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Review

Application of hydrophilic interaction chromatography for the analysis of polar contaminants in food and environmental samples

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ABSTRACT

For the analysis of highly hydrophilic and polar compounds, Hydrophilic Interaction Chromatography (HILIC) has been established as a valuable complementary approach to reversed-phase liquid chromatography (RPLC). Moreover, the use of mobile phases with a high percentage of organic solvent in HILIC separation is beneficial for mass spectrometric (MS) detection, because of enhanced ionization which results in an increased sensitivity. In this review, various applications of HILIC are described for a number of environmental and food contaminants together with detailed methodological descriptions and the advantages or drawbacks of HILIC compared to other LC methods are critically discussed. In the first part of the review, an overview is given of the work that has been carried out with HILIC for the analysis of pharmaceuticals and pesticides in environmental samples. HILIC has shown its applicability for polar pharmaceuticals, such as antibiotics, estrogens and their metabolites, drugs of abuse, cytostatics, metformin and contrast agents. In the pesticide group, HILIC chromatography was helpful for polar phenylurea and organophosphorus pesticides. The second part of the review focuses on the analysis of antibiotic residues in food and feed with HILIC, while in the pesticide group, HILIC experiments have been reported for dithiocarbamates and quaternary ammonium compounds. The last chapter gives an overview of the analysis by HILIC of miscellaneous analytes in aquatic and food/feed samples.

Contents

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1. Introduction

Reversed-phase liquid chromatography (RPLC) has been the alternative method of choice for the analysis of various classes of compounds difficult to analyze by gas chromatography (GC). A large number of compounds have been measured by RPLC using C_{18}-based silica stationary phases. However, highly polar compounds undergo early elution on traditional RP stationary phases, leading to lower sensitivity of the mass spectrometric (MS) detection due to (1) high matrix effects and (2) high water percentage in the mobile phase at the beginning of the run resulting in lower ionization efficiency in the MS interface. The analysis of highly hydrophilic and polar compounds by hydrophilic interaction chromatography (HILIC) coupled to MS has been demonstrated as a valuable complementary approach to RPLC [1]. The use of a low aqueous and high organic mobile phase in HILIC separation is almost ideal for electrospray ionization in many cases, leading to increased sensitivity [1]. In this review, the analysis of environmental and food contaminants, such as pharmaceuticals, drugs of abuse and pesticides, by HILIC coupled to different detectors (e.g. MS or UV) is discussed.

2. Pharmaceuticals in environmental samples

Several studies have pointed out that excreted human or veterinary pharmaceuticals or drugs of abuse (DOA) end up in the environment through insufficient elimination during treatment of wastewater in wastewater treatment plants (WWTPs) [2–5]. Since these compounds can cause negative effects on the aquatic environment, considerable interest has been focused nowadays on their detection and quantification in waste- and surface water. Table 1 shows an overview of the analytical methods based on HILIC for the determination of pharmaceuticals in environmental samples.

2.1. Estrogens

Estrogens have been found in the environment resulting from natural sources (human and animal excretion, plants, or fungi) or as by-products of synthetic chemicals. Estrogens pose a risk as they act as endocrine disruptors which can lead to fish feminization [19] and can influence plant growth or human health. Their monitoring has become therefore important. The analysis of estrogens (e.g. estrone) and their glucuronide and sulfate conjugates at low ng/L levels in the aquatic environment can be done by immunoassays, GC–MS or LC–MS. Immunoassay methods are sensitive and specific, but only few antibodies are available and cross-reactions remain a problem. GC–MS methods require derivatization and hydrolysis of the estrogen conjugates, so the substitution group is lost before analysis. LC–MS/MS is the most advantageous technique and it is widely used today.

To detect estrogens (e.g. estrone, estriol, estradiol) and their glucuronide and sulfate conjugates in surface water in a single run, Qin et al. [6] used a column-switching method involving a C_{18} and a HILIC stationary phase. The analytes were isolated and pre-concentrated from 500 mL surface water with solid-phase extraction (SPE) on Oasis HLB cartridges. Free estrogens were eluted with ethyl acetate, while estrogen conjugates were eluted with methanol (MeOH) containing 2% ammonium hydroxide. After elution of the SPE cartridge, the estrogen fraction was further derivatized with dansyl chloride and the reaction mixture was further purified on an Oasis HLB cartridge. The last methanolic eluate was mixed with the estrogen conjugates fraction and the solvent was evaporated. Analytes were reconstituted in mobile phase and injected in the LC–MS system.

RPLC–MS was optimal for the separation and detection of the hydrophobic dansyl derivatives of free estrogens, while HILIC–MS showed good performance for the highly hydrophilic estrogen con-

Table 1
Pharmaceuticals, drugs of abuse and pesticides analyzed in HILIC mode from environmental samples. A brief review of employed columns, mobile phases and type of samples.

Compound/class of compounds	Matrix	Column	Mobile phase	Refs.
Pharmaceuticals				
Estrogen conjugates	River water	SeQuant ZIC–pHILIC (100 mm × 2.1 mm, 5 μm)	AcN/ammonium acetate 5 mM in water; gradient	[6]
Cytostatics	Wastewater	SeQuant ZIC–HILIC (150 mm × 2.1 mm, 3.5 μm)	AcN/ammonium acetate 30 mM in water; gradient	[7]
Spectinomycin, lincomycin	Liquid manure Rainfall run-off	Alltech Alltima HP HILIC (150 mm × 2.1 mm, 3 μm)	AcN/formic acid 0.1% in water; isocratic	[8]
Metformin	Wastewater Surface water	SeQuant ZIC–HILIC (150 mm × 2.1 mm, 3.5 μm)	AcN/ammonium formate 10 mM in water (pH 3 with formic acid); gradient	[9]
Albuterol, cimetidine, ranitidine, metformin	Water, sludge	Waters Atlantis HILIC Silica (100 mm × 2.1 mm, 3 μm) Agilent Zorbax HILIC Plus (100 mm × 2.1 mm, 3.5 μm)	AcN/0.1% acetic acid/ammonium acetate; gradient AcN/ammonium acetate 10 mM in water; gradient	[10] [11]
13 pharmaceuticals	Wastewater	Phenomenex Luna HILIC (150 mm × 3 mm, 5 μm)	AcN/MeOH (87.5/12.5, v/v)/ammonium acetate 5 mM in water; gradient	[12]
Gd chelates	Wastewater	SeQuant ZIC–HILIC (150 mm × 2.1 mm, 3.5 μm)	12.5 mM ammonium formate and 12.5 mM formic acid in AcN/water (76/24, v/v, pH 3.75)	[13]
Drugs of abuse				
Cocaine and metabolites	Wastewater	Agilent Zorbax RX-Sil (150 mm × 2.1 mm, 5 μm)	AcN/ammonium acetate 2 mM in water (pH 4.5 with acetic acid); gradient	[14]
9 drugs of abuse	Wastewater	Phenomenex Luna HILIC (150 mm × 3 mm, 5 μm)	AcN/ammonium acetate 5 mM in water; gradient	[15]
Pesticides				
Organophosphorus pesticides	Water	Waters Atlantis HILIC Silica (150 mm × 2 mm, 5 μm)	AcN/isopropanol/ammonium formate 200 mM (pH 3); isocratic	[16]
Diquat, paraquat	Drinking water	Waters Atlantis HILIC Silica (150 mm × 2.1 mm, 3 μm) Waters Atlantis HILIC Silica (150 mm × 2.1 mm, 3.5 μm)	AcN/ammonium formate 10 mM, pH 3.7; isocratic AcN/ammonium acetate 250 mM, pH 3; isocratic	[17] [18]

jugates. The use of HILIC–MS compared with RPLC–MS for the determination of conjugated estrogens resulted in a 10-fold higher sensitivity [20]. A column switching set-up was used, employing a binary pump connected to a quadrupole-linear ion trap MS with an ESI interface operated alternatively in positive (for the detection of the dansylated estrogens) and negative (for estrogen conjugates) ions mode, in the same run. A 10-port 2-positions switching valve served to link up the two columns: Phenomenex Luna C18(2) (100 mm × 2.0 mm, 3 μm) for the separation of dansylated estrogens and SeQuant ZIC-pHILIC (100 mm × 2.1 mm, 5 μm), consisting of a silica-based zwitter-ionic stationary phase, for the estrogen conjugates. The mobile phase consisted of A) acetonitrile (AcN)/aqueous ammonium acetate 5 mM pH 6.8 (75/25, v/v) and B) AcN/aqueous ammonium acetate 5 mM pH 6.8 (95/5, v/v). The sample extract was injected with an initial mobile phase composition of 40% A and the valve connected both columns. Only estrone (0.04 ng/L) and estrone-3-sulfate (0.84 ng/L) were detected in river water samples.

2.2. Cytostatic drugs

The release and accumulation of cytostatic drugs in the environment is a risk factor for organisms since they have a cytotoxic effect on all fast-dividing cells [21]. The presence of chemotherapeutic drugs in waste- and surface water has recently received increased attention. Anti-metabolites are the most used cytostatic agents and have polar structures. They were analyzed in biological and environmental samples, at very low concentrations, by GC/MS, often with derivatization [22], and by ion-pairing RPLC or using specific stationary phases, such as porous graphite or HILIC.

Cytostatics and metabolites (5-fluorouracil, cytarabine, gemcitabine, α-fluoro-β-alanine, uracil 1-β-D-arabinofuranoside, 2,2′-dihydrodeoxyuridine) were extracted by Isolute ENV$^+$ cartridges [7]. Water samples (50 mL) were adjusted to pH 6, loaded onto the cartridges and eluted with MeOH. For the investigation of the cytostatics in hospital wastewater, samples were filtered through glass fiber and a cellulose acetate membrane, and then stored at −20 °C until analysis. The system employed by Kovalova et al. [7] to analyze cytostatics and their metabolites was composed of a binary pump connected to a triple quadrupole MS equipped with an ESI interface, operated in positive or negative ion mode (in two separate runs). Analytes were separated in gradient on a SeQuant ZIC-HILIC column (150 mm × 2.1 mm, 3.5 μm), with a mobile phase consisting of (A) aqueous ammonium acetate 30 mM/AcN (2/3, v/v) and (B) AcN. Method limits of quantification (LOQ) were in the range of 0.9–9 ng/L. To confirm the masses of the analytes and elucidate an interfering peak observed in hospital wastewater, a high resolution Orbitrap MS was used. Hospital wastewater samples were analyzed with the analytical method and 5-fluorouracil was detected in 76% of samples, while gemcitabine and 2,2′-difluorodeoxyuridine were found in 65% and 88% of the samples, respectively. The presented method was the first to analyze the metabolites of cytostatics in the aquatic environment. For 5-fluorouracil and cytarabine, methods based on GC–MS [22], capillary electrophoresis [23] and RPLC–UV [24] were earlier described. The HILIC–MS method presents clear advantages for the analysis of 5-fluorouracil with a LOQ (5 ng/L) that is 1000-fold lower than the RPLC–UV and capillary electrophoresis method. The LOQ of the GC–MS method is comparable (10 ng/L), but it requires a laborious and time-consuming derivatization step which is not necessary in the HILIC–MS method. For cytarabine, the HILIC–MS method is not preferable because of the presence of an interfering substance in wastewater.

2.3. Antibiotics

Antibiotics have the potential to affect the microbial community in sewage systems. The inhibition of wastewater bacteria may seriously affect organic matter degradation and, therefore, effects of antibacterial agents on the microbial population are of great interest. Antibiotics have been also in evidence as well in surface water where they may affect organisms of different trophic levels and can lead to resistance [25]. Antibiotics have been determined by bioassays, GC–MS and RPLC, often with derivatization, with ion-pair agents in the mobile phase, or even by ion-exchange techniques.

Spectinomycin and lincomycin are commonly used to control diarrhea produced by *E. coli* infections in pigs. The liquid manure resulting from livestock farming is often used as crop fertilizer and can be a source of veterinary pharmaceuticals into the environment.

Peru et al. [8] studied the environmental fate and transport of these veterinary antibiotics and developed an HILIC–MS/MS method for their detection and quantification from liquid manure and rainfall run-off. Sample preparation was achieved by SPE. For spectinomycin, the samples were loaded onto Oasis HLB and WCX cartridges connected in series and, after washing with citrate buffer (pH 5) and AcN, eluted with AcN containing 3% formic acid. For lincomycin, samples (at pH 9) were loaded onto Oasis HLB cartridges, and eluted with AcN. The extracts were evaporated to dryness and reconstituted with AcN. The chromatographic separation was performed on a silica-based Alltech Alltima HP HILIC column (150 mm × 2.1 mm, 3 μm), in isocratic conditions (35%A, 65%B), at 0.2 mL/min. The mobile phase consisted of A) 90/10 water/AcN + 0.1% formic acid and B) 90/10 AcN/water + 0.1% formic acid. MS detection was carried out using an atmospheric pressure chemical ionization (APCI) interface in MRM positive ions mode. Good retention times and separation from the matrix components were achieved with HILIC, and LOQs were 6.0 and 0.040 μg/L (liquid hog manure supernatant) and 0.2 and 0.008 μg/L (run-off water) for spectinomycin and lincomycin, respectively. The concentrations measured in the supernatant of a liquid manure hog collected from a nursery barn in Elstow, Canada ranged from 64 to 105 μg/L for spectinomycin; and from 93 to 216 μg/L for lincomycin. Analysis of simulated rainfall run-off samples from fields treated with swine manure gave positive results for lincomycin (0.027–0.160 μg/L), while spectinomycin was not detected [8].

A wide variety of chromatographic methods coupled to MS detection, most RPLC-based, are available for the separation of both compounds from different matrices (milk, honey, tissue, . . .). Because of the complex nature of the liquid hog manure matrix and the low concentrations, such methods pose some problems. Both compounds suffer from low retention in RPLC, leading to high matrix interference. Increased retention times were obtained by ion-pairing with heptafluorobutyric acid (HFBA), but this method leads to poor MS detection and thus low sensitivity [26]. The use of HILIC–MS/MS for the separation and detection of both compounds has some advantages: (a) both compounds are well separated from the complex matrix components, resulting in low matrix interference; b) the use of a high amount of organic phase in the mobile phase leads to a high ESI ionization efficiency, resulting in increased sensitivity.

2.4. Miscellaneous pharmaceuticals and medical reagents

Metformin is a widely used anti-diabetic drug from the biguanide class, and one of the most prescribed overall. Metformin is highly polar and as a consequence low retention is obtained in RPLC. For the determination of metformin in waste- and surface water, Scheurer et al. [9] have optimized a method based

on HILIC in order to obtain a good separation. A sample preparation with Strata X-CW SPE cartridges followed by separation on a SeQuant ZIC–HILIC column (150 mm × 2.1 mm, 3.5 μm) with a mobile phase consisting of (A) 10 mM ammonium formate in water (pH 3) acid and (B) AcN, in gradient starting with 95% B was used. The chromatographic system was coupled to a 4000 Q-Trap mass spectrometer equipped with an ESI ion source. Van Nuijs et al. [12] also applied HILIC–MS/MS for the determination of metformin in influent wastewater from Belgium. Besides metformin, 12 other top-prescribed pharmaceuticals were simultaneously analyzed in wastewater with a Phenomenex Luna HILIC column (150 mm × 3 mm, 5 μm) and a mobile phase composed of (A) ammonium acetate 5 mM in water and (B) AcN/MeOH (87.5/12.5, v/v), in gradient. Concentrations of metformin up to 129, 21 and 1.7 μg/L were measured in influent wastewater, effluent wastewater and surface water, respectively. Both methods are the first to describe in detail the quantification of metformin in water samples.

The high concern of measuring pharmaceuticals as an important group of emerging contaminants in the environment was also expressed by the US Environmental Protection Agency (EPA) that issued a method (EPA 1694) for the analysis of pharmaceuticals and personal care products in water, soil, sediments and biosolids by HPLC–MS/MS [10]. Several classes of pharmaceuticals, 71 compounds in total, and 19 internal standards were determined mainly by RPLC, but a HILIC approach was preferred for the most polar (e.g. albuterol, metformin, cimetidine, and ranitidine) [10]. Albuterol (salbutamol) is widely used as bronchodilator, while ranitidine and cimetidine are histamine H_2-receptor antagonists that inhibit stomach acid production, being in the top 20 list of most prescribed drugs [27]. In the EPA 1694 method, SPE was involved in the extraction of water and sludge samples [10]. An ultrasonic extraction with AcN was performed for the soil samples or the solid particles filtered from water. A volume of 500–1000 mL filtered water was spiked with labeled internal standards, adjusted to pH 10 and loaded on an Oasis HLB cartridge for isolation of metformin, albuterol, ranitidine, and cimetidine. The elution was done with MeOH and then with 2% formic acid solution in MeOH. The extract was concentrated to nearly dryness, then 3 mL of MeOH was added and spiked with labeled internal standards and finally brought to 4 mL with 0.1% formic acid in MeOH solution. The EPA method was set up using a HPLC system connected to a triple quadrupole MS, equipped with an ESI ion source operated in positive ion mode [10]. The chromatographic separation was carried out on a Waters Atlantis HILIC Silica column (100 mm × 2.1 mm, 3 μm) eluted in gradient with a mobile phase consisting of A) ammonium acetate/acetic acid buffer 0.1% in water and B) AcN. The acquisition was carried out in MRM mode using one transition per compound.

An alternative to the EPA setup was proposed by Agilent Technologies [11]. The sample pretreatment was the same, but the separation was performed on an Agilent Zorbax HILIC Plus column (100 mm × 2.1 mm, 3.5 μm) with a gradient of (A) aqueous ammonium acetate 10 mM and (B) AcN. A second MRM transition was added for confirmation of each compound.

Gadolinium-based contrast agents are widely used in magnetic resonance imaging (MRI), which can result in the widespread distribution of gadolinium (Gd) in the environment. Due to the toxicity of various Gd species, it is important to measure not only the total Gd concentrations, but also the different Gd species. For their trace separation, techniques like size-exclusion chromatography, ion-exchange chromatography, capillary electrophoresis, RPLC–MS and HILIC–MS have been described. Künnemeyer et al. [13] studied the distribution of Gd chelates in hospital effluent, municipal sewage sludge and wastewater samples. They developed an analytical method for the separation of 5 Gd chelates: Gadovist (Gd-BT-DO3A), Magnevist (Gd-DTPA), Omniscan (Gd-DTPA-BMA), Dotarem (Gd-DOTA), and Multihance (Gd-BOPTA) exploiting the benefits of a HILIC approach. The column employed was a SeQuant ZIC-HILIC (150 mm × 2.1 mm, 3.5 μm) with a precolumn with similar stationary phase. The analytes were eluted at 0.3 mL/min in isocratic conditions with a mobile phase consisting of a solution of 12.5 mM ammonium formate and 12.5 mM formic acid in AcN/water (76/24, v/v, pH 3.75). Detection was performed by ICP-MS. The LOQ of the HILIC-ICP-MS method was 3.3 nmol/L and the LOQ was 1 nmol/L. Samples were collected from Münster University Hospital (UKM), from the sewage pit 1 km downstream of the hospital, and from Münster main WWTP, in silylated glass bottles. Gd-BT-DO3A, the contrast agent mainly used in UKM, was measured at an average concentration of 21 nmol/L, while the other species were below the LOD. In the wastewater samples, Gd-BT-DO3A was found in concentrations up to 7.3 nmol/L; the other contrast agents, less used by radiological centers, had concentrations between LOD and LOQ. The method allows the study of the environmental fate of Gd chelates and their removal in WWTPs.

2.5. Drugs of abuse (DOA)

The analysis of DOA residues in waste- and surface water has become in recent years a tool to estimate the consumption of these illicit substances in different countries, using the excretion patterns and considering the stability of the unchanged DOA or of their metabolites in the environment [3,4].

RPLC methods for DOA have been published [3,28]. Considering the highly polar structures, HILIC could be a good alternative for the detection and quantification of DOA in water samples. A method for the determination of cocaine and its principal metabolites in waste- and surface water using HILIC was developed, validated and applied to samples collected from 41 WWTPs and rivers across Belgium [14,29–31]. A significant increase in sensitivity for all analytes was found when HILIC–MS/MS was compared to RPLC–MS/MS [14]. The target compounds were isolated by SPE on Oasis HLB cartridges. Analysis was carried out on a system comprised of a binary pump and ion trap detector with an ESI interface operated in positive ion mode [14]. HILIC separation was performed on an Agilent Zorbax Rx-SIL column (150 mm × 2.1 mm, 5 μm), eluted in gradient with a mobile phase composed of (A) ammonium acetate 2 mM in water (adjusted to pH 4.5 with acetic acid) and (B) AcN, and the flow was 0.25 mL/min. With this method, 162 samples were analyzed and the concentrations measured in Belgian wastewater samples ranged from 9 to 753 ng/L for cocaine and from 37 to 2258 ng/L for benzoylecgonine [29–31]. Ecgonine methyl ester concentrations were below the method LOQ.

Based on these results, a new HILIC method was developed for the simultaneous determination of nine DOA and metabolites (amphetamine, methamphetamine, MDMA, methadone, EDDP, 6-monoacetyl morphine, cocaine, benzoylecgonine and ecgonine methyl ester) in wastewater, with improved LOQs (down to 1 ng/L for all compounds except for ecgonine methyl ester, amphetamine and 6-monoacetyl morphine for which the quantification limit was 2 ng/L) [15]. The method involves separation on a Phenomenex Luna HILIC column (150 mm × 3.0 mm, 5 μm) and a gradient with (A) ammonium acetate 5 mM in water and (B) AcN, at a flow rate of 0.4 mL/min and starting with 95% B. The sample pretreatment was performed by SPE on Oasis MCX cartridges. All compounds except 6-monoacetyl morphine could be quantified in a set of 12 wastewater samples from Belgium. Ecgonine methyl ester was for the first time quantified in wastewater samples.

3. Pesticides in environmental samples

Pesticides can cause unwanted dangerous effects on the human health and environment if misused or even if used according to

official directions. The European Commission states that there is a risk associated with the use of pesticides and this has led to the setup of regulations in developed countries and to constant monitoring. Furthermore, studies have been executed to reveal their environmental fate and risks [32]. To evaluate these risks, analytical techniques have been validated to quantify low concentrations of pesticide residues in environmental samples. Because of the polar character, lack of chromophore or fluorescent groups and low molecular weights of some pesticides, their analysis by conventional analytical techniques is often impaired. Table 1 shows an overview of the analytical methods based on HILIC for the determination of pesticides in environmental samples.

3.1. Organophosphorus pesticides

Organophosphorus pesticides (OPPs) are cholinesterase inhibitors widely used for crop protection. The contamination of drinking water with OPPs is monitored in the European Union and a maximum concentration of 0.1 μg/L is allowed [33]. Most OPPs are easily analyzed by GC, but some have a more polar chemical structure or are thermolabile, hampering thus the use of RPLC or GC [34]. For these compounds, HILIC could be a good alternative as showed by Hayama et al. [16], who analyzed six polar OPPs (acephate, methamidophos, monocrotophos, omethoate, oxydemeton-methyl, and vamidothion) in water samples using HILIC–MS/MS. Sample preparation was carried out with GL-Pak activated carbon cartridges. Water samples (50 mL) were directly loaded on the cartridges and back-flush eluted with 5 mL of 0.2% (v/v) formic acid in AcN/iso-propanol (95/5, v/v). A deuterated internal standard ($^2H_6$-acephate) was added to the eluate for quantification. A Waters Atlantis HILIC Silica column (150 mm × 2.0 mm, 5 μm) was used for the separation of the OPPs using a mobile phase consisting of AcN/iso-propanol/200 mM ammonium formate in water (pH 3) (92:5:3, v/v) in isocratic conditions. Good retention times were obtained for all compounds, ranging from 3.4 to 4.9 min. The advantage of the HILIC–MS/MS method is that the SPE eluate can directly be injected in the HILIC–MS/MS system. The method was applied to a set of surface water samples from the Chikugo River in Fukuoka (Japan). Only acephate was detected in three river water samples at low concentrations (<100 ng/L). Ingelse et al. [34] reported the use of RPLC–MS for the determination of OPPs in aqueous samples. However, they had low retention for the analytes, resulting in high matrix interference and thus poor MS detection. They also described a chromatographic separation of OPPs with a polar endcapped C18 column which offered additional retention and also higher sensitivity. However, the detection limits of the HILIC–MS/MS method [16] were significantly lower than those obtained by RPLC–MS [34].

3.2. Quaternary ammonium salt herbicides

In the US alone, the estimated use of the quaternary ammonium salt herbicides paraquat and diquat in 1997 was over 1500 tons and 100 tons, respectively. Because of their ionic structures, only ion-pairing RPLC methods can be applied [35]. To avoid the use of ion-pair reagents, which may lead to decreased sensitivity and increased complexity of the analytical procedure, HILIC was reported as an easier alternative in a Waters Application Note [17] and by Makihata et al. [18]. The isolation procedure for paraquat and diquat was based on SPE using Oasis WCX cartridges [17]. After loading, the cartridges were washed with phosphate buffer pH 7, water and MeOH and then the analytes were eluted with ACN/water/TFA (84:14:2). The dried extracts were reconstituted in mobile phase and 20 μL of the final solution was injected in the HILIC–MS/MS system. The analysis of diquat and paraquat was performed on a Waters Atlantis HILIC Silica (150 mm × 2.1 mm, 3.5 μm) column, with a mobile phase consisting of 40% AcN and 60% 250 mM ammonium formate buffer (pH 3.7).

Makihata et al. [18] applied a similar approach to determine diquat from Japanese tap, river, lake, shallow well and deep well water samples. A Waters Atlantis HILIC Silica column (150 mm × 2.1 mm, 5 μm) was used and the mobile phase consisted of 50% AcN and 50% 10 mM ammonium formate buffer (pH 3.7). In these conditions, the achieved limit of detection was 50 ng/L, which is 100 times lower than the target value for diquat residues in Japan. Diquat was found only in one of the shallow well water samples at a concentration of 2 μg/L.

4. Pharmaceuticals in food and feed samples

In the European Union maximum residue limits (MRLs) of pharmacologically active substances in various foodstuffs of animal origin, including meat, fish, milk, eggs and honey have been established [36] and an international database of MRLs was issued by FAO's Codex Alimentarius [37]. Table 2 presents a brief list of the compounds analyzed using HILIC methods in food and feed samples.

Antibiotics are the group of highest concern and their monitoring in food products is compulsory. RPLC, ion-pair chromatography or HILIC are techniques employed for their separation.

Avoparcin has an activity against Gram-positive bacteria and has a structural similarity with the antibiotic vancomycin, which can lead to resistance to vancomycin in bacteria. It has never been licensed for use in the USA and Canada and it has been prohibited since 1997 in the EU. Curren et al. [38] described a novel extraction procedure of avoparcin from swine kidney with pressurized hot water (75 °C and 50 atm), followed by a cleanup on polyhydroxyethyl aspartamide SPE sorbent. Chromatographic separation was carried out on a PolyLC PolyHYDROXYETHYL A column (200 mm × 4.6 mm, 5 μm), with a mobile phase consisting of 47% aqueous 15 mM triethylammonium phosphate in AcN at 1 mL/min. Detection was performed in UV at 225 nm.

Aminoglycoside antibiotics are among the best candidates to be analyzed in the HILIC mode and such methods were developed with matrices as plasma, kidney or meat [39]. Due to their ototoxicity and nephrotoxicity, the use of aminoglycoside antibiotics was limited and MRLs in food products were established [39]. The quantification method for seven aminoglycosides from swine and bovine meat and kidney employed a SeQuant ZIC–HILIC column (100 mm × 2.1 mm, 5 μm) maintained at 32 °C with a mobile phase composed of (A) ammonium acetate 150 mM + 1% formic acid in water and (B) AcN, at a flow rate of 0.3 mL/min [39] (Fig. 1). MS detection was performed in positive mode. For the sample preparation, homogenization and solvent extraction with a mixture of potassium dihydrogen phosphate, EDTA and trichloroacetic acid was first carried out, and the extract was further purified by SPE on CBX (carboxypropyl weak cation exchanger) cartridges. LOQs in swine bovine kidney (the matrix with the highest suppression effect) were 25 ng/g for gentamicin, 50 ng/g for spectinomycin, dihydrostreptomycin, kanamycin and apramycin, and 100 ng/g for streptomycin and neomycin, well below the existing MRLs [37].

Milk is also strictly controlled for the presence of antibiotics. Inoue et al. [40] reported a HILIC–MS/MS method for the determination of bicozamycin, used for the treatment of non-specific diarrhea in animals. Milk samples were extracted with AcN/water (4:1, v/v), then the supernatant was evaporated to dryness, reconstituted with water and subjected to centrifugal ultrafiltration using Amicon Ultra-15 (Ultracel-10 K regenerated cellulose 10,000 M.W.). The resulting solution was directly injected into the LC–MS/MS system. Chromatographic separation was performed on a Tosoh TSKgel Amide-80 (150 mm × 2.0 mm, 3 μm) column maintained at

Table 2
Pharmaceuticals and pesticides analyzed in HILIC mode from food/feed samples. A brief review of employed columns, mobile phases and type of samples.

Compound/class of compounds	Matrix	Column	Mobile phase	Refs.
Pharmaceuticals				
Avoparcin	Kidney	PolyLC PolyHYDROXYETHYL A (200 mm × 4.6 mm, 5 μm)	AcN/15 mM aqueous triethylammonium phosphate; isocratic	[38]
Aminoglycoside antibiotics	Swine/bovine meat and kidney	SeQuant ZIC–HILIC (100 mm × 2.1 mm, 5 μm)	AcN/150 mM ammonium acetate + 1% formic acid in water; gradient	[39]
Bicozamycin	Milk	Tosoh TSKgel Amide-80 (150 mm × 2 mm, 3 μm)	AcN/50 mM ammonium acetate in water (pH 4.0 adjusted with acetic acid); gradient	[40]
Oxytetracycline, tetracycline, chlortetracycline and doxycycline	Food	Thermo Hypersil Silica (50 mm × 4.6 mm, 3 μm) Thermo Hypersil APS-2 (50 mm × 4.6 mm, 3 μm)	MeOH or AcN/citrate, acetate, oxalate and formiate in water; isocratic	[41]
Streptomycin	Distillers grain	Waters Atlantis HILIC Silica (100 mm × 2.1 mm, 5 μm)	AcN/0.1% aqueous formic acid; gradient	[42]
Carbadox and olaquindox	Swine feed	Waters ACQUITY UPLC BEH HILIC (100 mm × 2.1 mm, 1.7 μm)	Ammonium acetate 10 mM in a mixture of AcN/water (95:5, v/v); isocratic	[43]
Pesticides				
Dithiocarbamates	Fruit and vegetables	SeQuant ZIC–pHILIC (150 mm × 4.6 mm, 5 μm)	AcN/10 mM aqueous ammonia; gradient	[44] [45]
Chlormequat and mepiquat	Fruit, juices, bread, vegetables, baby food, mushrooms, beer, coffee powder	Waters Atlantis HILIC Silica (150 mm × 2.1 mm, 3 μm)	AcN/50 mM ammonium formate buffer (pH 3.75); gradient	[46]

40 °C, with a mobile phase consisting of (A) 50 mM ammonium acetate in water (pH 4.0 adjusted with acetic acid) and (B) AcN, in gradient conditions. The calculated LOD and LOQ were 2.5 and 5 ng/mL, respectively. All analyzed commercial milk samples gave negative results [40].

Oxytetracycline, tetracycline, chlortetracycline and doxycycline are used to treat general infectious diseases in animals and as growth additives in animal feeds. As a consequence, measuring the levels of tetracycline in food is compulsory and requires high sensitivity. The chromatographic separation of tetracyclines is a difficult task, because their poor retention on RPLC and peak tail due to complexation with metal ions and to adsorption on silanol groups. Valette et al. [41] have tested two HILIC columns: Thermo Hypersil Silica (50 mm × 4.6 mm, 3 μm), and Thermo Hypersil APS-2 (50 mm × 4.6 mm, 3 μm), with various mobile phases containing citrate, acetate, oxalate and formate in water and MeOH or AcN as organic modifier. The detection was carried out with UV. Tetracyclines were separated in approximately 2 min, with good peak shape and the method could be applied for the analysis of food samples [41].

Distillers grain, a major co-product resulting from the ethanol processing for biofuel, contains proteins, fats, minerals, vitamins and therefore it is a useful feed supplement for livestock. Use of antibiotics as processing aids in ethanol production has been a

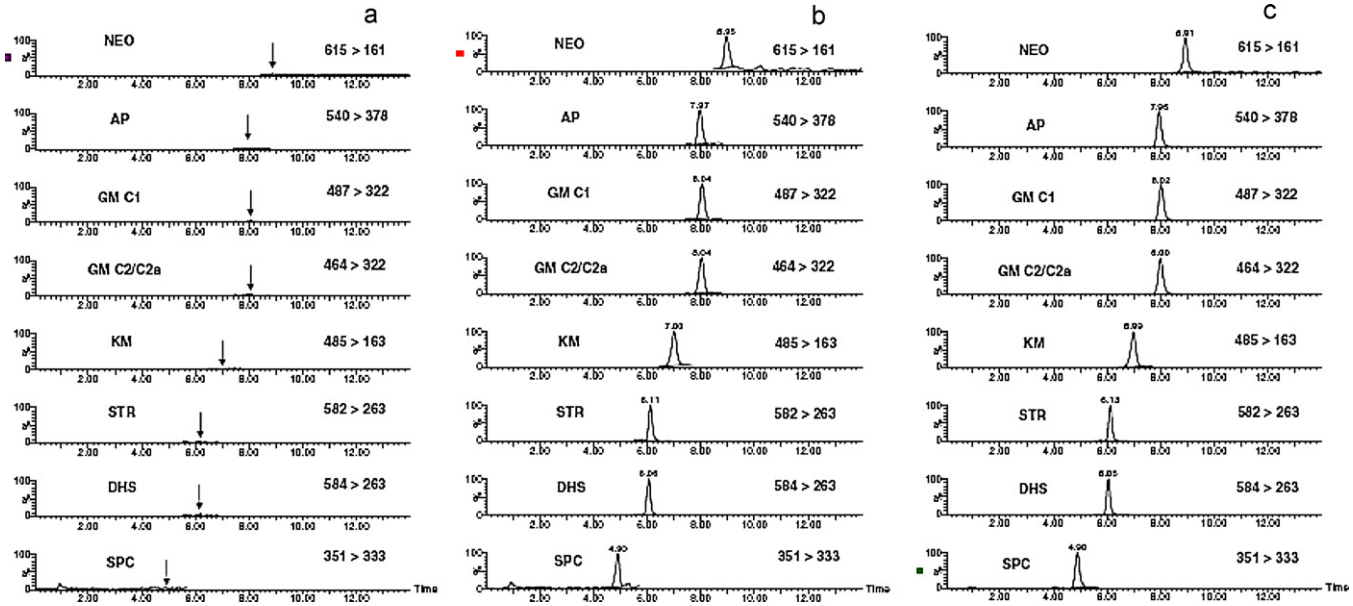

Fig. 1. MRM chromatograms of a blank swine kidney sample (a), a swine kidney sample spiked with gentamicin at 25 ng/g, spectinomycin, dihydrostreptomycin, kanamycin and apramycin at 50 ng/g, and streptomycin and neomycin at 100 ng/g (the level of the limit of quantitation) (b), and a swine kidney sample spiked with aminoglycosides at 500 ng/g (c). The arrow indicates the retention time of each aminoglycoside. Scales were taken from the intensities of the largest peak in each transition. Reproduced with permission from Ref. [39].

common practice in the dry-grind process, to increase efficiency of fermentation by preventing undesirable bacterial growth. The Food and Drug Administration Center for Veterinary Medicine (FDA/CVM), which approves drugs used in animal feeds and establishes limits for feed contaminants, has published a method to determine antibiotic residues in distillers grain [42]. The method was focused on 13 frequently used antibiotics with different structures: ampicillin, penicillin G, tetracycline, oxytetracycline, chlortetracycline, bacitracin A, virginiamycin M1, chloramphenicol, erythromycin A, clarithromycin, tylosin A, monensin A and streptomycin. The sample preparation prior to LC–MS/MS analysis was based on a two-step solvent extraction (aqueous EDTA and TCA, MeOH) from the distillers grain. The two extracts were combined, then split in two aliquots; one was subjected to SPE on Oasis HLB (eluted with MeOH), the other was adjusted to pH 8 and loaded on Isolute CBA cartridges (carboxylate weak cation exchanger, eluted with glacial acetic acid). After evaporation and reconstitution, the extract from Oasis HLB was injected on a phenyl LC column (50 mm × 4 mm, 3 μm), and eluted at 0.45 mL/min with a mobile phase composed by (A) 0.1% aqueous formic acid and (B) AcN, in RP mode gradient. Streptomycin, isolated with the Isolute CBE cartridge, was analyzed on a Waters Atlantis HILIC Silica column (100 mm × 2.1 mm, 5 μm), using the aforementioned mobile phase, and the following gradient, at 0.325 mL/min: 0–0.5 min at 70% B, decreased to 30% B in 3 min and held at 30% B for 7 min, then increased to 70% B in 8 min and held till 14 min. The LOQ for streptomycin achieved with distillers grain matrix was 0.5 μg/g, with good precision and accuracy [42].

Another example is represented by carbadox and olaquindox, members of the quinoxaline-1,4-dioxide antibacterial group, prohibited in the EU since 1998 due to their carcinogenic, mutagenic and photoallergenic effects [47]. Keisunaite et al. [43] developed an HILIC method with UV detection for the quantification of the two antibiotics in swine feed. Sample preparation was based on matrix solid phase dispersion extraction (0.25 g of feed and 0.5 g of C_{18} sorbent were blended in a mortar and then placed in a syringe and eluted with 10 mL of AcN-MeOH mixture (8:2, v/v)). After evaporation and reconstitution with mobile phase, 5 μL of the extract were injected onto an Waters ACQUITY UPLC BEH HILIC column (100 × 2.1 mm, 1.7 μm) maintained at 30 °C and eluted in isocratic conditions with a mobile phase consisting of ammonium acetate 10 mM in a mixture of AcN/water (95:5, v/v). Absorbance was measured at 307 nm for carbadox and 384 nm for olaquindox. LODs of 20 and 30 ng/g and LOQs of 70 and 100 ng/g for carbadox and olaquindox, respectively, were obtained. Quantification was performed using matrix-matched calibration standards prepared by adding the appropriate volume of antibiotic standard to 0.25 g of sample, which was mixed and kept for 24 h at room temperature in the dark. Good linearity and extraction recovery were achieved. After testing method performances, the authors analyzed 18 feed samples collected in Lithuania in 2007. Carbadox or olaquindox were not detected in any of them [43].

The above described methods demonstrate that the use of HILIC for the analysis of antibiotics from food and feed samples is a valuable approach in solving particular problems (e.g. the tetracycline class). Reversed-phase and HILIC chromatography are applied together when a large number of compounds with various structures and polarities need to be measured.

5. Pesticides in food and feed samples

The presence of pesticides in food products as a result from their application in agriculture could have severe and undesirable effects on humans or animals. The European Commission has therefore set MRLs for around 1100 pesticides in all agricultural products intended for food or animal feed [48]. The detection and quantification of pesticide residues in food and feed require sensitive and specific analytical methods. To discriminate in subclasses, to be sensitive and specific and to analyze multiple analytes in one analytical run, chromatographic methods, such as LC, seem appropriate. Because of the often highly polar character of pesticides, considerable efforts (e.g. derivatisation, ion-pairing) have to be made to develop suitable analytical RPLC methods and thus the use of HILIC-based methods would be an easier solution for their analysis [49].

5.1. Dithiocarbamates

Dithiocarbamates (DTCs) are extensively used as fungicides in agriculture, but also in paper manufacturing, the rubber industry, in the treatment of chronic alcoholism, and as anticancer or antitoxic drug agents [50,51]. Because of these multiple applications, it is not surprising that DTCs may contaminate food and feed samples.

DTCs can be categorized into three subclasses depending upon their structural backbone: dimethyldithiocarbamates (DMDs), ethylenebis(dithiocarbamates) (EBDs) and propylenebis(dithiocarbamates) (PBDs). The routine analysis of DTC residues in foodstuff has for decades consisted of a hot-acid digestion of the food samples followed by the spectrophotometric determination of the evolving CS_2. MRLs, expressed as mg CS_2/kg food, however can derive from different dithiocarbamates with different toxicities and therefore do not reflect a Good Agricultural Practice. Hence, several techniques for the direct determination of intact DTCs have been published. A number of LC–UV methods are presented in the literature [52–55], but requirements for selectivity and sensitivity can only be met when LC–MS is applied. Consequently, Crnogorac et al. [44,45] have performed experiments with HILIC–MS for the analysis of DTCs in foodstuff.

In first instance, they developed and validated a method based on HILIC–MS for the analysis of all three subclasses of DTCs in fruit and vegetables [44]. Whole samples were surface extracted using a sodium hydrogen carbonate buffer containing D,L-penicillamine to stabilize DTCs. The separation was obtained with a SeQuant ZIC-pHILIC column (150 mm × 4.6 mm, 5 μm) and a mobile phase consisting of (A) 10 mM aqueous ammonia and (B) AcN, in gradient conditions. Detection was performed with a single quadrupole MS. The three subclasses were fully separated within 14 min with LOQ of 50 ng/g. Subsequently, Crnogorac et al. [45] improved the method by applying MS–MS detection. This led to a 10-fold decrease of the LOQ (5 ng/g). Several fruits and vegetables ($n = 12$) were analyzed with the HILIC–MS and the HILIC–MS/MS method and compared with the routine CS_2 analysis. EBD was present in all samples, while DMD was only detected in 2 samples and PBD was never detected. In only one sample, the MRL of 50 ng/g CS_2 was exceeded. In general, results of HILIC–MS and HILIC–MS/MS were in good agreement with the CS_2 analysis, but they were more reliable and informative. The determination of DTC residues in foodstuffs by HILIC–MS/MS shows clear advantages, such as shorter times and multi-analyte simultaneous determination. The analytical methods based on HILIC–MS/MS are simple, rapid, sensitive and can discriminate between different DTCs; therefore they could be an alternative for the routine CS_2 analyses which are laborious and time-consuming, and which cannot distinguish between DTC subclasses.

5.2. Chlormequat and mepiquat

Chlormequat and mepiquat are two quaternary ammonium compounds used as plant growth regulators on cereals, vegetables and fruit. Nowadays, ion-pairing with HFBA is the preferred method for the determination of these compounds in food samples

Table 3
Miscellaneous compounds analyzed in food and environmental samples using HILIC methods. A brief review of employed columns, mobile phases and type of samples.

Compound/class of compounds	Matrix	Column	Mobile phase	Refs.
Dichloroacetic acid	Drinking water	Phenomenex Luna Amino (150 mm × 2.1 mm, 5 μm)	AcN/40 mM ammonium formate in water; gradient	[60]
Aromatic amines	Surface water	EKA Chemicals AB Kromasil SIL (250 mm × 4.6 mm, 5 μm)	AcN/10 mM NaH_2PO_4 (pH 1.5 with H_3PO_4); isocratic	[61]
Moniliformin	Maize plants	SeQuant ZIC–HILIC (150 mm × 4.6 mm, 3.5 μm)	AcN/water; gradient	[62]
Melamine	Edible fish tissues	Waters Atlantis HILIC Silica (50 mm × 3 mm, 3 μm)	AcN/20 mM ammonium formate; gradient	[63]
Melamine and cyanuric acid	Catfish, pork, chicken, pet food	Thermo BioBasic AX (150 mm × 2.1 mm, 5 μm)	AcN/isopropanol/50 mM ammonium acetate and AcN/water; gradient	[64]
Melamine and cyanuric acid	Animal feed and feed ingredients	SeQuant ZIC–HILIC (150 mm × 2.1 mm, 5 μm)	50 mL 0.1% aqueous formic acid + 950 mL AcN/20 mM aqueous ammonium formate + AcN; gradient	[65]
Melamine, cyanuric acid and metabolites	Milk and infant formula	Agilent Polaris NH_2 (150 mm × 3 mm, 5 μm)	AcN/10 mM ammonium acetate + 0.1% glacial acetic acid (solvent B 22%); isocratic	[66]
Melamine	Milk powder	SeQuant ZIC–HILIC (150 mm × 4.6 mm, 5 μm)	AcN/25 mM ammonium acetate 25 mM (pH = 6.8); isocratic	[67]
Melamine	Animal tissues, crop and soil	SeQuant ZIC–HILIC (150 mm × 2.1 mm, 3 μm)	50 mL of 5 mM aqueous ammonium acetate and 950 mL AcN + 0.1% formic acid/50 mL AcN, 950 mL of 5 mM aqueous ammonium acetate + 0.1% formic acid; gradient	[68]

[56–59]. However, in tandem with a mass spectrometer, this HFBA results in high signal suppression and background noise. Therefore, Esparza et al. [46] developed a sensitive and selective HILIC–MS/MS method for the determination of chlormequat and mepiquat in food samples, using a low ionic strength mobile phase, thus overcoming the above mentioned problems. Food samples were cut up and homogenized, then extracted with a mixture of MeOH and 100 mM ammonium formate buffer (pH 3.5) by ultrasonication and centrifugation. After adding an internal standard, the extract was further cleaned-up on ENVI-18 SPE cartridges and the resulting extract was analyzed. Chromatographic separation was achieved with a Waters Atlantis HILIC Silica column (150 mm × 2.1 mm, 3 μm) and a mobile phase consisting of (A) 50 mM ammonium formate buffer (pH 3.75) and (B) AcN, in gradient. Chlormequat and mepiquat were separated within 4 min and detection limits of low ppb levels were obtained. The two pesticides were analyzed in 28 food samples including fruit, juices, vegetables, mushrooms, bread, baby food, beer and coffee powder. Chlormequat and mepiquat were detected in seven and four samples, respectively. All concentrations were below the established MRLs, except for a coffee powder which had concentrations of mepiquat higher than the MRL for coffee beans (100 ng/g) [46].

6. Miscellaneous contaminants from environmental and food samples

A number of other applications involving HILIC and food or environmental contaminants and which cannot be categorized in the previous chapters are briefly summarized below. Table 3 presents a short list of the HILIC methods employed.

6.1. Dichloroacetic acid

Dichloroacetic acid (DCA) is a small and polar compound which is found in drinking water as a disinfection by-product of chlorination or deriving from metabolism of chlorinated solvents, such as trichloroethylene. The occurrence of DCA in drinking water is of concern because it has been shown to be carcinogenic in laboratory animals. The most common method for the analysis of DCA in water samples has been GC after derivatization. This methodology can cause problems since the strong acids that are used during derivatization also transform the present trichloroacetic acid into DCA, which eventually leads to inaccurate measurements of the DCA concentrations [69]. To overcome the GC related problems, separation of DCA from the aqueous matrix can be done with LC. In addition, for low concentrations, a specific and sensitive detection based on MS should be used. In the literature, two approaches using LC–MS can be found: RPLC–MS with ion-pairing reagents in the mobile phase [70,71], and HILIC–MS, as presented by Dixon et al. [60]. With the HILIC approach, no other sample preparation than dilution was performed with the water samples. A Phenomenex Luna Amino column (150 mm × 2.1 mm, 5 μm) was used for HILIC-ion exchange (the retention is based on the affinity of the polar analyte for the charged end group on the stationary phase), at a flow of 0.7 mL/min. The mobile phase was: (A) 40 mM ammonium formate and (B) AcN. The gradient run was as follows: 90% B at 0 min, 30% B at 5 min, and 90% B at 6 min. A triple quadrupole MS with ESI interface was used, monitoring the transition from m/z 127 to 83 in MRM mode. A LOQ of 5 ng/mL water was established with this method, which was similar to those for ion-pairing RPLC–MS. The analysis of real samples showed that bottled water contained much less DCA than tap water samples, which is not surprising, DCA being a disinfection by-product of chlorination.

6.2. Aromatic amines

Toxic aromatic amines, such as aniline and other substituted derivatives, are important industrial chemicals used to make dyes, synthetic polymers, rubbers, pesticides, cosmetics, medicines, and many other chemicals. Since they may be released from these manufacturing processes, their residues have become significant contaminants in environmental waters and are especially problematic given their toxicity and biological activity. GC has proven to be a suitable approach for the analysis of amines in water; however a derivatization step is always necessary and this is sometimes challenging [72]. Therefore, LC is regarded as the most convenient available technique for aromatic amines analysis in water samples. Because of their low retention in RPLC, ion-pairing reagents such as alkylammonium salts are often necessary to obtain efficient separations of the different amines [73]. As an alternative to such methods, a simple, precise, and accurate method based on HILIC was developed for the determination of

five aromatic amines (1-naphthylamine (1-NA), aniline (AL), N,N-dimethylaniline (N,N-DMA), N,N-diethylaniline (N,N-DEA), and benzidine (BZ)) in environmental water samples [61]. Chromatographic separation was carried out on a EKA Chemicals AB Kromasil SIL column (250 mm × 4.6 mm, 5 μm), using a mixture of AcN and a buffer of NaH_2PO_4–H_3PO_4 (pH 1.5, containing 10 mM NaH_2PO4) (85:15, v/v) as a mobile phase at a flow rate of 1 mL/min. Analytes were detected by UV absorbance at 254 nm. The LOD was within 0.02–0.2 mg/L (S/N = 3). The retention mechanism under the optimum conditions was determined to be a combination of adsorption, partition and ionic interactions. Aromatic amines were isolated from aqueous samples by SPE with Oasis HLB cartridges. Recoveries greater than 75% with RSD < 12% were obtained for amine concentrations of 5–50 μg/L in river water and influent wastewater. The present HILIC technique proved to be a viable alternative method for the analysis of aromatic amines in environmental water samples [61].

6.3. Moniliformin

Moniliformin is a mycotoxin frequently occurring in cereals and maize and is produced by *Fusarium* sp. [62]. Although it has an acute toxicity, comparable to that of other *Fusarium*-derived mycotoxins (e.g. type A trichothecenes), the levels of moniliformin are not regulated in food or feed in the EU, USA or any other country. The determination of moniliformin is very different from other mycotoxins since it is a small (MW = 98), highly polar (log Kow = 0.03) and acidic molecule (p*K*a = 0.5), and therefore a very good candidate for HILIC. Positively charged ion-pairing methods seem to be suitable as well for RPLC separation, but the ion-pairing reagent may suppress ionization in the case of an MS coupling [74]. A zwitterionic stationary phase (ZIC–HILIC) was used for the retention of moniliformin followed by UV or negative MS/MS detection [62]. For sample preparation, 2 g maize were extracted with 30 mL AcN/water, followed by a clean-up on Strata SAX cartridges (500 mg) eluted with 2 mL HCl 1 M. The column used was SeQuant ZIC–HILIC (150 mm × 4.6 mm, 3.5 μm), with (A) milli-Q water and (B) AcN as mobile phase, at a flow rate of 0.5 mL/min, in gradient. The LOQ in the MS/MS mode was five times lower than that achieved by UV and comparable with the LOQ reported for the RPLC method [74]. A polyhydroxyethyl aspartamide column was also tested, but, although achieving promising results with standard solutions, interfering substances present in maize coeluted with moniliformin and prevented a sensitive analysis.

6.4. Melamine and cyanuric acid

In March 2007, several North American manufacturers of pet food voluntarily issued nationwide recall notices for some of their products because of reports that they were associated with renal failure in cats and dogs [63]. Subsequent analyses revealed that the suspect foods were contaminated with the industrial chemicals melamine (MEL) [75] and cyanuric acid (CYA) [76]. In September 2008, more than 50,000 Chinese babies were found to be contaminated with melamine through the consumption of adulterated powdered milk [66]. This has led to raised public awareness and to the development of analytical methods for the determination of MEL and its metabolites in food and feed.

Three methods based on HILIC for the analysis of MEL and CYA in fish, meat and animal feed have been published [63–65]. Andersen et al. [63] first described a quantitative and confirmatory method based on HILIC to determine MEL residues in edible tissues from melamine-fed fish. Edible tissues were extracted with acidic AcN, defatted with dichloromethane, and cleaned up using mixed-mode cation exchange SPE. Extracts were analyzed by HILIC–MS/MS with ESI in positive ion mode. A Waters Atlantis HILIC Silica column

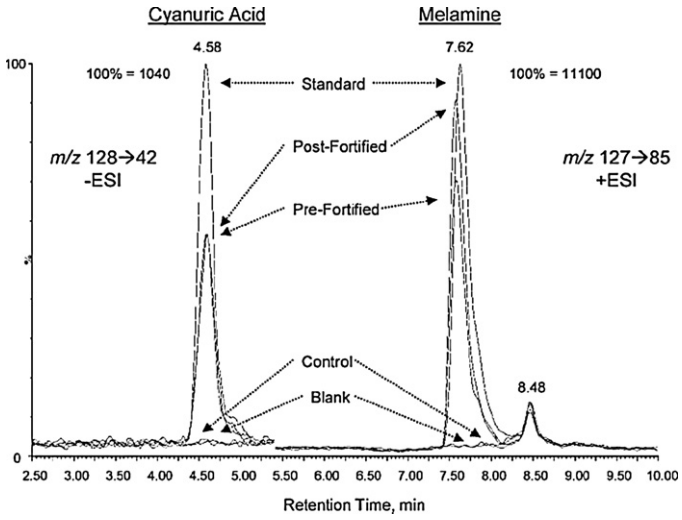

Fig. 2. Overlay of five analyses of wheat flour: solvent blank, control flour, control flour fortified at 1 μg/g MEL and CYA, extract of control flour post-fortified at 1 μg/g equivalent and standards in solvent at a concentration equivalent to 1 μg/g in flour (7 ng/ml actual). Reproduced with permission from reference [65].

(50 mm × 3 mm, 3 μm) was used for the chromatographic separation with a mobile phase consisting of (A) 20 mM ammonium formate and (B) AcN, in gradient. The method LOD was 5 ng/g. MEL residues (range 11–210 μg/g ww) were found in edible tissues from fish (catfish, trout, tilapia, and salmon) fed with MEL for 1–14 days. This proves that MEL can easily be taken up into the tissues of animals fed with contaminated feed and can build up to high concentrations. It is important to monitor both animal feed and tissues to prevent harmful residues from entering the human food supply.

Varelis and Jeskelis [64] have described a method based on isotope dilution LC–MS for the simultaneous determination of MEL and CYA in catfish, pork, chicken, and pet food. The method involved extraction into aqueous MeOH, liquid–liquid extraction (LLE) and ion exchange SPE (Strata SCX) followed by HILIC chromatography. A Thermo BioBasic AX column (150 mm × 2.1 mm, 5 μm) was employed, with the initial mobile phase composed of AcN/isopropanol/ammonium acetate (50 mM) (85:10:5, respectively). After 5 min, the mobile phase composition was changed to 9:1 water/AcN. The HILIC method had a LOD of 10 μg/g for both MEL and CYA with CV < 10%. Compared with the method from Andersen et al. [63], this method allows not only the determination of MEL, but also CYA in samples. LODs of both methods are comparable, but the sample preparation in the method from Varelis and Jeskelis [64] is more complicated and time-consuming.

In another application, MEL and CYA were determined in animal feed and feed ingredients [65]. The method permitted the simultaneous extraction and detection of MEL and CYA, whether they were present as free compounds or bound together as a melamine–cyanurate complex. A chromatographic system with a SeQuant ZIC–HILIC column (150 mm × 2.1 mm, 5 μm) enabled the separation and detection of both compounds in less than 8 min (Fig. 2) without extensive sample preparation. The initial mobile phase was composed of A) 0.1% aqueous formic/AcN (5/95, v/v) and B) 20 mM ammonium formate in AcN. Samples were extracted with a strong aqueous acid which was then diluted to bring the concentration within the working range of the method. The method LOD was 0.5 μg/g, comparable with the lowest calibration point, but the authors mentioned that it is possible to measure lower concentrations. The data confirm the presence of both compounds according to criteria recommended by the US Food and Drug Administration (FDA) [63]. The LC–MS/MS method provides an alternative to derivatization and GC–MS for regulatory analysis of feed sam-

ples. Compared with the two other methods, this method has a 10-fold higher detection limit, but an important advantage is that no extensive sample preparation has to be applied.

Two methods based on HILIC for the determination of MEL and CYA in milk products have been published [67,68]. Jiang et al. developed a simple and rapid method based on HILIC coupled to UV detection of MEL at 210 nm [67]. A SeQuant ZIC–HILIC (150 mm × 4.6 mm, 5 μm) column was used with a mobile phase consisting of 85% AcN and 15% ammonium acetate 25 mM, pH 6.8. LOQ was 4 μg/g for 2 g milk powder. Sample preparation was based on extraction with AcN and perchloric acid, centrifugation and appropriate dilution of the supernatant. Chang et al. [66] developed a more sensitive method based on HILIC–MS for the simultaneous analysis MEL, CYA and two of their major metabolites (ammeline and ammelide) in milk products. This method was primarily based on the FDA method [63] with minor modifications, such as the inclusion of a clean-up step during sample preparation. A Bond Elut Plexa cartridge (60 mg, 3 mL, Varian) is used as a filter to retain matrix interference, while the polar analytes passed through. An Agilent Polaris NH_2 column (150 mm × 3 mm, 5 μm) was used isocratically with a mobile phase consisting of 78% AcN and 22% 10 mM ammonium acetate + 0.1% glacial acetic acid. Baseline separation of all four analytes was obtained within 7 min. Compared with the HILIC–UV method, the method LOQ was more than 10 times lower. However, the HILIC–UV method was faster (no extensive sample preparation), simpler and cheaper compared with the HILIC–MS method. A choice between the two methods has to be made balancing the costs and the sensitivity needs.

Recently, another HILIC-based method was developed for the determination of MEL in animal tissues, crop and soil samples [68]. Following homogenization, a 2 g sample was spiked with [^{15}N3]-MEL standard and further extracted with 2 mL AcN and 8 mL 2% acetic acid. After sonication, the AcN layer was delipidated with n-hexane and filtered through a 0.22 μm filter. For analysis, a SeQuant ZIC–HILIC column (150 mm × 2.1 mm, 3 μm) was used and MEL was detected in MRM positive ESI mode. The mobile phase was (A) 50 mL AcN, 950 mL of 5 mM aqueous ammonium acetate, and 1 mL formic acid and (B) 50 mL of 5 mM aqueous ammonium acetate, 1 mL formic acid, and 950 mL AcN, in gradient. A flow rate of 0.3 mL/min was used and the total run time was 12 min. The method was used to evaluate the extent of MEL contamination in the aquatic samples and food products and possible risks of consuming MEL-contaminated diets [68]. Water, soil and crop samples from 21 Chinese provinces were tested, and concentrations in wastewater and soil samples collected near MEL-producing factories were 227 and 41 μg/g, respectively.

7. Future perspectives and concluding remarks

The routine analysis of aquatic and food pollutants, such as pesticides and pharmaceuticals, has to meet stringent requirements nowadays. Analytical methods have to be sensitive, specific and insofar as possible pollutants must be analyzed in one analytical run. Methods based on chromatographic separation coupled to mass spectrometric detection seem optimal to meet these requirements. GC–MS needs laborious clean-up and often derivatization and it can only be applied for thermally stable compounds. LC–MS is a suitable alternative in many cases. In applications with polar, hydrophilic and ionic analytes, classical RPLC analysis may require ion-pairing reagents, derivatization, post-column addition of organic solvent for optimal ionization, etc. For these compounds, HILIC methods are easier, faster and performing better. Since sensitive and specific multi-analyte runs often involve MS-based detection, the strong advantage of HILIC is the suitability for ESI–MS due to the mobile phases with high amounts of polar organic solvent, which lead to higher ionization efficiency and thus higher sensitivity. The work discussed in this review shows that HILIC techniques are easy to use and hould be explored more in the future. Most probably they will earn their place in the routine analysis of certain compounds in environmental and food samples, replacing older non-specific techniques.

The presented review suggests that HILIC is a useful tool in every chromatography laboratory. This is confirmed by the burgeoning of HILIC applications in parallel with an increase in commercially available brands of HILIC columns in recent years. Today, HILIC-based analytical methods are changing permanently the perception of how LC separations should be performed and in the future they will be strongly anchored in the field of chromatographic science as routine separation methods for highly polar compounds.

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