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Selective multiresidue determination of highly polar anionic pesticides in plant-based milk, wine and beer using hydrophilic interaction liquid chromatography combined with tandem mass spectrometry

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ABSTRACT

In this work, an easy and fast procedure for the selective multiresidue determination of 14 highly polar pesticides (including glyphosate, glufosinate, ethephon and fosetyl) and metabolites in beverages is presented. After an initial sample dilution (1:1, v/v), the extract is shaken and centrifuged, further diluted and then injected directly into the LC-MS/MS system, using hydrophilic interaction liquid chromatography (HILIC) and tandem mass spectrometry. No clean-up procedure was needed. The method was validated according to the current European guidelines for pesticide residue analysis in food and feed and linearity, limits of detection and quantification, matrix effects, trueness and precision were assessed. For plant-based milk, wine and beer samples, 10, 11 and 12 analytes, respectively, out of 14 were fully validated at 10 μg kg^{-1}, the lowest spike level tested. The matrix effect was negative in most of the cases, showing for some compounds, such as HEPA, up to 80% suppression when compared to the response from standards in solvent. The use of isotopically labelled internal standards is required for the optimal quantification, as it compensates for high and varying matrix effects and also for recovery losses during extraction.

1. Introduction

Herbicides are widely used in agriculture in order to increase the yield and quality of crops. They act by reducing/inhibiting weeds growth and also play an important role as desiccant in several crops as cereals (eg. wheat, barley, oat), oilseeds (e.g. soybean) and pulses (e.g. beans, peas) [1]. Highly polar ionic compounds are a group of herbicides frequently used worldwide in this kind of crops. Especially glyphosate is considered non-persistent and safe in the environment due to its rapid degradation in water and soil [2]. However, despite limited evidence of carcinogenic or mutagenic effects, it can cause serious eye damage and can be potentially toxic to aquatic biota [2]. Aminomethylphosphonic acid (AMPA) is the major metabolite of glyphosate and its toxicity is comparable to its precursor [3]. Glyphosate is currently the most produced herbicide in the world and its use has increased in the last few years since genetically modified crops were introduced to be resistant to glyphosate [4]. Chlorate has pesticide and biocide activities and, as perchlorate, can be produced as by-product of disinfection agents of drinking water. In plant-based beverages, chlorate and perchlorate could arise from the chlorinated water. Bromide, on the other hand, can be present in food samples from natural sources [5,6].

The glyphosate based herbicides (GBH) are applied mainly in agriculture in order to accelerate harvest, and residues of these substances can be found in the raw crops and also in its processed products. In the case of plant-based beverages, olive oil and orange juice, no maximum residue levels (MRLs) are established. For those products, MRLs have to be derived from the MRLs of the raw commodity, after applying processing factors [7].

Nowadays, cow milk allergy, lactose intolerance and the search for vegan diets has influenced consumers to choose an alternative for cow milk consumption [8]. Plant-based milks are fluids resulting from the mixture of milled material (cereals, pseudo-cereals, oilseeds, nuts) with water, followed by filtration [9]. The production of plant-based milk is basically based on the milling of the grain and water addition for a slurry preparation, followed by an enzymatic hydrolysis. After this process, the mixture is filtrated in order to obtain the final milk [10]. Soya milk is the most consumed plant-based milk and its use is reported since about 2000 years ago in China [9]. Due to the high fat content, the production process of soya milk involves some extra steps in order to remove

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the beany flavour caused by the action of lipoxygenase enzyme on unsaturated fatty acids. Such steps involve soaking the grain with water, heating, homogenization under pressure and neutralization [11].

Beer is a beverage made with malted cereal grains (barley, wheat, rye, corn or rice), hops and water that is fermented by addition of yeasts [12]. After enzymatic hydrolysis, the mixture is filtrated and boiled in order to extract the flavour from hops added in this step. After maturation and carbonation, the beer is cooled, clarified, filtered and placed in bottles [13].

Winemaking is among the oldest techniques known to civilization and it is one of the most commercially prosperous biotechnological processes involving alcoholic fermentation. Wine is an alcoholic beverage made from the complete or partial spontaneous fermentation of grape juice [14]. The grapes are crushed after stems removal and the mixture is fermented and further maturated and clarified [15].

One common step during production of plant-based milk and beer is water addition to the raw product. Due to the high polarity of the glyphosate-based herbicides, these compounds can be extracted from the product during homogenization and remain in the water until the final product. The process to make wine, on the other hand, does not involve water addition. However, wine is about 90% water coming from grapes which can contain GBH residues if they were applied in the field. The fact that water is the main component of this kind of matrices explains the importance of highly polar pesticides determination in plant-based beverages, especially those based on cereals/vegetables where GBHs have been applied in the field.

However, the determination of these compounds is not an easy task. These substances are usually not amenable to multiresidue methods due to their high polarity, which leads to poor extractability into typical organic extraction solvents and poor retention in reversed-phase chromatography during analysis [16,17,18]. To solve these issues, alternative extraction procedures have been widely used based on polar solvents such as water and methanol [19]. In order to improve retention and peak shapes, hydrophilic interaction liquid chromatography (HILIC) has been introduced for these polar compounds [20,21]. However a lack of robustness of HILIC columns has been repeatedly mentioned in previous publications [3,21]. Our group [22] has solved these drawbacks by applying high dilution factors and the most sensitive mass spectrometry system in the market. This finally optimized Dutch Polar Pesticides (NL-PP) method was successfully validated for 14 polar pesticides/metabolites in fruits, vegetable and cereals. Isotopically labelled internal standards (ILIS) were used for quantification purposes. This approach corrects for losses during extraction, for matrix effects and response drift in the detection system.

In the present study, the extraction method had to be adapted in order to analyse the plant-based beverages studied: oat milk, rice milk, soya milk, Pilsen beer, wheat beer and (red and white) wine. An initial acetonitrile/water (6:4, v/v) addition was applied to induce the precipitation of proteins in these sample types. Subsequently, the extracts were centrifuged and further diluted in the autosampler vials, just before direct injection in the LC-MS/MS system. A similar approach, using direct dilution of wine samples with acetonitrile/methanol was successfully applied for the multiresidue method of pesticides and mycotoxins via LC-MS/MS [23]. The use of HILIC and tandem mass spectrometry combined with low sample matrix equivalent concentrations (0.02 g mL^{-1}) in the final injected extracts lead to the selectivity, sensitivity and proven robustness of the method. To the best of our knowledge, this is the first publication describing a fully validated, fast and efficient method for 14 analytes (4 pesticides and their 7 metabolites, plus bromide, chlorate and perchlorate) in plant-based beverages.

2. Experimental

2.1. Chemicals

Acetonitrile and methanol, both UPLC/MS grade, were purchased from Biosolve (Dieuze, France). Formic acid, purity ≥ 99%, was from VWR Chemicals (Lutterworth, UK) and trifluoracetic acid (TFA), for synthesis grade, from Merck. Ultrapure water was obtained from a purification system, Millipore (Burlington, MA, USA). High purity (> 98%) analytical grade reference materials of ethephon, fosetyl, glufosinate, glyphosate, 2-hydroxyethyl phosphonic acid (HEPA) and phosphonic acid were purchased from LGC-Dr. Ehrenstorfer (Augsburg, Germany). 3-methylphosphinicopropionic acid (MPPA), N-acetyl-glufosinate, N-acetyl-glyphosate and N-acetyl-AMPA were from Toronto Research Chemicals, TRC (North York, Canada). Aminomethylphosphonic acid (AMPA) was from Sigma-Aldrich (Steinheim, Germany). Bromide, chlorate and perchlorate were from Inorganic Ventures (Christiansburg, USA). Isotopically–labeled internal standards, AMPA ^{13}C ^{15}N, ethephon D_4, fosetyl–aluminum D_{15}, 2-hydroxyethyl phosphonic acid (HEPA) D_4, glufosinate D_3 hydrochloride, N-acetyl-glufosinate D_3, 3-methylphosphinicopropionic acid (MPPA) D_3, glyphosate 1,2-$^{13}C_2$, ^{15}N, N-acetyl-glyphosate D_3, were purchased from LGC-Dr. Ehrenstorfer and from Toronto Research Chemicals. Phosphonic acid-$^{18}O_3$, $^{18}O_3$-chlorate, $^{18}O_4$-perchlorate were supplied by the EURL-SRM in Stuttgart, Germany.

2.2. Instrument

Chromatographic analysis was performed by a Shimadzu LC-system equipped with two Nexera X2 LC-30AD pumps and a SIL-30AC autosampler, coupled to a hybrid quadrupole/linear ion trap mass spectrometer (6500+ QTRAP, Sciex Instruments, Concord, ON, Canada) with an electrospray ion source (ESI). Chromatographic separations were carried out on an Obelisc N (5 µm, 100 A, 150 mm x 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% in one minute. This condition was kept during 11 minutes. 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 minutes. The flow rate was set at 0.5 mL min^{-1} and the injection volume was 15 µL.

The LC-ESI-QTRAP-MS system was used in the triple-quadrupole mode operating in the multiple reaction monitoring (MRM) mode with a unit mass resolution set for Q1 and Q3. Declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP) were optimised using flow injection analysis. Optimal parameters for each pesticide are described in our previous publication [22].

2.3. Sample extraction

Blank samples of oat milk, rice milk, soya milk, Pilsen beer, wheat beer, red wine and white wine were purchased in local supermarkets in the city of Wageningen, The Netherlands. Before weighing of the samples, the bottles were shaken in order to provide good homogenization. Then, two grams (± 0.1) of homogenised sample were weighed into a 50 mL centrifuge tube and spiked with 50 µL of isotopically-labelled internal standard (ILIS) solution of 10 µg mL^{-1}. Then, 2 mL of dilution solvent (mixture of acetonitrile/water, 6:4, v/v, containing 0.2% trifluoroacetic acid) were added and the tubes were shaken in an automatic axial extractor (Agytax®) during 5 min followed by centrifugation at 4150

R.C.F for 10 min, at 10°C. Finally, the extracts were extra diluted directly in an autosampler vial by mixing 20 µL of extract with 480 µL of dilution solvent. This results in a matrix concentration of 0.02 g mL^{-1} in the final extract, which corresponds to a final dilution factor of 50.

2.4. Method validation

Validation was carried out following the procedure established in the EU SANTE guidelines [24]. The method was assessed for linearity of calibration curves, instrument limits of detection and method limits of quantification, matrix effects, accuracy (trueness and precision) according to the performance criteria.

Linearity was checked by injecting standards solutions at 5 concentration levels (0.2, 0.5, 1, 5 and 50 ng mL^{-1}), four times each, in order to evaluate the determination coefficient (r^2) and deviation of back calculated concentrations. The instrument detection limit (LODi) has been determined based on the lowest detectable concentration level measured with a repeatability RSDr < 33%. Calibration curves have been constructed from calibration standards in solvent and in matrix extracts.

Matrix effects were determined by comparison of slopes obtained from calibration curves from standards in solvent (n=4) and in matrix extract (n=1) at the same range described above, using the following formula:

$$Matrix\ effect\ (\%) = \left(\frac{slope\ cal.\ curve\ matrix - slope\ cal.\ curve\ solvent}{slope\ cal.\ curve\ solvent} \right) \times 100$$

Trueness and precision were evaluated by spiking blank samples at 10, 20, 50 and 200 µg kg^{-1}. Six replicates of each concentration were performed. According to the EU SANTE document, recoveries between 70 and 120% and relative standard deviations lower than 20% should be achieved for method validation. From the recovery experiments, method limits of quantification (LOQm) were determined as being the lowest spike level that fulfilled the requirements for recovery and precision.

Identification criteria were based on the EU SANTE guidelines for retention time (tolerance of ± 0.1 min) and product ions response ratio (MRM_2/MRM_1, tolerance of ± 30%), where the reference values were based on the average of the standards in solvent.

3. Results

3.1. Extracts dilution

In our previous published work [22], we have proven that considerable dilution of extracts before injection is essential to obtain better retention time stability, peak shapes and reduction of matrix effects for fruits, vegetables and cereals. Due to the presence of solid particles and/or proteins in some of the liquid sample types studied in the current work, an initial dilution with solvent and a centrifugation step was necessary. Acetonitrile is known to be a favourable solvent to precipitate proteins [25]. Acetonitrile is already present in the dilution/injection solvent (acetonitrile/water [6:4] with 0.2% TFA) for the HILIC run. Therefore, to 2 g of homogenised liquid sample, 2 mL of this dilution/injection solvent was added, mixed, shaken and then centrifuged. Direct injection of the 1:1 diluted samples was tested and also several dilution factors (5, 10, 20 and 50-fold) were evaluated again in this study. As expected, direct injection of the 1:1 diluted sample extracts still caused problems with peak splitting and less good detectability of some of the more problematic analytes. In traditional reversed-phase chromatography, this peak splitting is normally caused by a combination of a high injection volume and a difference in modifier strength in the injected volume and the composition of the starting mobile phase of the gradient programme. However, this effect is usually only observed with early eluting analytes, which is not the case in our present application. Also, the acetonitrile/water ratio of injection solvent and mobile phase is the same. Therefore, the peak splitting must most likely be caused by a too high sample matrix load / analyte concentration ratio, which disturbs a regular start of the chromatographic process (exchange of analyte between mobile and stationary phase) from the start of injection. Higher dilution factors improve significantly the results for the most problematic pesticides. For example, figure 1 shows the chromatograms for perchlorate spiked at 100 µg kg^{-1} in plant-based milks, beer and wine. From this figure, it is clear that high dilution factors are essential to achieve better results. Perchlorate showed peak splitting when the dilution factor is less than 20 times for the plant-based milks and wine and less than 10 times for beer. In the same figure, one can observe that retention time and peak shape also improve when increasing the dilution factor of extracts. After evaluating all analytes, a total dilution of 50 times was applied for the final method to be validated for all matrices.

3.2. Validation

3.2.1. Linearity

Linearity was assessed by repeated injections (n=4) of calibration standard solutions at 0.2, 0.5, 1, 5 and 50 ng mL^{-1} prepared in matrix extract and in solvent. For plant-based milks, with exception of N-acetyl-glyphosate that presented a determination coefficient (r^2) of 0.98, all the evaluated compounds showed r^2 higher than 0.99 and deviations of back calculated concentration lower than 20%. For these matrices, the estimated instrument detection limit (LOD_i) was set as the lowest measured concentration (0.2 ng mL^{-1}). For beer and wine, all the analytes showed determination coefficients higher than 0.99 and deviations of back calculated concentration lower than 20%. Therefore, the LOD_i for beer and wine was set at 0.2 ng mL^{-1}, as for the plant-based milks. The linear dynamic range is 0.2 – 50 ng mL^{-1}, which corresponds with a range of 0.01 – 2.5 mg kg^{-1} for residue levels in the samples.

3.2.2. Matrix effect

The matrix effects of the 14 analytes for the seven matrices studied are summarized in figure 2. The majority of the compounds showed a negative matrix effect for all the matrices studied. AMPA, glyphosate and HEPA showed the highest response suppression (negative matrix effects) of all analytes. Besides, it is important to highlight that HEPA showed the highest suppression (around -80%) for oat, rice and soya milks. MPPA showed the highest response enhancement for beer samples (54% on average). Furthermore, opposite matrix effects were observed for MPPA, +39% for oat milk and -37% for soya milk. This effect was also observed in our previous study [20], where it was concluded that the matrix effect was typically dependent on the individual matrix and not on a commodity group. Soya drink and oat drink are both considered as plant-based milks, however, they showed different matrix effects. This behaviour shows that some matrices, even being similar,

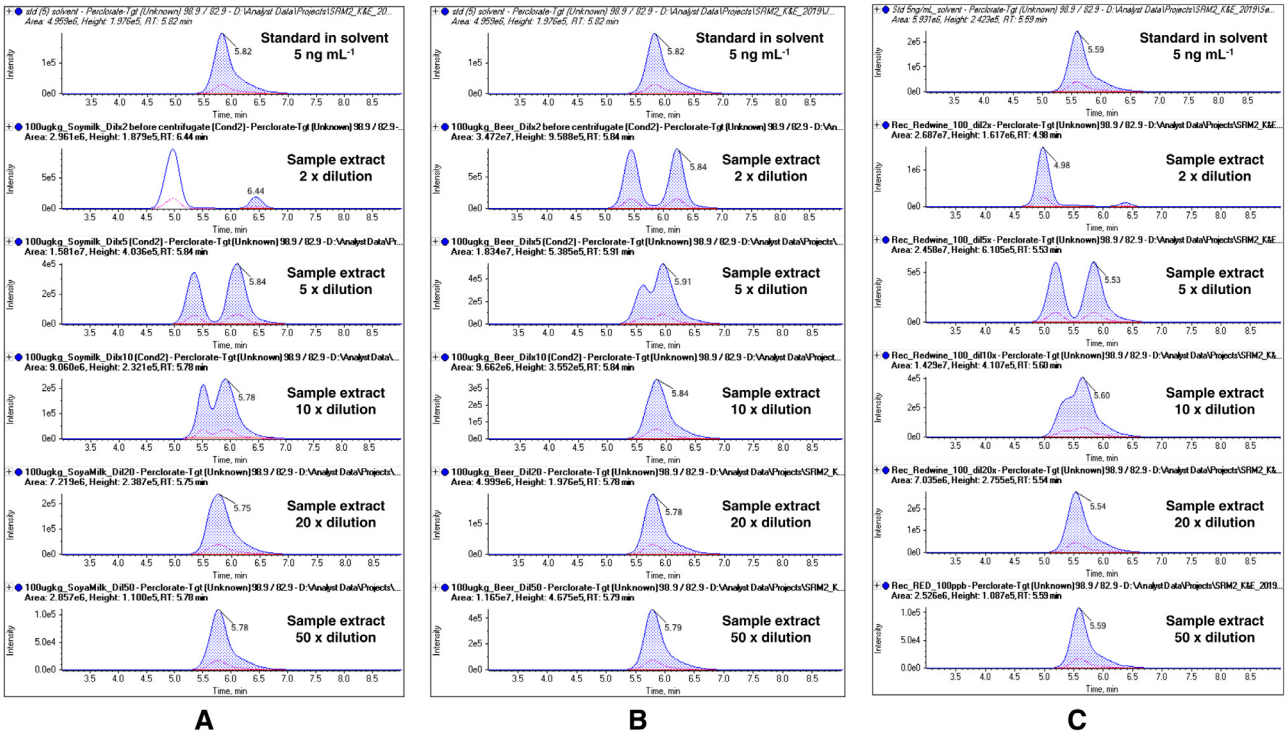

Figure 1. Extracted MS/MS ion chromatograms for perchlorate, spiked at 100 µg kg^{-1} to blank samples of plant-based milks (A), beer (B) and wine (C), applying different dilution factors for the extracts.

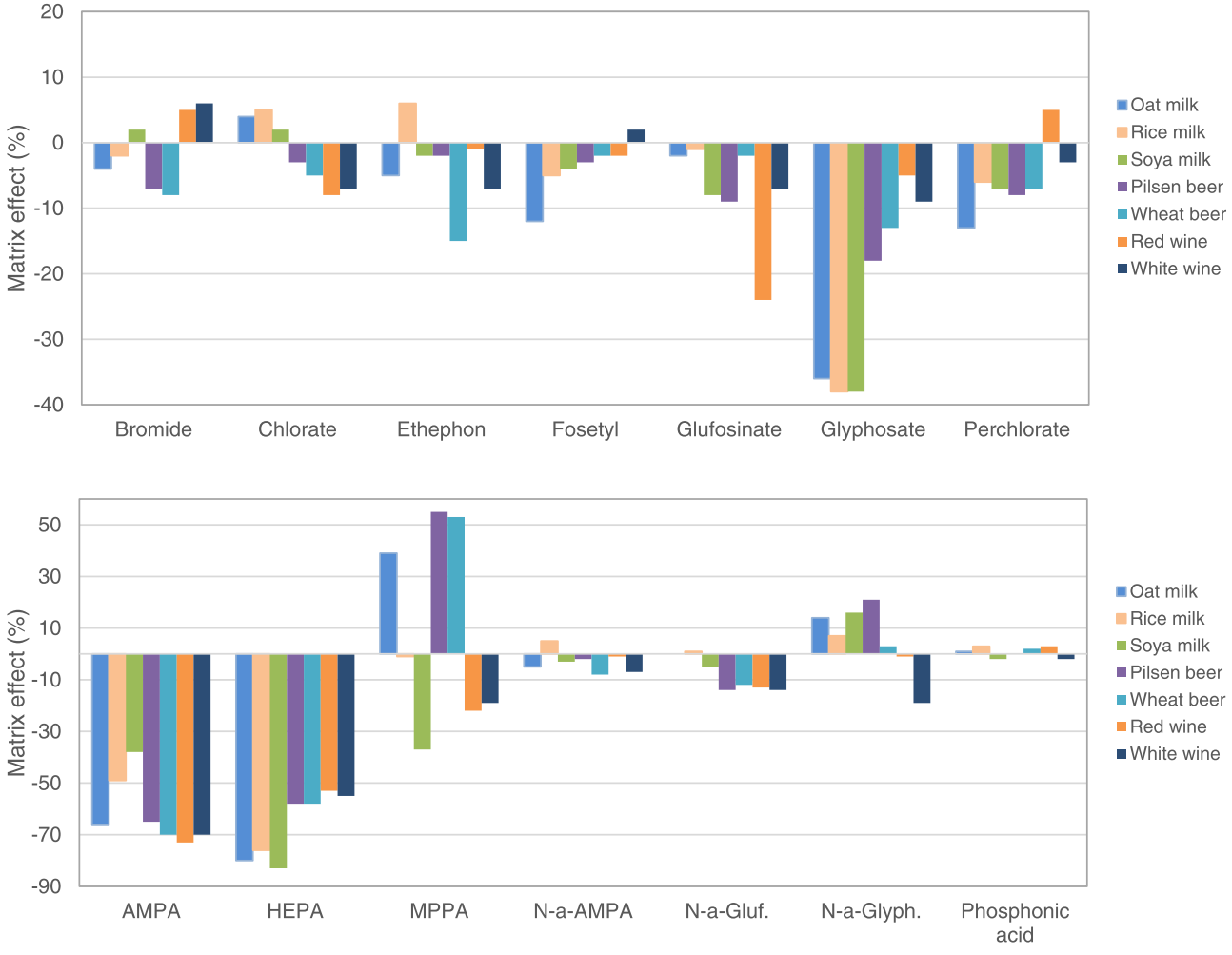

Figure 2. Matrix effects (%) for parent compounds (A) and metabolites (B) in 7 different matrices.

Table 1
Recovery (%), RSD (%) and LOQm (µg kg^{-1}) for the polar pesticides spiked to plant-based milks at 10, 20, 50 and 200 µg kg^{-1}.

Pesticide	Spike level (µg kg^{-1})*								LOQm (µg kg^{-1})
	10		20		50		200		
	Rec (%)	RSD (%)	Rec (%)	RSD (%)	Rec (%)	RSD (%)	Rec (%)	RSD (%)	
AMPA	100	8.2	100	9.7	100	1.8	99	2.7	10
Bromide	n.a.	-	n.a.	-	89	14.9	98	8.7	500
Chlorate	n.a.	-	91	18.1	100	8.5	100	3.1	20
Ethephon	107	11.7	105	9.5	104	5.9	103	6.7	10
Fosetyl	96	17.8	94	17.6	99	11.4	102	7.7	10
Glufosinate	96	18.9	104	12.8	98	6.3	96	7.2	10
Glyphosate	65	26.4	80	18.4	76	11.8	76	8.1	20
HEPA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	92	11.7	200
MPPA	89	9.4	100	7.6	94	6.7	100	2.8	10
N-ac-AMPA	96	11.6	104	3.6	101	7.5	107	4.1	10
N-ac-glufosinate	81	12.3	91	7.6	100	4.8	105	3.0	10
N-ac-glyphosate	108	5.3	105	10.5	99	6.2	103	4.7	10
Perchlorate	106	6.8	104	4.9	102	2.1	101	6.2	10
Phosphonic acid	94	8.5	97	3.9	99	4.0	98	1.6	100

* For bromide and phosphonic acid, the spike levels were 100, 200, 500 and 2000 µg kg^{-1}.
n.a.: not analyzed due to high blank value.
n.d.: not detected.

Table 2
Recovery (%), RSD (%) and LOQm (µg kg^{-1}) for the polar pesticides spiked to beer at 10, 20, 50 and 200 µg kg^{-1}.

Pesticide	Spike level (µg kg^{-1})*								LOQm (µg kg^{-1})
	10		20		50		200		
	Rec (%)	RSD (%)	Rec (%)	RSD (%)	Rec (%)	RSD (%)	Rec (%)	RSD (%)	
AMPA	101	6.1	92	8.8	90	6.7	89	3.4	10
Bromide	n.a.	-	n.a.	-	96	17.6	95	3.5	500
Chlorate	115	19.4	97	11.3	94	11.7	99	3.9	10
Ethephon	112	15.7	105	7.4	95	9.6	99	7.0	10
Fosetyl	97	14.6	110	15.1	104	12.8	100	6.2	10
Glufosinate	119	4.6	114	7.6	106	4.2	98	4.0	10
Glyphosate	108	14.1	92	12.7	80	9.2	76	9.1	10
HEPA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	95	3.1	200
MPPA	104	12.0	98	6.2	102	1.8	98	2.2	10
N-ac-AMPA	102	4.8	100	12.4	97	5.1	99	5.6	10
N-ac-glufosinate	87	11.0	88	11.4	95	6.4	94	5.0	10
N-ac-glyphosate	116	10.7	104	12.5	100	1.2	101	2.1	10
Perchlorate	105	7.5	105	2.4	101	1.7	97	1.5	10
Phosphonic acid	94	6.2	92	6.2	97	4.4	98	2.0	100

* For bromide and phosphonic acid, the spike levels were 100, 200, 500 and 2000 µg kg^{-1}.
n.a.: not analyzed due to high blank value.
n.d.: not detected.

interfere more with some of the analytes resulting in very opposite results, as shown for MPPA. N-acetyl-glyphosate showed negative matrix effects only for wine samples and the highest response suppression was in white wine (-19%), but still within the range considered as being not significant. Only for glyphosate, glufosinate (red wine), AMPA, HEPA and MPPA, significant matrix effects ($<$ -20% or $>$ +20%) could be observed for different matrices. All these different behaviours, combined with the extreme suppression for some compounds, confirm the importance of using ILIS for quantification.

3.2.3. Trueness and precision

Recovery experiments were carried out at spike levels of 10, 20, 50 and 200 µg kg^{-1} and six replicates for each level. Bromide and phosphonic acid were spiked at ten times higher levels (100, 200, 500 and 2000 µg kg^{-1}), because these compounds occur generally at much higher concentrations in real samples. For plant-based milk samples, two replicates of each matrix (oat, rice and soya milk) were employed for each level, in total six replicates. Even though the raw material belongs to different commodity groups according to the SANTE document (Group 4 – high oil content, for soya; group 5 – high starch content, for oat and rice), we assumed that the final product (plant-based milks) are similar matrices because fat is removed from soya milks during the production process. For beer, three replicates of Pilsen beer and three of wheat beer were used. For wine, three replicates of red wine and three of white wine were used. In this way, it is possible to validate the method for different matrix varieties of one product of a commodity group in a single validation set. The recovery experiments were planned this way, assuming that it could be the worst case scenario, where RSDs could be higher than in case of validation of an individual matrix. The acceptance criterion for the RSD is $<$ 20%, as described in the SANTE document. In the tables 1, 2 and 3, the results for the recovery experiments performed for plant-based milks, beer and wine, respectively, are shown.

Bromide was present at various background levels in all matrices tested (260, 600 and 260 µg kg^{-1} for plant-based milk, beer and wine, respectively) and could thus not be validated at the lower levels, which resulted in higher validated LOQs of 500 µg kg^{-1} for plant-based milks and beer, and 200 µg kg^{-1} for wine. Chlorate in plant-based milk, and fosetyl and phosphonic acid in wine could also not be validated at their lowest spike levels (10, 10 and 100 µg kg^{-1}, respectively), due to the presence of background levels (36, 80 and 770 µg kg^{-1}, respectively). Although the

Table 3
Recovery (%), RSD (%) and LOQm (µg kg^{-1}) for the polar pesticides spiked to wine at 10, 20, 50 and 200 µg kg^{-1}.

Pesticide	Spike level (µg kg^{-1})*								LOQm (µg kg^{-1})
	10		20		50		200		
	Rec (%)	RSD (%)	Rec (%)	RSD (%)	Rec (%)	RSD (%)	Rec (%)	RSD (%)	
AMPA	105	17.1	103	7.5	103	4.7	101	4.5	10
Bromide	n.a.	-	116	14.3	116	12.0	112	10.8	200
Chlorate	102	10.2	95	13.6	103	4.6	100	3.5	10
Ethephon	95	16.4	103	12.5	108	4.6	101	4.7	10
Fosetyl	n.a.	-	99	12.3	80	14.9	106	2.8	20
Glufosinate	106	14.7	104	13.4	105	1.3	106	3.8	10
Glyphosate	82	19.3	109	8.3	89	7.9	90	5.0	10
HEPA	105	19.9	110	17.7	94	12.0	95	4.4	10
MPPA	101	14.9	109	8.4	106	2.4	106	1.8	10
N-ac-AMPA	101	8.5	99	2.9	101	5.3	101	4.5	10
N-ac-glufosinate	92	17.3	99	12.3	104	3.6	103	5.0	10
N-ac-glyphosate	124	3.4	116	7.1	94	8.1	87	5.1	10
Perchlorate	103	8.1	101	4.5	102	1.7	100	1.5	10
Phosphonic acid	n.a.	-	98	13.1	96	7.0	94	5.4	200

* For bromide and phosphonic acid, the spike levels were 100, 200, 500 and 2000 µg kg^{-1}.
n.a.: not analyzed due to high blank value.
n.d.: not detected.

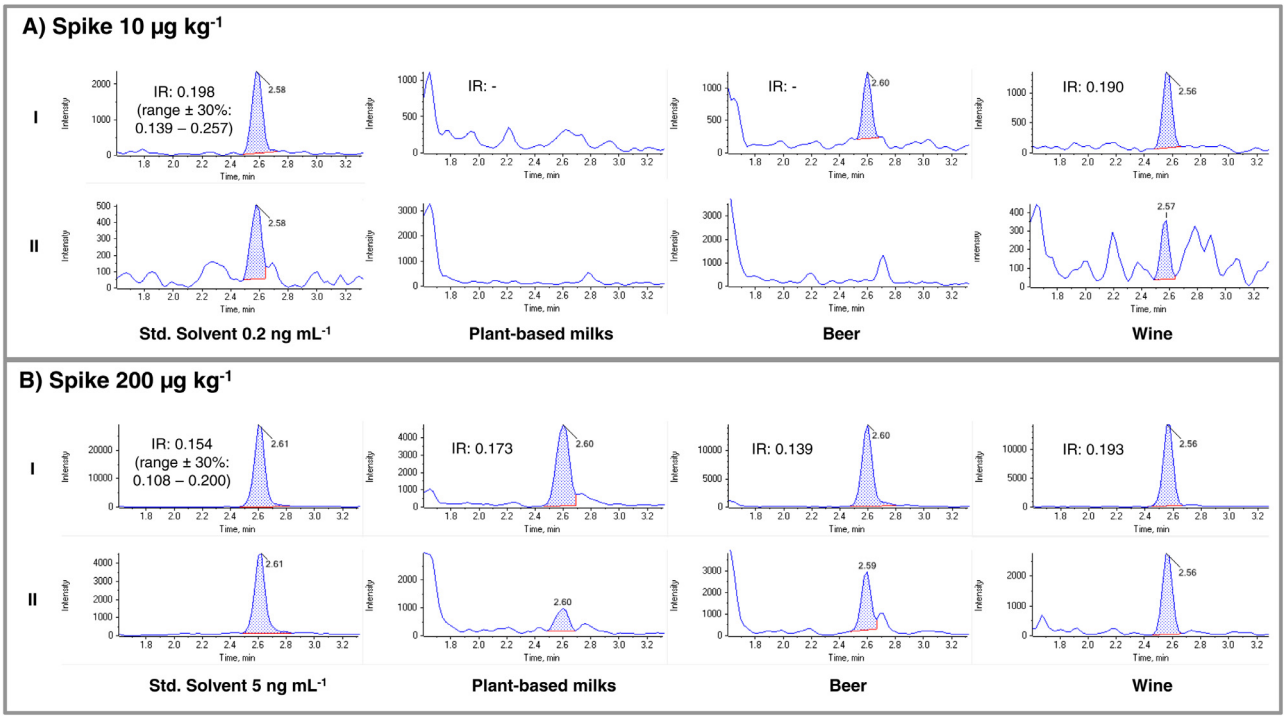

Figure 3. Chromatograms of the quantification ion (I) and qualifier ion (II) of HEPA in solvent and spiked to samples at 10 µg kg^{-1} (A) and 200 µg kg^{-1} (B).

lowest spike levels could not be established as validated LOQ for these 4 compounds, an estimated LOQ could be set as the lowest concentration of the calibration curve in solvent (0.2 ng mL^{-1}, corresponding to 10 µg kg^{-1}) due to the insignificant matrix effect, ion ratio and retention time stability.

For plant-based milk matrices, 10 out of the 14 compounds were successfully validated at the lowest spike level (10 µg kg^{-1}). Glyphosate showed a RSD > 20% at the lowest spike level and, therefore the validated LOQ is 20 µg kg^{-1}. Due to the high suppression of the response for both product ions, HEPA could only be validated at 200 µg kg^{-1}, the highest spike level. However, despite acceptable ion ratios, the confirmation ion for this compound at this concentration is at the limit of sensitivity.

For beer samples, from the 14 analytes evaluated, 12 were meeting the criteria for quantification and therefore were fully validated at 10 µg kg^{-1}, the lowest spike level. HEPA showed similar results as for the plant-based milk samples and it was only validated at 200 µg kg^{-1}, the highest spike level. However, other than with the plant-based milks, where the suppression affected both ions, in beer the quantification ion is sensitive enough, even at 10 µg kg^{-1}. The sensitivity for the qualifier ion, though, was very low and its detection at low levels was not possible.

For wine samples, 11 compounds were fully validated at the lowest spike level. HEPA showed a completely different behavior for this matrix than for the other ones. Very good peak shape and sensitivity for both quantification and qualifier ion were observed, allowing full identification at 10 µg kg^{-1}. In figure 3, the chromatograms for HEPA spiked to three matrix types at 10 and 200 µg kg^{-1} are presented. The different behavior of this compound in

Table 4
Ion ratio differences (%) for plant-based milks, beer and wine

Pesticide	Plant-based milks				Beer				Wine			
	Spike level (µg kg^{-1})*				Spike level (µg kg^{-1})*				Spike level (µg kg^{-1})*			
	10	20	50	200	10	20	50	200	10	20	50	200
	Δ Ion ratio standard matrix/standard solvent (%)											
AMPA	-2.3	-6.3	-6.3	-0.5	5.6	8.2	1.0	-2.9	-7.3	-5.0	-0.9	-4.0
Bromide	9.8	6.4	10.9	1.0	-0.3	1.4	3.9	-1.4	1.9	-3.0	-3.9	-1.4
Chlorate	-3.4	-1.1	4.7	2.3	-1.4	-8.3	-4.1	-4.5	-10.9	2.7	-7.3	1.2
Ethephon	5.8	1.1	4.9	8.3	1.9	0.5	17.7	2.0	-5.6	-4.4	-3.3	-3.4
Fosetyl	-3.4	-6.4	-2.3	-3.6	-7.2	-4.7	0.6	-1.0	-28.4	-23.7	-22.1	-11.2
Glufosinate	5.7	-2.2	-1.4	2.7	-6.9	-5.7	-5.3	-6.4	16.1	6.8	-0.6	0.0
Glyphosate	20.7	9.6	6.2	11.2	1.6	4.8	2.4	8.5	14.2	0.3	7.0	11.3
HEPA	n.d.	n.d.	n.d.	5.8	n.d.	n.d.	n.d.	-19.2	11.3	10.4	-3.1	-1.7
MPPA	-2.0	-2.7	1.4	-5.3	-17.7	0.7	-8.2	-2.0	2.6	-1.6	-1.0	-1.7
N-ac-AMPA	12.9	13.4	7.0	1.2	16.6	5.0	0.5	2.1	12.3	19.5	4.3	5.1
N-ac-glufosinate	6.3	-3.1	3.7	-0.7	16.9	7.5	7.3	1.4	12.9	15.6	3.0	1.6
N-ac-glyphosate	3.2	0.4	4.5	-4.6	-1.3	-0.9	-2.8	1.5	13.3	-5.3	-0.5	-0.3
Perchlorate	-9.1	-6.4	-4.9	-0.6	-7.0	-8.6	-1.2	0.7	-3.6	-2.3	-1.6	0.2
Phosphonic acid	-1.0	-5.1	-2.0	-0.7	0.5	2.6	2.2	-0.5	1.7	1.1	1.5	1.0

* For bromide and phosphonic acid, the spike levels were 100, 200, 500 and 2000 µg kg^{-1}.
n.d.: not detected.

the different matrices, due to the ion suppression, especially for the second ion, is clearly illustrated.

In summary, it can be concluded that for 8 out of 14 analytes, the validation criteria for a quantitative method (recovery, 70-120%; RSD < 20%) were successfully met for all matrices tested, at all 4 spike levels. Glyphosate had a validated LOQ of 10 µg kg^{-1} for beer and wine samples, but 20 µg kg^{-1} for vegetable milk. HEPA could be validated at all levels for wine, but for plant-based milks and beer only at the highest level of 200 µg kg^{-1}.

3.2.4. Identification criteria: retention time and ion ratio

Some studies have reported poor robustness and poor retention times stability when HILIC columns were used for polar pesticides analysis [3,18]. For this reason, an extensive retention time stability study was carried out by our group, especially for the Obelisc N column, as described in our previous publication [22]. It was demonstrated that this column has good retention time reproducibility and robustness under our experimental conditions (high extracts dilution factors), because more than 8000 injections on the same column were performed with good results. The retention time stability was continued to be monitored throughout this validation study.

According to the EU SANTE document [24], the retention time in the chromatograms of the analyte in a sample extract should be compared with the retention time of the analyte in the calibration standard. The allowed tolerance for retention time deviations is ± 0.1 min. On the other hand, the ion ratio of the confirmation ion and quantification ion for analytes in sample extracts should not deviate more than ± 30% (relative) from the average ion ratio of calibration standard solutions from the same sequence [24].

For all matrices evaluated, retention time variations were much lower than 0.1 min, demonstrating the robustness of the chromatographic column, even after long sample sequences. It is important to highlight that the column used for this validation is the same as used for routine samples in our laboratory, as well as for many other validation studies. A total of 8000 injections have been done with the same column and only two reconditioning procedures were necessary during all these batches of samples injected on it. The reconditioning procedure included flushing the column with a mixture of ammonium formate solution and acetonitrile and finally with EDTA solution.

Ion ratio deviations (%) are shown in table 4. As can be seen, all the compounds showed ion ratios within the acceptable range of ± 30% for all spike levels. As HEPA was only quantified at 200 µg kg^{-1} in plant-based milks and beer, the ion ratios were only calculated for this concentration.

4. Conclusion

In this study, a simple, robust and reliable selective multiresidue method for the determination of highly polar pesticides in plant-based beverages was developed and validated. Samples were diluted, shaken and centrifuged before extra dilution, followed by HILIC-MS/MS analysis. The method was successfully validated for most of the pesticides and their metabolites at the lowest spike level (10 µg kg^{-1}), obtaining good accuracy and precision. Despite of the high signal suppression for some analytes, like AMPA and HEPA, quantification was performed properly even for these analytes due to the use of isotopically labelled internal standards. This study proves once again that large dilution factors and a highly sensitive mass spectrometer are essential for good retention time stability and peak shapes.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Sonia Herrera Lopez: Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Writing - review & editing. **Jonatan Dias:** Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Validation, Writing - original draft. **Hans Mol:** Conceptualization, Investigation, Methodology, Project administration, Writing - review & editing. **André de Kok:** Conceptualization, Investigation, Methodology, Project administration, Writing - review & editing.

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