Profiling polyphenols and their derivates with triple-quadropol mass spectometry

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1. INTRODUCTION

Like everywhere in the developed world also in Hungary the so-called civilization diseases become a more acute problem. In Hungary the fruit- and vegetable consumption does not reach the recommended level that means that enough quantity of minerals, vitamins and provitamin could not get in into our body. These compounds are essential not only to the normal functions, but they are also important in the prevention of diseases along of the antioxidant compounds (polyphenols).

Polyphenols are secondary plant metabolites therefore they are not essential for the plant. Although they contribute to the survival of the plant with their several useful functions, like pigmentation, protection against UV radiation or against pests, regulation enzymes activities. The huge number of polyphenols and the fact that they occur in a complex way in food make difficult the investigation of their absorption or physiological effects. We need more and valid, information about the concentration of flavonoid in foodstuffs, about their formation and damage during the kitchen technique and about their effectiveness in human body. Investigation all of these are impossible without suitable analytical methods.

2. OBJECTIVES

During my research work I concentrated two main areas: developing screening- and target component methods. In the first part wine samples were investigated and the aim was to develop a target component method, with the following aspects:

- Applying the method we would be able to separate the investigated wine samples according to their geographical origin, and their vintage year.
  - With a fast, well reproducible method that can be used in further routine analyses.
- Investigation of the role of „rapid resolution" column in classification of wines according to their polyphenols, and investigation the cadence of the gained information to the reliability.

Afterward my aim was to develop a screening method that is suitable to determine unknown flavonoids in crude extacts of a sample. The following criteria are set up:

- Gaining the most information from the lowest number of measurements.

- Applying MRM method for the monitoring of in-source formed aglycone fragments.
- Identification mono- and diglycoside conjugates of aglycones without reference standards.
- Applying the developed method for real samples.

In the last part the aim was to develop a monitoring method, in which the experiences of the prevoius developed method was used:

- Decreasing the necessary steps for identifying unknown compounds.
- Expanding the method for the identification of more complex derivates (trisaccharides, acylated saccharised).
- Determination the structural isomer aglycones and their glycosides within one measurement.
- Applying the developed method for real samples

3. MATERIALS AND METHODS

For determination of the origin of the wine samples gallic acid, ferulic acid, caffeic acid, t-para-cumaric acid, myricetin, kaempferol, t-resveratrol, (+)catechin, (-)epicatechin and quercetin were measured. HPLC-QQQ-MS was the measuring system.

For the classification a simplified discriminant-analysis was used. The SPSS 15 (SPSS, Chicago, United States) was used for statistical analysis. The variations were the concentration of polyphenols. The classification was according to the year, growing area and the wine cultivars. The variation includes also the possible isomers. The research was supported by a Hungarian-Austrian project therefore authentic Austrian wines were investigated.

The other two part of my work flavonoid measuring systems were developed applying an HPLC-QQQ-MS coupled analytical system. During chromatography gradient elution was applied and the detection of the compounds was carried out by the combination of different Ms/Ms scanning techniques. The three-step method was developed using the following standards: apigenin, apigenin-7-O-glucosides, luteolin, myricetin, naringenin, naringin, quercetin, rutin. A commercially available fruit juice was the test sample. For the two-step method luteolin, myricetin, naringenin, naringin, quercetin, rutin, isoquercetin, hiperoside, daidzein, genistein, hesperitin, hesperidin, fisetin, robinin, luteolin-7-O glucosides, kaempferol-3-O-glucosides were used. The test samples were sour cherries grown in Hungary.

4. RESULTS AND DISCUSSION

4.1. Classification of of authentic red wines based on their minor polyphenols

There are several methods for determination of the origin of wine samples. In this work the determination based on the meaurements of polyphenols.

In red wines the anthocyanins and anthocyanidins are the dominant polyphenols. Using this for determination the origin is already well-known. Therefore, our aim was to focus on compounds that are not dominant in wines but might be characteristic too. The question was whether these compounds and their isomers possess the information related to the origin of the wines.

The selection of polyphenols was followed the next logic: it was important to represent that part of polyphenols that can fall into the wine during some natural reaction/way-from the grape or during the technological process from the oak barrel. The selected compounds were: gallic acid, ferulic acid, caffeic acid, t-p-cumaric acid, myricetin, kaempferol, t-resveratrol, (+)catechin, (-)epicatechin, quercetin and naringenin. Naringenin is not common in wines therefore it was used as internal standard.

For chromatographic separation, high resolution column was used so some isomers of certain compounds can be separated. This can be useful for profiling. The pre-exmperiments confirmed that the isomers carry „extra information" concerning of the origin of wines. The separation of all the compounds could be carried out within 15 minutes.

For the classification, a simplified discriminant-analysis was used. The SPSS 15 (SPSS, Chicago, United States) was used for statistical analysis. The variants were the concentration of polyphenols. The classification was according to the year, growing area and the wine cultivars. The variation includes also the possible isomers. This can increase the differences among the groups and also the reability of the data.

4.1.1. Observations related to the determination of authentic wines

1. Classification based on the geographical origin

In this part the relation among the investigated materials and the geographical origin was investigated. The two most important wines of Austria, the Zweigelt and Blau Frankisher provided the most important results. Zweigelt derived from the following area: Südsteiermark,

Kamptal, Donauland and Thermenregion whilst Blau Frankisher derived from Carnuntum, Neusiedlersee Hügelland and Mittelburgenland. The areas were the group variables and the polyphenol concentrations of the wines were the independent variables. The correctness of the results was analyzed applying „leave one out, (LOO) test" that resulted 100% correctness of the classification data.

The second analysis was based on the classification of Blau Frankisher derived from Carnuntum, Neusiedlersee Hügelland and Mittelburgenland. The reliability of the data was 84% according to the LOO test. This is lower than in the other case. The main cause can be that these areas are relatively close to each other (the radius of the area is 50 km).

The determination of origin of the grape cultivars motivated by economic reasons. The quality and the price of the wines derived from different areas can be relatively different. According to the results obtained the developed method is suitable for detection cheatings related to the origin of the wines.

2. Classifications based on the grape cultivars.

The statistical analysis was based on the data derived from Wines of Weinvirtel (Blauer Portugieser, Blauer Wildbacher, Sankt Laurent, Blauer Zweigelt, Blaufränkisch, Blauburger). The wine types were group variables and polyphenol concentrations of the wines were the independent variables. The preliminary results showed the data can be separated into two groups. The first group contains the Blauer Portugieser, Blauer Wildbacher, Sankt Laurent types, whilst the second contains Blauer Zweigelt, Blaufränkisch, Blauburger wines. Both groups were checked by LOO test. The first classification gave 100%, whilst the second gave 65% reability.

The first classification was suitable since the groups separated well from each other and the discriminant values were high. On the other hand, the second classification had low discrimination value, and the LOO value was also low.

3. Classification based on the vintage

In this case 3 different data group were analyzed. The first group contained all of the data of wines, the second contained the data of Zweigelts and the third contained the data of Blau Frankisher In this case the group variables were the vintages (2003-2007), the polyphenol concentrations of the wines were the independent variables. These two vine types were separated since they can represent well the chosen authentic wine samples but they can represent well generally the red vines in Austria.

The LOO test gave 95% reability in the case of first group and 100% in second and third group. This confirmed that the vintage can be separated well from each other based on the polyphenol concentration.

Prohibiting the cheating related to the origin of the wine is significant economically since some known and favorable wine can be sold only after a few years that have effect also to the price.

4.2. Three-step HPLC-ESI-MS/MS method for the tentative identification of flavonoid mono-and diglycosides

The method is based on the fact that each flavonoid contains the flavonoid aglycone, like a tag that can be used for the identification. For example every myricetin derivates contain the myricetin aglycone. Therefore, in the first step we focused on the aglycone, tag of the sample. The observed compounds were all derivates of the aglycone. But the number of the aglycones are also significant therefore selected derivates were chosen. In this case the investigated aglycones were the following: apigenin, myricetin, luteolin, naringenin, quercetin.

4.2.1. Introducing the steps

1. Introducing the first step

For the identification of aglycone frame MRM mode is used. In case of each compound two characteristic fragments were chosen for the identification. The set up of MRM mode was optimized by standards.

Identification of aglycones is impossible till it links to parent ions therefore the substituents have to be cleaved before they arrive to the mass analyzer. For this reason the fragmentation in the ion-source was exploited. There was a so-called declustering potential set up (DP) and its high value is responsible for the cleaving the substituents from the parent ion in the ion-source. A compromised DP value has to be found because of the different derivates of the same aglycone.

For this reason three chosen derivates were tested. In the ion source the fragmentation of apigenin (apigenin-7-O-glucosides), naringin (naringenin-7-O-rutinoside) and rutin (quercetin-3-O-rutinoside) were investigated.

The flavonoid standards were intriduced into the MS and the m/z value of actual aglycone ion was monitored by single-stage MS. As a result of the optimization a DP value of -120 V was chosen for compromised DP value and this value was set up in MRM mode.

2. Introduction of the second step

After the first step it can be decided which aglycones are found in the sample but there is no further information about it. This is the reason why a second step is needed, namely a full scan spectra is acquired in the range of m/z 250-900 with the same chromatographic conditions.

After this step the parent ion can be determined based on the mass spectrum. Based on the mass differences among the observed mass peaks the linked substituents can be determined. For example if the difference is 162 Da between the supposed parent ion and aglycone; it indicates a hexose loss. The theory behind that is that the lighter ion is a fragment of the heavier. On the other hand, the observed fragments can be independent from each other meaning that they are originating from two different compounds, which are partly or entirely eluated together because of the insufficient chromatographic separation. Therefore a third steep is required to avoid the unreliability.

3. Introduction of the third step

In this step those masses are targeted that are supposed to belong to the different derivates. This step is relying on product ion spectra of selected masses. This step cannot be included into the first two steps because the masses are unknown until now. Therefore, a third step is needed. Applying this step the relation between the supposed parent ion and the aglycone can be revealed. The characteristic ion peaks of the aglycone and also some other characteristic fragments of the aglycone appear in the mass spectrum.

4.3. Two-step method for identifying flavonoid derivates

Our aim was to develop a method that is suitable for identification of more complex derivates.

4.3.1. Principal of the method

Also in this case the fragmentation in the ion source was utilized. This method differed from the previous method in the followings. The DP value was determined according to compound groups. These values were the following for monoglycosides: (m/z 385-500) -125 V, for diglycosides (m/z 500-700) -160 V and for more complex compounds (m/z 700-1000) -195 V.

In the same time that aglycones can also be detected, which derived from smaller molecules and cleaved at high DP values. The situation of the isomers are special, like the

hesperetin-quercetin or luteolin-kaempferol.The nominal masses of the aglycones are the same in both cases (hesperetin-quercetin 302 Da, kaempferol-luteolin 286 Da) but the fragmentation process is different because of their different structure, therefore the product ion spectra are also different. For this reason their determination can be carried out simultaneously.

The next question is: what molecule the parent ion was. Answering the question the precursor ion scan was applied. With this one can answer whether which molecule is the parent ion of the certain fragments. We give the deprotonated mass of the aglycone in negative ion mode. We look for the parent ion of this mass. In general, the mass range where the mass of the parent ion is from m/z = 350 to m/z = 1000. This is the second parameters that should be given besides the mass of the cleaved fragment. The more efficiency the fragments falling down the more sensitive method is. Therefore the instrument set up should be carried out that most falling down compound be the aglycon in the cell. Also in this case groups are created.

In the first group the range was m/z 385-500, where we expected the simplest molecules, like the monoglycosides. The second range was m/z 500-700, where we the diglycosides were expected. The third range was m/z 700-1000 where we expected the most complex molecules. The parameter was different in all groups.

4.3.2. Discovered compounds

The developed method was tested on sour-cherry samples. Twelwe different flavonoid derivates could be detected based on the developed approach, which were the following: 1 kaempferol-hexose-deoxygexoside, 4 different luteolin hexosides (at different retentions), 4 different naringenin hexosides (also at different retention), 1 quercetin hexose-deoxyhexoside and 2 quercetin-triglycosides. Moreover, three genistein derivates could be detected. In the literature there are hints related to genistein content of sour-cherry but it could not be proved. During my work only the present of the aglycone could be proved, supposedly the derivates are derived from genisten.

5. THESIS STATEMENTS

1. I proved by statistical methods that the so-called minor polyphenols and their isomers that are in low concentrations in red wines possess information related to the origin of the wine. I confirmed that the ratio of the isomers shows significant differences among the wines derived from different areas.

2. I developed a three-step electrospray ionization- tandem-mass spectrometric technique that is suitable for the mapping the mono-O- and di-O-glycosides of flavonoids in real samples.

In the first step – using in-source fragmention – the aglycone can be determined. The second step is the selection of that masses that can be parent ions of the observed aglycones. The third step is the confirmation, namely which masses belong to the parent ion. Practically, the first two steps are conducted in one chromatographic run Applying the developed method 12 flavonoid derivates (aglycones and derivates) were identified in a black currant juice sample.

3. Applying electrospray-ionization tandem-mass-spectrometric method I developed a pilot protocol that is suitable for the selective determination of luteolin, kaempferol, and fisetin in negative mode. According to my experiments I can recommend the following transitions:

kaempferol: quantifier: 285/117, qualifier: 285/93

luteolin: quantifier: 285/133, qualifier: 285/151

fisetin:quantifier: 285/135, qualifier: 285/121

4. I worked out a two-step electrospray ionzation tandem-mass spectrometric method that is suitable for identifying flavonoid derivates and can eliminate the errors derived from the creeps of retention times caused by more chromatographic measurements.

a. The first step of the method is MRM mode, the determination of aglycone from by fragmentation in the ion-source. The next step is the monitoring of precursors that provides information related to the parention. Besides the precursor monitoring there is an MRM monitoring also only for the searched aglycons. So the derivates of flavonoid aglycones can be determined within 2 steps.

b. I proved that the fragmentation features of the flavonoid-derivates with various compositions can be significantly different. Therefore, selecting the suitable voltage value the method was suitable for determining more complex compounds like flavonoid-triglycosides.

6. Publications

In journals with impact factor

Rak, G.; Fodor, P.; Abrankó, L., Three-step HPLC-ESI-MS/MS procedure for screening and identifying non-target flavonoid derivatives. International Journal of Mass Spectrometry 2010, 290, (1), 32-38 **IF: 2,117**

Jaitz, L.; Siegl, K.; Eder, R.; **Rak, G.**; Abranko, L.; Koellensperger, G.; Hann, S., LC-MS/MS analysis of phenols for classification of red wine according to geographic origin, grape variety and vintage. Food Chemistry 2010, 122, 366-372. **IF: 3,146**

In conference proceedings

Hungarian abstract

Rak, G.; Béres, I.; Bognár, Á.; Abranko, L.; Fodor, P., Borok Pb-izotóp arányainak mérése ICP-TOFMS technikával. In *Lippay János – Ormos Imre – Vas Károly Tudományos Ülésszak*, Budapest, 2005.

Abranko, L.; Brunner, M.; Katona, R.; Jaitz, L.; **Rak, G.;** Fodor, P.; Hann, S.; Prohaska, T.; Stefánka, Z., Élelmiszerek eredet-meghatározása tömegspektrometriás módszerekkel. In *Magyar Spektrokémiai Vándorgyűlés*, Nyíregyháza, 2008.

International, full-text

Balogh, E.; **Rak, G.;** Stefanovits-Bányai, É.; Hegedűs, A.; Abranko, L. In Profiling flavonoid derivatives in Hungarian berry fruits, International Mass Spectrometry Conference Bremen, Germany, 30 Aug-4 Sept, 2009; Bremen, Germany, 2009.

International abstract

Rak, G.; Abranko, L.; Fodor, P., MULTI-ELEMENT ANALYSIS OF HUNGARIAN WINES BY COLLISION CELL ICP-MS AND THEIR CLASSIFICATION ACCORDING TO GEOGRAPHICAL ORIGIN, . In *European Winter Conference on Plasmaspectrochemistry* Taormina, 2007

Rak, G.; Abranko, L.; Fodor, P., Application of elemental fingerprinting for Hungarian wines
Pumpaya Conference, Nagymaros, 2007

Rak, G.; Abranko, L.; Fodor, P., Development of multicomponents flavonoid detection by HPLC-MS/MS, Pumpaya Conference, Dösendorf, 2008

Balogh, E.; Szilvássy, B.; **Rak, G.;** Engel, R.; Stefanovits-Bányai, É.; Hegedűs, A.; Abranko, L., Characterisation of selected compounds contributing to the antioxidant status of red and black currants grown in Hungary. In Young Scientistds Meeting - Future trends in phytochemistry in the global era of agri-food and health, Los Alcázares, Murcia, Spain, 2009.

Nagy, Á.; **Rak, G.;** Szilvássy, B.; Hegedűs, A.; Abranko, L., Characterization of compounds providing the total antioxidant capacity in apricot (Prunus armeniaca L.) cultivars and hybrids grown in Hungary. In The 4th International Conference on Polyphenols and Health, Harrogate, England, 2009.