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Determination of polar target components: HILIC-MS methods in food analysis

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1. SUMMARY

Hydrophilic Interaction Liquid Chromatography, abbreviated HILIC, has become one of the most dynamically developing branches of the liquid chromatography technique in recent years. Coupled with mass spectrometry detectors, HILIC-MS systems allow the separation of target components in complex samples, such as foodstuffs of plant or animal origin and feeds, that have been difficult or impossible to retain and detect using High Performance Liquid Chromatography (HPLC) earlier. This paper presents the possibility of using HILIC-MS to determine polar compounds through four specific examples. The test procedures presented include the analysis of a carcinogenic food contaminant, acrylamide, the determination of which in certain food groups is required by law. A further objective of this manuscript is to provide a detailed overview of the HILIC-MS method developed for the detection of water-soluble B vitamins added to foods. Several international standards have been developed for the analysis of B vitamins, which offer determinations for each vitamin B separately. in comparison, most B vitamins can be measured together using the HILIC-MS technique. This option could be a big step forward for laboratories that analyze B vitamins regularly in a large number of samples. For people living with food intolerance, it is important to know the lactose content of milk-based foods. The HILIC-MS coupled system can also provide a fast and accurate solution for the detection of lactose present in low concentrations, the presentation of which is also the subject of this dissertation. Finally, the paper includes the determination of urea in feed samples. The law prescribes a photometric measurement of urea, but the reliability of this procedure at low concentration levels has been questioned and thus the HILIC-MS method has become prominent as an alternative solution. The applicability of the present analytical methods has been confirmed by successful participation in international proficiency testing programs, sufficiently accurate concentrations detected in control samples, and the certification of fully validated methods by the National Accreditation Authority (NAH).

2. Introduction

Instrumental analytical chemistry is largely a collection of separation techniques, of which perhaps the most common is the use of HPLC. HPLC allows the separation, in the liquid phase, of target components from matrix components present in samples soluble in the HPLC eluent (e.g., foods). However, the use of HPLC may be limited by the polarity of the compounds to be determined, and this is especially true for high polarity, hydrophilic components **[1]**. In normal phase HPLC separation (NP-HPLC), the stationary phase of the HPLC column is of polar character, which would allow for interactions between the sorbent and the polar target compounds, however, due to the apolar nature of the mobile phase (e.g., alkane, halogenated alkane derivative, ether or acetate), hydrophilic components do not dissolve in the eluent

[1] ÉMI-TÜV SÜD Kft.

(mobile phase) and therefore cannot be analyzed by NP-HPLC. In reverse phase HPLC separation (RP-HPLC), the polarity conditions between the stationary and mobile phase change, so the hydrophilic target components are well soluble in the polar aqueous eluent, however, their retention on the alkyl-modified apolar stationary phase is low or nonexistent, and so they elute in the dead time. In HILIC-type separations, the difference in polarity between the stationary and mobile phases is not as sharp as in the case of NP-HPLC or RP-HPLC. HILIC can be characterized as an HPLC system where the stationary phase is more polar and the mobile phase is less polar **[1]**. This allows for the dissolution of the target components in the eluent on the one hand, and for the formation of the interactions necessary for their retention on the stationary phase on the other hand. Thus, HILIC is used for the HPLC separation of high polarity, ionic compounds, but not for apolar compounds or those with no proton function **[2]**.

HILIC columns are polar stationary phases, which can be based on silica gel, an be anion or cation exchange, double ionic stationary phases, or polarly modified phases. The silica gel stationary phase is the same as the stationary phase used in NP-HPLC, however, the HILIC column is not stored in apolar organic solvent but in a mixture of water and acetonitrile which may contain small concentrations (5 – 10 mM) of ammonium acetate. The essence of HILIC separation is that an interface layer is formed on the surface of the stationary phase from the physically sorbed water, as a result of the low water content eluent flowing through the column **[2]**. Hydrophilic target components dissolve in this layer and, depending on their polarity, they spend more or less time there during their migration on the stationary phase. This way, they can be distinguished on the basis of their retention properties. In the HILIC system, compounds with a higher polarity will have a longer migration time, so the higher the water solubility of a component, the more time it spend in the interface layer and thus on the stationary phase, i.e., the higher its retention will be. If the eluent does not contain water, the interface layer on the stationary phase does not form, therefore 100% organic solvent cannot be used as the mobile phase. HILIC mobile phases are mixtures of water and a water-miscible, low viscosity organic solvent. Due to the nature of HILIC, water is the strongest eluent as it is the most polar solvent. Acetonitrile is most often used as an organic eluent modifier because of its low viscosity, which results in narrow chromatographic peaks and a smaller pressure drop. The water content of the eluent varies between 2 and 40 v/v%; the lower the water content of the eluent that allows separation, the better the HILIC column is. At the same time, the eluent must contain at least enough water to dissolve the compounds to be analyzed. This condition limits the reduction of the volume fraction of water. In the case of gradient elution, the eluent can be made stronger by increasing the water content of the mobile phase. In the HILIC separation of basic components with a proton function that can be converted into an ionic state, such as amines, it is important that the pH of the mobile phase be acidic, because this way the target components will be in a polar (ionic) state and their retention will increase.

Acidic eluents with a high acetonitrile content are particularly advantageous when using mass-selective detectors, since the efficiency of electrospray ionization (ESI) can be greatly increased if the majority of the eluent is an organic solvent, which evaporates and dries in ESI faster than high water content eluates. A low pH further facilitates positive mode ionization and thus more ions are formed, which then enter the analyzer space, increasing sensitivity. Using tandem mass spectrometry (MS/MS) detection, in addition to the significantly improved ionization efficiency, utilizing the high degree of selectivity of the MS/MS instrument, previously unheard-of application possibilities can now be accessed. The HILIC-MS/MS coupled technique allows the determination of hydrophilic target components in biological matrices at low concentrations with high sensitivity. Examples include the analysis of polar pesticide residues, the determination of hydrophilic mycotoxins, or the separation of tenuazonic acid or flavonoids from plant extracts **[3, 4, 5]**. In this paper, three HILIC-MS/MS methods for food testing and one for feed testing are presented:

1. Determination of acrylamide in a variety of foods;
2. Determination of water-soluble B vitamins added to solid or liquid food samples;
3. Analysis of the lactose content of lactose-free products at low concentration levels;
4. Determination of urea in foodstuffs.

3. Determination of acrylamide in foodstuffs

Acrylamide is formed in foods during heat treatment above 120 °C in the presence of proteins and sugars; it is a carcinogen whose maximum level in foods is regulated by Commission Regulation (EU) 2017/2158 **[6, 7]**. A detailed and comprehensive domestic study on acrylamide was published in 2018 **[7]**. Acrylamide is widely analyzed, and the standard method developed for its determination (EN 16618:2015) is widely used by food testing laboratories worldwide **[8]**.

Acrylamide (NH_2-C(=O)-CH=CH_2) is a low molecular weight, polar compound (Mw: 71 g/mol; log*P*: 0.67) that can be analyzed by either gas chromatography or high performance liquid chromatography. For gas chromatographic separation, acrylamide must be derivatized **[9]**, but this is not necessary for the liquid chromatographic measurement.

An HPLC-MS/MS method is specified by the standard for the determination of acrylamide **[8]**. In the case of HPLC separation, however, it might present a problem that conventional RP-HPLC columns have essentially no retention for acrylamide. The standard recommends a porous graphitized carbon-based stationary phase (PGC, Hypercarb™), with adequate retention for acrylamide in 100% aqueous eluent (0.1%, v/v, acetic acid in water) **[8]**. The retention factor is *k'*=4, measured with a 100% aqueous eluent. The retention of other RP-HPLC columns for acrylamide is practically negligible, but even if it were higher, it would be only when using a 100% aqueous eluent. High water content eluents greatly reduce ionization during ESI ionization, as the more water there is in the mobile phase, the less efficient the evaporation and drying of the eluent in the ion source is. Thus, fewer ions are produced in the ion source, which reduces the sensitivity and deteriorates the limit of quantification that can be achieved for the compound **[10]**.

During sample preparation according to the standard, samples are extracted with water and the extract is first purified by multimode (anion and cation exchange in the same cartridge) solid phase extraction (SPE). The extract passed through the SPE column is then further purified by an ENV+ SPE column, having a retention for acrylamide, which can be eluted by a methanol/water mixture. Elution from the SPE column is followed by evaporation and redissolution. Taking into consideration the two SPE steps and the slow evaporation of the aqueous methanol, the conclusion can be drawn that this is a costly and time consuming sample preparation. In the case of coffee samples, the test standard even requires fat removal using hexane **[8]**.

3.1. Determination of acrylamide by a HILIC-MS/MS method

Due to the polar nature of acrylamide, it may have a retention on a HILIC column. However, being a small molecule, its interaction with the stationary phase is limited, so careful selection of the appropriate HILIC column is important in order to meet the minimum chromatographic condition of $k' \geq 1$, also prescribed by Commission Decision 2002/657/EC **[11]**. If this condition is met by adequate HILIC separation, then the separation can be performed with a mobile phase that is high in acetonitrile, compared to RP-HPLC. This aqueous eluent containing a large amount of acetonitrile results in a high ionization efficiency and effectively increases the sensitivity of the method.

In our experiments, several HILIC column were tested in order to achieve acceptable retention for acrylamide **[12]**. However, adequate retention was only achieved with an eluent having a very high acetonitrile content, which made method development difficult since, accordingly, the solvent of the samples to be tested also had to be high in organic solvent. The final choice was the TSKgel Amide-80 type column **[12]**. The stationary phase of the TSKgel Amide-80 contains carbamoyl groups, resulting in unique retention between the completely porous stationary phase and the polar target components. On the TSKgel Amide-80 column, $k' \geq 1$ could be achieved with 95% (v/v) acetonitrile, while for the other columns, 98% (v/v) acetonitrile was required in the eluent to achieve this. On the TSKgel Amide-80 column, with a mobile phase of water and acetonitrile (5/95, v/v) containing 0.1% (v/v) formic acid and 1 mM ammonium formiate, the retention factor of acrylamide was found to be 1.14 (***Figure 1***).

Sensitivity was decreased by an eluent with a higher ammonium formiate content (>1 mM), while it was not increased further by a higher acid content (>0.1%, v/v). Compared to the high water content (95%, v/v) eluent, the limit of detection was decreased by 1 order of magnitude by the mobile phase containing a large amount of acetonitrile during ESI-MS/MS detection. This allowed for the dilution of the sample and the omission of the SPE steps during the sample preparation, which led to the so-called dilute-and-shoot procedure. In dilute-and-shoot methods, following the exrtaction of the sample, the extract is diluted with an isotope-labeled internal standard, which is then injected into the LC-MS/MS instrument **[10]**.

In the case of the HILIC separation, it is important that the composition of the solvent of the sample injected is the same as that of the eluent, and is not stronger than it. Thus, the large solvent excess aqueous extraction in the standard cannot be used. This is because the aqueous extract would have to be diluted further with acetonitrile before injection. This, in turn, would increase the limit of quantification (LOQ) due to the further dilution of the sample by at least a factor of ten. Acetonitrile is a good solvent for acrylamide and is therefore often used to extract food samples. A particular advantage of acetonitrile extraction is that it is a solvent compatible with the HILIC system, which means that the extract can be injected directly into the HILIC-MS/MS system after acetonitrile extraction and filtration. An additional advantage of acetonitrile extraction compared to 100% aqueous extraction is that proteins and fats are precipitated and salts are not dissolved in high concentrations by acetonitrile, so that removal of these matrices from the sample during solid-liquid extraction is easily accomplished. Examining the appropriate extraction medium by an experimental plan, it was found that the highest amount of acrylamide can be extracted from naturally contaminated gingerbread using an acetonitrile – water – formic acid (69/30/1, v/v/v) mixture **[12]**. Gingerbread was an ideal sample matrix because due to its composition (honey, sugar, protein, starch) it may contain large amounts of acrylamide formed by heat treatment. Accordingly, the limit value for gingerbread (800 µg/kg) is high **[6]**. One of the typical areas of HILIC separation is the determination of sugars in foods **[1]**, so using HILIC,

the sugars remaining in the sample can also be separated from the acrylamide, as they do not elute on the HILIC phase with 95% (v/v) acetonitrile.

The extract of the sample extracted with the above-mentioned extraction mixture cannot yet be injected following centrifugation and syringe filtration, because it still has a high water content. Therefore, before injection, the extract must be diluted 1:1 (v/v) with acetonitrile. In the case of dilute-and-shoot methods, a large number of matrix compounds elute in the dead time in high concentrations, so it is advisable to use a diverter valve between HPLC and the MS/MS instrument to divert the components eluting at the dead time to a waste solvent collection tank, thus avoiding the contamination of the ion source. **Figure 1** shows that between minutes 3 and 5, the detector baseline drops to zero, because there is no input current to the MS/MS instrument. When extracting 2.0 g of the sample with 20 ml of extraction medium, and then diluting the decanted sample in a 1:1 (v/v) ratio with acetonitrile, the total dilution of the sample is 20-fold. The detection limit of the LC-MS/MS instrument used by us is 1 ng/ml for acrylamide, so without component loss during sample preparation, an LOQ of ~20 µg/kg can be achieved in the samples, which can be further reduced using more sensitive MS/MS systems. Tha accuracy of the method is 101% for naturally contaminated samples (crispbread) and 101% to 105% for spiked gingerbread samples (RSD<7.2%) **[12]**. To veify the method, a coffee sample from a proficiency testing program was examined. 183 µg/kg acrylamide was detected in the coffee. The value declared for the prficiency testing program was 242 µg/kg for acrylamide. The calculated z-score was thus -1.2, which indicates a satisfactory analysis.

3.1.1. Advantages of the HILIC method

1. By omitting sample clean-up, both SPE steps in the standard can be dispensed with. This simplifies samples preparation, and only a solid-liquid extraction step and the dilution of the decanted sample is needed. Omission of the two SPE clean-up steps significantly reduces the cost of the analytical test, both in terms of the SPE columns and the solvents.

2. According to the standard, after the second SPE step, the solvent of the water-methanol sample eluate has to be evaporated. However, evaporation of the solvent of the acrylamide-containing sample results in the loss of the target component **[9]**. In the case of the HILIC method, this concentration step can be omitted, because the extract is only diluted, no sample enrichment is requierd.

3. The acetonitrile-water-formic acid extraction solvent mixture precipitates the proteins and fat in the samples, so there is no need for the SPE step by which the proteins would be separated from the acrylamide, and fat removal with hexane is not required either. As acetonitrile is a HILIC compatible solvent, its use is optimal for both the extraction and the subsequent HILIC-MS analysis.

4. The mobile phase with 95% (v/v) organic solvent greatly increases ionization efficiency during ESI-MS/MS detection, and thus its sensitivity. This allows the use of the dilute-and-shoot procedure. Using isocratic elution, the analysis time is only 8 minutes **[12]**. The PGC column in the standard has a special stationary phase, its applicability is much more limited than that of a HILIC column, which can be widely used in other applications.

5. If an isotope-labeled internal standard of the target component is available and the sensitivity of the LC-MS/MS instrument allows the enrichment of the sample to be omitted, then the dilute-and-shoot procedure guarantees the simple, fast and cost-effective analysis of the target components in complex matrices **[10]**. HILIC-MS methods based on dilute-and-shoot procedures belong to a new trend in instrumental analytical chemistry, and their importance is constantly growing.

4. Determination of B vitamins in foods

Before the analysis of B vitamins, it should be clarified whether it is the analysis of the vitamin added to the food or whether it is necessary to determine the total vitamin content, including the original and added amounts. This is because naturally occurring B vitamins are present in the sample in a bound form, from which they can be released by hydrolysis or enzymatic sample preparation. In the present paper a method for the determination of the vitamin added to the samples is recommended.

Standards for the determination of B vitamins have been prepared separately for the each component, such as B1, B2, B6 etc. **[13, 14, 15]**. The reason for this is that to determine the total amount (naturally present and added) of a given vitamin (e.g., B1) a specific extraction is required during sample preparation. In addition, specific HPLC separation and detection, and for UV detection possible derivatization are required. If only the vitamins added to the sample are analyzed, their simultaneous multicomponent determination by RP-HPLC and an optical detector is still not feasible. Due to their polar nature, B vitamins can be measured conveniently by the HILIC method. Due to coelution interference and to determine vitamin B1, MS or MS/MS detection is required, resulting in the use of the HILIC-MS/MS coupled technique (***Table 1***). With this analytical solution, vitamins can be measured side by side,

and due to the high sensitivity, sample preparation is again limited to a solid-liquid extraction and a further acetonitrile dilution. Due to the hydrophilic nature of B vitamins, vitamins added to foods can be easily extracted with water. Due to the high concentrations (>0.06 mg/100 g), a large excess of solvent (20 ml water/1 g sample) can be used to extract B vitamins from solid samples, resulting in the complete extraction of added vitamins. In the case of liquid samples, samples may be further diluted with water and, at the end of sample preparation, with acetonitrile.

In the absence of isotope-labeled internal standards, the dilute-and-shoot procedure cannot be applied because the background cannot be compensated even at 100-fold sample dilution. Thus, matrix-free solvent calibration cannot be used for quantitative evaluation. The method thus requires matrix-matched calibration. This means that extracts of blank samples containing the target components below the limit of detection are spiked to certain concentration levels with vitamins, and these are used as calibration points **[10]**. This way the backgrounds of the sample and the calibration solutions are almost identical, and quantitative evaluation is not significantly affected by the background. It is important to note that matrix-matched calibration is only as accurate as the match between the matrices of the blank sample used for calibration and of the test sample. So with this calibration, the background cannot be compensated 100%, this can only be approximated. As an alternative solution, standard addition can be used, in which the test sample is spiked with vitamins, and the concentration of the non-spiked sample is calculated from the signal intensities measured in the spiked samples and the signal increase.

B vitamins can be measured well on silica HILIC stationary phases with a water-acetonitrile eluent containing 0.1% (v/v) formic acid (***Figure 2***), as they were detected in proficiency testing effervescent tablet and juice samples, as well as FAPAS breakfast cereal control samples by the HILIC-MS/MS method. Following dissolution of the effervescent tablet in water (4 g sample/200 ml), the sample was diluted 1:9 (v/v) with water, and then further diluted 1:9 (v/v) with acetonitrile. In the case of the juice, similarly to the effervescent tablet, it was first diluted with water and then with acetonitrile. In the proficiency testing program, vitamins B1, B2, B3, B5, B6 and B9 cold be detected in the samples in amounts ranging from 0.058 mg/100 g to 5.44 mg/100 g (***Table 2***). All z-scores calculated for the measured values were in the -2 to +2 range, so they were acceptable. Concentrations of B vitamins detected in the FAPAS control sample were also within the acceptable range (***Table 2***).

5. Determination of lactose in foods

Milk sugar, also known as lactose, is a disaccharide type compound. Due to its hydrophilic properties, it can be analyzed easily using HILIC separation. Since it does not have a proton function, it is not necessary to adjust the pH of the eluent. Lactose can be analyzed isocratically on a silica gel HILIC column with a mobile phase of acetonitrile-water (75/25, v/v) containing ammonium acetate (10 mM). With a refractive index (RI) detector, it is easy to measure lactose in milk, which is present in percent amounts (~ 4.5%), but for products marked “lactose-free” the limit value in Hungary is 0.1% (MSZ 1382/1-87). In Germany, the permissible lactose content is only 0.01% **[16]**. 0.1% in the sample corresponds to a lactose concentration of 1000 µg/ml or 1000 µg/g. If the dilution of the sample during sample preparation is taken into account, a lactose content of 0.01% to 0.1% can no longer be detected using a refractive index (RI) detector. This is when MS or MS/MS based detection can play an important role.

In the case of MS/MS detection, lactose can be measured as the acetate adduct $[M + CH_3COO]^-$ (401 *m/z*) with negative ionization (***Figure 3***). If a formiate salt is used in the eluent, the formiate adduct $[M + HCOO]^-$ parent ion is formed. The LOQ value of the HILIC-MS/MS instrument at the standard level is usually 0.1 µg/ml, i.e., four orders of magnitude lower than the Hungarian limit value. This allows for a high degree of dilution of the sample, which improves quantification in that background compounds eluting together with lactose do not greatly interfere with the ionization of lactose in the sample matrix (matrix effect). With this method, the fact of being “lactose-free” can be checked easily by extracting or diluting the sample with water and then further diluting the decanted sample with acetonitrile, and then filtering it before injection using a syringe filter. When analyzing milk, it is sufficient to dilute the sample with acetonitrile, which also removes the proteins and fats from the sample. After subsequent centrifugation and filtration, the sample can be injected directly into the HILIC-MS/MS system.

Another disaccharide, maltose, has the same mono-isotopic mass and structure as lactose (***Figure 4***). Thus, their ion transitions are the same, the MS detector cannot detect them on separate mass channels. However, measurement of the two sugars side by side is not difficult on an HILIC column, which, despite their similar structures, separates the sugars in the liquid phase using the above-mentioned eluent (***Figure 3***).

6. Determination of urea in feedstuffs

Urea ($(NH_2)_2CO$) is the diamide of carbonic acid and the end product of protein metabolism. Its molecular weight is 60 g/mol. Urea is also used as an additive in ruminant feeds. The maximum permitted urea content is 8,800 mg/kg **[17]**. Hoever, its use is prohibited in the feeding of other animals (other than ruminants), and the feeds must not contain detectable amounts of urea **[17]**. The accuracy of

the official spectrophotometric test used to quantify urea **[18]** is only appropriate for the detection of high levels of urea, and the suitability of the method for the analysis of small amounts of the target component is questionable. In 2019, an inter-laboratory comparison, a proficiency testing (PT UREA-19/2) was organized by the research laboratory of the EU Joint Research Centre (JRC, Geel, Belgium), the main objective of which was to determine which of the available analytical methods are the most appropriate for the determination of urea in feeds produced for non-ruminants (compound feeds).

The laboratories participating in the proficiency testing program could use any technique available to them. The method recommended by the JRC is a HILIC-MS/MS analysis, based on the dilute-and-shoot procedure and the method of the Institute of Food Safety, Animal Health and Enviroment Laboratórium (BIOR, Riga, Latvia). During sample preparation, the samples are extracted with water containing formic acid (1%, v/v), and the decanted samples are diluted with acetonitrile and an isotope-labeled internal standard (urea-$^{13}C^{15}N_2$). Urea can be measured well on a HILIC silica gel stationary phase with a water-acetonitrile mobile phase containing 0.1% (v/v) formic acid (***Figure 5***). The urea concentrations detected in the three feed samples analyzed ranged between 260 mg/kg and 890 mg/kg (***Table 3***), and the samples in the last batch (batch J) did not contain detectable amounts of urea (< 50 mg/kg). Based on the proficiency test report, batch J contained 18.4 mg/kg urea, which was below our LOQ level. For batch Ca nd batch G, the values assigned to the samples were 249 mg/kg and 935 mg/kg, respectively. Our z-scores calculated for the latter two samples were -0.2 and 0.2, respectively, which are adequate values.

7. Conclusions

The application of the HILIC technique has several advantages, only a few of which are mentioned in this paper. In addition to food analysis, the use of HILIC is also gaining ground in pharmaceutical and environmental analysis. For example, HILIC can be used to replace ion pair chromatography in the analysis of polar compounds with a proton function. HILIC-type separations are not among high resolution LC techniques, but by coupling the HILIC system with mass-selective detection, the signals of the components in the detector will be separable and polar target components can be analyzed with the high sensitivity mentioned above.

8. Acknowledgment

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9. Literature

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