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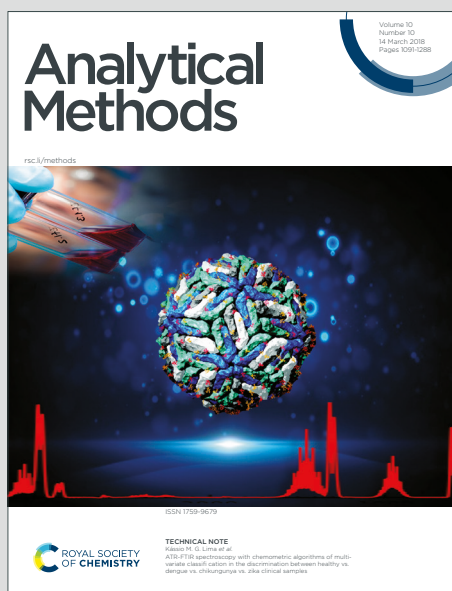
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3 **QuEChERS: a simple extraction for monitoring quaternary ammonium biocide** View Article Online
4 **pollution in soils and antimicrobial resistance.** DOI: 10.1039/D0AY01324B
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Abbreviations

BAC-C12 – Benzyltrimethylammonium chloride,

BAC-C14 – Benzyltrimethylammonium chloride,

d₇-BAC-C14 – d₇- Benzyltrimethylammonium chloride,

d₉-HDTMA - Hexadecyltrimethylammonium bromide-d₉, dSPE – Dispersive solid-phase

extraction, HDTMA – Hexadecyltrimethylammonium chloride, MgSO₄ – Magnesium

sulphate, NaOAc – Sodium acetate, QAC – Quaternary ammonium compound, QuEChERS

– Quick, Easy, Cheap, Effective, Rugged and Safe

Keywords

Environmental solids, Liquid chromatography-mass spectrometry, Biocides, Antimicrobial
resistance, QuEChERS

Abstract

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Quaternary ammonium compounds (QACs) are broad-spectrum disinfectants used in a range of everyday materials. Their high usage rates, limited regulation and reporting has meant their environmental release is largely uncontrolled and impact unknown. With links to antimicrobial resistance (AMR) and adsorption to wastewater solids (that are recycled), there is a need for more controlled disposal measures and monitoring. These environmental matrices are highly complex requiring methods that are often laborious and costly to undertake. Using a robust quantitative reversed-phase LC-MS/MS method, we have shown that an 'off the shelf' QuEChERS product can reliably extract (<10 %RSD) aromatic and aliphatic QACs anticipated within municipal, industrial and agricultural waste from water and soil, with reduced matrix effects of 95.7-104.4% for recoveries of up to 53% from soil when combined with extract dilution. Therefore, unlike current literature, this work has shown that, with minimal development, the QuEChERS product can provide a rapid, effective and low cost preparation for quantifying QAC pollution and monitoring AMR.

74 Introduction

75 With a move to a Circular Economy the reuse of solid sludge waste from wastewater (WW)
76 treatment is becoming increasingly popular, with the deposition of municipal waste on
77 agricultural land as fertiliser (80%).¹ However, given many pollutants can remain within this
78 organic material,^{2,3} the analysis of these environmental solids and soil is becoming more
79 important for environmental and public safety. Quaternary ammonium compounds (QACs) are
80 a common broad-spectrum disinfecting agent and preservative within a range of everyday
81 products⁴⁻⁶ and industries,⁷⁻⁹ used to inhibit microbial growth (at a minimum inhibitory
82 concentration (MIC) of 0.5-5 mg/L) or cause cell death (minimum bactericidal concentration
83 (MBC) of 10-50 mg/L).⁵ Limited regulation governing the reporting levels in the majority of
84 these products and poor efficacy of WW treatment for many chemicals,¹⁰⁻¹² along with their
85 adsorption to environmental solids,¹³ has meant that environmental exposure to these cationic
86 surfactants through domestic and industrial WW has been largely uncontrolled, with a need to
87 establish their fate and effects.^{7,14} However, studies concerning the use of QAC detergents
88 have shown an increase in biocide and multi-drug (antibiotic) resistance,^{4,15-20} via the
89 increased expression of genes for efflux pump proteins that actively remove biocides from the
90 cell.¹⁵ These antimicrobial resistance (AMR) genes have also been observed across species⁴
91 and further add to concerns that the unrestricted environmental release of QACs can result in
92 the over-exposure of bacteria to sub-MICs and AMR, highlighting the need for methods that
93 monitor the abundance and impact of these compounds in environmental solids.

94 The molecular analysis of environmental solids is highly challenging due to their
95 complexity and the sorption of trace material to more abundant (lipophilic) analytes in the
96 sample (e.g. fulvic and humic acids).² To displace the analyte from the matrix multi-step
97 sample preparations, that can take multiple hours per sample³ and often with variable
98 performance, are commonly used. Existing protocols to measure QAC biocides in
99 environmental solids are an example of this, with more successful and highly cited methods
100 needing more than an hour²¹⁻²⁴ per sample and/or do not report data concerning matrix
101 effects.^{21-23,25} The Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) method²⁶
102 offers significant flexibility for method development, and can facilitate screening of
103 environmental samples for pollutants by targeting the removal of abundant hydrophobic
104 interferences through selected dSPE materials (e.g. C18 or graphitised carbon black (GCB)).
105 Recent work with QuEChERS has shown the potential for measuring surfactants^{13,27-30} and
106 extracting environmental solids with low matrix interference.^{29,31} However, these protocols do
107 not cover the breadth of surfactant biocides anticipated in environmental solids,^{13,29-31} have
108 used a bespoke extraction product,²⁹ require additional steps to the protocol,¹³ or do not
109 address samples, such as soil.^{27,28} Given this, we believed QuEChERS could provide an
110 alternative single protocol extraction for the range of common QAC disinfectants from soil

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3 111 anticipated via exposure to municipal, industrial and agricultural waste, as a more rapid, cost
4 effective method that exhibits low matrix interference. We have therefore investigated the
5 112
6 113 usability of an 'off the shelf' QuEChERS product as part of a quantitative analytical workflow,
7
8 114 to measure these highly important QACs within fortified soil, as a much-needed monitoring
9
10 115 platform for AMR following WW contamination.
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117 **Experimental**

118 Chemicals and reagents

119 Aromatic and aliphatic QACs commonly used in disinfectants and preservatives were selected
120 for method evaluation. These included benzyldimethyldodecylammonium chloride (BAC-C12),
121 benzyldimethyltetradecylammonium chloride (BAC-C14),
122 benzyldimethylhexadecylammonium chloride (BAC-C16), didecylmethylammonium bromide
123 (DDMAB) and hexadecyltrimethylammonium chloride (HDTMA), obtained as solid standard
124 reference materials from Sigma Aldrich (Poole, UK). While stearalkonium chloride (BAC-C18)
125 was obtained from LGC (Teddington, UK) and the deuterated internal standards (ISs),
126 benzyldimethyltetradecylammonium chloride-d₇ (d₇-BAC-C14) and
127 hexadecyltrimethylammonium bromide-d₉ (d₉-HDTMA), were sourced from Toronto Research
128 Chemicals (Ontario, Canada). All reference materials were hygroscopic and stored under
129 argon gas in a vacuum desiccator. For the analysis and preparation of solutions and samples,
130 oxygen-free nitrogen (OFN) was purchased from BOC gas (Port Talbot, UK), with acetonitrile
131 (ACN), water (H₂O) and formic acid (FA) from Fisher Scientific (Loughborough, UK).
132 QuEChERS materials were obtained from Biotage (Uppsala, Sweden) and included standard
133 EN extraction and dSPE tubes of EN 'Waxed Fruit and Vegetables' (containing C18) and EN
134 'Pigmented Fruit and Vegetables' (containing graphitized carbon black (GCB) as detailed in
135 the Supplementary material. As a test environmental matrix, garden topsoil was collected from
136 an undisclosed location in West Wales, mixed and lyophilized to standardise the hydrophilic
137 (water) content of the soil matrix in readiness for sample preparation.
138

139 Instrumentation

140 Sample separation was undertaken using a Thermo Scientific Surveyor Autosampler (AS) and
141 MS PumpPlus LC system (Hemel Hempstead, UK) operated with a 3 µm 100 x 1 mm C18
142 Thermo Hypersil Gold column and a 5 µm 10 x 1 mm C18 Thermo Hypersil Gold guard
143 cartridge (Runcorn, UK). Mass analysis was performed with a Thermo Scientific LCQ Classic
144 ion trap (Hemel Hempstead, UK) operating with an electrospray ionization source in positive
145 mode. Both instruments were controlled using Xcalibur 2.0.
146

147 LC-MS methodology

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3 148 Mobile phases for the LC separation consisted of 0.1% FA in H₂O (A) and 100% ACN (B) with
4 an injection wash of 90% ACN and 10% H₂O (all 0.1% FA final concentration). Each sample
5 149 (5 µL) was injected on column at a mobile phase flow rate of 50 µL/minute operating with a
6 150 gradient elution; starting at 75% A:25% B, increasing to 100% B from 2-24 minutes, with a 20
7 151 minute wash prior to reconditioning at 75% A for 10 minutes. The mass spectrometer was
8 152 operated using a spray voltage of 4.5 kV and capillary temperature of 200 °C. Following
9 153 identification of the QAC precursor ion species, full mass scan (*m/z* 100-500) and multiple
10 154 reaction monitoring (MRM) analyses were used for the four BAC compounds, DDMAB, and
11 155 d₇-BAC-C14 (see Supplementary material for transitions and optimised collision energies
12 156 (%CE)). For HDTMA and d₉-HDTMA a single ion monitoring (SIM) acquisition was used as a
13 157 stable fragment ion could not be obtained. With a minimum number (≥10) of mass spectra for
14 158 quantitation, the data was processed using Xcalibur 2.0 and Microsoft Excel 2010.
15 159
16 160

161 Stock and working solutions

162 Individual 1 mg/mL stock solutions were prepared in 100% ACN and stored at -20°C prior to
163 use. For method evaluation a 'double blank' (S_b), a standard blank with IS (S₀), eight non-zero
164 calibration standards (2-100 ng/mL), and four quality controls (8, 20, 60, 90 ng/mL) were
165 prepared in 50:50 ACN:H₂O with relevant solutions containing an IS concentration of 30
166 ng/mL.
167

168 Sample preparation

169 The sample preparation procedure was tested using extracted quality controls (QCs) by
170 comparing the analyte peak area spiked before and after extraction³² (denoted SBE and SAE
171 respectively), initially in water (total 4 mL) and then soil (2.5 g based on preliminary in-house
172 screening). Performance figures of merit included percentage matrix effects (%ME), recovery
173 (%REC) and process efficiency (%PE), with the respective precision given as %RSD (see
174 Supplementary material). To confirm selectivity, additional solvent and matrix ('double') blanks
175 were prepared for the relevant samples. All samples were prepared in triplicate and fortified
176 by spiking with the QAC mixture and the IS sub-stock at an equivalent concentration of 60 and
177 30 ng/mL, respectively, with a further volume of H₂O (to equate a total volume of 4 mL) added
178 to the soil sample prior to extraction. To establish the effect of extract dilution the spike
179 concentration was increased proportionally for a 1:400 dilution, based on a mid-point
180 estimated from preliminary screening data for relevant environmental samples. For SAE
181 samples equivalent spike volumes of 50:50 ACN:H₂O and H₂O were added to the samples
182 and vortexed. To extract the samples 10 mL ACN and the EN extraction tube were added,
183 manually shaken for 1 minute and centrifuged at 4000 rpm for 5 minutes at room temperature.
184 The resulting supernatant was transferred to the dSPE tube, vortexed for 1 minute and

185 centrifuged at the same conditions. The final supernatant was transferred to a clean tube and
186 evaporated to dryness under a gentle stream of nitrogen. For undiluted samples, blanks and
187 SBE extracts were reconstituted in 2 mL 50:50 ACN:H₂O, while the SAE extracts were
188 reconstituted in an equivalent volume of ACN:H₂O, spiked with QAC and IS mixtures to
189 achieve a concentration of 60 ng/mL and 30 ng/mL, respectively. However, for diluted blanks
190 and SBEs, extracts were reconstituted as above but with a 1:400 reduction in concentration,
191 while for the SAE, the extract was diluted in a volume of 50:50 ACN:H₂O containing 60 ng/mL
192 and 30 ng/mL of QAC and IS mixtures, respectively.

194 Results and Discussion

195 Analytical method selectivity

196 During full scan analyses the base peak of each QAC showed a precursor ion consistent with
197 the loss of the halide ion, representing the anticipated singly-charged molecular cation of these
198 salt species ([M-X]⁺). Fragmentation of the aromatic QACs at optimised %CE primarily
199 generated product ion species related to the alkyl chain with the loss of the head group, and
200 for DDMAB, the loss of a single alkyl chain. Unfortunately, stable fragment ions for HDTMA
201 and d₉-HDTMA could not be obtained, and therefore SIM was used for quantitation (see
202 Supplementary material). Once established, the LC method was developed for the mixture of
203 standards based on initial in-house work; this employed a solvent gradient to achieve
204 appropriate resolution with analyte separation according to hydrophobicity (as anticipated for
205 reversed phase) and no clear evidence of aggregation. To minimise carryover, a compromise
206 was required between chromatographic resolution and analysis time, with significant washing
207 of the system needed. Following optimisation, the chromatographic selectivity was confirmed
208 for the sample types and the stability of the chromatographic method was characterised,
209 recording the mean, intra- and inter-precision (represented by %RSD and two-tailed F-test,
210 respectively) of the relative retention time and peak area. Pleasingly, the separation showed
211 good repeatability (<1.5 %RSD) and reproducibility, with a stable performance between the
212 two days (see Table 1 and Supplementary material). The chromatographic peak area was
213 also largely reproducible with only BAC-C14 showing a significant difference in precision
214 between day 1 and day 2. However, given this remained <7% RSD the method was
215 considered suitable for proceeding with further method evaluation.

217 Method evaluation for quantitation: Calibration homoscedascity, linearity, limit of detection 218 (LOD), accuracy and precision

219 A calibration graph of the analyte peak area normalised to a relevant IS was constructed for
220 the analytes over the anticipated quantitative range (e.g. 2-100 ng/mL, see Table 2). Given
221 analytical measurements are often heteroscedastic, exhibiting unequal variance across the

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3 222 concentration range, linear and weighted regression relationships were assessed for each
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5 223 analyte, and selected according to the regression factor that provided the lowest relative error
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7 224 (%RErr) (see Supplementary material). A 1/x weighted regression typically provided the
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9 225 lowest %RErr, with analytes showing excellent linearity ($R^2 > 0.99$) apart from BAC-C18,
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11 226 however this remained within acceptable levels at an $R^2 > 0.98$. Interestingly, HDTMA
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13 227 displayed excellent linearity with either IS, showing the potential of this protocol to operate
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15 228 with a single IS if needed. As a measure of sensitivity, the LOD was determined using both
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17 229 statistical and empirical methods (see Supplementary material). Of these, the empirical
18
19 230 approach was selected for method evaluation as those determined statistically were
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21 231 inconsistent with the required signal/noise ($S/N > 3$) due to the heteroscedasticity of the data³³.
22
23 232 The empirical LOD showed sensitivity < 1 ng/mL apart for HDTMA, however, this LOD
24
25 233 remained sufficient at ~ 1 ng/mL, with either IS, for the anticipated target application based on
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27 234 in-house data, and is on par with recent studies that have quoted this metric^{13,24}. For each
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29 235 analyte, the quantitative precision and accuracy were established using replicate QCs at four
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31 236 concentrations within the dynamic range (see Supplementary material and Table 2). Most
32
33 237 pleasingly, all QCs showed good precision $\leq 12.6\%$ regardless of concentration, including
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35 238 HDTMA with either IS, confirming that a single IS approach can be a viable quantitative
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37 239 method. Furthermore, good accuracy ($< 14.8\%$) was determined for all compounds at each
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39 240 concentration, providing confidence that the method is capable of performing quantitative
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41 241 measurements.

242

243 Applicability of sample extraction for QAC biocides: fortified water and soil

244 An ideal preparative protocol should reliably extract analytes ($< 15\%$ RSD) with a low %ME
245 (value of $\sim 100\%$) and at high %REC. However, where signal enhancement or suppression is
246 present, good precision is essential for accurate quantitation to enable a valid measure of
247 analyte response by normalising the recovery (and overall quantified amount) and account for
248 this change in signal. QuEChERS is a well-established protocol that exhibits these
249 performance characteristics, using specific reagent blends for recognised standards (e.g. EN
250 and AOAC) to extract acidic and basic pesticides²⁶. However, the flexibility of QuEChERS
251 facilitates the screening of environmental samples for other pollutants, by selecting
252 appropriate dSPE material (e.g. PSA, C18 and GCB) to target the removal of abundant organic
253 interferences, such as humic and fulvic acids. Therefore, in the interest of method accessibility,
254 standard 'EN kits' containing hydrophobic dSPE sorbents were selected to target these
255 lipophilic interferences, and tested with fortified water and soil to determine method viability
256 for treated effluent and contaminated soil (and sludges), respectively.

257 Pleasingly, repeatable %ME were observed for both C18 and GCB sorbents however,
258 this was in the form of significant signal enhancement that increased with analyte

hydrophobicity from 107.0 to 151.3 %ME (see Supplementary material). Given the biocides are spiked after extraction in the same solvent as the QC for this calculation, this result implies that co-extractives from the QuEChERS reagents have enhanced the analyte signal, potentially through limited (previously undetectable) aggregation of the biocide. Limited but repeatable recoveries were also observed for more hydrophobic analytes, although this was anticipated due to the more challenging measurement conditions (e.g. at lower recoveries) observed for aromatic BACs and the aliphatic QACs using GCB and C18 sorbents, respectively. However, given adsorption to the dSPE material is a competitive process, recoveries were expected to improve with matrices containing increased amounts of organic matrix (e.g. environmental solids). This was observed for the majority of analytes (apart from HDTMA) within soil, however, with a significantly lower proportional increase of %ME with analyte hydrophobicity (apart from BAC-C18), with values ranging from 106.1-152.6% regardless of dSPE sorbent (see Figure 1). This improved performance was also observed for the recovery precision, with most compounds showing RSDs <19%. An exception to this was the recovery of BAC-C18 using the GCB sorbent, where greater hydrophobicity and retention appears to result in a more variable interaction with the sorbent. However, given the positive overall performance, the products were explored further and optimised to reduce matrix enhancement.

Sample dilution can offer a quick and simple approach to reduce matrix effects,³⁴ although the success of this method is highly dependent on maintaining sufficient analyte within the extract for measurement. Given the high anticipated levels of QACs in environmental solids, we tested this approach by diluting the extract (rather than the initial sample), to offer greater flexibility in accommodating different dilution factors if further method optimisation was required. Based on a preliminary screen of relevant environmental solids carried out in-house, a dilution factor of 1/400 was to ensure the final concentration would reside in the middle of the analytical method dynamic range. This required a 400x adjustment of the fortification concentration of the soil samples, and these were extracted as per the 'undiluted' extracts. Disappointingly, this adversely affected the %ME precision for the GCB extraction however, experiments undertaken with C18 dSPE sorbent showed much improved matrix enhancement versus existing studies,^{13,29} at 95.7-104.4 %ME for all analytes and a similar precision to undiluted extracts (see Figure 2). Pleasingly, this provides considerable confidence that the recovery measurements are representative of the amount of analyte extracted without the need for 'correction' or additional steps to the protocol. Again, recovery did decrease with hydrophobicity however, this loss was significantly lower (indicative of a competitive retention process), and equivalent to past work involving more rapid protocols,²⁴ including QuEChERS approaches that do not include C18 dSPE to remove organic interferences prone to environmental solids.^{13,30} This data therefore, confirms a standard

296 QuEChERS product can extract, with reasonable and repeatable recoveries and minimal
297 %ME, the range of aromatic and aliphatic QACs anticipated within environmental solids that
298 have links to AMR,¹⁵⁻²⁰ as a quick and cheap ‘off the shelf’ method to determine their
299 environmental distribution and impact, as part of a future monitoring programme for AMR.

300

301 **Conclusion**

302 There is an increasing need to establish exposure levels and sources of QAC pollution due to
303 their high usage rates with limited regulation and reporting of biocide levels, sorption to
304 environmental solids following WW treatment and links to bacterial cross- and co-resistance
305 mechanisms. The extraction of QACs from environmental solids can require laborious
306 preparative methods to achieve precise data with minimal %MEs and high recovery. Recent
307 QuEChERS work has shown potential for a more limited selection of surfactant biocides
308 anticipated in environmental solids (and those used in this study) however, these methods
309 require additional steps to the protocol, have used a bespoke extraction product, or alternative
310 matrices. Using a robust quantitative reversed-phase LC-MS/MS method, we have shown that
311 an ‘off the shelf’ QuEChERS product can recover the range of anticipated QACs for municipal,
312 industrial and agricultural waste, to values up to 53% and with <4% suppression and
313 enhancement, as a repeatable single extraction for soil operating within <10 %RSD by using
314 a simple extract dilution. With minimal method development, this provides a much needed
315 rapid sample preparation method for quantifying the breadth of QAC pollution and monitoring
316 the progression of AMR.

317

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319 **Conflicts of interest**

320 The authors confirm that there are no conflicts to declare and ethical approval was not required
321 for this work.

322

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327

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Figures and Tables

Table 1: Summary of physiochemical data and chromatographic reproducibility of adjusted retention times for each analyte (day 1: n=11, day 2: n=7). Chromatographic repeatability is represented by %RSD and reproducibility by two-tailed F- test; F-stat were $F_{(10,6)} 5.461^{\wedge}$, $F_{(6,10)} 4.072^{\S}$.

Analyte	Molecular Formula	logP	m/z Precursor (SRM fragment)	Chromatographic Stability		
				Repeatability		Reproducibility
				Day 1	Day 2	
BAC-C12	C ₂₁ H ₃₈ N	1.69	304 (212)	1.49	1.20	1.50
BAC-C14	C ₂₃ H ₄₂ N	2.55	332 (240)	0.87	1.21	1.96
BAC-C16	C ₂₅ H ₄₆ N	3.42	360 (268)	0.58	0.95	2.72
BAC-C18	C ₂₇ H ₅₀ N	4.28	388 (296)	0.59	0.91	2.43
DDMA	C ₂₂ H ₄₈ N	2.51	326 (186)	0.72	1.07	2.28
HDTMA	C ₁₉ H ₄₂ N	2.40	284 (n/a)	0.88	1.05	1.45
BAC-C14-d ₇	C ₂₃ H ₃₅ D ₇ N	2.55	339 (240)	0.85	1.21	2.05
HDTMA-d ₉	C ₁₉ H ₃₃ D ₉ N	2.40	294 (n/a)	0.90	0.96	1.16

Table 2: Summary table of the quantitative performance of the weighted ($1/x$) regression with linearity represented by the coefficient of determination (R^2) limit of detection (LOD), mean percentage accuracy and precision of quality control (QC) samples at 8, 20, 60 and 80 ng/mL for each analyte ($n=5$). Interestingly, the latter compound was also assessed using d_7 -BAC-C14 to scope the possibility of using a single IS and has showed good linearity, accuracy and precision results for quantitation.

Analyte	MS Data (m/z)	Regression Function	Internal standard	Linearity (R^2)	LOD (ng/mL)	QC concentration (ng/mL)							
						Accuracy (%)				Precision (%)			
						8	20	60	90	8	20	60	90
BAC-C12	304>212	$1/x$	d_7 -BAC-C14	0.995	0.06	-7.3	6.9	0.2	-5.2	5.5	8.0	3.1	7.7
BAC-C14	332>240	$1/x$		0.994	0.83	4.0	7.1	-3.6	-0.5	12.4	5.4	2.7	6.3
BAC-C16	360>268	$1/x$		0.991	0.21	-3.5	2.3	3.6	-1.0	8.0	9.1	3.0	7.1
BAC-C18	388>296	$1/x$		0.980	0.38	-7.9	-12.2	-2.8	-0.4	12.4	12.6	9.2	7.3
DDMA	326>186	$1/x$		0.993	0.23	1.8	6.9	4.6	0.5	8.8	6.1	5.5	4.6
HDTMA	284	$1/x$		0.994	1.02	-14.8	-3.8	1.6	2.5	7.1	9.2	4.8	5.4
HDTMA		$1/x$	d_9 -HDTMA	0.996	1.51	-7.7	3.8	-1.7	-1.0	8.1	7.7	6.0	2.7

Figure 1: Mean percentage matrix effects and recovery of each analyte and internal standard (with standard error bars) for spiked soil samples following QuEChERS extraction (n=3) with C18 and GCB dSPE sorbent.

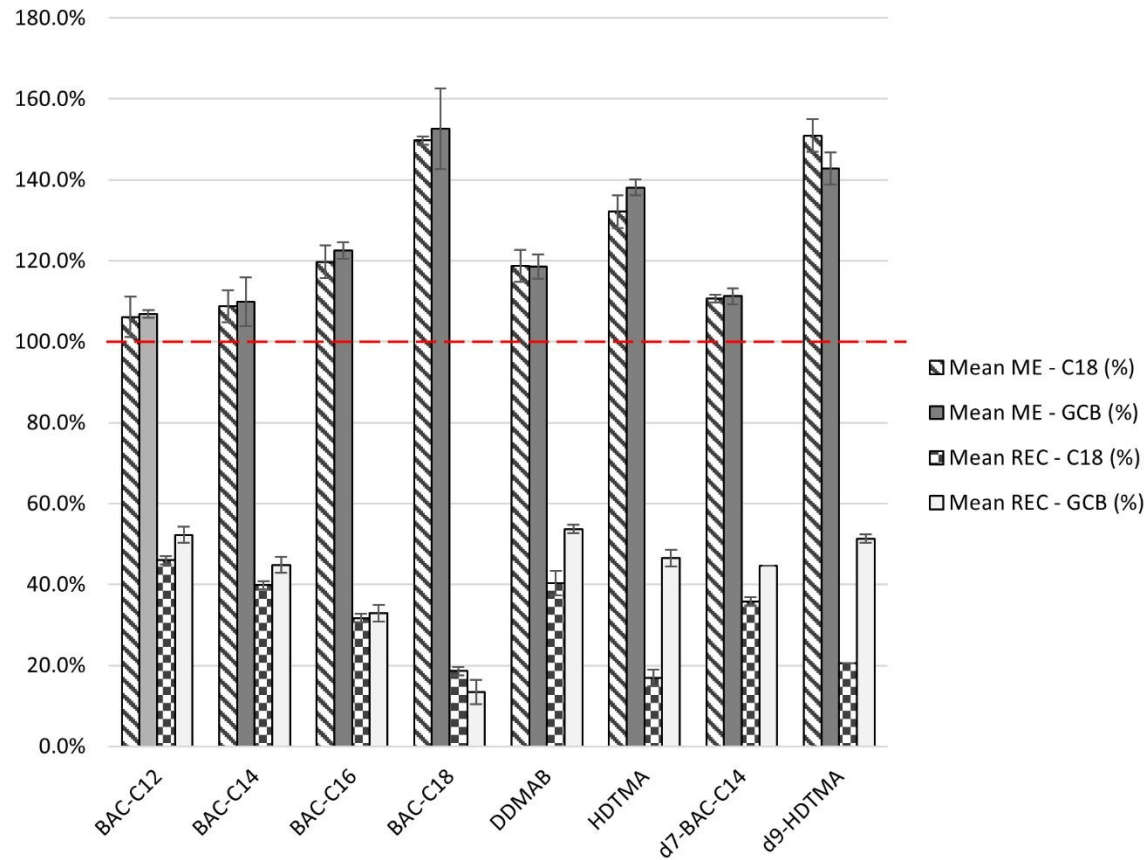
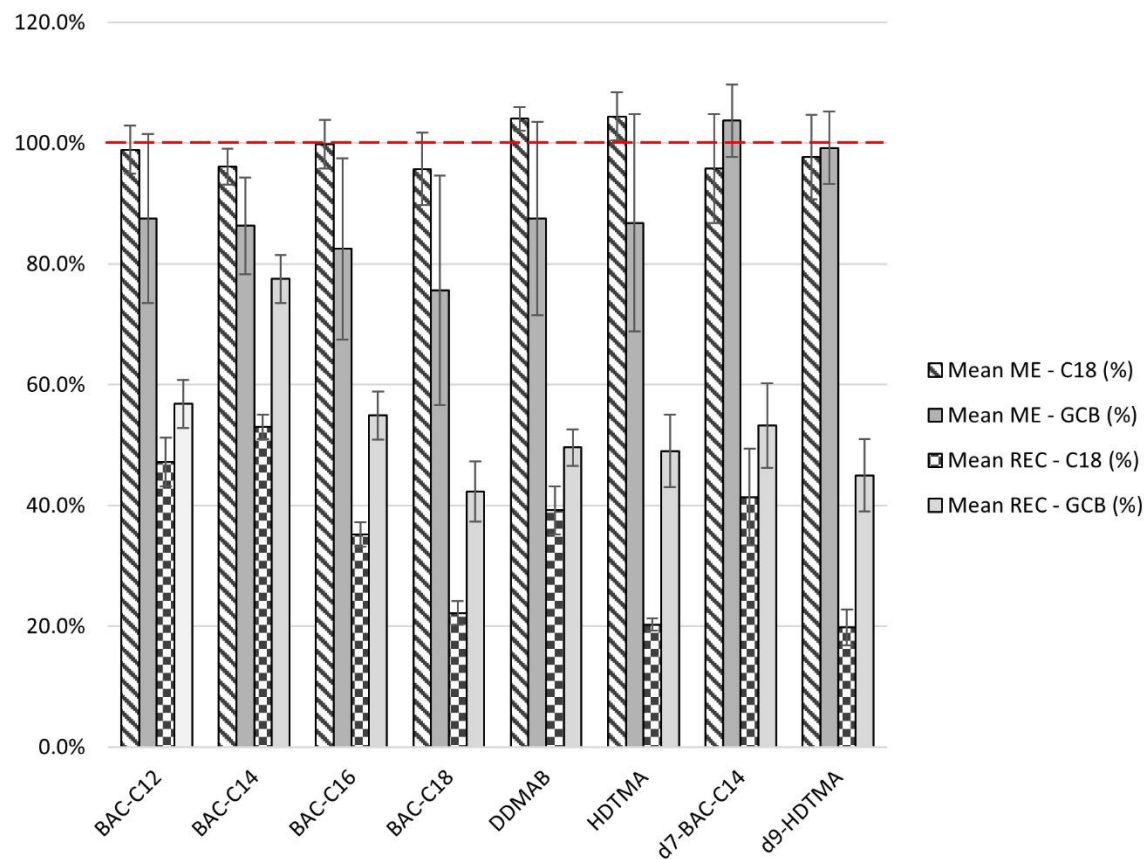
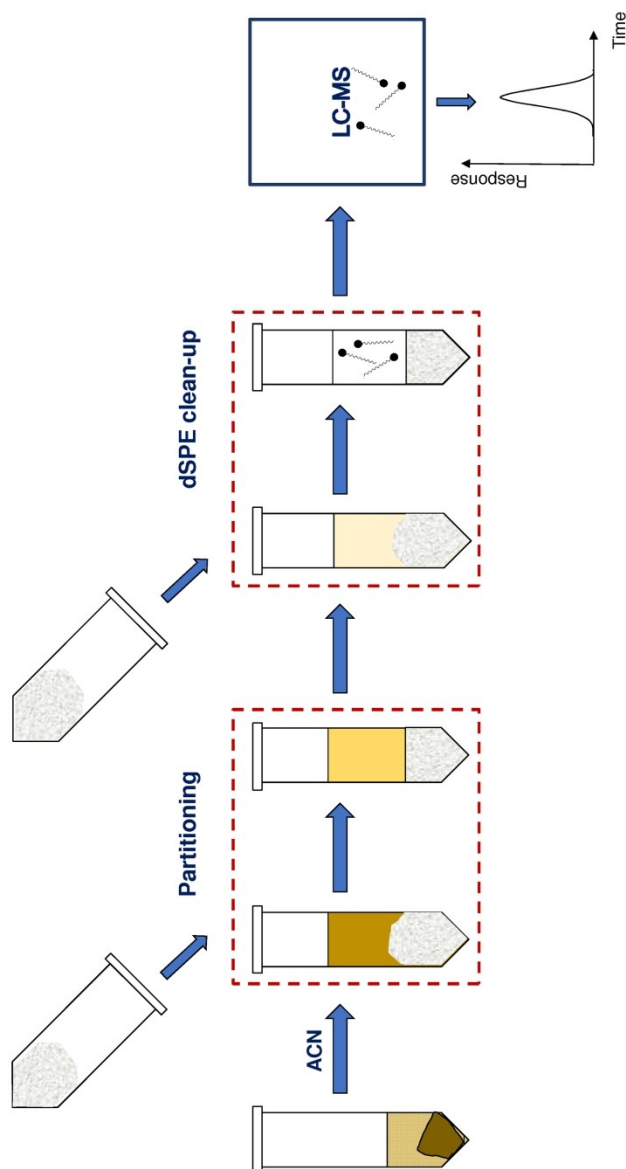


Figure 2: Mean percentage matrix effects and recovery of each analyte and internal standard (with standard error bars) for spiked soil samples following QuEChERS extraction (n=3) with C18 and GCB dSPE sorbent and subsequent extract dilution (1:400).





A rapid, robust 'off-the-shelf' preparation for extracting quaternary ammonium biocides from soil with low matrix interference.

209x297mm (300 x 300 DPI)

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