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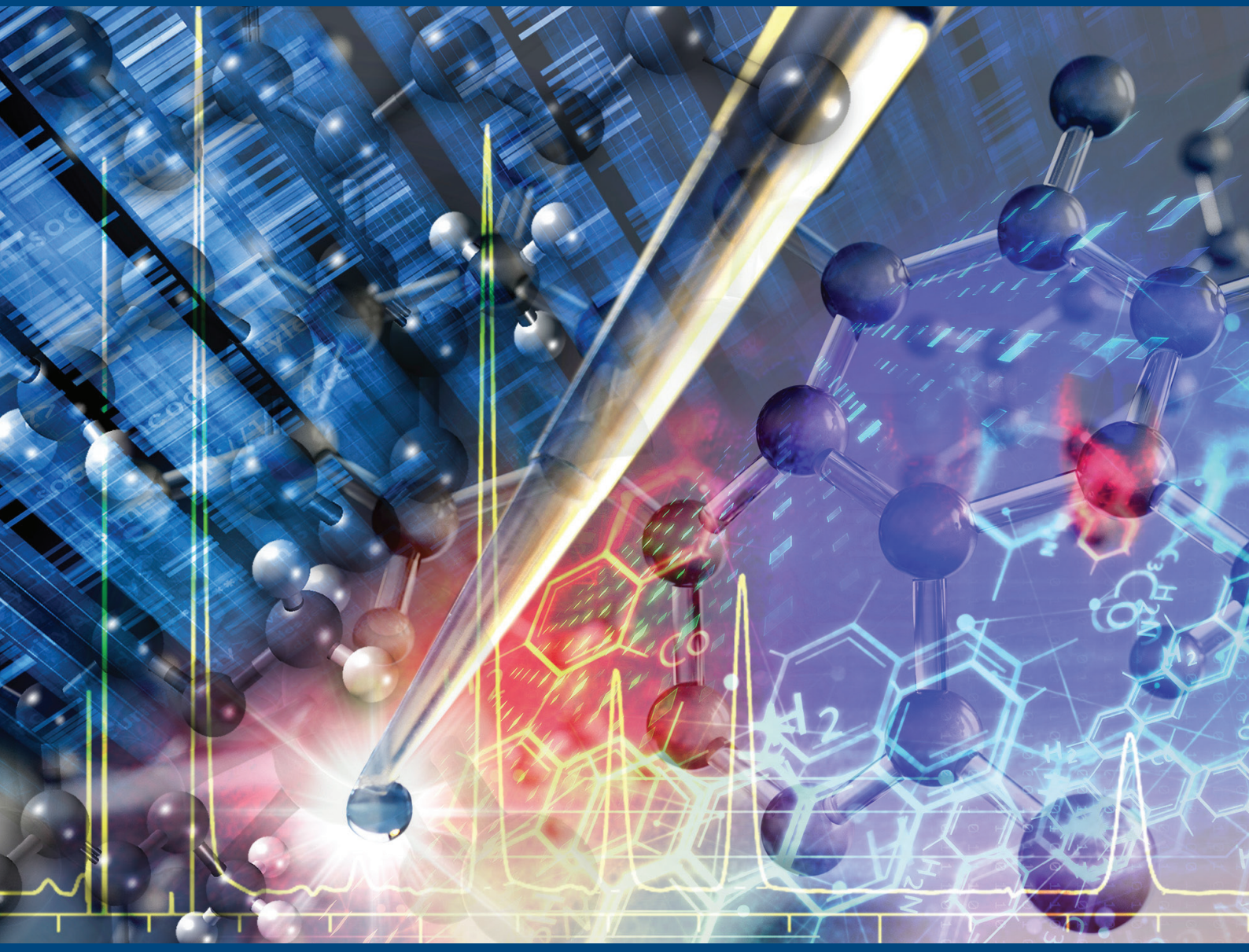

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RESEARCH ARTICLE

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Influence of different hydrophilic interaction liquid chromatography stationary phases on method performance for the determination of highly polar anionic pesticides in complex feed matrices

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Hydrophilic interaction liquid chromatography is an alternative liquid chromatography mode for separation of polar compounds. In the recent years, this liquid chromatography mode has been recognized as an important solution for the analysis of compounds not amenable to reverse phase chromatography. In this work, we evaluated three different hydrophilic liquid chromatography stationary phases for the determination of 14 highly polar anionic molecules including pesticides such as glyphosate, glufosinate, ethephon and fosetyl, their main metabolites, and bromide, chlorate, and perchlorate. Several mobile phase compositions were evaluated combined with different gradients for the chromatographic run. The two columns that presented the best results were used to assess the performance for the determination of the 14 compounds in challenging highly complex feed materials. Very different matrix effects were observed for most of the compounds in each column, suggesting that different interactions can occur. Using isotopically labeled internal standards, acceptable quantitative performance and identification could be achieved down to 0.02 mg kg^{-1} (the lowest level tested) for most compounds. While one column was found to be favorable in terms of scope (suited for all 14 compounds), the other one was more suited for quantification and identification at lower levels, however, not for all analytes tested.

KEYWORDS
glyphosate, hydrophilic interaction chromatography, isotopically labeled standards, polar pesticides

1 | INTRODUCTION

Hydrophilic interaction liquid chromatography (HILIC), formerly also known as aqueous normal-phase LC, is an alternative HPLC mode for separation of very polar compounds [1]. This type of chromatography can be characterized by using a normal-phase type stationary phase in combination with a reversed-phase type mobile phase, where the percentage of organic solvent in water is higher than 50% [2, 3]. HILIC separation is based on the use of polar stationary phases in combination with water-miscible organic solvents, generally acetonitrile, containing a small percentage of water.

Article Related Abbreviations: APP, anionic polar pesticide; ME, matrix effect; NPC, normal phase chromatography; RPLC, reversed phase LC

HILIC is a technique in which the separation mechanism is mainly due to the partitioning of the analyte between a water-rich layer near the hydrophilic surface of the stationary phase and a hydrophobic-rich bulk mobile phase. Polar stationary phases retain water strongly on their surface and in these conditions, a partitioning phenomenon is created, in which the compounds will move from the water rich layer near the hydrophilic surface of the stationary phase to an acetonitrile rich bulk, based on their hydrophilicity. The more hydrophilic the analyte, the more the partitioning equilibrium is shifted toward the immobilized water layer on the stationary phase, and thus, the more the analyte is retained [1]. The water-rich layer and the acetonitrile-rich bulk constitute a liquid–liquid separation system of HILIC mechanism [4]. If only the mechanism of liquid–liquid partitioning would exist in the stationary phases of HILIC columns, complementary interactions would be expected only on a small scale and the differences in separations would be explained basically by the thicknesses of water layer in each material and the polarity of the compounds [5]. However, the overall HILIC retention is more complex than the mechanism described above. It normally involves several processes and that is why it is often described as a mixed-mode retention mechanism. Several interactions/mechanisms (partitioning between a aqueous-rich layer at the stationary phase and the bulk of mobile phase; hydrogen bonding between polar functional groups and aqueous layer and/or stationary phase; electrostatic interactions of ionized functional groups; and ion exchange) can contribute simultaneously to the final retention of the compounds [6,7].

The main benefits of the use of HILIC are the better retention of very polar compounds that are usually difficult to retain in reversed phase liquid chromatography (RPLC) [8]. As in normal phase chromatography (NPC), polar stationary phases are usually used to retain polar analytes in HILIC mode and several different silica-based or polymer-based stationary phases are available (amide-, cyano-, diol-, polyethylene glycol-, cyclodextrin-bonded phases). Some of these stationary phases can be used for HILIC but also for RP applications [2]. In the last years, many new materials for HILIC separation have been developed [9]. The basic types of materials for HILIC columns include plain silica, neutral polar chemically bonded phases, and ion-exchange and zwitterion materials [2].

The columns evaluated in this work are mixed mode HILIC columns but with different stationary phases. Obelisc N is a zwitterionic-type, originally intended for ion exchange but frequently applied to HILIC separations [9]. It has internal negatively charged groups and outer positively charged groups separated with a hydrophilic chain. It exhibits very polar characteristics and has been shown to work well for polar and charged analytes. When Obelisc N is operating in the ion-exchange mode, charged analytes interact with oppositely charged groups of the stationary phase [10]. Poroshel 120 HILIC-Z is also a zwitterionic stationary phase column with porous particles and its charges distribution are opposed to the Obelisc N [11]. The Waters anionic polar pesticide (APP) column is a very new column specifically designed for analysis of polar pesticides such as glyphosate. The stationary phase contains diethylamine functional groups and it can be operated in HILIC and weak anion exchange modes.

HILIC has some advantages over NPC regarding the eluent used. When NPC is used, nonpolar solvents, like hexane, are required. However, in the case of HILIC, mixtures of water with acetonitrile or methanol can be used, although acetonitrile is preferred, because methanol could interfere in the solvation of the silica surface due to the similarity with water (both are protonic solvents) [2]. In LC-MS, the use of acetonitrile is preferable over hexane because ionization is not easily achieved when nonpolar organic solvents are used [12]. In addition, the use of acetonitrile allows higher flow rates than methanol, due to the lower viscosity and, consequently, lower column back pressure. Furthermore, as the percentage of organic solvent is higher, compared to RPLC, the HILIC mode ensures efficient desolvation in the MS ionization process, which leads to lower detection limits. Nevertheless, HILIC has some drawbacks when compared to RPLC, including limited applicability, slow column equilibration, lack of robustness for some applications, and high organic solvent consumption [8, 13].

Glyphosate and related pesticides such as glufosinate, ethephon, and fosetyl are not amenable to usual reversed phase chromatography due to the lack of retention of these highly polar compounds. One solution that has been proposed as workaround for this issue is to do flow injection [14]. However, this can only be used for screening and the required limits of quantification (LOQs) cannot be achieved in all cases. In order to increase the retention of this type of compounds in RPLC, several applications make use of derivatization to reduce the polarity of the derivatives [12]. However, derivatization is time consuming and some compounds, such as the "N-acetyl" metabolites of glyphosate, AMPA, and glufosinate, are difficult to derivatize [15]. HILIC appears in this context as a powerful tool to solve retention issues, providing adequate retention when these compounds are analyzed directly without derivatization.

Animal feed materials are often by-products obtained after processing of cereals, pulses, and oilseeds. Pesticide residue analysis in animal feed is considered challenging due to the complexity and diversity of this kind of matrix, combined to a wide variety of constituents and additives such as grains, milling products, added mineral, vitamins, and fats, besides many other nutritional and energy components [16, 17]. The biggest difficulty in pesticides

residues determination in feed samples is attributed to the presence of co-extractives which make the identification and quantification more complicated if compared to high water content matrices such as fruits and vegetables, for example [18].

The goal of this work was to evaluate the influence of the stationary phases of three different mixed mode HILIC columns, under several mobile phases compositions, on the analysis of 14 anionic highly polar pesticides and metabolites in the very challenging matrix feed. To the best of our knowledge, no paper has ever been published for these difficult feed matrices and our analytes chosen. The 14 target compounds were selected based on the priority of these compounds in European coordinated pesticide monitoring programmes and the lack of fast and efficient analytical methods that can be applied for even the most challenging matrices, such as feed samples. Similar work was recently published that also evaluated different HILIC columns, but in that case only for the relatively easier matrices of fruits and vegetable [19]. The Quick Method for the analysis of numerous highly polar pesticides in food involving extraction with acidified methanol and LC-MS/MS measurement (QuPPe-method) [20], developed by the European Reference Laboratory for Single residue methods, also makes use of various HILIC methods, but the columns applied are different from those in our current research and appeared to be less robust in real practice. In another study, different HILIC columns were also tested for direct analysis of (only) glyphosate in rice, maize, and soybean [21]. The previously published Dutch Polar Pesticides method (NL-PP) [15, 22, 23] has been used as the basis for our current study, with slight modifications in the extraction procedure. The ultimate goal of the evaluation of the HILIC columns and various LC conditions was to develop a fast, reliable, and robust method with practical applicability in all types of difficult matrices. A full validation was carried out with two of the three evaluated HILIC columns, one zwitterionic (Obelisc N) and one non-zwitterionic (APP), and the method performance, in terms of trueness, precision, LOQ, and matrix effect (ME), was compared for all validated matrices (sunflower seed cake/meal, dried peas, and soya cake/meal). Isotopically labeled internal standards were used for all the compounds in order to ensure optimal quantification.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

High purity pesticide (>98%) standards of ethephon, glufosinate, fosetyl, phosphonic acid, glyphosate, and hydroxyethyl phosphonic acid (HEPA) were purchased from LGC-Dr. Ehrenstorfer (Augsburg, Germany), N-acetyl-AMPA, N-acetyl-glufosinate, N-acetyl-glyphosate, and 3-methylphosphinicopropionic acid (MPPA) from Toronto Research Chemicals, TRC (North York, Canada), aminomethyl phosphonic acid (AMPA) was purchased from Sigma–Aldrich (Steinheim, Germany) and bromide, chlorate, and perchlorate were obtained from Inorganic Ventures (Christiansburg, Virginia, USA). Isotope–labeled internal standards, AMPA ^{13}C ^{15}N, ethephon D_4, fosetyl–aluminum D_{15}, HEPA D_4, glufosinate D_3 hydrochloride, N-acetyl-glufosinate D_3, MPPA D_3, glyphosate 1,2-$^{13}C_2$, ^{15}N, and N-acetyl-glyphosate D_3 were purchased from LGC-Dr. Ehrenstorfer was obtained from Toronto Research Chemicals. Phosphonic acid-$^{18}O_3$, $^{18}O_3$-chlorate, and $^{18}O_4$-perchlorate were supplied by the EURL-SRM in Stuttgart, Germany. HPLC-grade water from a Water Purification System of Millipore (Burlington, MA, USA) was used. Formic acid was purchased from VWR (Lutterworth, United Kingdom) and trifluoroacetic acid (TFA) was obtained from Merck (Darmstadt, Germany). UPLC-grade acetonitrile and LC-grade methanol were supplied by Biosolve (Dieuze, France) and Merck (Darmstadt, Germany), respectively.

2.2 | Instrumentation

Chromatographic analysis was performed by a Shimadzu LC-system equipped with two Nexera X2 LC-30AD pumps and an SIL-30AC autosampler (Shimadzu, Kyoto, Japan) coupled to a hybrid quadrupole/linear ion trap mass spectrometer (6500+ QTRAP, Sciex Instruments, Concord, Ontario, Canada) with an electrospray ion source (ESI). For method 1, chromatographic separations were carried out on an Obelisc N (5 μm, 100 A, 150 mm × 2.1 mm) HILIC column (SIELC, Wheeling, IL, USA), kept at a constant temperature of 35°C. Mobile phases were water with 1% formic acid (mobile phase A) and acetonitrile (mobile phase B). The mobile phase gradient ranged from 20% A increasing linearly to 80% A in 1 min. This condition was kept during 11 min. Then, the mobile phase was changed to the initial condition in 0.2 min and maintained until the end of the chromatographic run time of 15 min. The flow rate was set at 0.5 mL min^{-1} and the injection volume was 15 μL.

For method 2, chromatographic separations were performed on a Waters (Milford, MA, USA) APP Column (5 μm, 130 Å, 100 mm × 2.1 mm), kept at a constant temperature of 35°C. The LC system was operated with mobile phase A (water with 0.9% formic acid) and mobile phase B (ACN with 0.9% formic acid). The gradient started at 10% A going to 85% A in 4 min. This condition was kept during 11 min. Then, the initial condition was set and kept until

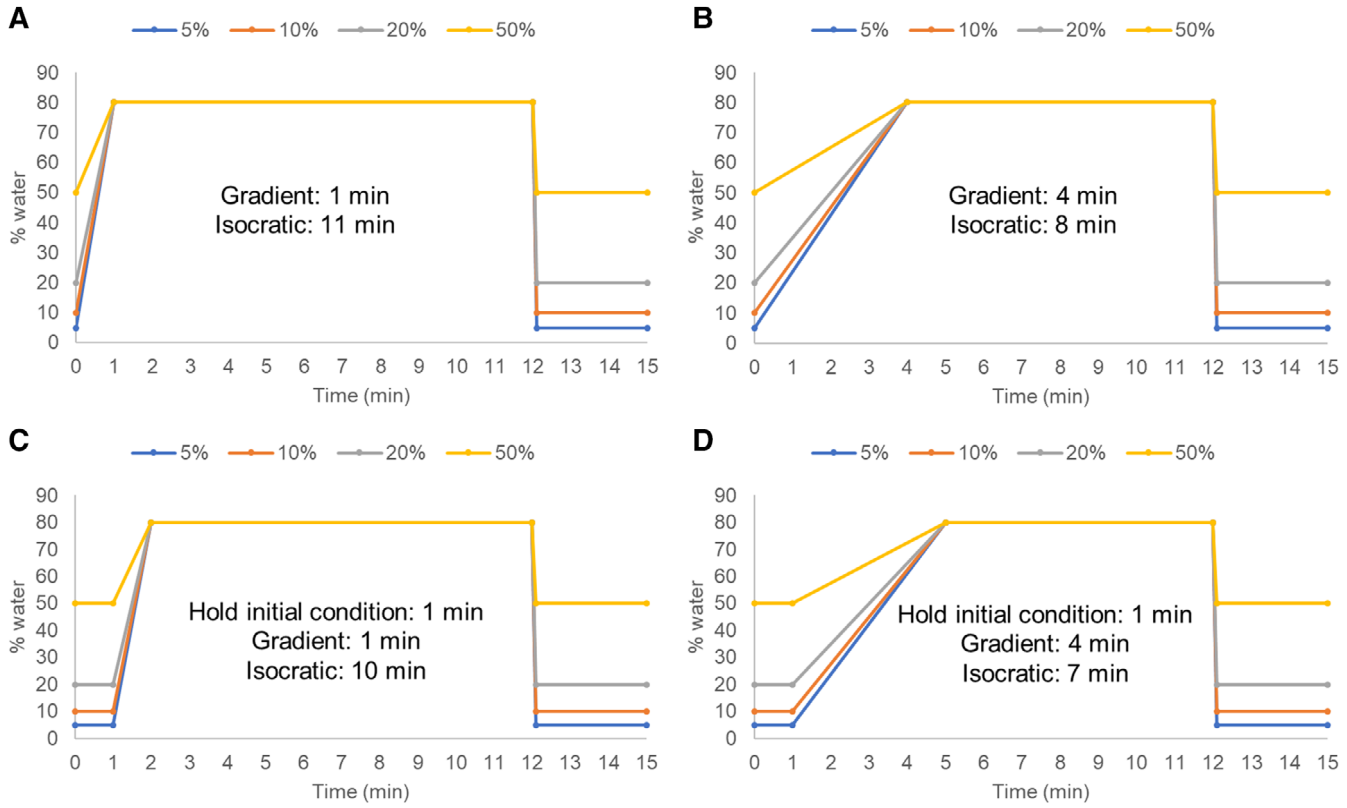

FIGURE 1 LC gradient composition for chromatographic analysis with HILIC columns

the end of the chromatographic run time of 20 min. The flow rate was set at 0.5 mL min^{-1} and the injection volume was 10 μL.

The LC-ESI-QTRAP-MS system was used in the triple-quadrupole mode operating in the multiple reaction monitoring mode with a unit mass resolution set for Q1 and Q3. Declustering potential, entrance potential, collision energy, and collision cell exit potential were optimized using flow injection analysis. Optimal parameters for each pesticide are described in our previous publication [15].

2.3 | Sample preparation and extraction

Two grams (± 0.1) of homogenized sample were weighed into a 50-mL centrifuge tube and 10 mL of HPLC-grade water were added. After 30 min, samples were spiked with 100 μL of isotopically labeled internal standard (ILIS) solution of 10 μg mL^{-1}. After that, 10 mL of MeOH with 1% of formic acid were added. The tubes were shaken in an automatic axial extractor (AGYTAX; Cirta Lab.S.L., Spain) during 5 min and placed in the freezer at −80°C for 15 min. Thereafter, the tubes were centrifuged at 4000 rpm for 10 min.

For method 1, the extracts were diluted 10 times extra prior to injection into the LC–MS/MS system. A 50 μL aliquot of the extract was diluted with 450 μL of dilution solvent (mixture of ACN/H_2O (60:40) and 0.2% TFA). The final sample concentration in the vial was 0.01 g mL^{-1}. For method 2, the extracts were diluted two times extra with dilution solvent. A 250 μL aliquot of the extract was diluted with 250 μL of dilution solvent (mixture of H_2O/MeOH, 1% formic acid). The final sample concentration in the vial was 0.05 g mL^{-1}.

2.4 | Experimental procedure for column comparison

Three different HILIC columns have been evaluated as to the retention capacity using different HILIC gradient starting conditions (see Figure 1). The eluents used for the gradient were water with 1% of formic acid (mobile phase A) and acetonitrile (mobile phase B). Considering that the maximum percentage of water for working in the HILIC mode is 50% [2], four different initial percentages of water have been evaluated (5, 10, 20, and 50%) in order to show the effect in the creation of the water layer of the liquid/liquid partitioning system, which is responsible for the retention of the polar compounds in the column when it is working in the HILIC mode. Two of the columns tested had stationary phases with zwitterionic functionalities:

Obelisc N (5 μm, 100 Å, 150 mm × 2.1 mm) from Sielc and Poroshell 120 HILIC-Z (2.1 μm, 100 × 2.7 mm) from Agilent. With Obelisc N, the positively charged groups in the active layer are placed externally and with Poroshell 120 HILIC-Z internally. The third tested column was from Waters, the APP column (5 μm, 130 Å, 100 mm × 2.1 mm), containing a ethylene bridged hybrid type stationary phase with bonded tertiary amine groups without zwitterionic properties.

2.5 | Validation

The method was validated for three different feed matrices (sunflower seed cake/meal, dried peas, and soya cake/meal) according to the SANTE guidelines [24] and linearity of calibration curves, instrument LODs and method LOQs, MEs, and accuracy (trueness and precision) were assessed. Two of the three evaluated columns (Obelisc N and APP) were employed for a full validation. From the zwitterionic columns, Obelisc N was chosen due to the better results in terms of peak shape and sensitivity. Besides, Obelisc N was already used in our previous publications for other matrix types [15, 22, 23]. The APP column showed better results regarding peak shape and retention in comparison to the Obelisc N and HILIC-Z, and for this reason it was also used for validation. Linearity was evaluated by injecting solvent standard solutions at 5 concentration levels (0.2, 0.5, 1, 5, and 50 ng mL^{-1}), four times each, and the determination coefficient (r^2) and deviation of back calculated concentrations were determined. Matrix effects were calculated by comparison of slopes obtained from calibration curves of standards in solvent ($n = 4$) and in matrix extract ($n = 1$) at the same range as described above. Trueness and precision were evaluated by spiking blank samples at 0.02, 0.05, 0.1, and 0.5 mg kg^{-1}. Six replicates of each concentration were performed. From the recovery experiments, method LOQs were determined as being the lowest spike level that fulfilled the requirements for recovery (between 70 and 120%), precision (RSD < 20%), and identification (ion ratios and retention time stability).

3 | RESULTS AND DISCUSSION

3.1 | Comparison of HILIC columns

As expected for columns working in the HILIC mode, the retention times for most of the compounds were higher when the initial percentage of water was lower (5%). However, no big differences were observed between 5, 10, or 20% of water at the beginning of the gradient program. In Supporting Information Table S1, a summary is given of the retention times for all compounds under all conditions evaluated. In the zwitterionic columns, a retention time difference of not more than 0.5 min was observed when 5 or 20% water as starting mobile phase was used and the faster gradients (see Figure 1A) were applied (see Figure 2). When slower gradients (see Figure 1B and D) were used, a difference of less than 1 min was observed. With the APP column, the compounds are strongly retained and the differences in retention times were more pronounced than for the zwitterionic columns. Different retention times are observed when the zwitterionic columns are compared to the nonzwitterionic ones. However, for all three columns, the lower the water concentration is at the beginning of the gradient, the more retention is observed. Thus, although the column manufacturers claim that the columns could work with a mixed-mode mechanism, it seems that the main retention mechanism involved under our LC conditions is the HILIC mode. In Supporting Information Figure S2, the chromatograms are presented for all three columns evaluated under condition B (Figure 1) with 5, 10, and 20% water at the start of the mobile phase gradient. In Supporting Information Figure S4, the reconstructed total ion chromatograms for the 14 compounds are presented for all the four gradients evaluated with 5% water at the start of the gradient for all columns.

In both zwitterionic stationary phase columns, the same behavior was observed when the gradient started with 50% of water (see Figure 3). With the HILIC-Z column, all compounds were eluting within 2 min and AMPA even within 1 min. With Obelisc N, most of the compounds also eluted within 2 min, except bromide, chlorate, fosetyl, and N-acetyl-glyphosate, which eluted within 3 min and perchlorate at 4.5 min. This indicates that, apparently, the HILIC mechanism is not acting in these columns when a high percentage of water is used at the initial conditions, which causes that the compounds are not retained. However, the APP column showed a completely different behavior than the zwitterionic columns. Compounds like AMPA and glufosinate are eluting at 0.7 and 2.5 min, respectively, but there are other compounds, as glyphosate, HEPA, N-acetyl-AMPA, for which the retention time ranges from 4 to 9 min depending on the gradient used (see Figure 3). Considering these retention times, it is possible to conclude that the major mechanism acting under these conditions is other than HILIC. Hydrophilic interactions are still possible considering that 50% water is still in the recommended range for HILIC separations [2]. However, the water layer created on the surface of the stationary phase under this condition would not be as effective as the one created with 5% water, suggesting that a combination of mechanisms is probably active where the major one is

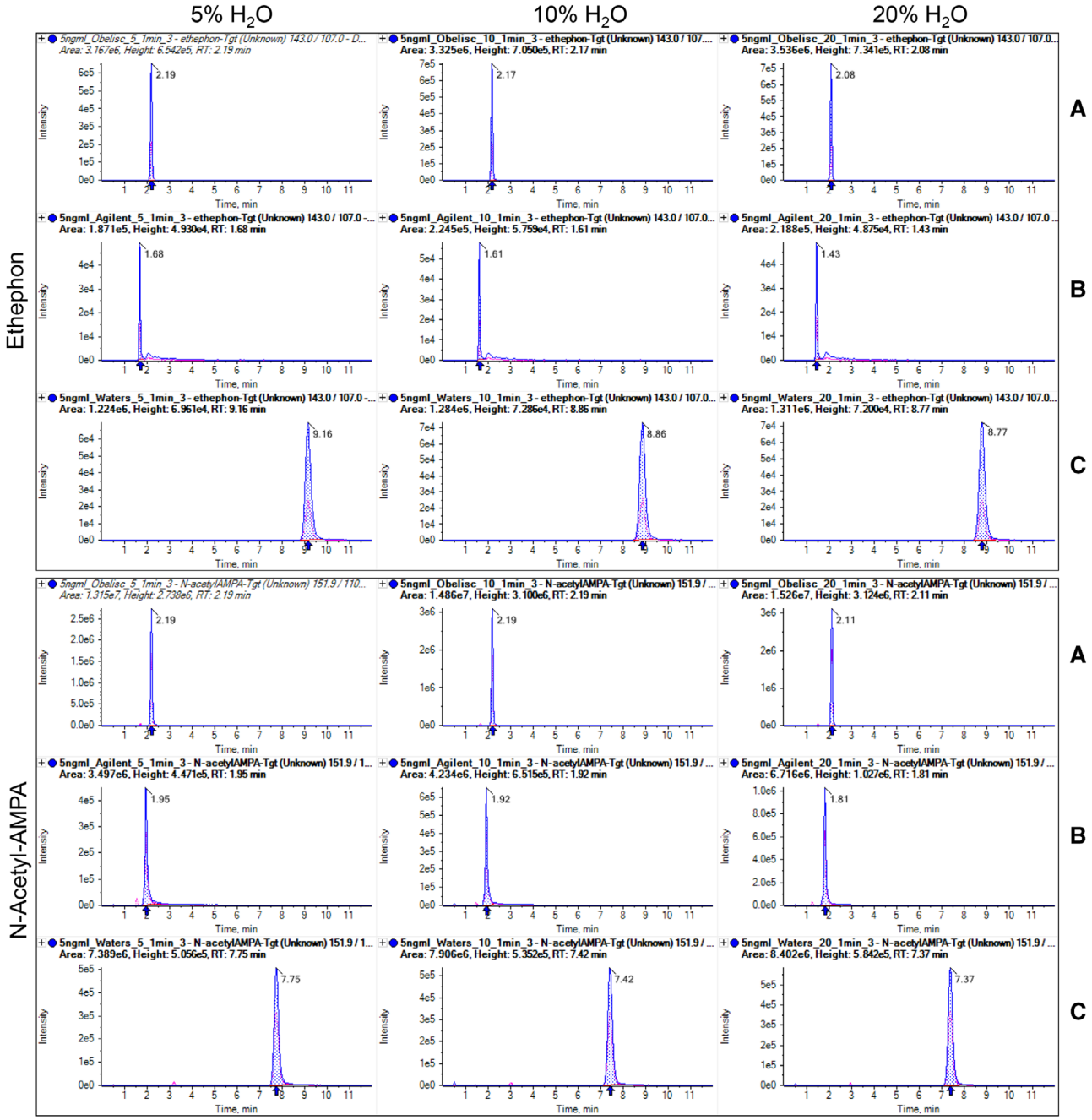

FIGURE 2 Chromatograms of standards of ethephon and N-acetyl-AMPA (5 ng mL^{-1}), when analyzed using 5, 10, and 20% water at the start of the gradient run (Figure 1A), in the Obelisc N (A), HILIC-Z (B), and APP (C) columns

not HILIC, like under the previous condition, but normal phase and/or anion exchange. With the HILIC-Z column, when operated under the condition where the gradient takes more time to reach the maximum water percentage (Figure 1B and D), the peaks showed peak splitting and tailing/fronting, indicating that the compounds are not properly eluted with lower amounts of water. This behavior was not observed when a fast gradient was used (Figure 1A and C).

3.2 | Effect of different columns on method performance—ME

The ME was evaluated for each pesticide by comparison of the slopes of calibration curves from standards prepared in organic solvent and in matrix extract. Figure 4 shows the ME results for all the compounds for each column. It is important to highlight that different dilution factors were used for each method. While for method 1 a 100-fold

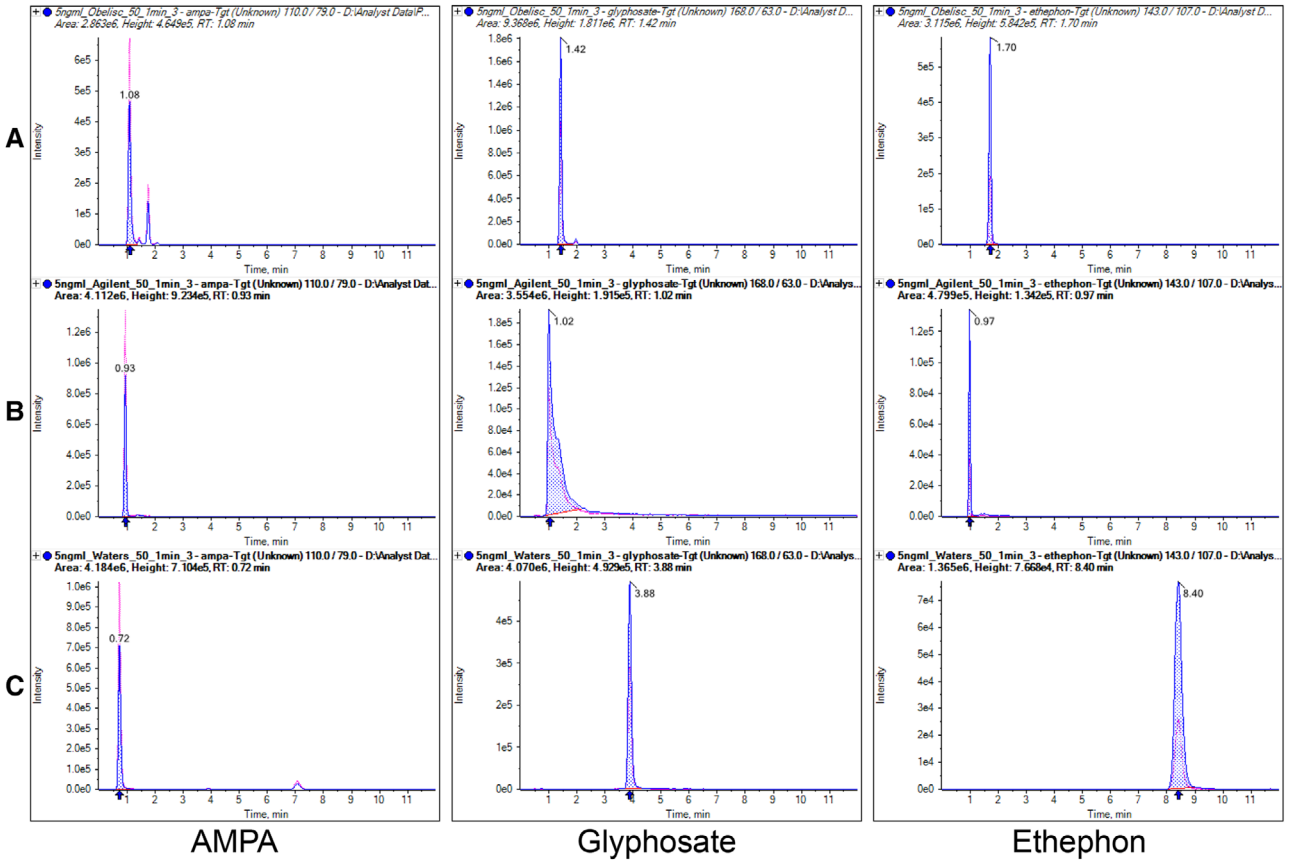

FIGURE 3 Chromatograms of standards of AMPA, glyphosate, and ethephon (5 ng mL^{-1}) using 50% water at the start of the gradient run (Figure 1A), in the Obelisc N (A), HILIC-Z (B), and APP (C) columns

dilution was applied, for method 2 a dilution factor of 20 was used instead. For method 1, high dilution factors are essential in order to ensure optimal peak shapes and sensitivity as it was already proved in previous publications [15, 22]. The more the extracts are diluted, the lower the ion suppression is, due to the reduction of the amount of matrix components in the ion source. Therefore, it would be logical to assume that for method 2, where a higher equivalent amount of matrix is injected onto the column, the suppression would be higher compared to method 1. This can be confirmed only for AMPA, where −55% ME (on average) was obtained for method 1, while almost 100% suppression was observed for method 2. For seven other compounds, namely ethephon, fosetyl, glyphosate, HEPA, N-acetyl-AMPA, N-acetyl-glufosinate, and phosphonic acid, an improvement of the ME is visible with method 2. While for method 1, a significantly higher ME was obtained, for example almost 80% for fosetyl, the reduction with method 2 was so significant that almost no ME was observed. MPPA showed almost 100% suppression for method 1 in sunflower seed cake/meal and dried peas, and was even not detected at all in soya cake/meal. Method 2 showed some improvement, because for soya cake/meal a peak was detectable despite the still high suppression. Glufosinate was the only compound for which no differences were observed when the two methods were compared.

Considering the results obtained, the most probable explanation for the differences of MEs between the two columns is the different interaction of the analytes/co-extracted substances from the matrix with the stationary phase, resulting in a different selectivity of the chromatographic separations. Method 2 obviously provides the more efficient separation of analytes from the interfering matrix components. A more detailed explanation for the typical retention behavior of the 14 analytes, which differ widely in terms of structure and functional groups, cannot be given.

3.3 | Effect of different columns on method performance—recovery

In order to prove the suitability of the methods with the Obelisc N (method 1) and APP (method 2) columns and to complete the accurate comparison/evaluation, the two methods were fully validated for all feed matrices according to the SANTE guidelines [24]. In Supporting

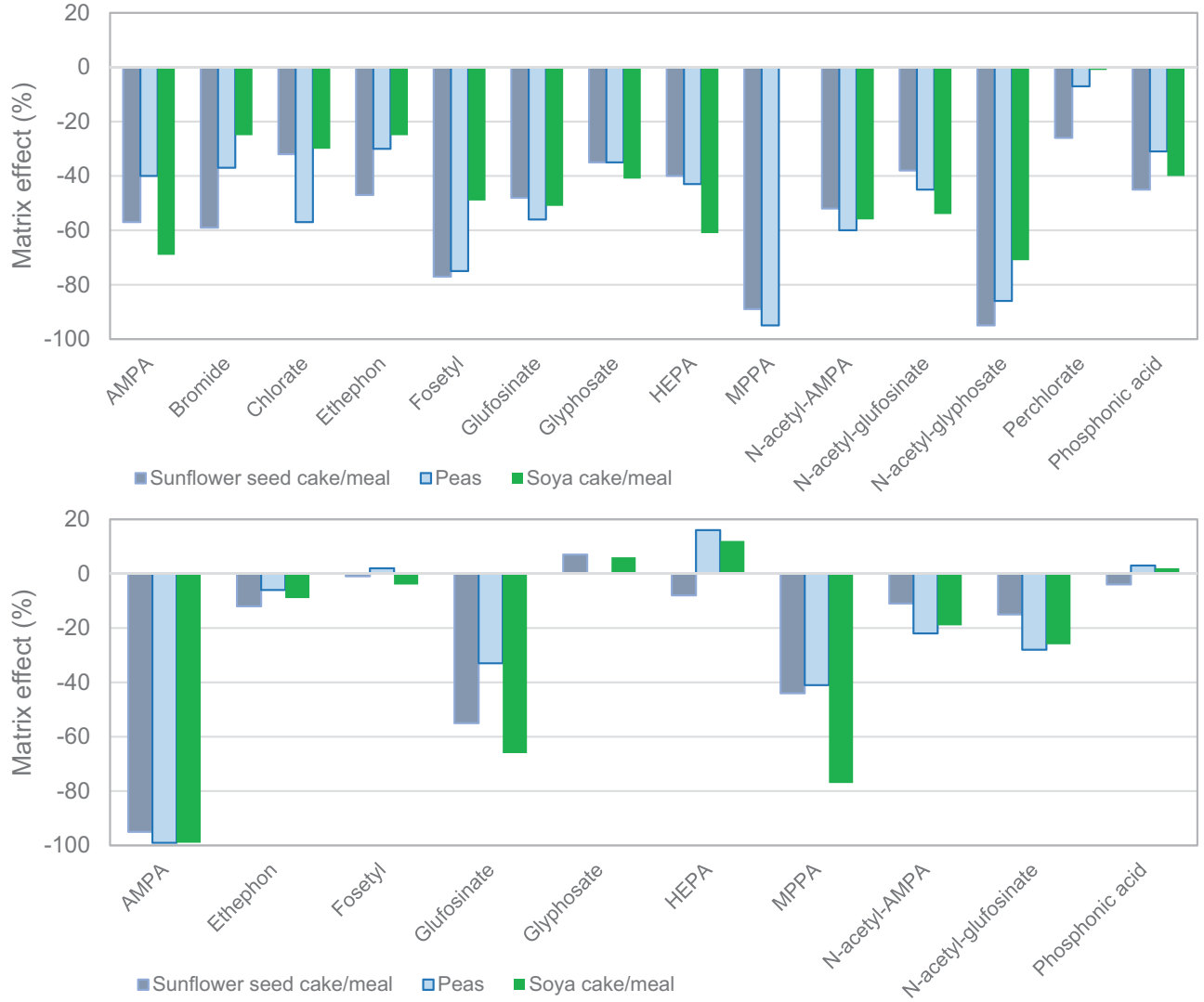

FIGURE 4 Matrix effects (%) for all matrices evaluated using (A) Obelisc N column and (B) APP column

Information Table S3, the results of the recoveries experiments are shown for all four spike levels and all matrices. Despite each method has its own dilution procedure with the optimal solvent for injection, the extraction is exactly the same. This means that the extractability of the analytes and consequently also of the interferences are the same for both methods. Table 1 shows the method LOQ (definition see 2.5) for each compound in all matrices for both methods. For seven (namely AMPA, fosetyl, glyphosate, HEPA, MPPA, N-acetyl-AMPA, and phosphonic acid) out of the 14 compounds, improvements regarding the LOQ were achieved when method 2 was applied. Fosetyl, for example, showed an interference eluting at the same retention time for method 1 and, for this reason, a high LOQ was obtained for sunflower seeds cake/meal. Furthermore, for dried peas and soya cake, the coeluting matrix components interfered in such a way that no useful data were obtained, even for the higher spike level for this compound.

Very different behavior was also observed for glyphosate. In Table 1, it can be seen that for method 1, only screening detection limits could be set at the lower spike levels for sunflower seeds cake/meal and dried peas due to the lack of sensitivity and selectivity for the product ion of the second MS/MS transition. On the other hand, for method 2, all requirements were met at the lower spike levels (0.02 or 0.05 mg kg^{-1}) resulting in lower LOQs. In Figure 5, which shows the MS/MS extracted ion chromatograms for glyphosate spiked at all concentrations to all sample types, this scenario can be seen in more detail. In this figure, the peak of the quantification transition (in blue) for glyphosate in method 1 is perfectly visible and no interferences are present at its retention time. However, the signal of the product ion from the second MS/MS transition (in pink) is completely masked/interfered by co-eluting matrix compounds, and thus, the ion ratio in the sample extract did not match with that of the standard. The

TABLE 1 Limits of quantification (mg kg^{-1}) for all matrices using two HILIC methods

	Method 1			Method 2		
Compound	Sunflower seed cake	Peas	Soya cake	Sunflower seed cake	Peas	Soya cake
AMPA	0.05*	0.05*	0.05 (0.02*)	0.02	0.1	0.5
Bromide	n.a	n.a	0.05	n.d	n.d	n.d
Chlorate	0.02	0.02	0.02	n.d	n.d	n.d
Ethephon	0.02	0.02	0.02	0.02	0.02	0.02
Fosetyl	0.5	n.f.r	n.f.r	0.02	0.02	0.02
Glufosinate	0.02	0.02	0.02	0.02	0.02	0.02
Glyphosate	0.1 (0.02*)	0.5 (0.02*)	0.02	0.05	0.02	0.02
HEPA	0.1 (0.02*)	0.02	0.5	0.02	0.02	0.02
MPPA	0.1 (0.02*)	0.1	n.f.r	0.02	0.02	0.02
N-Acetyl-AMPA	0.02	0.1	0.1	0.02	0.02	0.02
N-Acetyl-Glufosinate	0.02	0.02	0.02	0.02	0.02	0.02
N-Acetyl-Glyphosate	0.5	0.02	n.f.r	n.d	n.d	n.d
Perchlorate	0.02	0.02	0.05	n.d	n.d	n.d
Phosphonic acid	0.2	0.2	0.02	0.02	0.02	0.02

*Screening detection limit, only taking the quantifier into account.
n.a., not analyzed due to high background levels in the blank; n.d., not detectable in the same run; n.f.r., not fulfilling requirements for a quantitative method.

acceptance criterion (relative ion ratio difference <±30%) could not be met and consequently, no identification in line with SANTE/12682/2019 is possible. At higher concentrations (0.1 or 0.5 mg kg^{-1}), it is still possible to observe some interference, but nevertheless, the ion ratios matched the identification criterion, even though the peak is not completely separated from the interferences. Thus, the LOQ for method 1 was set at these concentrations. For method 2, on the other hand, low LOQs were achieved and as can be seen in Figure 5, the method appears to be very selective, which is illustrated by the clean chromatograms for both quantification and confirmation ions.

MPPA, similar to glyphosate, presented high LOQs for method 1. Besides, for soya cake/meal, no peak for this compound was observed at all due to strong matrix interference. Figure 6 shows the extracted ion chromatograms for MPPA for the three matrices spiked at 0.02 mg kg^{-1}. In this figure, the performance of both methods can be clearly seen. At the same concentrations and for the same matrices, method 1 is not capable to achieve enough sensitivity for MPPA. Method 2, on the other hand, is sensitive (and selective) enough to quantify MPPA at very low concentrations.

We could observe that for some pesticides, such as fosetyl, glyphosate, and MPPA, the product ion of the second transition is more affected by the co-eluting matrix component than the product ion of the first transition applying method 1. Method 2, on the other hand, shows to be a good solution for this problem considering that even at the very low concentration of 0.02 mg kg^{-1}, very good peak shape and sensitivity could be achieved, as already shown in Figures 5 and 6.

The presumable explanation for this behavior is that, when using the APP column, the matrix components are interacting differently with the stationary phase and a different mobile phase gradient is used. Better separation between target pesticides and interferences is achieved and as a consequence better detectability of the MS/MS product ions from the analytes.

Considering the (limited) information from the manufacturer of each column, it is clear that for both of them, the analytes and also the extracted matrix compounds can have different possibilities of interaction with the stationary phase due to the different column materials employed, as mentioned above. Besides the interactions involved in HILIC separations, such as hydrophilic partitioning, hydrogen bounding, and electrostatic interactions, the stationary phase composition provides extra possibilities, such as ion exchange and normal phase behavior. Taking into account that Obelisc N is a zwitterionic stationary phase, different interaction mechanisms (e.g., ion exchange; ion pair) for analytes/co-extractives can occur, opposite to the APP column, which contains a non-zwitterionic material. Furthermore, while the manufacturer of Obelisc N claims that the column can be operated in the ion exchange mode, the APP column manufacturer claims that their product can be operated also in the weak anion exchange mode. Weak ion

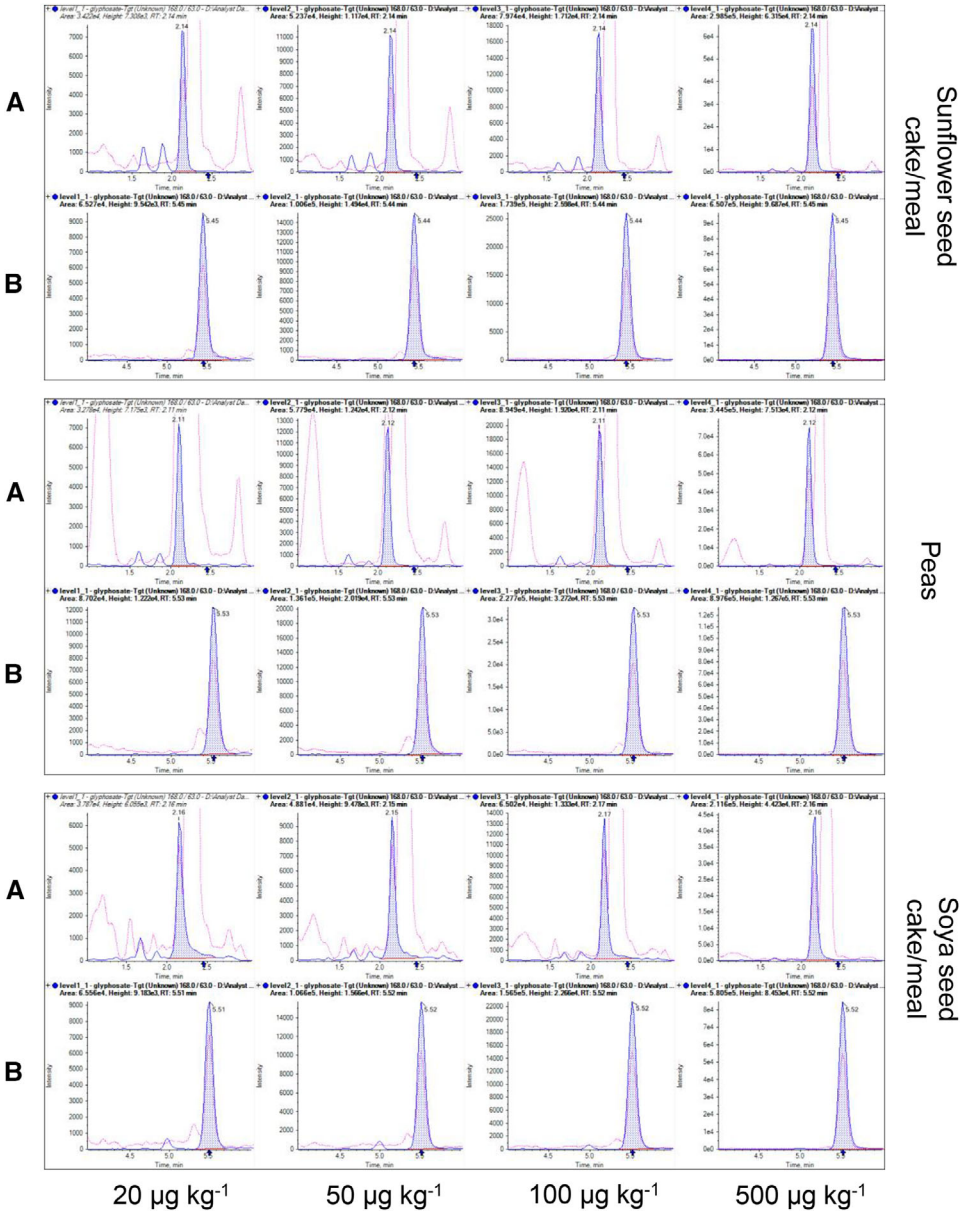

FIGURE 5 Extracted ion chromatograms for glyphosate spiked at 0.02, 0.05, 0.1, and 0.5 mg/kg^{-1} in sunflower seed cake/meal, dried peas, and soya seed cake/meal obtained via method 1 (A) and method 2 (B). Blue line (168→63 quantifier ion), pink line (168→81 qualifier ion)

exchangers have less ability to bind/retain impurities and consequently enhance resolution [25], which can explain the results obtained in the present research.

4 | CONCLUSION

HILIC appears to be a powerful technique to apply for the analysis of 14 target analytes of highly polar pesticides and metabolites, which present very poor retention in RPLC. Three HILIC columns from different vendors were evaluated and the two best performing columns (Obelisc N and APP) were applied for a full validation study of three representative, difficult feed matrices. Trueness and precision were determined by spiking blank samples at 0.02, 0.05, 0.1, and 0.5 mg kg^{-1} and analysis of six replicates at each level.

After evaluation of the validation results, we could observe significant differences in performance of both columns. The Obelic N column provides a wider scope of compounds (14 in total) with LOQs of 0.02–0.05 mg kg^{-1} for most analytes/matrix combinations. However, quantitatively validated results are not possible for some other analyte/matrix combinations and/or only screening detection limits could be set because of poor sensitivity/selectivity of the qualifier ion needed for identification. On the other hand, the APP column showed to be very efficient in terms of selectivity, so that substantial

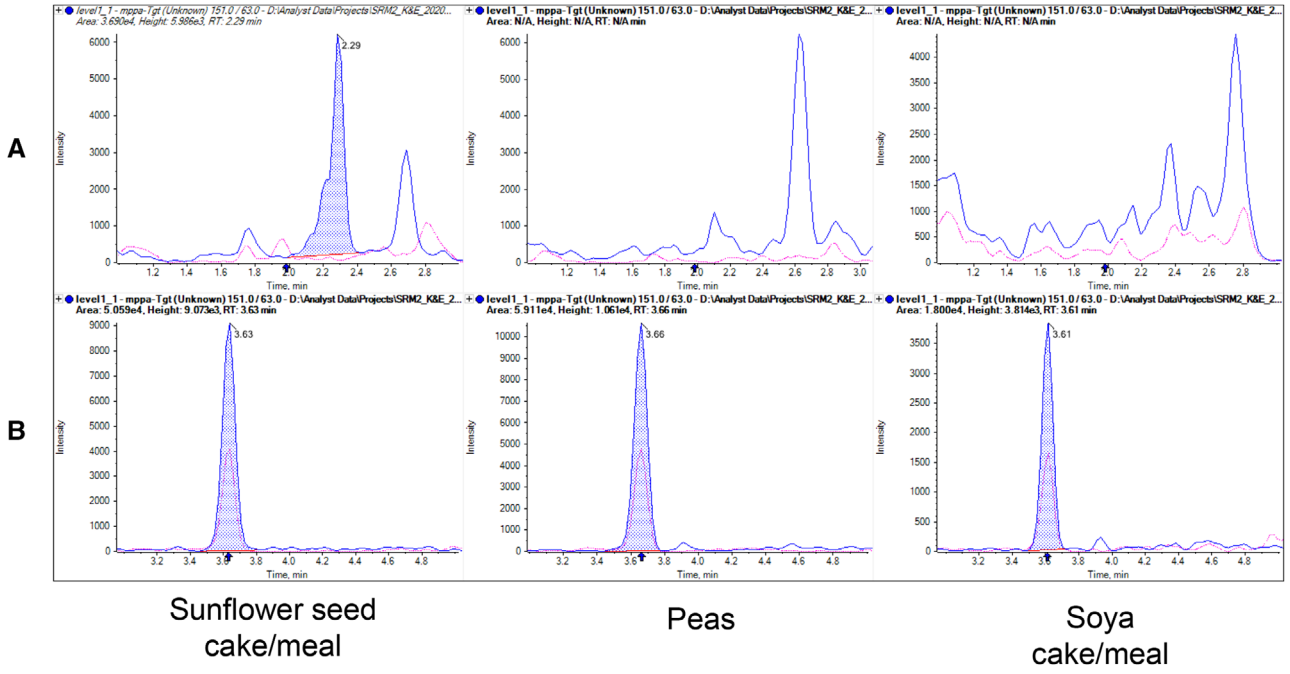

FIGURE 6 Extracted ion chromatograms for MPPA spiked at 0.02 mg kg^{-1} in sunflower seed cake/meal, dried peas, and soya seed cake/meal obtained via method 1 (A) and method 2 (B). Blue line, quantifier ion (151→63), pink line, qualifier ion (151→107)

reduction of MEs were obtained for most of the compounds, except for AMPA, which still showed an extremely high suppression of almost 100%. Moreover, LOQs of 0.02 mg kg^{-1} were obtained for ten analytes/matrix combinations, except for glyphosate in sunflower seed cake and AMPA in dried peas and soya cake.

Four analytes, chlorate, bromide, N-acetyl-glyphosate, and perchlorate could not be analyzed with the APP column using acidic mobile phase conditions. With a different eluent, it might be possible to analyze these in a separate chromatographic run.

Finally, we are able to conclude that both columns have advantages and drawbacks. Obelisc N can be used as screening method, as it covers a larger number of compounds (14 in total). In the cases where residue concentrations are above the LOQ, it can also be used for quantification. The APP column has the advantage of more selectivity and lower validated LOQs, but is not able to cover all the 14 pesticides/metabolites in one run, using acid conditions. Thus, the final choice for the preferred method will depend on the required scope and/or method LOQs and the frequency of occurrence of positive residues to be expected. Both methods have been successfully applied in routine analysis of the target analytes in feed samples in the yearly monitoring programme.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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