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Detection of polyvinylpyrrolidone in *Daphnia magna*: Development of a refractive index quantification method for water-soluble polymers in aquatic organisms

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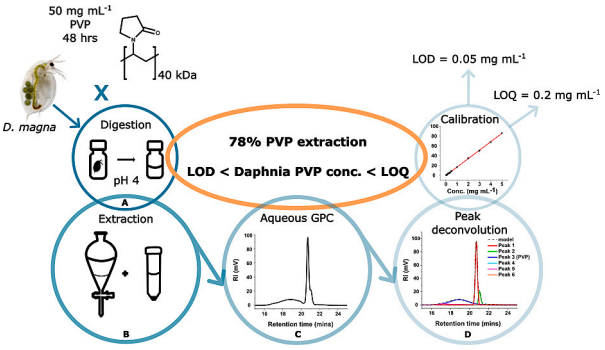
HIGHLIGHTS

- Novel method developed to detect and quantify PVP using *Daphnia magna*.
- Refractive index GPC coupled with Fityk 1.3.1 enabled deconvolution of PVP peak.
- Method had LOD and LOQ of 0.05 and 0.2 mg mL^{-1}, respectively.
- Recovery of 78 % of PVP from spiked *Daphnia magna*.
- PVP detected in *Daphnia magna* for the first time.

GRAPHICAL ABSTRACT



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ABSTRACT

The water-soluble polymer polyvinylpyrrolidone (PVP) is an established ingredient in pharmaceutical and personal care product (PPCP) formulations. Due to its high usage and lack of biodegradability, it has been detected up to 7.0 mg L^{-1} in wastewater and 0.1 mg L^{-1} in the receiving freshwaters, with several studies showing detrimental sub-lethal effects in a range of aquatic species. A lack of simple analytical methods to detect and quantify PVP currently impacts further investigation into the cause of these sublethal effects. In this paper we propose a refractive index gel-permeation chromatography (GPC) method to quantify PVP, which includes the processing of raw chromatograms using line deconvolution to calculate peak area. The method was then applied to *Daphnia magna* exposed to PVP for 48 h. A limit of detection (LOD) and limit of quantification (LOQ) of 0.05 and 0.2 mg mL^{-1} respectively was determined, with a recovery of 78 % from spiked *Daphnia magna*. PVP was detected in the samples above the LOD but below the LOQ. This suggests PVP is ingested by *Daphnia magna*, which warrants further investigation into whether bioaccumulation of PVP could be causing the sublethal effects seen in other studies.

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1. Introduction

The discussion around plastic pollution has become synonymous with insoluble plastics or plastic fragments, termed micro- or nano-plastics. The large research sphere and debate surrounding insoluble plastics, and the polymers that are their main component, has led to multiple legislative breakthroughs, from the restriction of intentionally added microplastics to the more recent ban on single-use plastics across the UK and European Union (ECHA, 2019; European and Directorate-General, 2021; Hartmann et al., 2019; UK Department for Environment, 2023). However, over the last few years, soluble polymer pollution has gained traction as another branch in the field of polymer-based pollution.

Of particular concern are water-soluble polymers (WSPs) that are resistant to biodegradation. Polymers such as polyethylene glycol (PEG) are mostly biodegradable at varied molecular weights under the Organisation for Economic Co-operation and Development (OECD) guidelines; however, polymers like polyvinylpyrrolidone (PVP, Fig. 1) have proved resistant to biodegradation and are therefore likely to enter freshwaters, for example via wastewater treatment plants (WWTPs) (Bernhard et al., 2008; Eubeler et al., 2010; Julinova et al., 2012; Loraine, 2008; Trimpin et al., 2001). Thus, the assumption that WSPs will simply degrade in wastewater treatment and therefore not pose an environmental threat is incorrect (Zumstein et al., 2022).

PVP is used in a variety of products and is well established as an ingredient in pharmaceutical formulations (Buhler, 2005; Pourmadadi et al., 2023). Its apparent low toxicity ($LD_{50} > 100$ g kg^{-1} in rats and guinea pigs), amphiphilic nature, biocompatibility and its ability to cater for hydrophilic and lipophilic drugs mean this polymer is frequently chosen for drug delivery systems (Kurakula and Rao, 2020). Alongside its use in the pharmaceutical industry, PVP is a key ingredient of STRESS COAT™, a product used by the aquaculture industry to form a synthetic slime coating on fish, to promote epidermal healing (Robison-Smith et al., 2024).

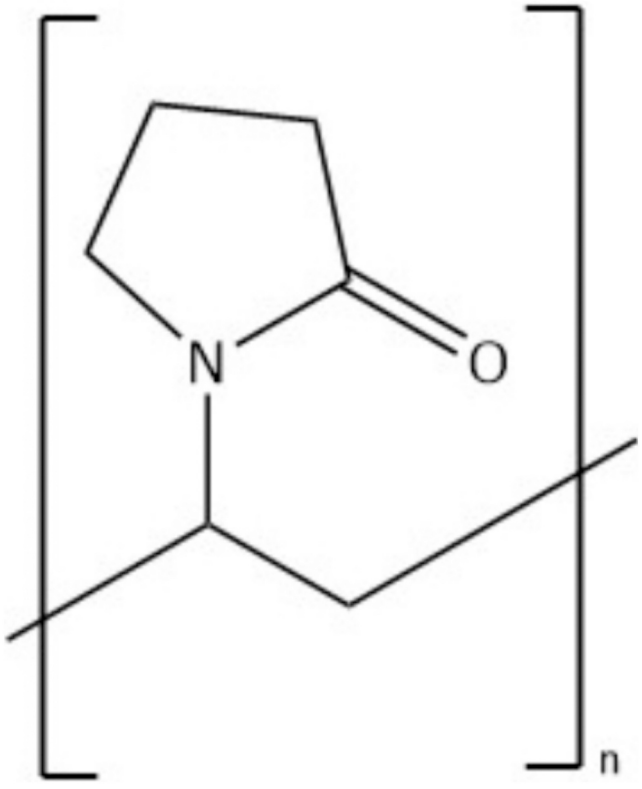

Fig. 1. Polyvinylpyrrolidone repeat unit.

PVP was first detected in the environment using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) and then in later studies, quantified using pyrolysis-gas chromatography–mass spectrometry (Py-GC–MS) up to 7.0 mg L^{-1} in wastewater effluent and 0.1 mg L^{-1} in the receiving freshwater 50 m downstream (Antic et al., 2011; Antic et al., 2012; Trimpin et al., 2001). In terms of the ecotoxicological effects, several recent studies have highlighted the sublethal effects of PVP on multiple organisms. Effects such as a decreased mobility and swimming behaviour in *Danio rerio* have been noted at concentrations as low as 0.001 mg L^{-1} (Binelli et al., 2024; Nigro et al., 2023). Decreased parasite survival and disruption to biotic interactions in *Poecilia reticulata* were also observed alongside the complex impact of PVP and polyvinyl alcohol (PVA) on host-parasite relationships (Robison-Smith et al., 2024). Additionally, PVP at 5 mg L^{-1} had chronic reproductive effects on *Daphnia magna*, and PEG at different molecular weights appeared to cause different toxic effects, implying molecular weight may impact polymer toxicity (Mondellini et al., 2022).

A significant barrier to determine the mechanisms by which PVP affects aquatic organisms is a lack of suitable analytical techniques to detect and quantify WSPs. There is difficulty in using conventional and advanced small molecule environmental techniques, such as liquid chromatography-mass spectrometry (LC-MS) due to polymer complexity, large molecular weight, multitude of end groups and branching (Knol et al., 2021). Most recently, a method was developed for the detection and quantification of polyethylene oxide (PEO): hyphenating size-exclusion chromatography (SEC) with electrospray ionisation (ESI) high-resolution mass spectrometry (SEC-ESI-HRMS), where PEO was quantified in wastewater and surface water down to 20 μg L^{-1} and 11 μg L^{-1}, respectively (Pauelsen et al., 2023). While hyphenation of SEC with ESI provides a useful tool for sensitive analysis of lower molecular weight compounds, multiple difficulties are encountered when the molecular weight is increased, which is the case for most biodegradation-resistant synthetic polymers. ESI of polymers can lead to multiply charged species, making accurate mass determination complex and, in the case of some polymers, the polymer is not present in the spectrum (Crotty et al., 2016; De Bruycker et al., 2020).

While hyphenation brings many benefits, SEC alone has advanced rapidly over the last few decades as a promising technique for WSP analysis, particularly with regards to quantification using refractive index (RI), which is directly proportional to the concentration of the polymer (Cheong et al., 2015; dos Santos et al., 2017; Gómez-Ordóñez et al., 2012; Izumi et al., 2013). A recent review on quantification methods for PVP-based products and copolymers discussed the success but cost and complexity of techniques previously used for PVP quantification, such as Py-GC–MS (Antic et al., 2011; Antic et al., 2012), with SEC identified as a preferable and accessible quantification method, either separate or hyphenated (Horváth et al., 2023).

Recent studies on toxicity and the challenges remaining for the analysis of WSPs in general have given rise to many questions regarding the environmental impact of PVP, each of which would require multiple studies to investigate. Firstly, why is PVP behaving the way it does when interacting with aquatic organisms? Secondly, if PVP is indeed ingested, is it likely to accumulate due to its recalcitrance and could this explain the effects discussed earlier? And finally, what are the wider implications of this for other WSPs; is their "non-toxic" assessment accurate (Kurakula and Rao, 2020)? In this study, we provide the analytical basis to begin to address these problems, by developing a method to extract PVP from a model freshwater organism - *Daphnia magna* - exposed to PVP 40,000 Da and quantifying the extracted polymer using refractive index gel-permeation chromatography (GPC), otherwise known as SEC. This method will couple the work carried out in previous ecotoxicity studies and the development of novel WSP extraction, detection and

quantification techniques (Mondellini et al., 2022; Pauelsen et al., 2023; Tarring et al., 2024). It is hoped that by exploring quantification within organisms, this method could be applied alongside future ecotoxicity studies to investigate whether PVP is inherently toxic, bioaccumulative or if, as Robison-Smith et al. proposed, PVP could be interacting with the mucosal layer or the gastrointestinal tract of organisms, preventing natural synergistic relationships or nutrient uptake (Robison-Smith et al., 2024).

2. Materials and methods

2.1. Chemicals

Polyvinylpyrrolidone (PVP) (M_n = 40,000) was purchased from Sigma-Aldrich (Gillingham, UK), sodium nitrate from Honeywell (St Helens, UK), and sodium phosphate monobasic and potassium hydroxide were supplied by ThermoFisher scientific (Loughborough, UK). Hydrochloric acid (37 %), hexane (petroleum ether fraction), diethyl ether and methanol laboratory grade reagents, alongside HPLC grade water, were supplied by Fisher Scientific (Loughborough, UK).

2.2. Daphnia magna exposure

Live *Daphnia magna* were purchased from Fish & Fins, UK in 2009 and thereafter maintained at Cardiff University at a constant temperature and light exposure (20 °C ± 1.0 °C, 12:12 h light: dark cycle). The breeding stock is 50 % water changed and fed every alternate day with a *Spirulina*-yeast solution.

2.2.1. Daphnia magna for method validation

Two ~1.0 g samples (wet mass) of *Daphnia* were isolated, rinsed with dechlorinated water in 500 μm metal sieves for 5 min and placed in glass containers and stored at −20 °C until analysis.

2.2.2. Daphnia magna exposure study

PVP was used to make a 1 g L^{-1} stock solution with autoclaved water to prevent any potential microbial degradation. Separating funnels were used to maintain two ~1.0 g (wet mass; equivalent to 0.076 ± 0.002 g dry mass) cultures of *Daphnia* in continually oxygenated freshwater. 50 mL of the PVP stock solution was added to 950 mL of the *Daphnia* cultures to achieve a concentration of 50 mg L^{-1}. *Daphnia* were fed once with 1 mL Spirulina-yeast solution at the start of the exposure and were not water changed/re-dosed. After 48 h exposure, the funnels were drained, and the *Daphnia* collected and stored in glass containers. *Daphnia* were rinsed in a 500 μm metal sieve for 1 min with dechlorinated water before being stored at −20 °C until analysis. This allowed for the separation of *Daphnia* from other matter within the tank and ensured no PVP remained on the surface of the *Daphnia*.

Control *Daphnia* followed the above protocol with 1 L cultures without the addition of the PVP solution.

2.3. GPC measurements and line deconvolution

GPC analysis was conducted using an Agilent InfinityLab LC series 1260 Infinity Quaternary system connected to an Agilent 1260 Infinity GPC/SEC Multi Detector Suite (MDS). The MDS comprised of a dual angle light scattering detector (15° and 90°), refractive index detector and viscometer in order. The eluent comprised of HPLC water containing 0.2 M $NaNO_3$, 0.1 M NaH_2PO_4 at pH 3, with two PL aquagel-OH MIXED-H 8 μm columns (300 × 7.5 mm) in series selected to analyse the samples. For all GPC samples, the extracts were filtered through 0.2 μm nylon filters prior to analysis.

2.3.1. Calibration, LOD and LOQ

Calibration of the refractive index detector was carried out in duplicate using PVP 40 kDa at 11 concentrations (0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 mg mL^{-1}). An additional three samples were analysed for the concentration range 0.05–0.3 mg mL^{-1} for limit of detection determination. The refractive index peak area was determined using Fityk 1.3.1 line deconvolution software (Table S1). This average peak area was then plotted against concentration of the sample to obtain a linear calibration curve. The limit of blank (LOB) and limit of detection (LOD) were calculated using the equations below as per the methodology of Vashist and Luong (Eq. (1), Eq. (2), Table S2) (Armbruster and Pry, 2008; Vashist and Luong, 2018). The LOB was calculated out by analysing five blank eluent samples. The limit of quantification (LOQ) was determined at the lowest concentration at which the refractive index response was five times the response of the blank and had a standard deviation of <20 % (Armbruster and Pry, 2008; Vashist and Luong, 2018).

$$\text{LOB} = \text{mean of blank samples} + (1.645 \times \text{standard deviation (SD) of blank samples}) \quad (1)$$

$$\text{LOD} = \text{LOB} + (1.645 \times \text{standard deviation (SD) of 0.1 mg mL}^{-1}\text{samples}) \quad (2)$$

In order to determine concentration, the straight line equation from the PVP calibration curve was used to calculate the corresponding concentration to peak area (Eq. (3), Table S3).

$$\text{Concentration}\left(\text{mg mL}^{-1}\right) = \frac{\text{refractive index peak area (mV.}min\text{)} - \text{intercept (mV.}min\text{)}}{\text{gradient}\left(\frac{\text{mV.}min}{\text{mg mL}^{-1}}\right)} \quad (3)$$

2.4. PVP extraction and quantification

For samples containing *Daphnia*, the sample was rinsed onto filter paper (185 mm diameter, 11 μm porosity) using distilled water and air dried before transferral into a glass sample vial (14 mL) and weighed. For all samples, HPLC water, acidified to pH 4 using dilute hydrochloric acid, was added to the sample in a 1:5 wt (g):v (mL) ratio. The sample was then digested for 24 h under vigorous stirring and the resulting extract made to 50 mL with distilled water. Organics were extracted from the sample using solvent/solvent extraction with hexane (1 × 100 mL) followed by diethyl ether (1 × 100 mL). During each wash, the organic layer was washed three times with distilled water and the aqueous portions added to the main aqueous layer. At the end of both the hexane and diethyl ether washes, any emulsion formed between the aqueous and organic layer was collected in a Corning® centrifuge tube (50 mL) and centrifuged (4400 rpm, 5 min). The aqueous fraction from this was pipetted into the main aqueous layer. The resulting aqueous layer was made to pH 5 using either dilute hydrochloric acid or potassium hydroxide solution. The sample was transferred and washed into to a round-bottom flask (500 mL) and concentrated via rotary evaporation (~30 mL) and centrifuged (4400 rpm, 5 min) in a Corning® centrifuge tube (50 mL). The aqueous extract was decanted and washed into a round-bottom flask (250 mL) and the pellet formed via centrifugation

washed with distilled water and re-centrifuged twice, with the washings added to the main aqueous extract. This extract was then rotary evaporated to dry and reconstituted in methanol (5 mL). The methanol extract was transferred to a glass sample vial (14 mL), with the round-bottom flask washed with methanol (2 × 2 mL) into the sample vial to ensure complete transferral. Methanol was evaporated under nitrogen and the final extract dissolved in GPC eluent (2 mL).

The above procedure was applied to two procedural blanks (*Daphnia* and PVP) and a spiked extraction of PVP from *Daphnia*. The *Daphnia magna* procedural blank contained only *Daphnia magna*. The PVP procedural blank contained only PVP 40 kDa in HPLC water (1 mL, 3 mg mL^{-1}), with the same amount of acidic water added as for the *Daphnia magna* procedural blank. As this sample did not contain any tissue, no emulsion was formed at the organic/aqueous interface during solvent/solvent extraction. Therefore, the interface was centrifuged as a replacement for this.

This method was also applied to duplicate *Daphnia magna* samples for the exposure study. The sample masses and corresponding acid volumes are given in Table S4.

The tank water from the replicate exposed *Daphnia magna* samples alongside a control tank water (spirulina and PVP 40 kDa (50 mg L^{-1})) were frozen in liquid nitrogen before the water was removed via freeze-drying. Each sample was extracted using methanol (5 mL) and washed (2 × 2 mL) into a 100 mL round-bottom flask. The methanol was removed via rotary evaporation and the residue dissolved in methanol (5 mL) and washed (2 × 2 mL) into a glass sample vial (14 mL). Methanol was evaporated under nitrogen and the final extract dissolved in GPC eluent (2 mL).

2.4.1. Sample quantification

For sample analysis, the same methodology applies as to the calibration samples. The refractive index peak area was determined using Fityk 1.3.1 line deconvolution software using the Exponentially Modified Gaussian (EMG) line shape. This value was then used to calculate the corresponding concentration of PVP within the sample using the straight line obtained from the PVP 40 kDa calibration curve (Eq. (3)).

3. Results and discussion

3.1. Refractive index quantification method

To quantify PVP within *Daphnia magna* samples, several WSP-specific challenges needed to be addressed. Firstly, extracting the polymer from the tissue sample requires the digestion of the tissue without degrading the polymer. Several methods are available for the extraction of microplastics from tissue samples, however these utilise strong bases or peroxides, both of which are capable of degrading PVP, which is susceptible to basic and free-radical depolymerisation (Dehaut et al., 2016; Karami et al., 2017; Loraine, 2008; Taghizadeh and Nasirianfar, 2020; Tsangaris et al., 2021). Following this, the polymer needs to be separated from organic matter within the tissue sample. Solvent/solvent extraction has been employed for the extraction of PVP from wastewater, however here the fatty content in a tissue sample provides additional challenges due to the emulsion stabilising property of PVP (Antic et al., 2011; Antic et al., 2012). Due to its amphiphilic nature, PVP stabilises the organic/aqueous layer interface, creating a large emulsion that can prevent complete extraction of PVP into the aqueous layer (Ezaki et al., 2017; Kurakula and Rao, 2020; Woo et al., 2010). Additionally, PVP is characteristically polydisperse, producing a broad peak when analysed by GPC (Malvagna et al., 2002; Technologies, 2015). Therefore, it is not possible to fully separate PVP from all low-molecular weight material remaining in the sample. Alongside this, aqueous GPC can be susceptible to system peaks, due to the addition of salts into the mobile phase to stabilise the signal (Levin and Grushka, 1986). While these peaks can be reduced, they cannot be removed due to the interaction of these salts with the stationary phase.

Due to the multitude of difficulties in analysing and quantifying PVP, a tailored WSP method is required to address and reduce these methodological concerns and enable the quantification of PVP within tissue samples. A four-step method was developed involving the digestion of the sample, extraction of PVP, analysis by aqueous GPC and line deconvolution data processing (Fig. 2).

In step A, the sample was digested using pH 4 water and stirring to enable the complete digestion of the tissue without degrading the polymer. Following this, the polymer was extracted into water (step B), adapting the protocol developed by Antic et al. (2011) via solvent/solvent extraction with hexane and diethyl ether (Antic et al., 2011). Due to the formation of a PVP-stabilised emulsion layer at the water/organic interface, the emulsion was centrifuged, which allowed the complete separation of the layers, reduced solvent requirements and increased PVP extraction. The samples were then analysed using the refractive index (RI) detector of an aqueous GPC system (step C). To account for the incomplete separation of PVP from low-molecular weight matter and the presence of system peaks in the detector, additional data processing was required, using line deconvolution software to fully separate the PVP peak from the remaining peaks in the trace (step D). Fityk 1.3.1 enabled the area of PVP extracted from tissue samples to be compared to a PVP peak area calibration curve for quantification. This software has previously been used for the resolution of complicated spectral patterns with multiple signal peaks (Schulze et al., 2022; Wojdyr, 2010).

3.2. Calibration and method validation

To use RI as a polymer quantification method, a range of PVP concentrations were analysed. The chromatograms of these PVP samples were processed using Fityk 1.3.1 to identify the PVP peak and plot this peak area to create a linear calibration (Fig. 3). The retention time for the calculated peak areas are similar, with increasing uniformity with concentration. This suggests the line shape deconvolution provides accurate peaks areas across the concentration range. When these peak areas are plotted to create a calibration curve, there is a strong linear relationship, with an R^2 value of 0.9996. Alongside this, the

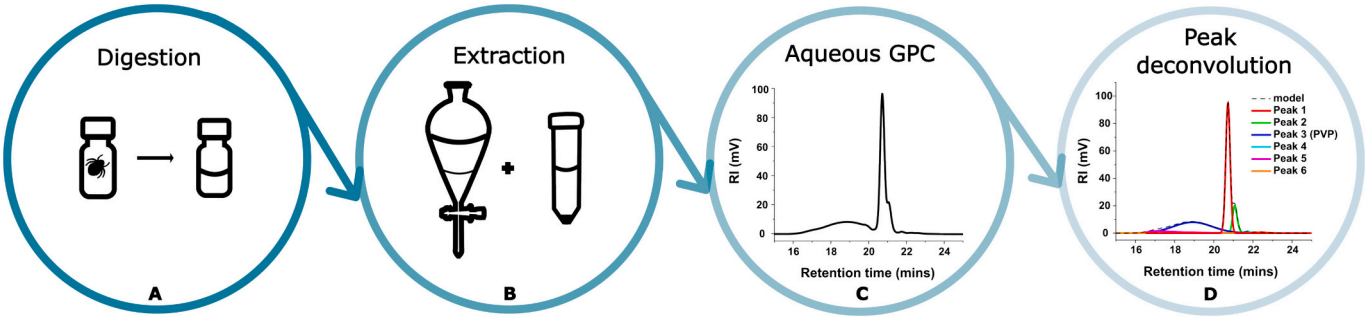

Fig. 2. An overview of the four-step method to quantify PVP within aquatic organisms.

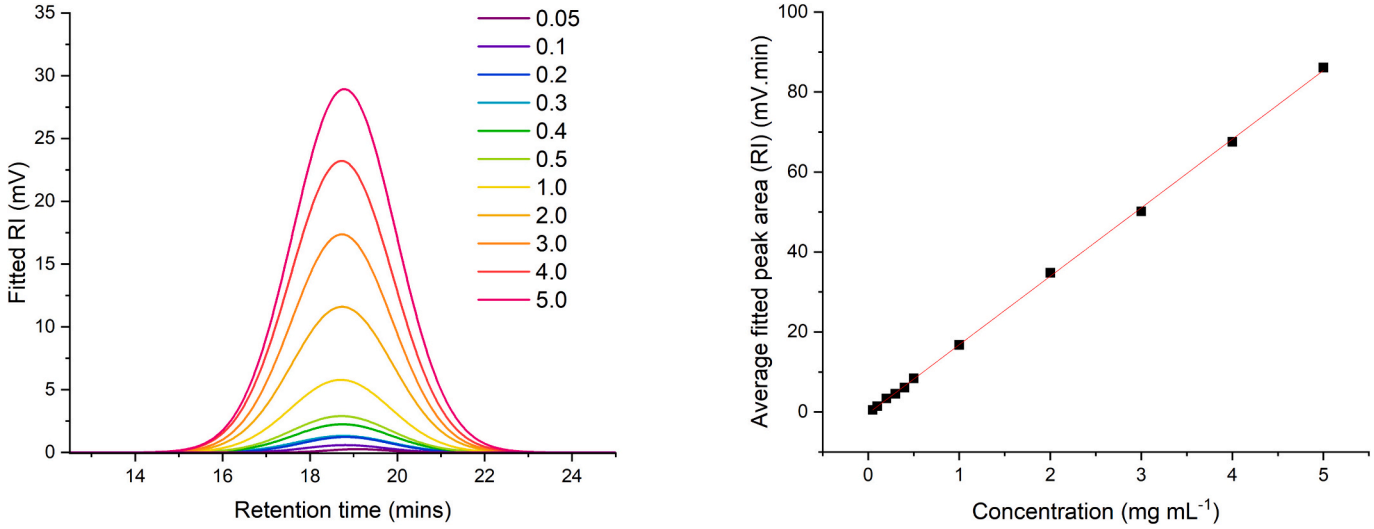

Fig. 3. Left) Retention time for PVP at different concentrations between 0.05 and 5.0 mg mL^{-1} against the fitted refractive index. Right) Corresponding calibration curve of the PVP concentrations against fitted refractive index peak area values ($R^2 = 0.9996$).

Table 1
The limit of blank, limit of detection and limit of quantification for PVP 40 kDa detected using aqueous gel-permeation chromatography. The recovery of PVP from a blank sample (water) and a *Daphnia magna* (tissue) sample.

Analytical performance	PVP 40 kDa
LOB (mg mL^{-1})	0.039 ± 0.014
LOD (mg mL^{-1})	0.050 ± 0.014
LOQ (mg mL^{-1})	0.200 ± 0.015
Procedural blank recovery (%)	80 ± 3
Spiked tissue recovery (%)	78 ± 1

reproducibility of these peak areas indicated that RI is a robust tool for polymer quantification.

The LOB, LOD and LOQ concentrations for the analysis of PVP 40 kDa using RI were calculated using the equations outlined in Section 2.3.1 and are shown in Table 1. The specific LOD and LOQ value calculated for this polymer can only be prescribed to this molecular weight as LOD/LOQ can be affected by molecular weight of the polymer (Pauelsen et al., 2023). This suggests that analysis of polymers provides additional complications to the analysis of individual molecules of a specific molecular weight. Furthermore, it is not clear what the implications of different manufacturers or methods of synthesis would have on peak area, as polymer formulations tend to come only with an average molecular weight, which can vary in precision. Despite this, the LOD suggests that RI analysis of polymers within biological/environmental samples is possible but may require pre-concentration, depending on the exposure of the sample to polymeric material. Alongside this, LOD/LOQ values are similar to other SEC polymer studies (Cheong et al., 2015; Gómez-Ordóñez et al., 2012).

Following detector calibration, method performance was assessed through the recovery of a known amount of PVP from water and *Daphnia magna* revealing similar (78–80 %) recovery rates (Table 1). A negative control containing only *Daphnia magna* was also analysed to check for polymer contamination.

Interestingly, the low molecular weight matter peak from 20 min is at a much higher intensity in the PVP tissue sample than in the tissue procedural blank, despite a similar amount of initial tissue mass in the samples. Considering this low molecular weight peak in the tissue procedural blank mirrors the same peak in the PVP procedural blank, this implies the addition of PVP to the tissue sample could be reducing its separation and removal from the sample. Alongside this, the PVP peak has a greater overlap with the low molecular weight peak. This is not entirely unsurprising due to the emulsion stabilising properties of PVP which could be binding to organic matter in the sample, reducing separation efficiency (Ezaki et al., 2017; Woo et al., 2010). This highlights the difficulty faced when analysing WSPs in comparison to other polymeric contaminants such as microplastics; soluble polymers are designed to bind to small molecule systems as part of their function as surfactants and emulsifiers and will consequently be harder to separate and analyse. Additionally, this raises concerns about the ability of PVP to bind to organic matter, such as nutrients, which could affect the food uptake of aquatic organisms if they ingest PVP (Robison-Smith et al., 2024).

Deconvoluted PVP peaks in the RI chromatograms suggest the software is capable of fitting PVP peaks in blank extracted samples and in spiked tissue samples, despite the greater overlap, with only minor differences in peak shape (Fig. 4 (right)). Additionally, no PVP peak was detected in the tissue procedural blank, suggesting there is no polymeric contamination in the samples. These PVP peaks were compared to the calibration curves at 1.0 and 2.0 mg mL^{-1}, as 100 % extraction of PVP in the 2 mL sample would be 1.5 mg mL^{-1}.

3.3. Application to Daphnia magna exposure study

Following calibration and method validation, the extraction and quantification method was applied to *D. magna* exposed to PVP 40 kDa for 48 h, with the corresponding RI traces and fitted peaks shown in Fig. 5. Additional fitted peaks of PVP extracted from the tank water and control water samples are shown in Fig. S2 to demonstrate the lack of biodegradation of PVP over the acute study period.

The aim of this study was to identify if PVP could be detected and quantified after a short-term exposure experiment and the implications this would have on future studies assessing the effects of WSPs on organisms. For both *Daphnia magna* samples, PVP 40 kDa was identified above the LOD but below the LOQ. This means a numerical value could not be assigned to the concentration of PVP within the samples but that they fall between 0.05 and 0.2 mg mL^{-1}.

It is clear that the limit of quantification is still high in comparison to other methods used to quantify PVP in environmental samples and PVP-containing products, and that this was a barrier to determining the actual uptake of PVP within *D. magna* samples in this study (Antic et al., 2011; Cheong et al., 2015; Horváth et al., 2023). However, the LOD and LOQ of this method were derived from the linear calibration of samples directly injected into the instrument. Therefore, other samples of

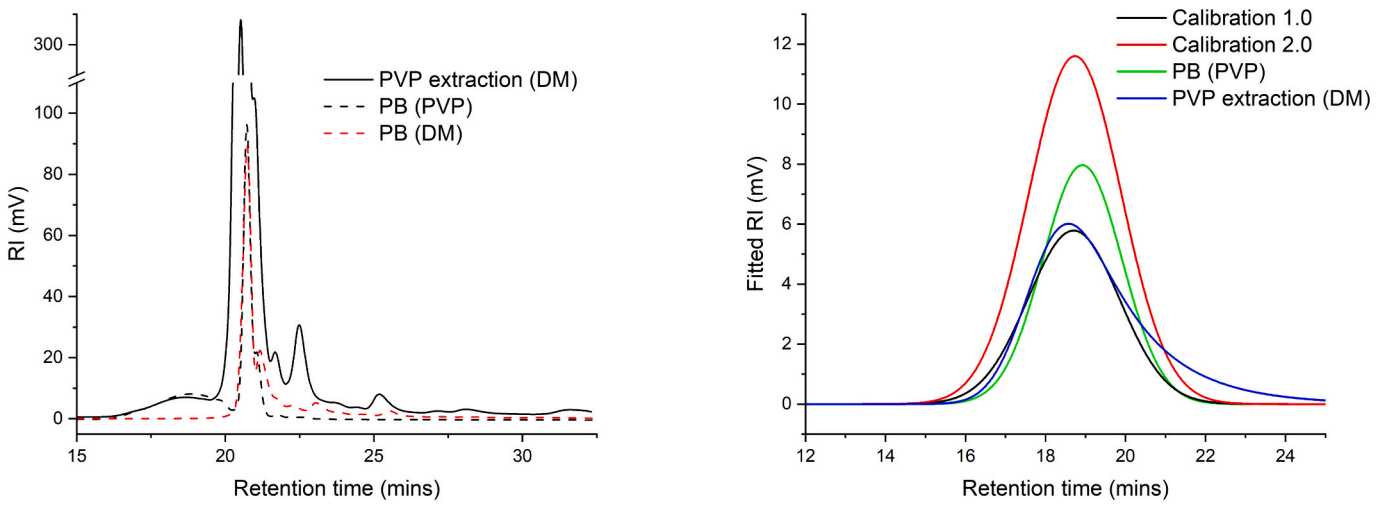

Fig. 4. Left) Refractive index traces of two procedural blanks (PVP and *Daphnia magna*) and extraction of PVP from *Daphnia magna*. Right) Fitted refractive index traces for PVP calibration samples at 1.0 and 2.0 mg mL-1, the procedural blank (PVP) sample and the extraction of PVP from *Daphnia magna* sample demonstrating extraction efficiency of the method.

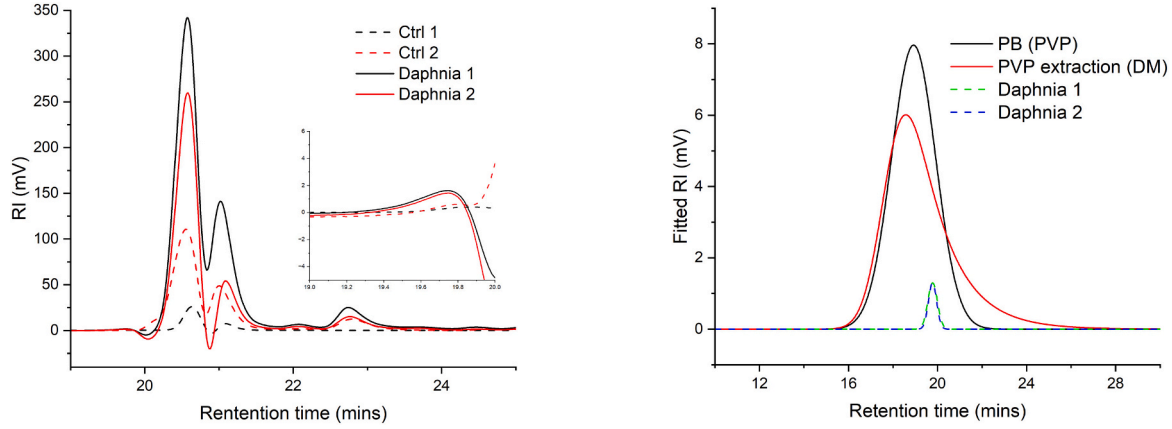

Fig. 5. Left) Refractive index traces of two procedural blanks (PVP and Daphnia magna) and extraction of PVP from Daphnia magna. Right) Calculated refractive index traces for PVP calibration samples at 1.0 and 2.0 mg mL^{-1}, the procedural blank (PVP) sample and the extraction of PVP from Daphnia magna sample demonstrating extraction efficiency of the method.

environmental interest, such as water samples, could be pre-concentrated to levels well above the quantification limit in this study (Antic et al., 2011). Further studies in this area should focus on greater pre-concentration of samples prior to analysis to ensure values above the LOQ can be achieved.

While the acute concentration of PVP could not be quantified in the *D. magna* samples, the RI chromatograms and their corresponding fitted peaks were able to be detected and identified as PVP 40 kDa. This suggests PVP is present within *D. magna* and, over 48 h, they are ingesting a polymer that is known to be resistant to biodegradation (Julinova et al., 2018; Trimpin et al., 2001). While the concentration tested in this study is likely to be above environmentally relevant levels, the aim of the study was to investigate if ingestion of the polymer was possible, with it still remaining uncertain whether PVP can bio-accumulate within organisms (Antic et al., 2011). Alongside this, another acute study of the ecotoxicological effect of WSPs at 50 mg mL^{-1} detected no adverse effects on *Daphnia magna*, however this does not mean the polymer is not interfering with other key behaviours (Mondellini et al., 2022). Chronic PVP exposure does appear to impact *Daphnia magna* reproductive cycles and maximum growth, implying constant exposure to PVP can affect this invertebrate or indeed the polymer could be accumulating, thus increasing its effect (Mondellini et al., 2022). Other studies have focussed on not just the chronic toxicological effect but biochemical modulation and host-parasite interaction (Binelli et al., 2024; Nigro et al., 2023; Robison-Smith et al., 2024). These studies discuss the various modes of action PVP could have, but it is still unclear if the PVP is ingested is inherently toxic or if, through its behaviour as an emulsifier, it has the potential to coat the organism, interacting with the mucosal layer and potentially internal organs to prevent feed uptake, or in the case of Robison-Smith et al., alter biotic interactions within food webs (Robison-Smith et al., 2024).

Results in the current study show PVP is present within the test organism, indicating its negative effects are likely to be or to include direct ingestion of the polymer. Further chronic studies are needed, in tandem with analytical methods able to investigate the cause of WSP sublethal effects. The newly developed method has proved a relatively simple alternative to more complex methods and promotes GPC as a versatile method in the identification of WSPs as chemicals of emerging concern (Antic et al., 2011; Horváth et al., 2023; Pauelsen et al., 2023).

This method could also be transferred to other WSPs, with aqueous GPC having previously been used for both natural and synthetic WSP quantification, some investigating the use of a universal calibration for application to unknown samples, which would remove the use for individual calibration curves (Cheong et al., 2015; Gómez-Ordóñez et al.,

2012; Ruiz et al., 2019). These universal calibration curves are determined from the refractive index increment (*dn/dc*), which is a polymer-specific parameter that can be used for direct concentration calculation. Universal *dn/dc* values have been employed with good accuracy to natural WSPs, such as polysaccharides, where light scattering was used in conjunction with RI (Cheong et al., 2015). While it is unclear whether this could be applied to PVP, it would be interesting to explore an alternative to requiring standards for polymer quantification using GPC, which would enable a greater range of applications. Alongside this, light scattering detectors could provide additional accuracy in quantifying polymeric material.

Investigating a universal method would be necessary if this method were to be applied to environmental samples, where many different natural and synthetic polymers could be present. It is unlikely that the use of line deconvolution software would be able to separate multiple overlapping polymers peak areas. Therefore, this method would need to be tested on more complex environmental samples, where additional techniques such as MALDI-TOF MS, infrared spectroscopy (IR) or additional GPC detectors such as multi-angle light scattering (MALS) could aid the identification and quantification of these samples (Cheong et al., 2015; Fandrich et al., 2010; Horváth et al., 2023; Ruiz et al., 2019; Tarring et al., 2024; Trimpin et al., 2001).

Both universally calibrating the RI detector and investigating the use of the method proposed in this study for other WSPs should be the focus of future aqueous GPC studies for WSP quantification.

Alongside PVP, multiple other WSPs have been identified as a cause for concern, mostly with regards to sublethal effects (Acharya et al., 2010; Binelli et al., 2024; Buczek et al., 2017; Connors et al., 2023; Cumming et al., 2008; Liber et al., 2005; Mondellini et al., 2022; Robison-Smith et al., 2024). PVP is likely to be common in the environment due to its high usage and lack of biodegradability, however, there are still only a handful of studies investigating and quantifying WSPs (Antic et al., 2011; Pauelsen et al., 2023; Tisler et al., 2021; Vidovic et al., 2023). Recent studies on WSPs predominantly focus on toxicological assessment, with limited detection or quantification methods to explore further how WSPs are impacting organisms. Interdisciplinary collaboration is required to address both the questions around polymer quantification and ecotoxicological effect.

4. Conclusion

Here, we develop a new method using GPC refractive index to detect and quantify PVP above 0.05 mg mL^{-1} and 0.2 mg mL^{-1}, respectively. A combination of GPC with line deconvolution enabled the identification of polydisperse PVP before its separation from multiple, complex lower molecular weight peaks using line deconvolution with good accuracy and linearity. This method therefore addresses the significant challenges of broad polymer distributions in chromatography.

PVP recovery of 80 % and 78 % within a procedural blank and a spiked *Daphnia magna* sample, respectively, demonstrates the method can be applied to its quantification in freshwater samples. When applied to *Daphnia magna* samples exposed to PVP at 50 mg L^{-1} for 48 h, PVP was detected above the LOD but below the LOQ. This highlights that GPC quantification could be used in tandem with observed sublethal effects in ecotoxicological studies to understand the increased sublethal effects seen in chronic studies.

While this method enabled the detection of PVP within *Daphnia magna* for the first time, limitations such as the quantification limit and necessity for polymer standards require further exploration. It is unclear whether this method would be able to be applied to environmental samples, where many different polymers may be present. However, investigation into a universal calibration and pre-concentration of the sample may enable further environmental application.

Nevertheless, this method could now be applied to chronic ecotoxicological studies, and should be capable of demonstrating whether PVP can bioaccumulate, as well as other WSPs, with minor alterations made to eluent and extraction. Importantly, it could also be applied to other freshwater organisms under lab or field conditions to understand how PVP interacts with freshwater ecosystems, and whether regulations of WSP are required, which is a key next step for research.

CRediT authorship contribution statement

Eve Tarring: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Charlotte Robison-Smith:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Jo Cable:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Isabelle Durance:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Michael Harbottle:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Benjamin D. Ward:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization.

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.

Data availability

The data supporting the results presented in this article are freely available via the Cardiff University data catalogue at https://doi.org/10.17035/d.2024.0318661497.

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Appendix A. Supplementary data

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