MASS SPECTROMETRIC METHODS FOR THE ANALYSIS OF POLYPHENOLIC COMPOUNDS IN PLANT FOODS

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INTRODUCTION

The beneficial health effects of polyphenols have been confirmed in numerous experiments. Epidemiologic studies provided convincing evidence that high flavonoid administration has a positive effect on general state of health, e.g. it decreases the development of coronary diseases, asthma and chronic lung diseases, it has an anti-carcinogenic effect and reduces the risk of apoplexy. However, it is not yet clarified in many cases what are the true mechanisms of action underlying the positive properties of these compounds. In the past, only the anti-oxidant effect of polyphenols has been considered important in this respect. Nowadays, however, the connection between the anti-oxidant properties of polyphenols observed in chemical assays and the processes underlying the biological effects shown under *in vivo* conditions is more often questioned. As a result, recently, an increasing number of studies are focusing on the elucidation of the true modes of action of polyphenols. The biological effects on the organism have been tested first on aglycones in order to study the metabolism and mode of action. It turned out, however, that this is not the right approach either, since polyphenols are administered into the human body by consumption of plants, and these compounds are not present alone (as aglycones) in plants but rather in the form of conjugates i.e., attached to sugars and organic acids. Due to the high number (close to ten thousand) of possible conjugate-varieties, in many cases it is not exactly known which polyphenol-conjugates are present in plants used as raw materials in many foodstuffs. Therefore, it is essential to know the exact chemical composition of polyphenols consumed as a part of the diet. To achieve that analytical methods that are capable of providing information not only about the aglycones but also about the derivatives are highly needed. This is very important, since the effect of certain polyphenols in the organism is highly dependent on the exact form, in which the given polyphenol is present. The investigation of intact forms of polyphenol-conjugates, however, is a complex task, and mass spectrometry combined with liquid chromatography is the most efficient approach for such studies. In my PhD work, I used and developed special mass spectrometry-based polyphenol analytical methods that are suitable for more detailed determination and profiling of polyphenol conjugates present in certain plants.

AIMS

First, my goal was to analyze polyphenol compounds of domestically grown herbs. So, goals were:

- quantitative determination of phenolic acids in plant extracts in free and bound form and comparing the phenolic components of the "thymol type" and "non thymol type" thyme populations, and the common and Greek oregano populations.
- In addition, to explore intact flavonoid components of both species by HPLC-MS/MS screening method.

In the second part of my work during the scholarship period in Spain, I studied oleuropein in olive oil and olive plants. My goal was to:

- Identify supposed oleuropein aglycone isomers more accurately by TOF-MS instrument.
- Furthermore, to compare olives, olive leaf and olive oil with respect to these derivatives.

The aim of the third part of my work was to find a solution to the challenges relating to the determination of constitutional isomers of flavonoid aglycones. For this purpose, my goal was to develop a selective HPLC-MS/MS method in case of flavonoid glycoconjugates which contain isomer aglycones. My goal was to:

- Examine the mass spectra of isomeric aglycones in order to find diagnostic product ions, whichare sufficient for discrimination.
- To justify the adequacy of the developed method using real samples.

MATERIALS AND METHODS

Aqueous extracts of different plant materials were analysed for polyphenolic components. Different chemotypes (thymol-type and non-thymol type selected population) of *Thymus vulgaris* L. (common thyme) and oregano (*Origanum vulgare* L. *subsp. hirtum* – greek oregano, *Origanum vulgare* L. *subsp*. *vulgare* – common oregano) were selected for the experiments. These samples were collected at the time of full blooming in Soroksár, at the Corvinus University of Budapest, Department of Medicinal and Aromatic Plant, Experimental Farm in May, 2010.

The aqueous extract was obtained by the Hungarian Pharmacopoeia (8th edition) standards. Thereafter, two types of sample preparation were used in order to measure the phenolic acids which are present in the free and derivative form in the sample. The first type was a simple dilution, and the other was a hydrolyzed sample preparation in order to liberate the phenolic acids present in conjugate form and become detectable for the target method. Experiments were performed by Agilent ® (Agilent Technologies, Waldbronn, Germany) 1200 HPLC system coupled with an Applied Biosystems ® (Foster City, CA, USA) 3200 Q-Trap hybrid triple quadrupole / linear ion trap MS / MS instrument, with a Turbo electrospray-V ® (electrospray, ESI) ion source used in negative ionization mode.

Local olive leaves and olives (Campiña Norte) were used for the analysis the oleuropein, collected at the campus of the University of Jaén, Spain. Among the tested olive oils, the one labelled as "Fuenroble" brand originated from Jaén province, while "Borges" were made by different olives of Spanish provinces. In both cases, the extra virgin type olive oils were used. The phenolic components from the extra virgin olive oil were extracted by solid-phase extraction, while components in olive leaves and olives were extracted by accelerated solvent extraction. The evaporation was performed by Caliper Turbovap LV Concentration Workstation (Caliper LifeSciences, Barcelona, Spain). The ASE sample preparation was done on a Dionex ASE ® 200 (Dionex GmbH, Idstein, Germany) system. The SPE sample purification was carried out by Visiprep SPE vacuum bath (Supelco, Bellefonte, PA, USA). Separation of the phenolic components was performed by an Agilent 1200 (Agilent Technologies, Santa Clara, CA) HPLC, which contained a vacuum degasser, an autosampler

and a binary pump. The HPLC system was connected to an Agilent 6220 (Agilent Technologies, Santa Clara, CA) time-of flight, (TOF) mass spectrometer equipped with an ESI ion source.

After the development of a method, which enables the determination of constitutional isomers of flavonoid aglycones, different plant samples were measured to verify the adequacy of the method. Local cherries, commercially available frozen sour cherries, oregano and thyme varieties (presented in the first part), were used as samples. In the sample preparation, 200 mg of lyophilized sample was measured in a 15-ml plastic centrifuge tube and homogenized with 10 ml of MeOH: H_2O: HCOOH 60:39:1 v/v mixture. It was placed in an ultrasonic bath for an hour and then centrifuged. Subsequently, 8 ml of the supernatant was measured which was evaporated to 1.5 ml. To the concentrated sample 200 µl of acetonitrile and 20 µl of water containing 50 V/V% formic acid were added and completed with 2 ml of water. Finally, it was filtered through a 0,45 mm PTFE filter. Experiments were performed by Agilent ® (Agilent Technologies, Waldbronn, Germany) 1200 HPLC system coupled with an Applied Biosystems ® (Foster City, CA, USA) 3200 Q-Trap hybrid triple quadrupole / linear ion trap MS/MS instrument , which contained a Turbo electrospray-V® (electrospray, ESI) ion source, used in positive ionization mode.

RESULTS AND DISCUSSION

The analysis of polyphenol conjugates in intact forms is a complex task. The liquid chromatography mass spectrometry coupled system provides the most effective approach for these measurements. In this PhD work, special mass spectrometric methods were developed and used which are suitable for the better understanding and exploring of polyphenol conjugates found in plants.

At the beginning of the PhD study, I worked with oregano and thyme in order to analyze their polyphenolic components. In particular, two populations (thymol-type and non-thymol type) of domestically-grown thymes (*Thymus vulgaris* L.) as well as domestically-grown oregano (Greek (*Origanum vulgare* L. subsp. Hirtum) and regular (*Origanum vulgare* L. subsp. Vulgare)) populations were compared taking into account the phenolic acid and flavonoid content. During the experiments, I was able to examine the ratio of the most abundant derivative forms and free phenolic acids. A targeted mass spectrometric method in combination with two types of sample preparation procedures were used to analyze phenolic acids. First, a simple aqueous extraction was used to gently extract the components found in herbs. In addition, an alkaline hydrolysis of the aqueous extract was used to convert the complex (conjugated) components to their free forms, thus they became visible for the targeted mass spectrometry method.

The results showed that this step was necessary, since, the amount of phenolic acids extracted by the simple aqueous extraction showed 20 to 100 times smaller concentrations compared to the ones that were measured after hydrolysis. In accordance with the literature, in the tested herbs, rosmarinic acid was present at highest concentrations. However, ferulic acid and p-coumaric acid and, in the case of thyme, syringinic acid also occurred in significant amounts. The concentration of chlorogenic acid was 3-40 times higher, the concentration of caffeic acid was ~ 10 times higher in the oregano than in the thyme, depending on the populations. Furthermore the concentration of chlorogenic acid, ferulic acid, and synapic acid was five times, two times and three times higher respectively in the Greek oregano sample, than in common oregano. In the case of thyme, the concentration of syringic acid and rosmarinic acid were two times higher in the thymol-type than in the non thymol-type populations.

The method enabled the provision of information on the ratio of conjugated / free forms of phenolic acids. However this approach gives no information about the type of moieties conjugated to the aglycone. This is why I additionally used a different approach for studying flavonoids. I aimed to obtain information on the type of conjugated glycans. The results showed that both types of tested plants contained components with apigenin and luteolin aglycone in the largest amount. In thyme, three luteolin derivatives, one apigenin derivate and a naringenin conjugate were present, while in oregano, four luteolin and two apigenin derivates were identified.

In the second part of my work, the goal was to examine oleuropein oleuropein aglycone (the latter is the free form of oleorupein), which are the typical polyphenols in olive oil, olives and olive leaf. The motivation to this research was given by the observations reported in the literature, where numerous isomeric forms of oleuropein aglycone and ligstroside aglycones have been shown as a result of a non-targeted analysis. Nonetheless, we cannot find any convincing biological explanation for the existence of these isomers. Thus the question is, whether these considered and recognized components are truly isomeric forms. Or they might not be isomers, but oleuropein derivatives, which have misidentified because of an incorrect application of the analytical methods? In this work, non-targeted mass spectrometric investigations were carried to examine the aforementioned hypothesis. Our results demonstrated that a part of some assumed oleuropein isomers are actually not isomers, but molecules which have a CH_4O moiety attached to the original aglycone. We do not yet have information on the exact forms of these compounds. A possible explanation can be that they are endogenous components formed during olive ripening, or they also might be derivatives formed during processing. However the theory of Karkoula *et al.* should not be overlooked claiming that artifacts can be generated during sample preparation.

Furthermore - as a criticism of the used technique and for the sake of caution - it is also worth mentioning that if we narrow the mass window in order to increase the accuracy of compound picking, neither the oleuropein aglycone, nor its conjugates were found, which indicates the limits of this technique. If a recent (06:00 B) version of the software was used, the *m/z* 377.1242 (oleuropein aglycone) have been identified as a $C_{21}H_{22}N_4O_5$ molecule. On one hand this may happen due to the limited capability of the software as mentioned above. On the other hand it is also possible that these components are not oleuropein components but other,

yet unknown molecules which have very similar weight to oleuropein. However, the probability of the latter assumption is rather low if the nature of the sample is taken into account, because oleuropein is one of the most typical molecule of polyphenolic components in olive.

In the third part of my Ph.D. work I presented a specific mass spectrometry method development, which is capable of distinguishing and determining flavonoid glycoconjugates which contained aglycones with the same ion mass. Such a method may be required for the selective identification of constitutional isomers because the determination of these components is not possible even with the use of high mass resolution MS devices. It was possible to find a solution to this problem by using a tandem mass spectrometry-based method, in which the flavonoid aglycone ions produced from the glycoconjugates by in-source fragmentation were further fragmented in order to increase selectivity. Subsequently, these characteristic product ions of the aglycones were chosen for further investigation. These were proved to be specific for a given aglycone component, regardless of being in the form of aglycone or as derivative in the sample. Cyanidin, kaempferol, luteolin, apigenin, genistein, pelargonidin,delphinidin-hesperetin, quercetin standards and those of their glycosides were used for method development. Fragmentation patterns observed for in-source formed aglycones and those of free aglycones were the same, meaning that conjugation does not significantly affect the applicability of the method.

Four diagnostic product ions were sucessfully identified in each molecular group (*m/z* 271, *m/z* 287, *m/z* 303) and the abundance ratio of these ions were proven to be specific, therefore they can be used for selective determination of flavonoid glycoconjugates having isomeric aglycones. The developed method was verified on four plant species. In the case of local black cherry, two cyanidin, two pelargonoidin, four hesperetin and one kaempferol derivatives could be identified. In the case of a commercially available sour cherry, two pelargonidin, three genistein, four cyanidin, one kaempferol and six quercetin derivatives have been identified. In thyme samples, which were presented in chapter 5.1., two luteolin and one apigenin compound could be identified, while in the oregano, two apigenin and two luteolin derivatives have been found. These results are in agreement with the componunds presented in the chapter 1.5. In summary, it can be concluded that the developed method allows the identification and can distinguish between aglycones with same chemical formula (isomers) regardless of whether they are present in their conjugated forms or as free aglycones.

NEW SCIENTIFIC RESULTS

1) I verified that the vast majority of phenolic acids in aqueous extracts of thyme and oregano are present in the form of conjugates.

This statement was proved by the analyses of syringinic acid in thyme, sinapic acid in oregano as well as ferulic acid and p-coumaric acid which are typical of both plants, and by examination of more chemotypes in both plants.

2) My results have proved that the biological effects associated with phenolic acids in thyme and oregano should not be judged only by examining the biological effects of free (deconjugated) forms.

3) High mass resolution and high accuracy mass spectrometric analysis proved that the oleuropein aglycone isomers arising from oleuropein by the processing technology are not actually isomers, but oleuropein aglycone derivatives.

The precise structure of these compounds is not known, however it can be concluded, based on these investigations, that the presence of the molecules with the chemical formula $C_{20}H_{25}O_9$ is proved in several cases.

4) I developed a mass spectrometric method which is suitable for the selective identification of flavonoid glycoconjugates with isomer aglycones.

With apigenin-genistein-pelargonoidin, cyanidin-kaempferol-luteolin and delphinidin-hesperetin-quercetin standards and with those of their glycoconjugates it was proved that with the analysis of fragment ions derived from the isomeric / isobaric aglycones, which are the core of these components, the flavonoid glycoconjugates having the same chemical formula can be clearly distinguished.

I also proved that the relative abundances of selected diagnostic ions of isomeric / isobaric aglycones are well reproducible and informative, even if the aglycone ions that are the subject of MS/MS experiments were prepared by ion source fragmentation from glycoconjugates.

5) The applicability of the method was verified by real samples.

In the course of this study, in home-grown black cherry one cyanidin-hexose, one cyanidin – hexose-dezoxyhexose, one kaempferol-3-*O*-rutinoside, one pelargonidin-3-*O*-glucoside, and one pelargonidin-hexose-dezoxyhexose were found. In addition, four quercetin molecule, such as quercetin-dihexose-dezoxyhexose, quercetin-hexose-dezoxihexose, a quercetin-hexozide and a clearly identified quercetin-3-*O*-glucoside were determined.

In sour cherry two pelargonidin (pelargonidin-dihexose-dezoxihexose, pelargonidin hexose-dezoxihexose) and three genistein (genistein-7-glucoside, and two other genistein-hexoside) component were determined. In addition, three cyanidin derivatives have also been identified. One of them is a cyanidin derivative with pentose-hexose-dezoxyhexose conjugate, which can be probably identified as cyanidin-3-*O*-xilosil-rutinoside based on the results. This component has been identified until now only in berries. In addition, one kaempferol-3-*O*-rutinoside, two quercetin-dihexose-dezoxyhexose, two quercetin-hexose-dezoxyhexose, and two quercetin-hexose derivative were identified, one of which is a quercetin-3-*O*-glucoside.

In addition, I also used samples of herbs to discover patterns of flavonoids and I managed to identify the main components.

PUBLICATIONS

Papers with impact factor :

Papp, N., **Szilvássy, B**., Abrankó, L., Szabó, T., Pfeiffer, P., Szabó, Z., Nyéki, J., Ercisli, S., Stefanovits-Bányai, É., Hegedűs, A. (2010): Main quality attributes and antioxidants in Hungarian sour cherries: identification of genotypes with enhanced functional properties. Int. *J. Food Sci. Tech*., 45 (2) 395-402. p. **IF 1,065**

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Abrankó, L.; Nagy, Á.; **Szilvássy, B**.; Stefanovits-Bányai, É.; Hegedűs, A., (2014) Genistein isoflavone glycoconjugates in sour cherry (*Prunus cerasus* L.) cultivars, *Food Chemistry*, 2014, megjelenés alatt. **IF: 3.334**

Hungarian conference (summary)

Szilvássy B. Sárosi Sz., Novák I., Pluhár Zs., Abrankó L. (2011): Kakukkfüvek vizes kivonatában található nem-illó polifenolok vizsgálata, MKE 1 Nemzeti Konferencia, Sopron 2011. május 22-25. ISBN 978-963-9970-11-3, 240.o.

International conference (summary)

Blanka Szilvássy - Dr. Szilvia Sárosi - Ildikó Novák - László Abrankó (2011): Effects of different drying treatments on phenolic acids in Thymes (Thymus vulgaris). Euroanalysis2011. 2011. szeptember 12-15., Belgrád, Szerbia.

Blanka Szilvássy - Abrankó László (2011): Mass spectrometric profiling of flavonoid glycoconjugates having isomeric aglycone nuclei. 5th International Conference on Polyphenols and Health. 2011. október 16-20., Barcelona-Sitges, Spanyolország.