[3] Palecek, E., Tkac, J., Bartosik,, M., Bertok, T., Ostatna, V., Palecek, J., *Chem. Rev.* 2015, *115*, 2045-2108.
[4] Vacek, J., Zatloukalova, M., Geleticova, J., Kubala, M., Modriansky, M., Fekete, L., Masek, J., Hubatka, F., Turanek, J., *Anal. Chem.* 2016, *88*, 4548-4556.

## EXTRACTION, SEPARATION AND IDENTIFICATION OF BIOACTIVE COMPOUNDS IN BARLEY USING LC-MS/MS

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**Summary**
An extraction procedure, based on alkaline and acidic hydrolysis, was developed, using the *Box-Behnken* design of experiment, with the aim to achieve an exhaustive recovery of free and also linked polyphenols, i.e. bonded to cell structures, in barley (*Hordeum Vulgare L.*) samples. An LC-MS/MS (MRM transition mode) method was developed for analysis of the samples extracted through the optimized procedure, and to achieve a correct quali-quantitative characterization of a specific group of polyphenols.

The optimized procedure was also employed for the extraction and the identification of bioactive molecules in a dietary supplement composed of dried leaves of barley, commonly known as "young barley". A *selectivity* study was carried out employing some of the compounds identified in young barley, and by using six different columns (C18, silica, ES-CN and OH5 types). The results showed that different mechanisms are involved in the retention of the standards, especially in case of HILIC mode.

### 1 Introduction

In the last years, the market of functional foods and dietary supplements has increased with a compound annual growth rate of 7%; consequently, the characterization of *nutraceuticals* is nowadays a topic of great interest. At present, polyphenols appear to be among the most interesting bioactive compounds, thanks to their extraordinary antioxidant properties, but still there is not a standard method for their extraction. However, in recent years, the researchers focused on the basic or acid, or both, hydrolysis process [1] in order to recover also linked polyphenols. Polyphenolic compounds, in fact, may be present in plants in free or esterified/etherified soluble form as well as in insoluble form, bound to the cell wall constituent. It is widely known that more than 90% of polyphenols in cereals are present in bonded form and are considered the major contributors to the total antioxidant capacity of cereals [2]. Therefore, for a

correct quali-quantitative characterization, these processes are required. Moreover, liquid chromatography-mass spectrometry (LC-MS) technique is nowadays the best analytical approach to study polyphenols from different biological matrices. On the basis of these knowledge, one of the aims of this research was to develop a suitable extraction procedure and a fast RPLC-MS/MS method, for the quali-quantitative characterization of barley.

Some polyphenols identified in a dietary supplement consisting of an extract of "young barley", namely the plant in the first stage of growth (leaves), which is rich in minerals, vitamins and polyphenols [3], were chosen as standards to carry out a selectivity study. Separation selectivity is important fundamental parameter affecting resolution in LC, especially when the separation of complex mixture has to be achieved. The selectivity in various chromatographic modes strongly depends on types of interactions, and the combination of stationary and mobile phase is the key factor affecting the achieved results. The selectivity study performed enables comparison of different columns and stationary phases in order to select the best possible conditions, with the aim to develop a LC method suitable for the separation of all compounds.

## 2 Experimental

*Box-Behnken* design of experiment and *Statistica software version 12* (Stat Soft, Czech Republic) were used for the design and the evaluation of data, respectively, for the development of the optimized extraction procedure. LC-PDA analysis were performed using an HPLC system equipped with a binary gradient pump LC-20AD (Shimadzu), PDA detector (Shimadzu), column thermostat LCO 102 Single (Ecom). The LC-MS/MS analyses were performed using a Shimadzu modular liquid chromatograph consisting of two LC-20ADXR pumps, DGU-5 degassing unit, SIL-20ADXR autosampler and LCO-102 column thermostat (Ecom) coupled with QTRAP 4500 mass spectrometer operating in electrospray mode (AB SCIEX).

The column used was the Ascentis Express C18 (150 mm 3.0 mm, 2.7 μm). The injection volume was 1 μL, mobile phase consisted of HCCOH 0.4% (pH ~2.3) (solvent A) and $CH_3CN$ (solvent B) and the step-wise gradient profile was as follows: 0 min, 12% B; 4 min, 12% B; 8 min, 30% B; 12 min, 70% B; 14 min, 12% B; 24 min, 12% B; the flow rate was 0.5 mL/min and the temperatue was 30°C.

The column used for the selectivity study were the Ascentis® Express: HILIC (150 x 3 mm, 2.7 μm), ES-CN (100 x 2.1 mm, 2.7 μm), an OH5 (100 x 2.1 mm, 2.7 μm), a Kinetex® C18 (150 x 3 mm, 2.6 μm) and two Luna®: C18 (150 x 3 mm, 3 μm) and HILIC (150 x 3 mm, 3 μm). The standards used for characterization of barley and the solvents used for the optimization of the extraction procedure and the HPLC-PDA and LC-MS/MS analyses were purchased from Sigma-Aldrich. The standards used for characterization of young barley were purchased from Extrasynthese.

## 3 Results and Discussion

The parameters chosen for the extraction procedure were: 70% (*v/v*) of acetone, 100% of ethyl acetate and 25 min of sonication. With the first step of the procedure, only free

polyphenols can be recovered, so with the aim to have an exhaustive recovery, considering also the bound molecules, alkaline and acid hydrolyses were carried out, using ascorbic acid and EDTA to prevent eventual degradation phenomena. The extracted polyphenols of five different cultivars of barley were analyzed with the LC-MS/MS technique developed. Epicatechin was the only compound present exclusively in free form, while all the other have also be found after the basic and acid hydrolysis. Moreover, the presence of some polyphenols was observed only after the hydrolysis process, indicating that they are present only in bound form (catechin, myricetin, quercetin, kaempferol). These results are in agreement with the aim of this research, designed to establish the real need for a more complete extraction procedure, and for the correct evaluation of the phenolic content. The quali-quantitative composition of polyphenols presented in the five samples was quite similar, without significant differences. According to the literature data [4], *p*-hydroxybenzoic and ferulic acids were the most abundant, 624 μg/g and 198 μg/g, respectively (average of the five cultivars). It's important to underline that, although *p*-hydroxybenzoic acid is the most abundant compound among the free molecules, its larger quantity was obtained only after the alkaline hydrolysis (sample β). Myricetin, quercetin and kaempferol are not present in free form, but they were detected after alkaline and acid hydrolysis. Apigenin exists in free and linked form, but it was found only in samples γ, in this case the basic hydrolysis was not enough to break the bonds, which link this molecule to some cell components.

Among the RP columns tested, the Ascentis Express C18 column provided the best resolution and the shortest time of analyses. The gradient was optimized using the *LC Simulator* software and further evaluated at different values of pH.

Using the LC-MS/MS method, it was found that young barley is rich in soluble polyphenols (linked to one or more molecules of sugar). Anyhow, some of them were found after hydrolysis procedure.

The columns tested to evaluate the retentions of different standards showed linear correlation between concentration of solvent B and log *k*. However, in some cases, deviations from linearity were observed, demonstrating that there are many mechanisms involved in HILIC separation mode.

## 4 Conclusions

A new extraction procedure, aimed at the exhaustive recovery of free and also linked polyphenols was developed. The results showed the importance of the acidic and alkaline hydrolyses in this procedure. A HPLC-MS/MS method in MRM transition mode was developed for the correct quantitative characterization of polyphenols in barley. Young barley is rich in soluble polyphenols, while some of these compounds showed significant differences in retention, depending on the column used.

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**References**
[1] Chandrasekara, A., Shahidi, F. *J. of Functional Foods*, 2011, *3*, 144-158.
[2] Serpen, A., Gokmen, V., Pellegrini, N., Fogliano, V. *J.of Cereal S.*, 2008, *48*, 816-820.
[3] Park, M.J. *et al*. *Nat. Prod. Commun.*, 2014, *9*, 1469.
[4] Garrote, G., *et al*. *J. Food Eng*., 2008, *84*, 544-552.

## DETERMINATION OF DISTRIBUTION CONSTANTS OF ANTIOXIDANTS BETWEEN LIPOSOMES, ALKYLSULFATE MICELLES, OCTANOL AND WATER USING ELECTROKINETIC CHROMATOGRAPHY

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**Summary**
The *in vivo* effects of the antioxidants are dependent on their lipophilicity, which govern membrane and protein interactions. Two electrokinetic systems, i.e. micellar electrokinetic chromatography (MEKC) and liposome electrokinetic chromatography (LEKC), were used for studying of the lipophilicity of antioxidants. Micelles of sodium decyl sulfate (SDeS) and sodium dodecyl sulfate (SDS) were used as the pseudostationary phase in MEKC and the liposomes were composed of mixtures of 1-palmitoyl-2-oleyl-*sn*-glycerophosphatidylcholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-phosphatidylserine (POPS) or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidyl-DL-glycerol (POPG) in LEKC. The retention factors of the studied analytes were determined at pH 7.4. The micelle and liposome/aqueous distribution constants for studied antioxidants were experimentally determined and compared with values of octanol/aqueous distribution constants.

### 1 Introduction

Antioxidants are secondary metabolites of plants, which can protect organisms from the effect of free radicals. They are able to inhibit oxidative processes and improve the immune function [1]. Polyphenols, including flavonoids, are the most common natural antioxidants in a human diet. Flavonoids can be divided into six groups – flavones, flavonoles, flavanoles, flavanones, isoflavones, and anthocyanines. Phenolic acids can be divided into two groups based on the structure of cinnamic or benzoic acid. *In vivo* effects of these compounds are dependent on their lipophilicity and hydrophilicity. The logarithm of the octanol/water distribution constant, *log* $P_{o/w}$, is the most common way of describing hydrophobicity of compounds. For charged compounds, logarithm of the distribution constant between octanol and aqueous solution with the target pH value is