Environmental Research 197 (2021) 111103

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

Urinary excretion of herbicide co-formulants after oral exposure to roundup MON 52276 in rats

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A B S T R A C T

The toxicity of surfactants, which are an integral component of glyphosate-formulated products is an underexplored and highly debated subject. Since biomonitoring human exposure to glyphosate co-formulants is considered as a public health priority, we developed and validated a high-resolution mass spectrometry method to measure the urinary excretion of surfactants present in Roundup MON 52276, the European Union (EU) representative formulation of glyphosate-based herbicides. Quantification was performed measuring the 5 most abundant compounds in the mixture. We validated the method and showed that it is highly accurate, precise and reproducible with a limit of detection of 0.0004 μg/mL. We used this method to estimate the oral absorption of MON 52276 surfactants in Sprague-Dawley rats exposed to three concentrations of MON 52276 via drinking water for 90 days. MON 52276 surfactants were readily detected in urine of rats administered with this commercial Roundup formulation starting from a low concentration corresponding to the EU glyphosate acceptable daily intake. Our results provide a first step towards the implementation of surfactant co-formulant biomonitoring in human populations.

1. Introduction

Glyphosate is the most widely used herbicide active ingredient worldwide. It was initially sold in the 1970s as a commercial herbicide called Roundup. While the use of Roundup remained relatively stable in the first decades after its introduction, its application started to increase exponentially in the late 1990s when farmers massively adopted genetically engineered crops harbouring a transgene for glyphosate tolerance (e.g. Roundup-Ready soybeans) (Benbrook, 2016). The use of glyphosate also increased in other sectors after it became one of the cheapest weed control solutions when the patent protection period for glyphosate expired in 2000 (Perry et al., 2019). It was estimated in 2015 that glyphosate was used in more than 750 herbicides sold for agricultural, amenity or domestic use (Guyton et al., 2015).

Formulated glyphosate herbicidal products are always a mixture of glyphosate with other chemicals called co-formulants (Mesnage et al., 2019). A large number of co-formulants are included in commercial pesticides to adjust solubility, adherence, volatilization, penetration, rainfastness and foaming of the spray mixture. Since glyphosate is a hydrophilic herbicide, it cannot penetrate the waxy surface of plant leaves if it is not mixed with a surfactant.

The first Roundup formulation included a mixture of ethoxylated tallowamine surfactants (MON 0818), which was highly toxic and caused numerous adverse effects after accidental exposures (Blondell, 1986). Monsanto developed new glyphosate products in the 1980s and 1990s (Mesnage et al., 2019). Major milestones in formulation technology are the inclusion of phosphate esters to decrease the toxicity of ethoxylated tallowamine surfactants, the replacement of alkylamine surfactants with etheramines to increase glyphosate loading, or the introduction of propoxylated surfactants, which considerably reduced non-target toxicity. The most recent generation of glyphosate herbicides include alkylpolyglycosides or nitroryl (an alkoxylated etheramine oxide co-surfactant).

The contribution of co-formulants to the toxicity of herbicides is poorly investigated, not only because the chemical composition of formulations is considered as confidential business information, but also due to the fact they are often wrongly assumed to be an 'inert' part of spray mixtures leading to a lack of regulatory health risk assessment (Mesnage and Antoniou, 2018). Although glyphosate is one of the herbicide ingredients with the lowest degree of acute toxicity (Kniss, 2017), the surfactants included in formulated products have been held responsible for the toxicity of glyphosate-based herbicides in various organisms such fish species (Folmar et al., 1979; Wan et al., 1989), amphibians (Howe et al., 2004; Mann and Bidwell, 1999), crustaceans

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https://doi.org/10.1016/j.envres.2021.111103
Received 22 January 2021; Received in revised form 11 March 2021; Accepted 26 March 2021
Available online 1 April 2021

(Tsui and Chu, 2003), laboratory rodents (Adam et al., 1997) and humans (Sawada et al., 1988). This was the case for the oldest generations of Roundup herbicides (e.g. MON 2139), which contained the most toxic surfactants (i.e., MON 0818). While formulations containing variations of MON 0818 are still in use in the US, they have been banned from the European market. By contrast, new generations of Roundup have a more favourable toxic profile (Mesnage et al., 2019). In the European Union (EU), the representative formulation MON 52276 contains a propoxylated quaternary ammonium surfactant known as Dodigen 4022 and is sold under different trade names in Europe (e.g. Roundup BioFlow in Italy or Roundup Ultra in Belgium).

Limited information is available concerning MON 52276 mammalian toxicity. However, ecotoxicological assessments suggested that the use of a propoxylated quaternary ammonium in Roundup formulations reduces toxicity to many organisms. LD50 in rainbow trout for MON 52276 acute toxicity is 989 mg/L, and approximately 100 times lower for MON 2139 (8.2 mg/L). A similar order of magnitude is found for LD50 in water fleas (Mesnage et al., 2019). This is also supported by cytotoxicity tests on human cells which found that MON 52276 was 50-times less cytotoxic than Roundup GT plus (Mesnage et al., 2013). However, these acute exposure studies do not provide information of toxicity that could arise from chronic exposure in mammals. The only study which has compared the toxicity of MON 52276 and glyphosate found that MON 52276 but not glyphosate caused alterations in the blood metabolome suggestive of oxidative stress (Mesnage et al., 2021).

Treating pesticide co-formulants as inert results in these substances is being largely ignored by regulatory agencies and not being monitored for their presence either in the environment, the food chain, or in human body fluids (Mesnage and Antoniou, 2018). Surveys of the American mid-west have found that surfactants from MON 0818 are ubiquitously present in agricultural fields (Tush and Meyer, 2016) and beyond in watercourses (Tush et al., 2018). In addition, the use of glyphosate as a pre-harvest desiccant, for example on cereal crops such as oats and wheat, makes it likely that both glyphosate and its co-formulants will enter the food supply.

There is a need to conduct biomonitoring surveys for co-formulant residues in human blood and urine, in order to determine the daily intake and body burden of these compounds. Biomonitoring human exposure to glyphosate co-formulants has been defined as a priority by the EU human biomonitoring programme HMB4EU consortium (HMB4EU, 2018). A first step to evaluate possible surfactant intake from exposure to Roundup would be to evaluate this possibility by performing laboratory animal studies. It is not known if glyphosate surfactants can be absorbed from the gastrointestinal tract and also exert toxic effects in other tissues after accidental ingestions. We report here the development of a method for the accurate measurement of Roundup MON 52276 surfactants in urine of Sprague-Dawley rats administered with this herbicide via drinking water for 90 days. Our findings show that MON 52276 surfactants can readily be detected even in animals exposed to a dose equivalent to the EU acceptable daily intake.

2. Material and methods

2.1. Experimental animals

The animal study was performed as previously described on young adult female Sprague-Dawley rats (Mesnage et al., 2021), at the Cesare Maltoni Cancer Research Center, Ramazzini Institute (Bentivoglio, Italy), in accordance with Italian law regulating the use and humane treatment of animals for scientific purposes (Decreto legislativo N. 26, 2014. Attuazione della direttiva n. 2010/63/UE in materia di protezione degli animali utilizzati a fini scientifici. - G.U. Serie Generale, n. 61 del 14 Marzo 2014). The experiment was authorised by the ad hoc commission of the Italian Ministry of Health (authorization N. 447/2018-PR). In brief, groups of 12 female Sprague-Dawley rats of 8 weeks of age were exposed for 90 days with MON 52276 (purchased as Roundup BioFlow, Italy) via drinking water to give a daily intake of 0.5 mg, 50 mg and 175 mg glyphosate per kg body weight per day (mg/kg bw/day), which respectively represent the EU acceptable daily intake (ADI), the EU no-observed adverse effect level (NOAEL) and the US NOAEL (European Food Safety, 2015).

Urine samples were collected at the end of the treatment period by placing animals individually in metabolic cages (model 3701M081; Tecniplast spa Italy) for approximately 16 h. The morning of the day after, the volume of consumed water and the urine collected from each experimental animal was measured. For each animal, a sample of 5 mL urine was stored at −70 °C.

2.2. Instrumentation and experimental conditions

The UHPLC-HRMS instrument consisted of a Thermo Scientific™ Vanquish™ UHPLC coupled to a Thermo Scientific Exactive™ Orbitrap mass analyser with a heated electrospray ionization source (HESI-II). The mass spectrometer was operated in positive mode, at 35,000 mass resolution and data were acquired with Xcalibur software. The chromatographic separation was achieved using a Thermo Scientific™ Accucore C18 column (2.6 μm, 100 × 2.1 mm) maintained at 40 °C. Binary gradient profile was developed using water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) at a flow rate of 200 μL/min. HPLC grade acetonitrile and HPLC grade water were from Fisher Scientific, and LC-MS grade formic acid was from Merck.

Separations were conducted under the following chromatographic conditions: 95% solvent A for 0.1 min, decreased to 5% over 15 min, maintained for 10 min at 5% before being increased to 95% over 0.5 min.

Column equilibration time was 4.5 min, with a total runtime of 30 min. The injection volume was 20 μL. Mass spectrometric conditions were as follows: spray voltage 4.5 kV, capillary temperature 350 °C, sheath gas 55 au, auxiliary gas 5 au, heater temperature 300 °C. The quantitation of the different analytes was carried by analysing samples in full scan (range 100–2000 m/z) and comparing their accurate masses and retention times with the MON 52276 mixture.

2.3. Analyte quantitation

Due to the lack of suitable chemical standards, an accurate quantitation of the different surfactant species was not possible. Thus, to this end a Roundup MON 52276 mixture was used as a reference standard. According to the patent EP0498145B1, the total amount of surfactants in a typical formulated product is 120 mg/mL. Considering that surfactants belong to a homologous series, it can be reasonably assumed that they would ionise in the same way in the HESI source of the mass spectrometer. In the light of this observation, from the total ion scan spectrum (Fig. 1), the relative intensities of each ion have been calculated and used to estimate the relative amount of each compound in the mixture (Table 1).

2.4. Preparation of working solutions

A stock solution of Roundup MON 52276 (1 mg/mL in surfactants) was freshly prepared in water before being further diluted into a series of working solutions (in a matrix) and used for the preparation of the calibration standards and quality control samples (prior to the addition of propranolol used as an internal standard at the final concentration of 50 ng/mL). Calibration curves were plotted on the day of analysis across the concentration range of 0.006–15 μg/mL (total surfactant title). On the day of analysis, two quality control samples were plotted within the concentration range.

2.5. Sample preparation

A 10 μL aliquot of each urine sample was diluted with 40 μL of an

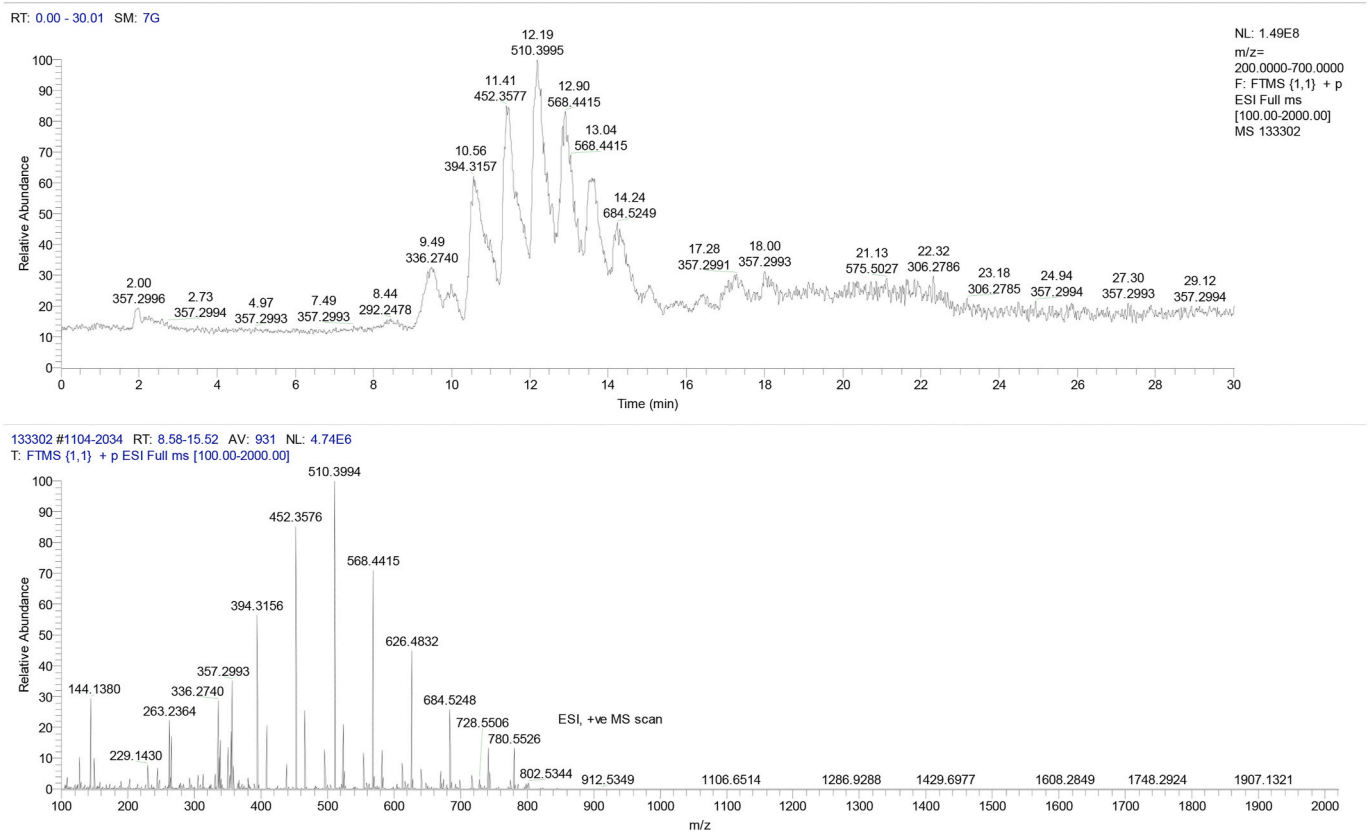

Fig. 1. Representative spectrum of MON 52276 obtained by liquid chromatography-high resolution mass spectrometry. The formulation MON 52276 was diluted in water to reach a concentration of 30 μg/mL Dodigen 4022. The chromatogram (top panel) shows that MON 52276 co-formulants are a mixture of compounds, which elute at different retention times. The mass spectrum (bottom panel) shows that peaks are separated by 58 m/z increments, suggesting that each peak corresponds to a different molecule belonging to a homologous series of surfactants with a different number of propoxylated units.

Table 1
Estimated amount of surfactant in Roundup MON 52276. We estimated the surfactant concentrations (mg/mL) for major ion peaks considering that the surfactant compounds are homologous and would ionise in the same manner in the HESI source of the mass spectrometer.

Ion (m/z)	Relative peak intensities (%)	surfactant (mg/mL)
336.2740	36	9.5
394.3156	70	18.4
452.3576	100	26.3
510.3994	97	25.5
568.4415	74	19.4
626.4832	44	11.6
684.5248	22	5.8
742.5661	9	2.4
800.6077	3.5	0.9
858.6495	1.5	0.4

aqueous solution of propranolol (50 ng/mL). Propranolol was purchased from Sigma.

3. Method validation

The method was fully validated following the FDA Guidance for Bioanalytical Method Validation (Ronquist-Nii et al., 2011) by evaluating accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ), linearity and matrix effect. This was performed by using spiked urine samples. A matrix effect was observed as a moderate ion suppression in the area under the curve (AUC) of analytes in urine, compared to deionised water (Fig. S1). To overcome this and perform an accurate quantitation of the analytes of interest, calibration curves and quality control samples, as well as all the working solutions, were prepared in spiked urine. Accuracy and precision were assessed using six determinations, covering the selected concentration range (two concentrations, three replicates each).

3.1. Statistical analysis

Surfactant urinary excretion is summarised as the mean ± SD. Accuracy was evaluated by calculating the percent deviation from the nominal concentration and is reported as relative error (RE) and precision was determined by calculating the relative standard deviation (RSD %) of replicates. LOD and LOQ were established by means of a method based on the calibration curve, where the standard deviation of the y-intercept was used as standard deviation. Data Changes in water consumption was evaluated by Dunnett's Test computed using STATA 10 software.

4. Results

The aim of this study was to evaluate the possible absorption of surfactants added as co-formulants in the EU representative glyphosate formulation Roundup MON 52276, by measuring their excretion in urine. Information on the quantity of Dodigen 4022 in MON 52276 is not directly available. The concentration of surfactants indicated on the material safety data sheet for MON 52276 is 16%. In a patent providing information on glyphosate formulations containing Dodigen 4022 (EP0498145B1), it is indicated that this surfactant is present at a proportion of 10.5%, which would correspond to a concentration of 120 g/L. We used this value as an indication of surfactant concentration in the MON 52276 formulation tested in this study to provide an order of

magnitude for its excretion.

No significant differences were observed in water consumption for animals exposed to the two lowest doses of MON 52276 (Table 2). However, a statistically significant decrease of mean urine volume, excreted/mean water consumption ratio (expressed as a percentage) in animals treated with the highest dose of MON 52276 [$p < 0.01$; Dunnett's test] was observed.

5. Method development and validation

First, we characterised MON 52276 by liquid chromatography coupled to high resolution mass spectrometry to identify the most suitable ions for surfactant quantification. The corresponding mass spectrum (Fig. 1) reveals peaks, which are typical of surfactant mixtures. A surfactant known to be included in MON 52276, Dodigen 4022, is a propoxylated quaternary ammonium compound. The delta of 58 m/z corresponds to a number of propoxylated moieties (CH2–CH2–CH2–O motif) of the different components in this surfactant mixture. The length of the most abundant surfactant in the mixture corresponded to the maximal m/z of the spectrum.

In validation experiments for all the compounds, a good linearity is shown in the range 0.006–15 μg/mL. Intraday typical R2 values, obtained by plotting 3 calibration curves prepared from different starting standard solutions, were between 0.9992 and 0.9889 (Table 3). Interday typical R2 values, obtained by plotting 3 calibration curves prepared from different starting standard solutions and run on three different days, were between 0.9986 and 0.9929 (Table 3). Limit of detection (LOD) and limit of quantitation (LOQ) values were found to be satisfactory and appropriate for the analysis of the samples (Table 3). Carryover was not observed as blank samples, injected after high concentration calibrants, do not show any detectable peaks. Overall, the analytical method developed resulted in highly accurate, precise and reproducible measurements of Roundup MON 52276 surfactants (Fig. 2).

5.1. Quantification of MON 52276 surfactants in rat urine

The amount of MON 52276 surfactants was then quantified using the method described above in urine samples of Sprague Dawley rats administered with this Roundup formulation for 90 days in their drinking water at 0.5 mg, 50 mg and 175 mg/kg bw/day glyphosate equivalent concentration. Quantification was performed by using the peak area ratio (PAR = AUC analyte/AUC IS where AUC is area under the curve) of the 5 most abundant compounds in the mixture (394.3156; 452.3576; 510.3994; 568.4415; and 626.4832 m/z; see Fig. 3). All ions were quantified in the groups exposed to the two higher doses of MON 52276 herbicide (50 and 175 mg/kg bw/day glyphosate equivalent) (Table 4). The lowest abundant ions were not detected in every urine sample from the group of animals exposed to the lowest dose of MON 52276 (0.5 mg/kg bw/day glyphosate equivalent) (Fig. 4). However, the ion 394.3156 m/z was found in all samples from this group. Based on the estimated amount of surfactant in Roundup MON 52276 (Table 1), we could estimate that the urinary concentrations of Dodigen 4022 were approximately 0.0078 ± 0.0068, 0.67 ± 0.19, and 3.29 ± 1.14 μg/mL for the three groups receiving 0.17, 17, and 58 mg/kg bw/day of this surfactant in the form of MON 52276, respectively. Collectively, our data show that the MON 52276 main surfactant can be detected and quantified in the urine of animals exposed to a glyphosate dose equivalent to the EU ADI.

We then attempted to estimate the proportion of surfactants that can be excreted in urine compared to concentrations in drinking water. Even though accurate calculations cannot be performed because the chemical structure of these compounds has not been fully disclosed or established, and thus chemical standards are not available, we estimated the urinary excretion rates for a standard rat. Considering that a rat excretes 58 mL of urine per kg bw/day, we could estimate that the urinary excretion of Dodigen 4022 was approximately 0.45 ± 0.39, 38.9 ± 11.1, and 190.7 ± 65.8 μg/kg bw/day for the three groups receiving 0.17, 17, and 58 mg/kg bw/day of this surfactant in the form of MON 52276, respectively. Although the absorption and metabolism of Dodigen 4022 could not be evaluated here, it can be estimated that urinary excretion of Dodigen 4022 represented 0.1% of the administered dose (Fig. 5).

Table 2
Mean urine volume and mean water consumption measured after 16 h in metabolic cage. Statistical significance was determined with Dunnett's test (*, $p < 0.05$; **, $p < 0.01$).

dose (mg/kg bw/day)	urine volume (mL)	water consumption (mL)	urine/water ratio (%)
0	20.50 ± 6.72	33.00 ± 7.98	62.07 ± 13.15
0.5	18.83 ± 6.79	35.00 ± 9.24	53.79 ± 12.73
50	16.58 ± 7.49	26.36 ± 5.12	58.19 ± 19.84
175	7.75 ± 3.93 **	23.83 ± 4.93 *	31.91 ± 11.58 **

6. Discussion

The toxicity of surfactants, which are integral components of glyphosate-formulated herbicide products remains a controversial and underexplored area of toxicology. Although their irritant properties on mucosal surfaces are well characterised (Martens et al., 2019), the extent to which these surfactants can be absorbed from the digestive tract and distributed among internal organs with consequent health implications is unknown. In order to provide the first insight into the possible absorption of the principal class of surfactants (Dodigen 4022) present in the EU representative glyphosate formulation Roundup MON 52276, we developed a quantitative MS method to measure their concentration in urine. Using urine samples from a subchronic toxicity test of three concentrations of MON 52276 in Sprague-Dawley rats (Mesnage et al., 2021), we reveal for the first time that MON 52276 Dodigen 4022 surfactant was excreted in the urine of these animals, which were administered with this Roundup formulation starting from a low concentration corresponding to the glyphosate EU ADI. We estimated that the excretion of Dodigen 4022 corresponded to approximately 0.1% of the amount ingested by the rats on a daily basis. This might indicate that absorption of this surfactant is low. However, we cannot exclude the possibility that Dodigen 4022 accumulates within the animal's body resulting in low levels of excretion, which in turn would result in an underestimate of the absorption rate based solely on measurements of urinary levels. In addition, we could not evaluate whether the surfactants are fully absorbed but then metabolised to other compounds and thus not detected by the method used in this investigation. Future studies adapting the method described here are needed to provide insight into the biodistribution of this and other surfactants.

The development of biomonitoring methods to evaluate human exposure to glyphosate co-formulants has been established as a priority by the EU program HMB4EU (HMB4EU, 2018). In its most recent report, the HMB4EU consortium referred to polyoxyethylene tallow amine (POEA) as the priority substance to evaluate exposure to glyphosate-based herbicide co-formulants. POEA is a family of surfactants with different composition, the most common being POE (15) tallow amine containing an average of 15 ethylene oxide units (Tush et al., 2013). However, since the use of this surfactant in glyphosate-based herbicides has been banned in the EU, we judged that it would be more relevant to focus biomonitoring efforts on the most recent generation of co-formulants such as the quaternary ammonium surfactant present in MON 52276, which was investigated in this study.

The oral absorption of glyphosate-based herbicide co-formulants is likely to differ depending on the structure of the surfactant. Some surfactants are considered to be rapidly absorbed by the gastrointestinal tract such as ethoxylated alcohols (ECHA, 2020a). After oral exposure, more than 75% of ethoxylated alcohols are absorbed by the

Table 3
Validation of the Dodigen 4022 detection method. The method was fully validated following the FDA Guidance for Bioanalytical Method Validation by evaluating limit of detection (LOD), limit of quantitation (LOQ), linearity R^2, accuracy and precision as the relative standard deviation (RSD%) for a low and a high surfactant concentration.

	394.3156 m/z	452.3576 m/z	510.3994 m/z	568.4415 m/z	626.4832 m/z
LOD (μg/mL)	0.0004	0.001	0.003	0.004	0.004
LOQ (μg/mL)	0.001	0.002	0.009	0.013	0.012
R^2 intraday	0.9969	0.9980	0.9992	0.9964	0.9889
R^2 inter-day	0.9963	0.9984	0.9986	0.9973	0.9929
Accuracy % low level	98 (at 0.23 μg/mL)	97 (at 0.33 μg/mL)	92 (at 0.32 μg/mL)	78 (at 0.24 μg/mL)	67 (at 0.14 μg/mL)
Accuracy % high level	99 (at 1.15 μg/mL)	94 (at 1.64 μg/mL)	97 (at 1.59 μg/mL)	96 (at 1.22 μg/mL)	92 (at 0.72 μg/mL)
RSD% precision low level	6 (at 0.23 μg/mL)	2 (at 0.33 μg/mL)	4 (at 0.32 μg/mL)	7 (at 0.24 μg/mL)	6 (at 0.14 μg/mL)
RSD% precision high level	1 (at 1.15 μg/mL)	5 (at 1.64 μg/mL)	5 (at 1.59 μg/mL)	8 (at 1.22 μg/mL)	4 (at 0.72 μg/mL)

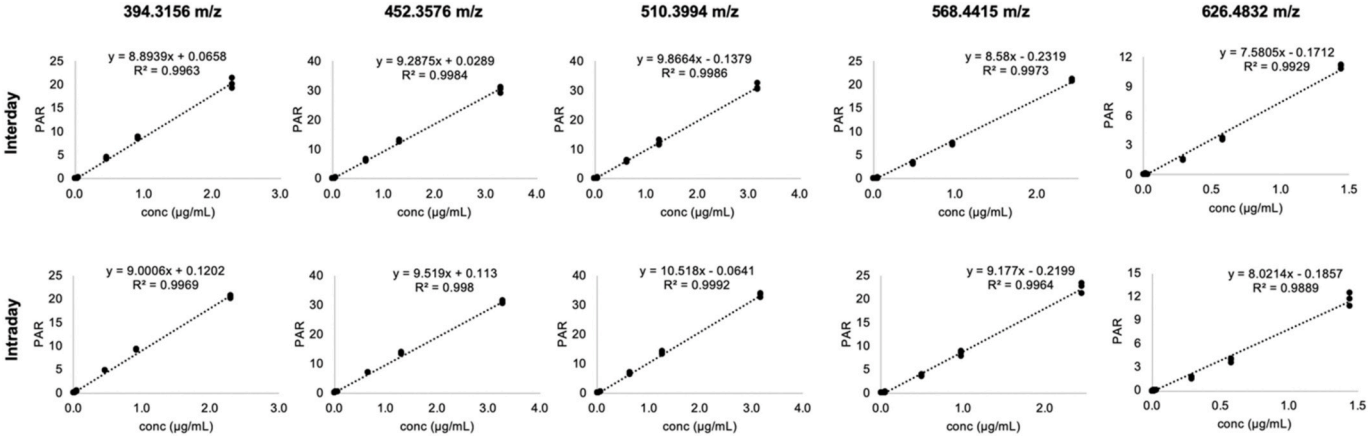

Fig. 2. Interday and intraday validation. A good linearity is shown for the 5 principal Dodigen 4022 ions. PAR, Peak Area Ratio (i.e., the area under the curve of the analyte divided by the area under the curve of the internal standard).

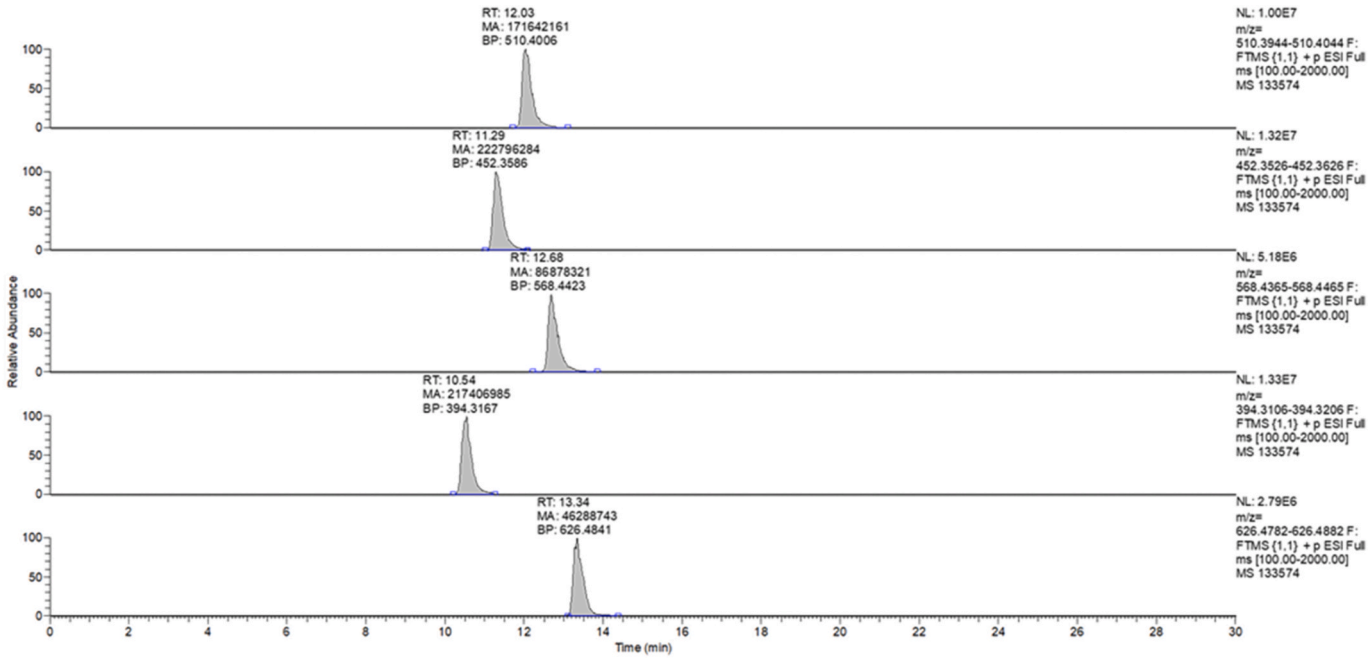

Fig. 3. Extracted chromatograms of the different ions selected for MON 52276 surfactant relative quantification in a representative Sprague-Dawley rat urine sample. The urine sample belongs to a Sprague-Dawley rat exposed to a medium dose of Dodigen 4022 (50 mg/kg bw/day glyphosate). The 5 chromatograms represent the elution time of the 5 principal Dodigen 4022 ions chosen to quantify this surfactant. RT, retention time in min; MA, manually integrated area of a detected peak; BP, base peak; NL, normalisation level describing the intensity of the base peak; FTMS, Fourier transform mass spectrometry; ESI, electrospray ionization.

Table 4
Summary statistics of the relative quantification of the MON 52276 surfactant for 5 main ions. The mean and standard deviations are reported with the number of samples below levels of quantification (BBLQ) and the number of samples in which the measured ion was not found (NF).

Group	Ion (m/z)	Mean (μg/mL)	SD	BLLQ	NF
Control	394.3159	–	–	3	9
	452.3578	–	–	8	4
	510.4000	–	–	7	5
	568.4416	–	–	9	3
	626.4831	–	–	0	12
MON 52276 **low dose (0.5 mg/kg bw/day glyphosate)**	394.3159	0.0024	0.0016	0	0
	452.3578	0.0031	0.0021	3	0
	510.4000	0.0025	0.0015	7	0
	568.4416	0.0017	0.0010	4	0
	626.4831	0.0012	0.0007	4	0
MON 52276 **medium dose (50 mg/kg bw/day glyphosate)**	394.3159	0.22	0.063	0	0
	452.3578	0.21	0.060	0	0
	510.4000	0.13	0.038	0	0
	568.4416	0.071	0.020	0	0
	626.4831	0.037	0.011	0	0
MON 52276 **high dose (175 mg/kg bw/day glyphosate)**	394.3159	1.03	0.33	0	0
	452.3578	0.96	0.31	0	0
	510.4000	0.66	0.24	0	0
	568.4416	0.40	0.16	0	0
	626.4831	0.24	0.11	0	0

gastrointestinal tract and approximately 1% is sequestered in the liver. The majority was found to be excreted in urine or feces, with excretion rates depending on the length of the ethoxylated chain (ECHA, 2020a). In contrast, quaternary ammonium surfactants such as Dodigen 4022 present in MON 52276, may have a lower oral bioavailability. Although the pharmacokinetics of alkyl chain quaternary ammonium salts can still be different from that of polyalkoxy chain quaternary ammonium salts, it is interesting to note that pharmacokinetics data for C12–C18 dialkyl dimethyl ammonium chloride show that only 1% of this molecule is detectable unchanged in urine (ECHA, 2020b). This could correspond to the difference between the excretion of Dodigen 4022 and the amount ingested by the rats #in our study. However, it is not clear to which extent this surfactant either bioaccumulates or is metabolised. The majority of surfactants are known to be readily biodegradable under aerobic conditions but cationic surfactants containing a quaternary ammonium have shown no or very poor primary biodegradation under anaerobic conditions in the environment (Ying, 2006). In fish, quaternary ammonium surfactants accumulated mostly in the gills but were nearly absent in liver or muscle, suggesting that their uptake is very slow (Kierkegaard et al., 2020). Further experiments will be required to develop full pharmacokinetic and pharmacodynamic modelling for this class of surfactants allowing the use of this information in health risk assessments. However, our data on MON 52276 should not be directly transposed to other glyphosate formulations containing other surfactants.

Although our results could be relevant to estimate surfactant absorption in cases of accidental ingestion, or suicide attempts, they are not representative of agricultural worker exposures, bystanders, or dietary consumption at low environmental levels. In order to evaluate the toxicity of co-formulant exposure, in the absence of biomonitoring data, health risk assessments are generally performed by monitoring glyphosate exposure with this information then used to extrapolate the systemic dose of glyphosate to the systemic dose of co-formulant (Martens et al., 2019). Since it is generally considered that approximately 20% of a glyphosate dose is absorbed (Anadón et al., 2009; Brewster et al., 1991), co-formulant toxicity would be over- or underestimated if their absorption is different to that of glyphosate. In the case of the MON 52276 surfactants, which may have a low oral absorption according to our study, using glyphosate absorption as a reference value would overestimate health risks.

Factors influencing human exposure to surfactants should not be restricted to studies within agricultural environments. A large number of quaternary ammonium compounds are commercially available. Although surfactants are widely used in agriculture, human exposure is likely to be predominantly through their use in household, workplace, and industry settings (Boethling, 1984). They are used in commercial products which are directly aimed at being used on human body surfaces such as disinfectants, hair conditioners, emulsifying agents, or deodorizers (Boethling, 1984). This peaked recently with their increased use in disinfectants during the SARS-CoV-2 pandemic (Hora et al., 2020). While the consequences of human exposure to quaternary ammonium compounds (QACs) are unknown, numerous studies have showed that they can harm both aquatic and terrestrial organisms since high volumes of these surfactants are released from effluents and sludge from sewage treatment plants (Zhang et al., 2015). Thus, these compounds can end up in the food chain, as some food surveys have found residues of benzalkonium chloride (a QAC) in milk and other dairy products (BfR, 2012). Exposure to surfactants, whether from agricultural use or not, may have effects beyond our current understanding. For instance, the use of QACs as disinfectants in animal facilities could affect the sensitivity of the animals and explain why the results of some studies are inconsistent. It has been hypothesized that the use of QACs to disinfect laboratory material caused fertility problems in some animal husbandries (Fallon, 2008).

More accurate information about the toxicokinetics of MON 52276 in rats could be obtained with a gavage study including repeated sampling of urine and feces (in metabolic cages) over 48–72 h. Examples of well-

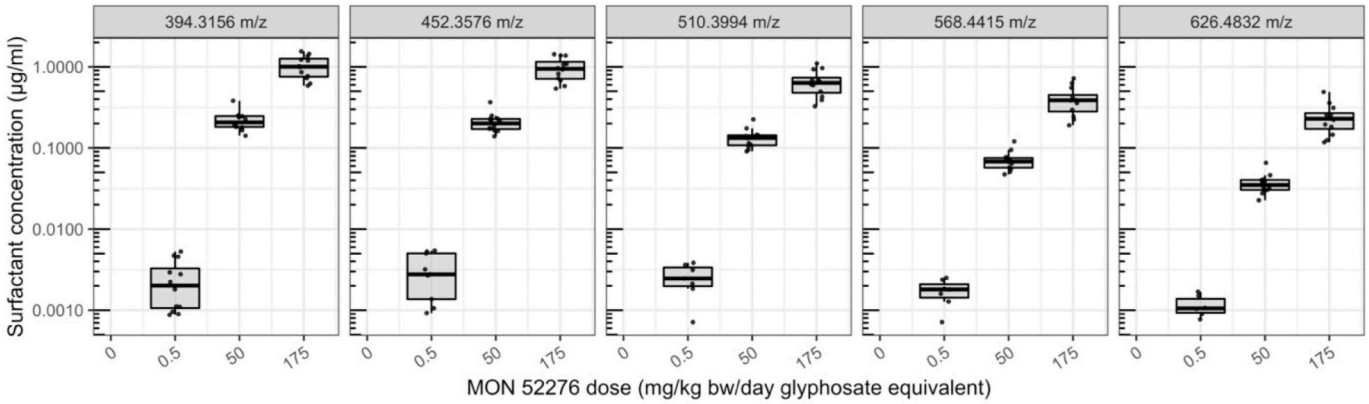

Fig. 4. Urinary excretion of MON 52276 surfactant in Sprague-Dawley rats by measurement of the 5 major Dodigen 4022 ions. Calculated surfactant concentrations are shown for the three groups of rats exposed to various doses of MON 52276. No surfactant was detected in the unexposed control group of animals. Individual values are shown together with a box plot; n = 12 per group.

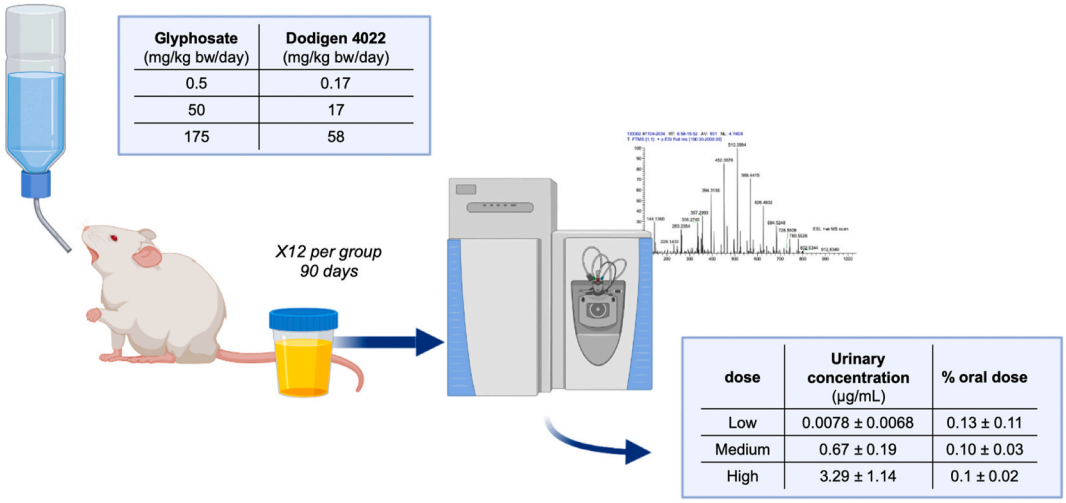

Fig. 5. Determination of oral absorption of MON 52276 surfactant Dodigen 4022 from urinary excretion in Sprague-Dawley rats. We estimated the excreted doses of Dodigen 4022 using measured urinary concentrations of its 5 main ions, as well as experimentally determined urine volumes. Created with BioRender.com. Values are the mean ± SD, n = 12.

designed toxicokinetics study using gavage are available for glyphosate (Anadón et al., 2009; Brewster et al., 1991). Another limitation of our study is that the validation method was not performed on a pure standard because we did not have access to certified or commercially available raw material (Dodigen 4022).Further studies could also test if urine hydrolysis could improve surfactant recovery, since glucuronidation frequently occurs prior to excretion. Glucuronidation has been demonstrated for the metabolites of other classes of surfactants such as N-Alkyl perfluorooctane sulfonamides (Xu et al., 2006).

In summary, the health risks posed by surfactants represents a vastly underexplored area of toxicology, which in the case of pesticides such as Roundup can result in a major underestimation of their potential toxicity (Mesnage and Antoniou, 2018). Given the almost ubiquitous exposure of human populations to surfactants from multiple sources, there is an urgent need to conduct biomonitoring studies to assess for their presence and overall body burden. The method described here offers a starting point for conducting such surveys of surfactant exposure in different population groups in both occupational and domestic settings.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: RM has served as a consultant on glyphosate risk assessment issues as part of litigation in the US over glyphosate health effects. The other authors declare no competing interests.

Acknowledgements

This work was funded by the Sustainable Food Alliance (USA) and in part by the Sheepdrove Trust (UK) whose support is gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2021.111103.

CRediT author statement

Robin Mesnage: Conceptualization, Investigation, Writing – original draft. Francesca Mazzacuva: Methodology, Investigation, Data curation, Validation, Anna Caldwell: Writing- Reviewing and Editing, Resources. John Halket: Writing- Reviewing and Editing, Resources. Michael N Antoniou: Writing- Reviewing and Editing, Project administration, Supervision.

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