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ABSTRACT

Urea represents a common form of organic nitrogen (N) which is added to agricultural soils in large quantities in both cropping (e.g. fertiliser) and livestock (e.g. urine) systems. In addition, there is a small, dynamic ambient pool of urea in soil associated with metabolic functioning in the microbial community. The diacetyl monoxime (DAM) colorimetric method is routinely used to quantify urea in soil, however, it lacks specificity due to the potential to react with the ureido group ($R_1NHCONHR_2$), a common structural moiety in soil organic matter. The aim of this study was therefore to critically evaluate the accuracy of this method for urea determination in soil. Using the DAM assay, we demonstrated significant cross-reactivity with a range of ureido compounds, many of which are ubiquitous in soil. We conclude therefore that the DAM assay is highly likely to overestimate urea concentrations in environmental materials. Such overestimation was confirmed using high resolution HPLC-Orbitrap MS to quantify urea in grassland soils using standard addition and the concentrations compared with those of the DAM assay. The results obtained show the DAM colorimetric method overestimated urea concentration by between 7.2 and 58 times for the sites studied. This significant overestimation of urea emphasises the need to validate the colorimetric method with reference to the LC-MS assay to ensure the robustness of measured urea concentrations. On this basis we recommend that reporting of the results from the DAM colorimetric method as "urea" concentration be curtailed and reported as "ureido-N" to recognise the contribution of unknown and variable contributions from other compounds. Indeed, given the problems with quantitatively assessing the latter contributions we would recommend the DAM method is now avoided in surveys of urea concentrations in soil and the wider environment.

Keywords: Analytical method, Dissolved organic nitrogen, Nitrogen cycling, Urea determination, Ureido-N

1. Introduction

Urea occurs ubiquitously in the environment, deriving from both natural and anthropogenic sources. It is an important compound in key biological processes and metabolic pathways of all living organisms (Berman and Bronk, 2003; Remsen, 1971). Due to the high abundance of the microbial enzyme urease and plant uptake mechanisms, urea is rapidly turned over in soils under ambient conditions, resulting in a small but dynamic urea pool, which is an important part of the soil N-cycle (Harder Nielsen et al., 1998; Lloyd and Sheaffe, 1973; Solomon et al., 2010). Elevated urea concentrations in the environment can indicate anthropogenic inputs, predominantly from agriculture (as fertiliser or from livestock urine) and wastewater treatment systems (Galloway and Cowling, 2002; Glibert et al., 2006). It is important to determine these anthropogenic inputs into the environment to assess human impacts. High concentrations of soluble low molecular weight nitrogen (N) compounds, such as urea, can have detrimental impacts on the environment, such as eutrophication and denitrification, leading to the release of the greenhouse gas, nitrous oxide (N_2O) (Galloway and Cowling, 2002). The latter also catalyses the production of ozone in the troposphere, which damages agricultural crops and natural ecosystems, alongside negative impacts on human and animal health (Galloway and Cowling, 2002; Heil et al., 2016). Such negative impacts on ecosystems, and on human and animal health, alongside the increasing importance of urea globally as a fertiliser, means it is important to quantify N inputs, particularly the urea component, into the soil environment (Gilbert et al., 2006).

There are two indirect methods commonly used to quantify urea in environmental matrices: enzymatic and colorimetric. The enzymatic method uses the bacterial enzyme urease to convert urea to ammonium, which is subsequently quantified using other enzymatic or colorimetric methods (McCarthy, 1970). A drawback of this method is the considerable background levels of ammonium in the environment (i.e. high blanks). Furthermore, the urease

inhibitors used in combination with urea fertilisers in agriculture reduce the accuracy of these determinations (Lambert et al., 2004).

The diacetyl monoxime (DAM) colorimetric method provides an often-used alternative for urea detection and quantification in both biological and environmental matrices (Douglas and Bremner, 1970; Stepanauskas et al., 2000; Taylor and Vadgama, 1992). The latter method is preferred for environmental matrices due to its sensitivity, with modified methods able to detect urea down to 0.03 μM N concentrations (Alam et al., 2017; Goeyens et al., 1998). It has been applied to a wide range of environmental matrices, including river water (Burford et al., 2011; Revilla et al., 2005; Satoh and Katoh, 1989; Stepanauskas et al., 2000), seawater (Cochlan and Bronk, 2001; Mulvenna and Savidge, 1992; Remsen, 1971), estuarine waters and sediments (Alam et al., 2017; Nakas and Litchfield, 1977; Torres-Valdés and Purdie, 2006; Twomey et al., 2005), rainwater (Cornell et al., 1998), and soil extracts (Daigh et al., 2014; Greenan et al., 1995; Sullivan and Havlin, 1991).

Interestingly, the DAM assay was first developed for the determination of the amino acid citrulline and subsequently adapted to detect urea, diacetyl. The assay relies on the production of diacetyl from diacetyl monoxime under acidic conditions, which reacts with urea to form an unknown chromogen, by an unknown mechanism, which is detected at 520 nm (Archibald, 1945; Butler and Walsh, 1982; Fearon, 1939; Lugosi et al., 1972; Veniamin and Vakirtzi-Lemonias, 1970). The reaction has been adapted for automation using continuous flow analysis and flow injection analysis (FIA), enabling high sample throughput and high reproducibility, at low reagent costs. However, such colorimetric methods are known to be non-specific, and the non-specificity of this method for urea detection has been noted previously in biological fluids (Archibald, 1945). However, the consequences of this have not yet been investigated in soil. Critically, this method is commercially marketed as a method to detect urea in

environmental samples, with few caveats to acknowledge potential interference or cross-reactivity in such settings. Other colorimetric methods, such as the molybdenum blue method for orthophosphate have addressed the potential for interference, e.g. known silicate interference prompted method development to address this (Murphy and Riley, 1962; Neal et al., 2000). In the urea method, no such adaptation is currently available, and the specificity/non-specificity of the method for determination of urea in solution is undefined. Significantly, citrulline and alloxan are expected to cross-react under the reaction conditions and adaptions of the DAM colorimetric method have been used to quantify these compounds in biological fluids (Archibald, 1945; Fearon, 1939; Satoh and Katoh, 1989). Citrulline interference has been evaluated in natural waters with the chromogen produced with the DAM colorimetric reaction conditions separated from urea by liquid chromatography (LC), however, the question of non-specificity with other compounds in relation to determined urea concentrations was not addressed (Satoh and Katoh, 1989). As far as we are aware, a thorough investigation of environmentally relevant compound classes cross-reacting under the reaction conditions used to produce a response at 520 nm has not been undertaken.

One approach to assessing the potential for cross-reactivity in the DAM assay would be to submit known concentrations of candidate compounds to the assay and determine their responses relative to known concentrations of urea determined under the same assay conditions. An alternative approach would be to employ an independent reference assay for urea and compare the results of this assay to the results of DAM based analyses. The reference assay of choice is HLPC-MS, with the latest generation of high resolution (HR) MS instruments, e.g. Orbitraps, offering the possibility for assessing urea concentrations in complex biological and environmental matrices at previously unattainable selectivity and specificity (Clark et al., 2007; Kind et al., 2007; Warren, 2014). Use of such an HPLC-HRMS

method for urea in the environment would allow unambiguous identification and quantification, facilitating rigorous evaluation of the DAM colorimetric method.

Herein, we assess the potential for a range of potentially cross-reacting compounds to interfere in the "off-the-shelf" DAM colorimetric method. The compounds chosen are likely to be present in environmental matrices and represent a range of compound classes that will allow a better understanding of the reasons underlying the non-specificity of this method. The results obtained are assessed in relation to previous determinations undertaken for biological matrices, but which up to now are lacking for environmental matrices likely to contain cross-reacting compounds. A reference HPLC-HRMS assay is then described which allows direct determination of urea in soil extracts. This allowed rigorous assessment of the degree of overestimation by the DAM colorimetric method and demonstrated an effective reference method is vital for cross-validation of the less specific and selective automated and low-cost colorimetric method.

2. Methods

2.1. Colorimetric detection of urea

Urea concentrations were determined using the Skalar San $^{++}$ system (Skalar Analytical B.V., The Netherlands), a continuous flow analyser with a series 1050 sampler and data collected and analysed on the San^{++} FlowAcess^{TM} V3 data acquisition Windows® software package. All reagents and standards were from Merck (Germany) and were prepared according to Skalar method for the analysis of urea (Catnr 612-001 issue 080714/MH/99290607). A urea stock standard solution was prepared monthly (100 mg N l^{-1}) and working standards prepared daily (0-1.0 mg N l^{-1}). The colour reagent was prepared with diacetyl monoxime (DAM; 4.125 g l^{-1}), semi carbazide hydrochloride (0.05 g l^{-1}), manganese (II) chloride ($MnCl_2.4H_2O$, 13.075 g l^{-1}) and potassium nitrate (KNO_3, 0.25 g l^{-1}). A saturated sodium chloride solution was also

prepared with Brij 35 (3 ml l^{-1}). The acid reagent was prepared from sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$, 50g l^{-1}) and sulfuric acid (H_2SO_4, 97 % *v/v*, 588 ml l^{-1}). All analyses were carried out in triplicate.

2.2. Analysis of standards to identify cross-reactivity

A range of commercially available compounds were used to test the specificity of the DAM colorimetric method. Initial standard solutions were prepared to identify compounds which showed a positive response. Nitrogenous compounds tested were: allantoin, xanthine, hypoxanthine, biuret, alloxan monohydrate, glucosamine, galactosamine, mannosamine, adenine, cytosine, guanine, thymine and uracil (Sigma-Aldrich, UK). Sugars, including ribose, glucose, sucrose, fructose, rhamnose, lyxose, xylose, arabinose, galactose and fucose (Acros Organics, UK) were also tested. Other compounds tested included myo-inositol and oxalic acid (Sigma Aldrich, UK). Solutions at a concentration of 2 mg l^{-1} were prepared in purified water (Millipore). Standard solutions were analysed using the described DAM colorimetric method and compounds which showed a positive response (allantoin, alloxan, biuret, hydantoin), or interference (xanthine, hypoxanthine, adenine, cytosine, guanine, thymine, uracil, fructose) were analysed at a range of concentrations 0.01–10 mg N l^{-1} or 0.1-10 mg l^{-1}, prepared weekly from stock solutions (100 mg N l^{-1} / 100 mg l^{-1}). Whilst this is higher than expected concentrations of such compounds in environmental matrices, this concentration range was selected to ensure compounds exhibiting cross-reactivity would be identified and the degree of response over a range of concentrations detectable once cross-reactivity had been identified.

2.3. UV-Vis absorbance

UV-Vis absorption measurements of the standard solutions (concentrations shown in table 1) were carried out on a Cary 60 UV-Vis spectrophotometer (Agilent Technologies Inc.,

US) between 200-800 nm and recorded with Cary WinUV Software (Agilent Technologies Inc., US). The absorption spectra were background corrected, and instrument wavelength accuracy confirmed using a holmium perchlorate standard (Starna Scientific, USA).

2.4. Sample collection and extraction

Soil samples were collected from eleven grassland sites in the UK (see table S1 for further site information). Three replicate soil samples from each site were collected at random, bulked and stored at 4 °C until extraction. Prior to extraction, plant material, macrofauna and stones were removed by sieving (2 mm). Triplicate soil samples (2 g) were treated with double distilled water (soil: double distilled water (DDW) ratio of 1:10 *w/v*) and shaken at room temperature (1 h at 400 rev min^{-1}). Following this, the soils were centrifuged (10 min, 1690 x g), the supernatant was removed, and residues washed with a further 5 ml of DDW and the supernatant combined to give an overall soil to extractant ratio of 1:12.5 *w/v*. Soil extracts were stored at 4 °C for 24 h prior to analysis, and were diluted with purified water (Millipore) at a ratio of 1:2 *w/v* soil extract to DDW for colorimetric urea determination.

2.5. Reference LC-MS assay

To directly determine urea concentration in soil extracts, a reference LC-MS assay was used. The LC separation was performed using a 150 mm x 2.1 mm i.d., 1.7 µm Synchronis HILIC column (Thermo Scientific) operated by a Dionex Ultimate 3000 HPLC system (Thermo Scientific). The mobile phase comprised of solvent A, 0.1 % (*v/v*) formic acid in water and solvent B, 0.1 % (*v/v*) formic acid in acetonitrile (ACN). The LC mobile phase was held at 80 % B for 2 min, followed by a linear gradient to 20 % B (15 min), 1 min ramp to 80 % B and a 15 min re-equilibration at 80 % B. The flow rate was 300 µl min^{-1}, column temperature was 30 °C and injection volumes were 20 µl. The column was directly interfaced to an Orbitrap

189 Elite mass spectrometer (Thermo Scientific). The FTMS was operated in full scan mode (*m*/*z*
190 50 to 400; 60,000 mass resolution) and to improve sensitivity to urea, the ion trap was set to
191 scan a range of *m*/*z* 60 to 62. The ion source parameters were: sheath gas (N_2) flow 60 arbitrary
192 units (arb), the auxiliary gas (N_2) flow and the sweep gas (N_2) flow both set to 0 arb. The
193 capillary temperature was 275 $^{\circ}$C. The electrospray was in positive ionisation mode, with a
194 source voltage of 3.00 kV. The data was acquired and analysed using Thermo Xcalibur (version
195 3.0). To ensure instrument performance, urea standards (80 % ACN; 0.1 to 5 mg l^{-1}) were
196 analysed and subsequent analyses only accepted when the calibration graph had R^2 >0.99.
197 Between each triplicate analysis of samples, a urea standard (1 mg l^{-1}) was analysed to check
198 instrument performance.
199 The soil extracts were prepared as above, and 5 ml sub-samples concentrated 10-fold
200 using a stream of nitrogen, and brought to 80 % ACN. Quantification of urea was achieved
201 using standard addition as ion suppression interfered with quantification using an external
202 calibration curve. A urea standard in purified water (Millipore) was prepared (8 mg ml^{-1}) and
203 added to the concentrated extracts to give three spike concentrations (0.4, 2.0 and 4.0 mg l^{-1})
204 to allow quantification by standard addition and analysed in triplicate.
205
206 *2.6. Statistical analysis*
207 All statistical analyses were performed using SigmaPlot 13.0 (Systat Software Inc.).
208 For all statistical analyses and regressions, data was tested for normality (Shapiro-Wilk) and
209 homogeneity of variance (Brown-Forsythe). A one-way ANOVA was used to evaluate
210 differences between treatments and the significance level was set at $P \leq 0.05$ for all statistical
211 analyses.
212
213 **3. Results**

3.1. Cross reactivity and interference of standards with the DAM colorimetric method

The reference compounds (as shown in Table 1) were analysed at concentrations above expected environmental concentrations to determine any cross-reactivity under the experimental conditions for the DAM colorimetric determination of urea. Compounds which showed a response at this concentration (allantoin, alloxan, biuret, fructose, hydantoin, sucrose) were subsequently analysed at a range of concentrations (0.1 to 10 mg N l^{-1} or mg l^{-1} compounds containing no N) to determine the linearity and degree of response under the experimental conditions. The other compounds tested did not exhibit cross-reactivity or interference with the detection of urea by this method, in agreement with Douglas and Bremner (1970).

The purine and pyrimidine derivatives (hypoxanthine, xanthine, adenine, uracil, thymine, guanine and cytosine) were analysed separately at concentrations between 0.01 to 5 mg N l^{-1}. It was determined that this group of compounds did not exhibit a linear response under the experimental conditions, however, they appeared to cause baseline instability. A similar baseline instability was observed for the sugars fructose and sucrose (0.1 to 10 mg l^{-1}), which had been previously observed (Prescott and Mangnall, 1976). It was not possible to quantify the interference, as the baseline interference caused by these compounds was irreproducible over triplicate determinations and separate analytical runs. Allantoin, alloxan, biuret and hydantoin showed a linear response with increased concentration under the conditions used in the DAM colorimetric determination of urea, as shown in Fig. 1. The response of these compounds relative to urea at the same concentrations varies across the concentration range tested as shown in Table 2.

To confirm there was no intrinsic absorbance at 520 nm for the selected standards which would result in a response under the analysis conditions without cross-reaction with the reagents, the reference compounds were analysed using UV-Vis at the concentrations shown in Table 1. There was no absorbance observed at 520 nm for any of the compounds tested,

239 indicating no intrinsic interference at this wavelength. Therefore, any interference or cross-
240 reactivity observed with these compounds is solely due to a response generated by the
241 analytical reagent conditions.
242
243 *3.2. Stability of colour reagent*
244 During analysis of standards to determine cross-reactivity and interference, it was noted
245 that there was a decrease in determined concentration over the course of a week (as shown in
246 Fig. S1). Therefore, repeated determinations over the course of one week with the same colour
247 reagent and working reference compounds made up daily were undertaken to assess the change
248 in response with colour reagent age for urea and the cross-reacting compounds. For urea, the
249 decrease in response after 4 d was within analytical error (less than 0.01 mg N l^{-1}), however,
250 for allantoin and hydantoin, the decrease in response on day 4 was greater than the acceptable
251 analytical error (±0.05 mg N l^{-1} for allantoin and alloxan; ±0.01 mg N l^{-1} for hydantoin). For
252 allantoin, alloxan and hydantoin, an analysis of variance showed the effect of reagent age on
253 the determined concentration was significant (one-way ANOVA; $P < 0.05$), although the
254 determined concentrations for urea and biuret did not differ significantly with reagent age ($P >$
255 0.05). The analyses were repeated using colour reagent prepared daily and no decrease in
256 response was observed after 4 d, indicating the colour reagent degraded over time and must be
257 prepared daily to ensure there is no decrease in response due to reagent degradation.
258
259 *3.3. Urea concentration in soil extracts determined using the DAM colorimetric method and*
260 *LC-MS*
261 Urea was directly detected in soil extracts using LC-MS following HILIC separation,
262 eluting at 2.1 min, after the initial column break-through. Fig. 2 shows a typical extracted ion
263 chromatogram at *m*/*z* 61.08 (±0.1 Da), the $[M+H]^+$ ion for urea, in a soil extract. Urea was

added at three concentrations to enable urea quantification in the soil extracts by standard addition (Fig. 2e). It was determined that the peak area for the $[M+H]^+$ ion was linear with respect to urea concentration for both urea standards (concentration range from 0 to 12 mg l^{-1}) and in spiked soil extracts, therefore it was not necessary to use other ions (such as *m/z* 121.2 $[2M+H]^+$) for quantification.

The determined urea concentrations in soil extracts for the DAM colorimetric method and the LC-MS assay are shown in Table 3. Determined urea concentrations for the DAM colorimetric method were between 1.5 (S.D. 0.08) and 17.5 (S.D. 0.1) µg g^{-1} soil whilst the urea concentration determined by the LC-MS method ranged between 0.067 (S.D. 0.0007) and 0.71 (S.D. 0.01) µg g^{-1} soil. To evaluate the degree of overestimation observed across the sites, the fold overestimation was calculated (Table 3) for the eleven grassland soils studied. UV-Vis determinations for soil extracts (soil-extract solution 1:12.5 *w/v*) showed no significant absorbance at 520 nm, indicating no intrinsic absorbance at the wavelength at which the chromogen is measured for the DAM colorimetric method. The limit of detection (LOD) for the LC-MS method was determined to be 0.1 mg l^{-1}, in line with similar LC-MS methods for urea (note soil extracts were concentrated 10 fold prior to analysis, thus the LOD accounting for the concentration step is 0.01mg l^{-1}; Warren, 2014).

4. Discussion

4.1. Cross-reactivity and interference with the DAM colorimetric method

The compounds tested for cross-reactivity and interference with the DAM colorimetric method are all compounds which might be expected in environmental matrices from a range of natural and anthropogenic sources (Table S2 shows the variety of compound classes investigated). Interference caused by purine and pyrimidine bases, found in the environment in the form of DNA, RNA, ATP, ADP, etc. and purine degradation products reduce base-line

289 stability and therefore the reliability of urea concentrations determined by this method for
290 environmental matrices. It is not practically possible to quantify the interference in
291 environmental matrices, due to varying concentrations of the interfering compounds.
292 Interference at less than 10 times the concentration of urea N has not been previously reported
293 (such as for uracil) indicating different reaction conditions (e.g. colour reagent concentration,
294 stabilising agent and acid reagent composition) may offer an opportunity to reduce this baseline
295 instability using continuous flow and flow injection analyses (Price and Harrison, 1987).

296 Current protocols for the "off-the-shelf" method suggest the colour reagent is stable for
297 one week, however, we have demonstrated here that colour reagent degradation occurs within
298 3 days of preparation, and a decrease in response is observed for cross-reacting compounds.
299 The observed decrease in response for analyses of urea standards is within acceptable analytical
300 error for this compound. However, it should be noted, the observed drop in the determined
301 concentration for cross-reacting compounds is greater than the acceptable analytical error (0.05
302 mg N l^{-1}). The concentration of cross-reacting compounds in environmental matrices is
303 unknown, and variable, therefore the changing response of these compounds with colour
304 reagent age reduces the reproducibility of ureido-N concentrations determined by the
305 colorimetric method. Therefore, it is recommended that the colour reagent is made up daily
306 and used for a maximum of 24 h to prevent degradation of the reagent to an extent that results
307 in variation in the measured urea (or ureido) concentration in environmental matrices.

308 Cross-reactivity for this method has been demonstrated before in biological samples
309 (Archibald, 1945, 1944; Fearon, 1939), however, the degree of cross-reactivity for the "off-
310 the-shelf" DAM colorimetric method has not been assessed for these compounds and others of
311 the same compound class, which this work has undertaken. The key structural moiety present
312 in these compounds is the ureido group (highlighted in Fig. 3). Compounds with the general
313 structure $R_1NHCONHR_2$ will react with diacetyl monoxime under acidic conditions to yield a

chromogen which absorbs at 520 nm. The degree of absorption at this wavelength depends on the substituents R_1 and R_2. It is important to note that whilst the chromogen structure has been hypothesised (e.g. substituted 1,2,4-triazine (Beale and Croft, 1961; Lugosi et al., 1972), imidazole ring (Lugosi et al., 1972; Veniamin and Vakirtzi-Lemonias, 1970), or a skipped diene susceptible to oxidation to generate a carbonium ion under acidic reaction conditions (Butler and Walsh, 1982)) it has not been confirmed. Elucidating the mechanism of chromogen formation and structure in future research may help understanding the relative response of cross-reacting ureido compounds compared to urea.

The ureido group is a common feature in biological compounds, such as citrulline and allantoin, shown in Fig. 3b, accounting for between 4-8 % of ruminant urinary-N (Bristow et al., 1992). Furthermore, compounds containing the ureido group are also used in agriculture, with biuret an impurity in urea fertiliser and utilised as a non-protein nitrogen (NPN) in animal feed and hydantoin derivatives applied as pesticides and fungicides (e.g. miprothin and iprodione). Allantoin, biuret and hydantoin can all be expected in inputs from agricultural settings, alongside urea, and therefore will contribute to urea concentrations determined by the DAM colorimetric method due to cross-reactivity in such sites. They are also likely to be available to the biota to support primary production, in much the same way as urea is. The size and composition of the ureido-N pool will vary with different land uses due to different inputs of ureido compounds (such as an arable crop, which may receive urea inputs as fertiliser, compared to a grazed grassland, which would have urea and allantoin inputs from ruminant urine). Subsequently, the different levels of response relative to urea of ureido compounds (Table 2) means there will be varying degrees of overestimation of urea depending on the concentration of individual compounds within the ureido-N pool, which extends beyond the compounds identified in this study due to the ubiquity of this moiety in both natural and anthropogenic compounds. Therefore, a direct method of determination for urea was required

to confirm the presence of cross-reactivity by comparison of urea concentrations determined by the colorimetric method and a direct LC-MS assay. Such analyses are needed to ensure the commercial "off-the-shelf" method has been sufficiently validated for the environmental application (Glibert et al., 2006).

4.2. LC-MS assay confirms overestimation of urea

The LC-MS assay described enabled direct determination urea in environmental matrices (using standard addition) with no interference as observed with the DAM colorimetric method. This provides a specific determination for urea, enabling investigation into the degree of overestimation of urea by the DAM colorimetric method. This is illustrated in Fig. 2, where the extracted ion chromatogram (*m/z* 61.08) for urea at three spike concentrations was used to construct the standard addition calibration curve and determine the urea concentration in the soil extracts. It was necessary to quantify using standard addition due to the presence of ion suppression in the area of elution in the chromatogram for urea. This was confirmed by spiking a soil extract with urea at a concentration equivalent to 12 mg l^{-1} with a spike recovery of 18 % (S.D. 3.2 %) indicating suppression of MS response. Ion suppression is widely recognised phenomenon which arises in the analysis of complex mixtures due to the presence of non-volatile analytes which reduce the efficiency of ionisation for the analyte of interest (in this case urea), Quantification via standard addition is advantageous as it can correct for ion suppression as the same sample matrix is present and a calibration is based on the known added urea concentration. Since ion suppression affects the analyte and calibrant urea equally the absolute ratio is maintained, hence, accurate quantification of urea in soil extracts is achievable.

Overestimation by the DAM colorimetric method is confirmed using the LC-MS reference assay, which detects urea based on *m*/*z* 61.08, due to lower concentrations observed by the direct method compared to the indirect method (Table 3). The degree of overestimation

varies across the grassland sites, with the urea concentration determined by the DAM colorimetric method between 7.2 and 58.9 times higher than the directly determined urea concentration using LC-MS. The degree of overestimation is not consistent with land cover (all sites were grasslands), soil texture or geographical location, and it is likely this variation in overestimation can be attributed to the varying composition of the ureido pool in soils, in both concentration and compounds. This has implications for our understanding of the importance of urea in soils as it is overestimated to different degrees in different settings and therefore urea concentrations determined by this method are not comparable, unless reported as ureido-N. Possible implications of this would be overestimation of the concentration of anthropogenic urea, or underestimation of urea utilisation by soil microbes. Ideally, it would be useful to characterise the nature of the of the ureido compounds present, however, this is impractical in such a complex matrix. It was for this reason we undertook to use known natural and anthropogenic compounds, representative of those likely to be present, to demonstrate that it is the ureido group that is responsible for DAM cross-reactivity. Other compounds with the ureido group include pharmaceuticals, mammalian metabolites, antibacterial and antifungal agents in personal care products and plastics, insecticides, pesticides and food products (Brausch and Rand, 2011; Lewis et al., 2016). Due to the widespread occurrence of the tested compounds and related ureido compounds, it can be expected a number of ureido compounds will be present in environmental matrices, contributing an unknown and variable degree to urea concentration when analysed using the DAM colorimetric method.

4.3. Recommendations for the DAM colorimetric method for urea determinations

The results presented above highlight considerable concerns in the use of the DAM colorimetric method for urea determinations in environmental matrices. Our findings using model compounds unequivocally confirm the cross-reactivity of the ureido group, a common

moiety in natural and anthropogenic compounds, alongside interferences from sugars and purine and pyrimidine derivatives. The colorimetric method should be considered a useful technique to quantify ureido-N as it is an inexpensive and quick tool (extraction and analysis of 60 soil extracts per day) relative to the more intensive and time-consuming LC-MS method (extraction and analysis of *ca.* 16 soil extracts per day as 3 analytical runs are required per soil for standard addition). The type of studies where this would be relevant include studies of the fate of added urea on a temporal or spatial scale, where the cross-reactivity can be accounted for by proper use of controls. It is also a useful tool for comparison of the ureido-N pool between natural systems, which is an environmentally relevant and biologically available pool. This is also the case for existing studies comparing urea in soil extracts, which should be considered as reporting ureido-N due to the unknown contribution of this pool to the reported urea concentration.

Given the ubiquitous occurrence of these compounds containing the ureido group in the environment the DAM method is an inappropriate choice for quantitative analysis of urea in environmental matrices. Due to the ubiquity and unpredictability of ureido and interfering compounds in environmental samples it is unfeasible to identify and quantify their contributions to the urea concentrations determined by the DAM method, negating any ideas of applying "corrections". It is therefore suggested that:

(i) Results reported from the DAM colorimetric method should include a caveat recognising the presence of cross-reactivity and interferences in environmental matrices, reporting determined concentrations as "ureido-N".

(ii) It is vitally important to monitor the stability of reagents used in the DAM colorimetric method since deterioration results in changing responses affecting reproducibility of ureido-N concentrations.

(iii) If accurate determinations of concentrations of urea are required, then HPLC-HRMS is the preferred approach.

(iv) It is important to properly validate the DAM method in all the environmental matrices this method is currently applied to.

We will be discussing our findings with the marketers of the commercial assay to help them to re-design their user guidelines to ensure reagent stability and sufficient method validation for the DAM colorimetric method for the matrix under analysis.

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562

563 **Figure legends**

564

565 **Fig. 1** Calibration curves for (a) allantoin, (b) alloxan, (c) hydantoin, and (d) biuret under the
566 DAM colorimetric reaction conditions at concentrations between 0.1 to 10 mg N l^{-1}.

567

568 **Fig. 2** Extracted ion chromatograms for urea $[M+H]^+$ (*m*/*z* 61.08) for a soil extract from
569 Merddwr, Conwy with (a) no added urea spike, and (b) 0.4 mg l^{-1}, (c) 2.0 mg l^{-1} and (d) 4.0 mg
570 l^{-1} added urea spikes used for quantification by standard addition and (e) the standard addition
571 calibration curve used to determine the urea concentration in the soil extract (0.709 µg g^{-1}).

572

573 **Fig. 3** (a) Ureido group responsible for the cross-reactivity with the DAM colorimetric method,
574 and environmentally relevant compounds containing the ureido group which cross-react: (b)
575 allantoin; (c) hydantoin and (d) biuret.