes, phenylethanoids, stilbenoids and related compounds. During the method development, several parameters were optimized, including the gradient elution profile and the detection parameters for the MRM mode of tandem mass spectrometry.

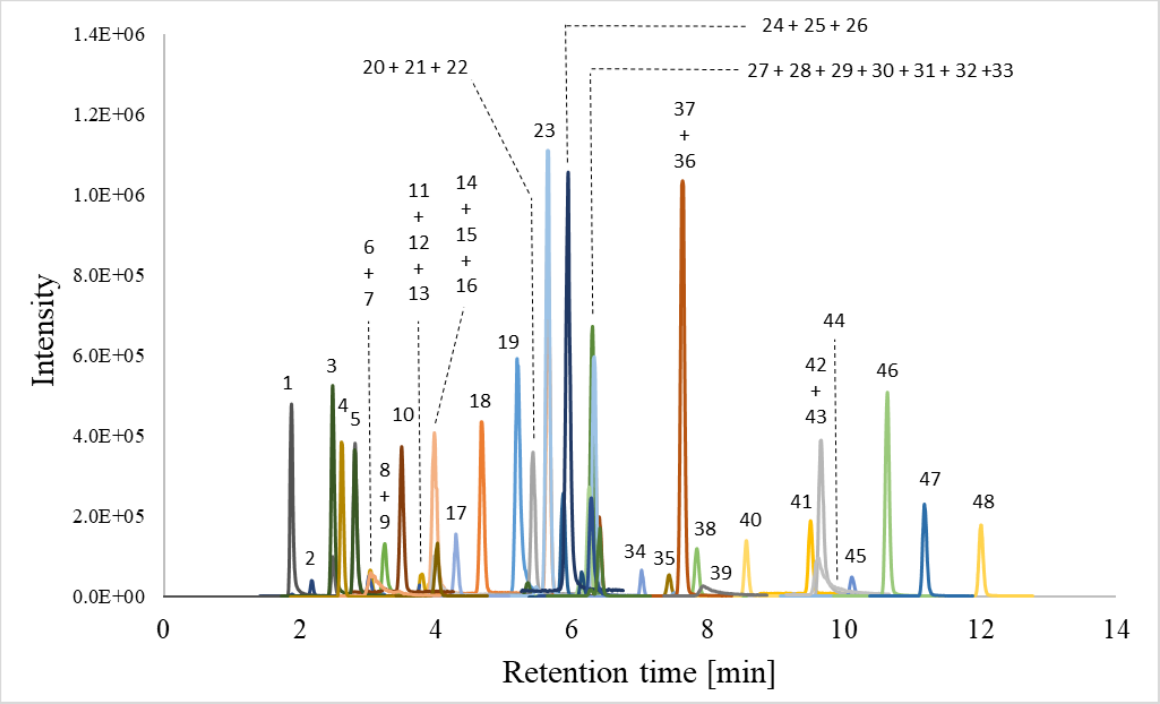

Figure 2: Chromatogram of the analysis of standards of 48 phenolic compounds (concentration of 0.1 mg/L of each).

The final method was validated in terms of accuracy, repeatability (intraday, interday), linearity, linear range, and limits of detection and quantification. The accuracy at the medium concentration level ranged from 89.4 % to 108.8 %, intraday repeatability ranged from 0.48 % to 7.55 % (RSD), and interday repeatability ranged from 0.54 % to 10.65 % (RSD) (38.26 % (RSD) for one compound). The linearity of the method was greater than 0.9973 for all compounds, with a linear range typically between 0.001 and 0.5 mg/L. Limits of quantification ranged from 0.31 to 134.8 µg/L.

3.3 Phenolic profile of different types of vinegar

LC-MS/MS analyses of the selected vinegar samples have been performed. Based on the results (Figure 3), it can be stated that different groups of vinegars significantly differ in the presence and amount of phenolic compounds. However, a relatively high concentration of tyrosol was found in all samples, and within each sample, tyrosol was often one of the main compounds. Its presence is characteristic for food products processed through alcoholic fermentation (wine, vinegar, etc.) since it is formed through the Ehrlich pathway by the gradual enzymatic transformation of the amino acid L-tyrosine by yeast. Significant differences in tyrosol concentration were found within individual groups, ranging from 0.06 to 23.4 mg/L.

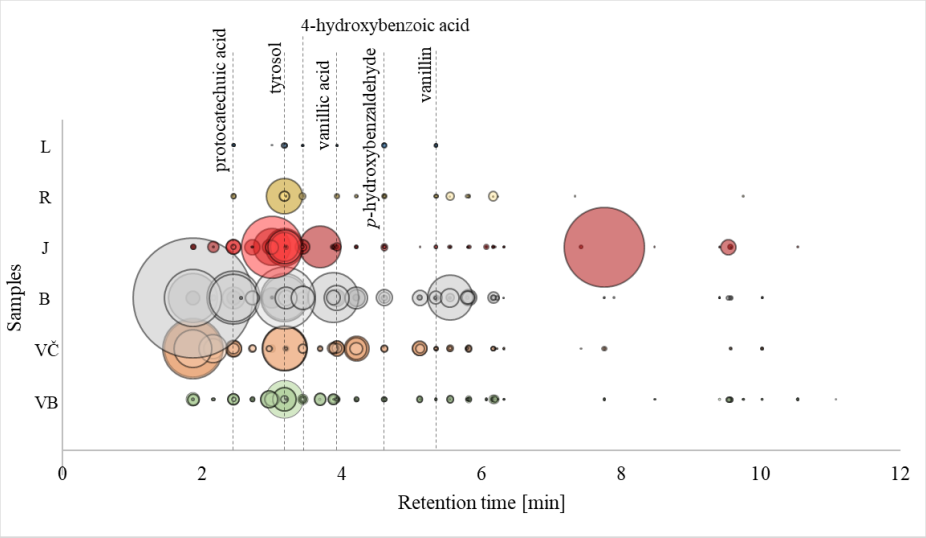

Figure 3: Chromatograms of the different types of vinegar presented as a bubble diagrams (bubble size indicates concentration). L – spirit vinegar; R – rice vinegar; J – apple vinegar; B – balsamic vinegar; VČ – red wine vinegar; VB – white wine vinegar.

In all types of vinegar, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, *p*-hydroxybenzaldehyde and vanillin were identified (Figure 3). However, this concludes the list of common compounds characteristic of the selected vinegar types, mainly due to the limited diversity of spirit and rice vinegars, where up to 7 and 19 phenolic compounds were identified. The highest number of phenolic compounds was identified in the group of apple vinegars (35) and white wine vinegars (35), followed by red wine vinegars (30) and balsamic vinegars (29).

The Venn diagram (Figure 4) shows that, except for rice (and spirit) vinegar, there is at least one specific compound for each group. The group of white wine vinegars contains the most specific compounds. The diagram also indicates that 10 phenolic compounds can be identified in every type of vinegar (with the exception of spirit vinegar).

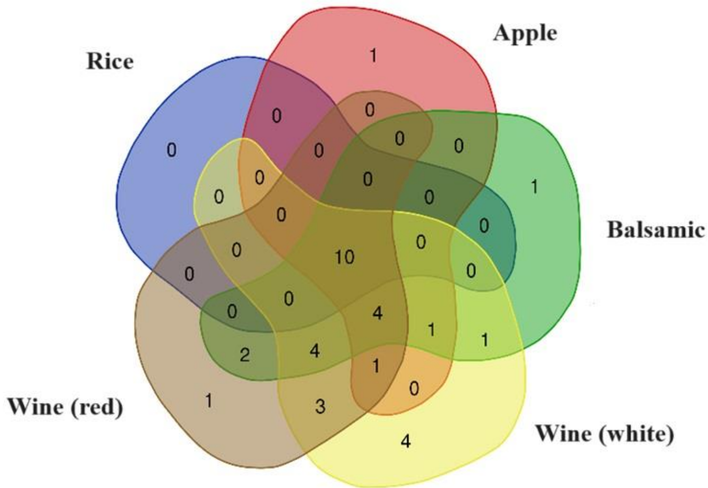

Figure 4: The Venn diagram summarizing the number of characteristic substances for a given vinegar group.

In terms of the content of phenolic compounds, balsamic vinegars had the highest values (average total phenol concentration $\bar{c}_{total}$ = 71.1 mg/L). This was followed by red wine vinegars ($\bar{c}_{total}$ = 39.6 mg/L), apple vinegars ($\bar{c}_{total}$ = 38.4 mg/L), and white wine vinegars ($\bar{c}_{total}$ = 8.3 mg/L). As expected, the lowest values were found in rice vinegars ($\bar{c}_{total}$ = 3.8 mg/L) and spirit vinegars ($\bar{c}_{total}$ = 0.3 mg/L).

3.4 Changes in the phenolic profile during roasting of coffee beans

Methanolic extracts of coffee beans were measured using a modified LC-MS/MS method. Of the original 48 compounds, 25 phenolic compounds were identified in the coffee beans. Among these, only pyrogallol and 4-methylcatechol were not identified in green coffee beans. These two compounds were formed during the roasting process, or their initial concentration was below the detection limit of the method. On the contrary, none of the monitored compounds completely disappeared during the roasting process.

On the basis of the results of this part, it can be asserted that during the roasting process, the amounts of individual phenolic compounds can either decrease or increase. Practically from the beginning of roasting, there was a reduction in the amounts of chlorogenic acid (Figure 5), caffeic acid, and ferulic acid (Figure 6). In the case of neochlorogenic acid and cryptochlorogenic acid (Figure 7), an initial increase in concentration was observed during roasting, followed by a subsequent decrease. During the roasting of the coffee beans, there was also a more or less steep increase in the concentration of some minor phenolic acids, aldehydes, alkylphenols (Figure 8), and pyrogallol.

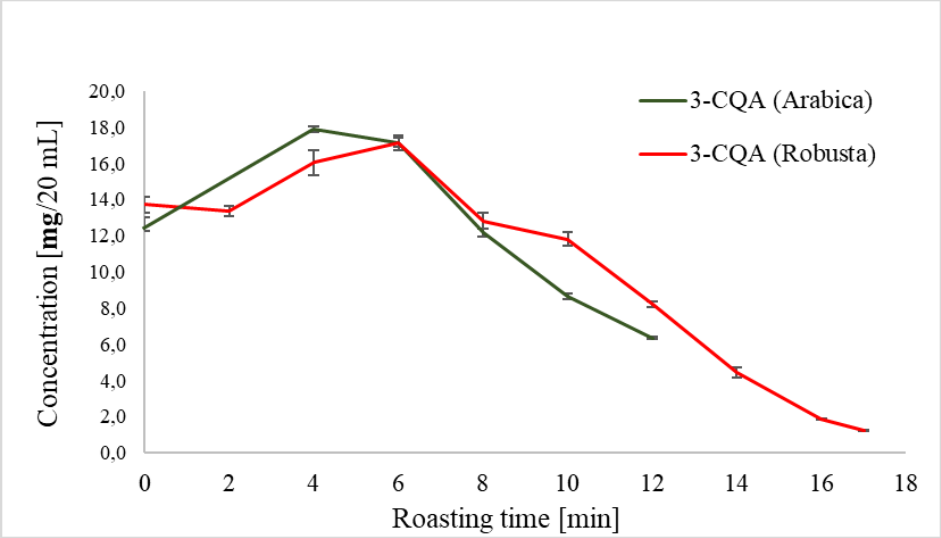

Figure 5: Changes in the concentration of chlorgenic acid (3-CQA) during the roasting of coffee beans (n = 3).

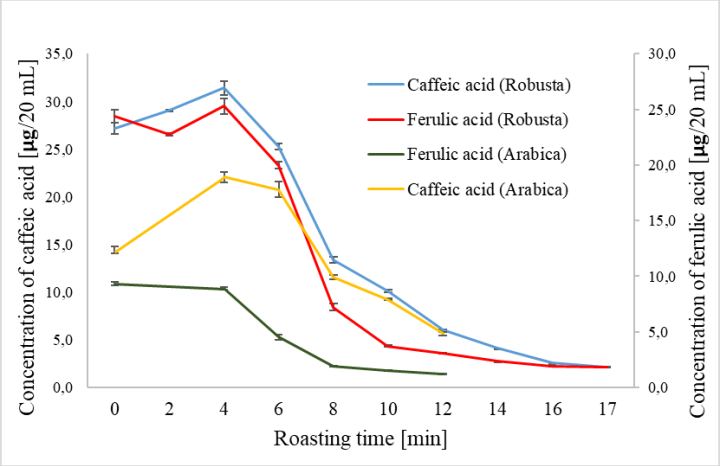

Figure 6: Changes in the concentration of caffeic acid and ferulic acid during the roasting of coffee beans (n = 3).

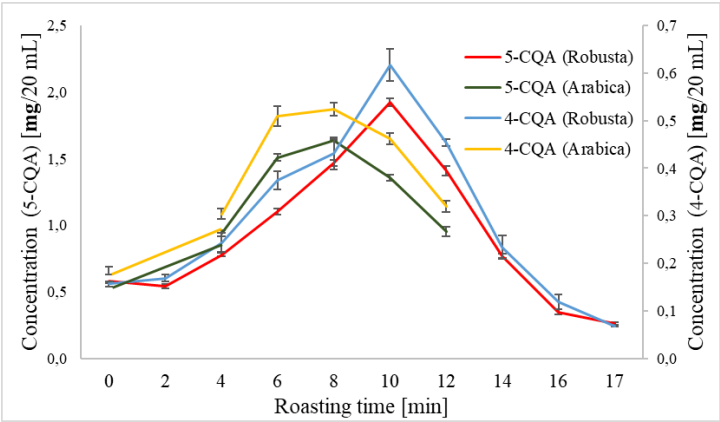

Figure 7: Changes in the concentration of neochlorogenic acid (5-CQA) and cryptochlorogenic acid (4-CQA) during coffee bean roasting (n = 3).

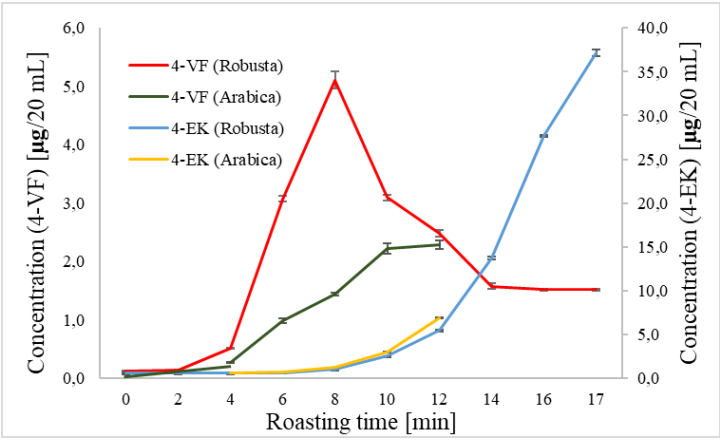

Figure 8: Changes in the concentration of 4-vinylphenol (4-VF) and 4-ethylcatechol (4-EK) during coffee bean roasting (n = 3).

Conclusion

This doctoral thesis provides a comprehensive overview of the determination of phenolic compounds in different plant-based matrices. The work is divided into four interrelated chapters, each beginning with a brief review of the literature to aid in better understanding of the topic. The theoretical parts of the doctoral thesis cover an introduction to phenolic compounds including their classification, modern trends in liquid chromatography, and the combination of this technique with mass spectrometry, the production and brief introduction of vinegars, and lastly, an introduction to the coffee bean roasting process.

The first experimental section describes the retention behavior of selected phenolic compounds and evaluates the tested columns in terms of efficiency and suitability for their use in the analytical determination of such substances. Based on the results of the first experimental section, a column was selected and used in the second experimental section, which deals with the development, optimization, and validation of the LC-MS/MS method for the determination of 48 phenolic compounds. Subsequently, the developed method was used to determine the phenolic profile of six different types of vinegar. The results of this section highlight the similarities and differences among various types of vinegar and compare the results obtained with those of already published studies. This part extends previously published work aimed at determining the phenolic profiles of balsamic vinegars of different qualities.

The final experimental section provides an expanded view on the fate of phenolic compounds during the roasting process of coffee beans. While previously published works focused primarily on the determination of chlorogenic acids, this doctoral thesis expands and elucidates the changes in the amounts of minor phenolic compounds in coffee beans. The results achieved from the fourth section will be used to create a publication that will include changes in the concentrations of not only phenolic compounds, but also selected furan compounds, amino acids, carbohydrates, organic acids, and volatile organic compounds.

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List of Students' Published Works

Scientific papers

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Posters

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