Supplementary Information

Seasonal variation dynamic and risk assessment for the presence of pharmaceuticals in Brazilian urban rivers

Miraldo Santa Rosa dos Santos[a], Cristiane Vidal[a], Vinicius Sarracini Santos[a], Ramon Domingues dos Santos[a] and Cassiana Carolina Montagner[a*]

[a] Instituto de Química, Universidade Estadual de Campinas – UNICAMP, Josué de Castro Street, - Cidade Universitária, Campinas/SP - Brazil, 13083-970.

**E-mail: ccmonta@unicamp.br*

Instrumental method development

MS/MS parameters

MS/MS parameters were optimized by direct infusion. For the infusion process, a 1 mg L^{-1} standard solution of each compound at a flow rate of 0.02 mL min^{-1} were combined with the mobile phase (ammonium formate 0.1% m/v:methanol, 1:1 m/v) at a flow rate of 0.2 mL min^{-1}. The optimization was conducted in product ion scan mode with capillary energy set to 1.75 kV, the parameters optimized were, cone voltage (CV) and collision energy. The collision gas was argon, the desolvation gas was nitrogen at 500 °C, at a flow rate of 200 L/h. The two most intense product ions were selected as quantification and confirmation ions, respectively. A summary of the optimized parameters is presented in Table S1.

Table S1: Optimized MS/MS conditions for the method.

Pharmaceutical	[a]IM	[b]IS	[c]CV (V)	[d]QT / [e]CE (eV)	[f]CT/ [e]CE (eV)	[g]RT (min)
Acetaminophen	ESM+	[h]ACT-d4	35	151.9 > 109.9 / 16	151.9 > 92.9 / 21	2.63
Caffeine	ESM+	[h]ACT-d4	45	195.0 > 138.0 / 17	195.0 > 110.0 / 21	2.90
Diclofenac	ESM-	[i]DIC-d4	22	293.8 > 249.8 / 12	295.8 > 251.9 / 12	3.66
Sulfathiazole	ESM+	[h]ACT-d4	30	256.1 > 92.1 / 26	256.1 > 156.1 / 17	2.78

[a]IM = Ionization mode, [b]IS = Internal standard, [c]CV = Cone voltage, [d]QT = Quantification transition, [e]CE = Collision energy, [f]CT = Confirmation transition, [g]RT = Retention time, [h]ACT-d4 = Acetaminophen-d4, [i]DIC-d4 = Diclofenac-d4.

On-line solid phase extraction (SPE) and chromatographic separation

Figure S1 illustrates the overall process, with the mobile phase pathway indicated by the black arrows. The online solid-phase extraction procedure and chromatographic separation were conducted in sequence in three main phases. In phase 1, the sample was loaded into the SPE column, in phase 2 the analytes were eluted from the SPE column, and in phase 3 the chromatographic separation and conditioning of both columns were performed.

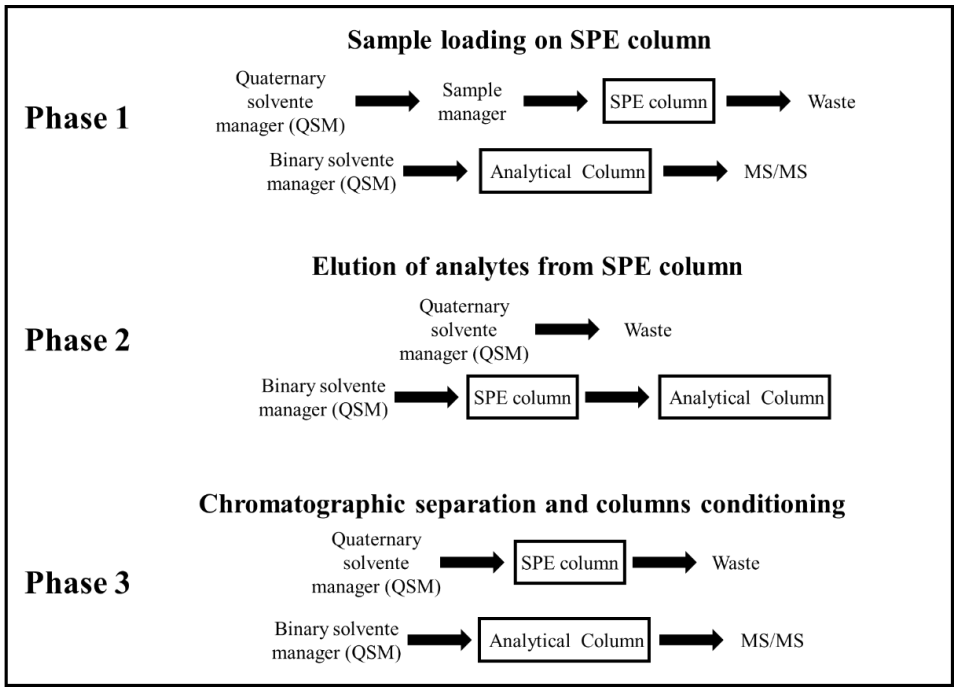

Figure S1: Overall process of on-line solid phase extraction and chromatographic separation. The black arrows indicate the mobile phase pathway.

The binary solvent manager (BSM) was provided with two solvents: B1, which was a 0.1% (m/V) water solution of ammonium formate, and B2, which was methanol. The quaternary solvent manager (QSM) was provided with two solvents: A, which was ultrapure water, and B, which was methanol. The solvent percentage or gradient for each step of the procedure and the solvent manager responsible are summarized in Table S2. The mobile phase flow rates were 0.3 mL min^{-1} for BSM and 0.9 mL min^{-1} for QSM.

Table S2: Solvent percentage and gradient for each step of the procedure and the solvent manager responsible. Flow rate was 0.3 mL min^{-1} for BSM and 0.9 mL min^{-1} for QSM.

Procedure			Time/min	% Methanol	
QSM	BSM			QSM	BSM
Loading sample			0	0	5%
			0.30	0	
	Chromatographic Separation	Elution	0.31	100%	[a]G
			2.00	100%	
			2.01	100%	
Cleaning SPE column			3.00	100%	70%
			5.00	100%	70%
	Analytical column conditioning		5.01	100%	5%
			6.00	100%	5%
SPE column conditioning			6.01	0	5%
			10.00	0	5%

[a]G = Gradient interval.

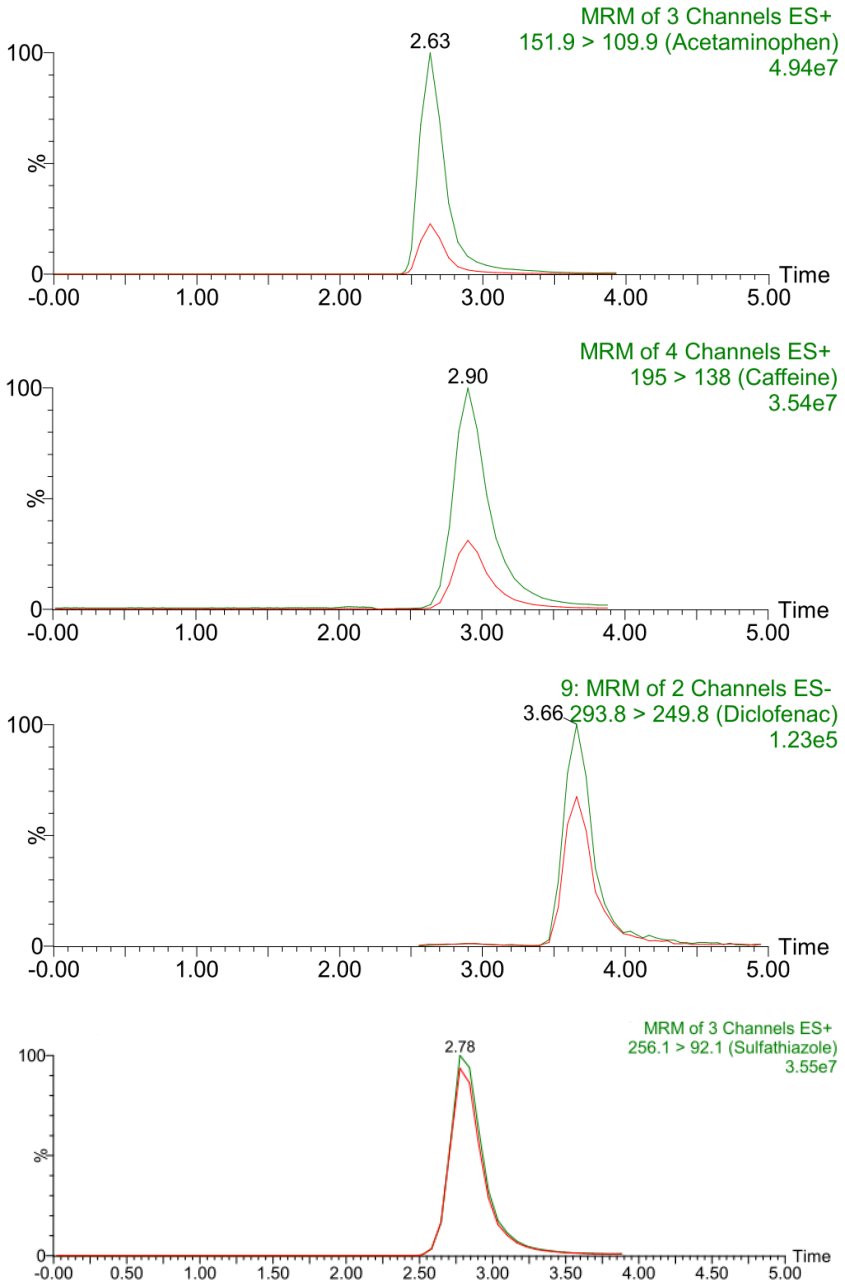

Figure S2: Chromatograms (MRM of the quantification and confirmation ions) of the target pharmaceuticals standard solution 100 μg L^{-1}.

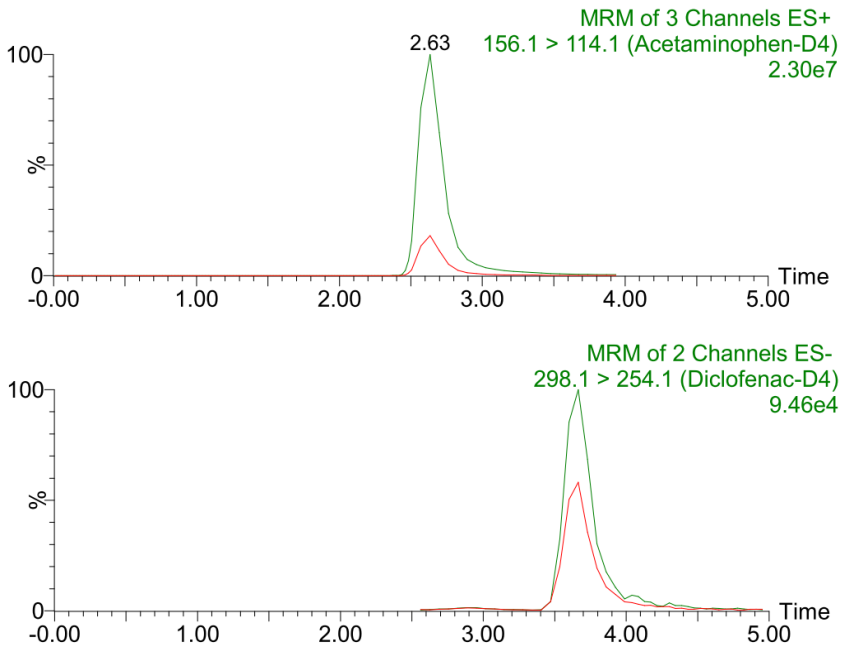

Figure S3: Chromatograms (MRM of the quantification and confirmation ions) of the pharmaceutical's internal standard solution 50 μg L^{-1}.

Table S3: Lowest Predicted No-Effect Concentration (PNEC) for Freshwater (ng L^{-1}), number of samples, frequency of detection, total number of samples, number of non-detected samples, number of samples with RQ > 1 and minimum, maximum, mean, and median concentration (ng L^{-1}) of pharmaceuticals quantified in rivers and creeks located in Belo Horizonte, Campinas, and São José do Rio Preto. acetaminophen = ACT, caffeine = CAF, diclofenac = DIC, and sulfathiazole = SUF.

	ACT	**CAF**	**DIC**	**SUF**
Lowest PNEC Freshwater* (ng L^{-1})	46000	1200	50	1920**
Belo Horizonte				
Minimum Concentration/ (ng L^{-1})	161	532	-	34
Maximum Concentration/ (ng L^{-1})	7449	122,520	-	40
Mean Concentration/ (ng L^{-1})	1742	35795	-	37
Median Concentration/ (ng L^{-1})	865	7798	-	37
Total Number of Samples	16	16	16	16
Frequency of Detection	94%	94%	0%	13%
Number of Non-detected Samples	1	1	16	14
Number of Samples with RQ > 1	0	15	0	0
Campinas				
Minimum Concentration/ (ng L^{-1})	157	113	62	-
Maximum Concentration/ (ng L^{-1})	320	2578	176	-
Mean Concentration/ (ng L^{-1})	221	787	127	-
Median Concentration/ (ng L^{-1})	186	451	144	-
Total Number of Samples	15	15	15	15
Frequency of Detection	20%	47%	20%	0%
Number of Non-detected Samples	12	7	12	0
Number of Samples with RQ > 1	0	7	3	0
São Jose do Rio Preto				
Minimum Concentration/ (ng L^{-1})	-	60	-	-
Maximum Concentration/ (ng L^{-1})	-	585	-	-
Mean Concentration/ (ng L^{-1})	-	271	-	-
Median Concentration/ (ng L^{-1})	-	220	-	-
Total Number of Samples	8	8	8	8
Frequency of Detection	0%	50%	0%	0%
Number of Non-detected Samples	8	4	8	8
Number of Samples with RQ > 1	0	3	0	0

* Lowest PNEC Fresh Water reference: NORMAN Ecotoxicology Database (consulted on July 16, 2024).

** PNEC that were predicted by Quantitative structure-activity relationships (QSAR).

Method validation

Analytical curves

Linear least-square regression (LSR) was applied to build analytical curves for the determination of unknown samples. The ratio compound area/internal standard area (A/AIS) was used as dependent variable for the LSR models, while compound concentration was set as independent variable (internal calibration). For the method validation, three independent sets of standard solution were prepared, while for routine analyses the analytical curves were obtained from a single set of standard solutions. All analytical curves employed in the method validation are presented in Figures S4 to S7.

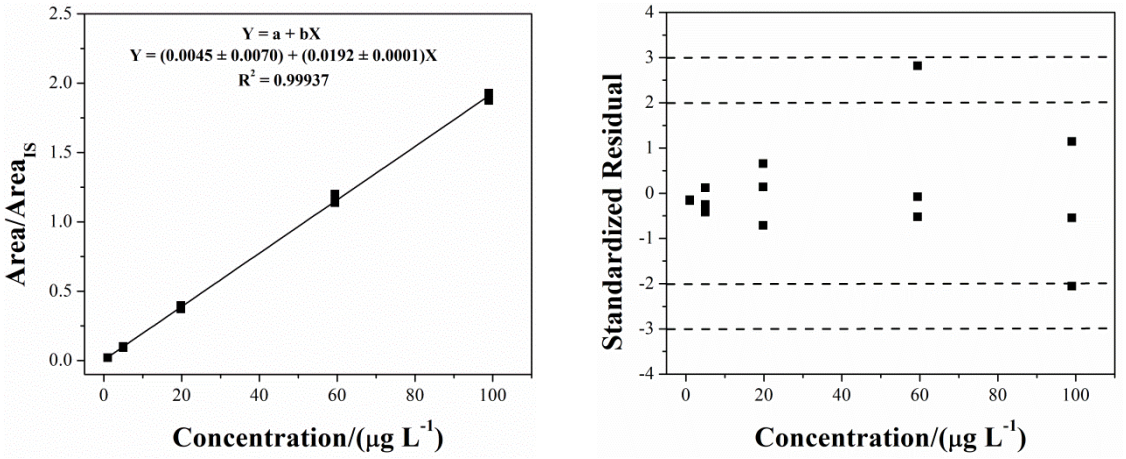

Figure S4: Analytical curve and standardized residuals vs. concentration plot for acetaminophen.

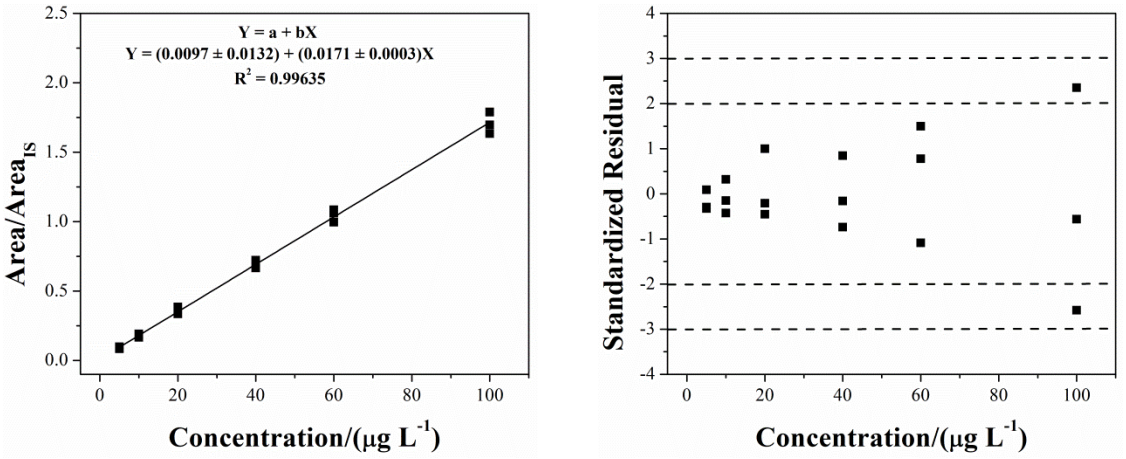

Figure S5: Analytical curve and standardized residuals vs. concentration plot for caffeine.

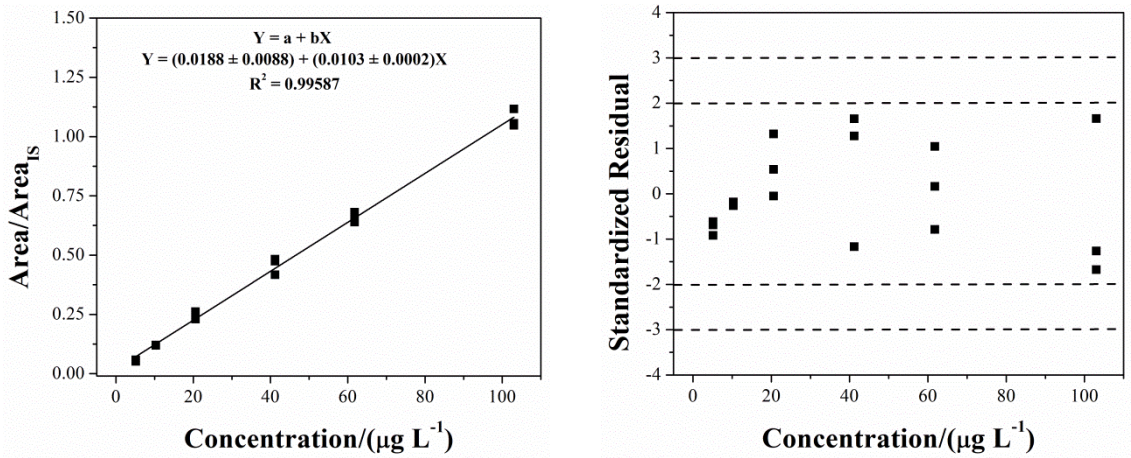

Figure S6: Analytical curve and standardized residuals vs. concentration plot for diclofenac.

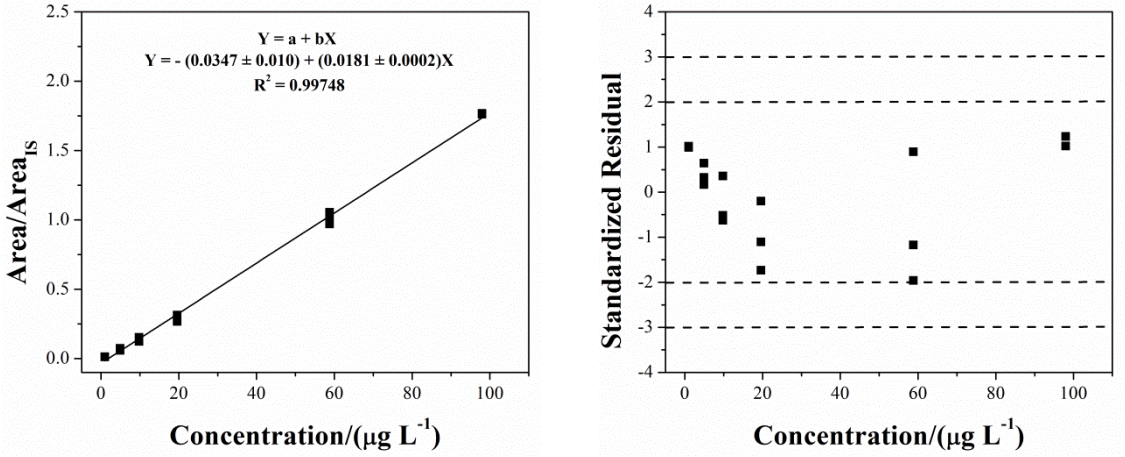

Figure S7: Analytical curve and standardized residuals vs. concentration plot for sulfathiazole.

Linearity of the least-square regression models

For the linearity evaluation, three independent sets of standard solutions were prepared from the stock solutions. Prior to assessing the linearity of the least-squares regression model, a Grubbs test (Equation S1) with a 5% significance level was performed to detect any outlier among the responses of replicates for each point of the LSR (Figure S8). One replicate response for Sulfathiazole presented a calculated G value (Gcalculated = 1.1541) slightly above the critical G value (Gn=3,α=0.05 = 1.153) and was hence excluded before proceeding with further linearity evaluation tests.

$$G_{critical} = \frac{|\overline{X} - X_i|}{S} \quad \text{(S1)}$$

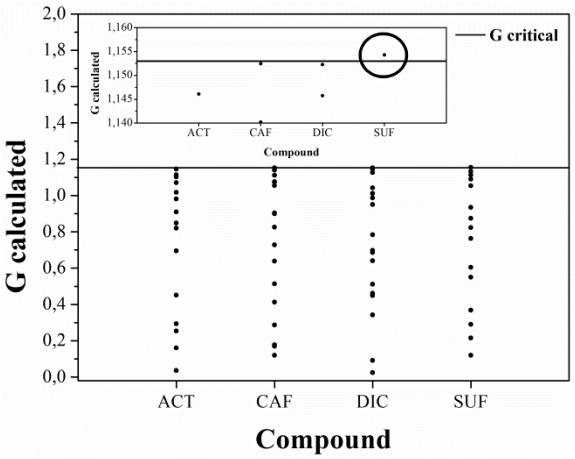

Figure S8: Grubb's test for outliers among linearity validation LSR replicate responses.

Linearity was evaluated based on homoscedasticity, regression significance, coefficient of determination (R2), and standardized residual plot of the least-squares regression.

Homoscedasticity was evaluated based on Cochran's C test (S1). Cochran's test assesses the homogeneity of a group of variance values by testing the group's largest variance value (S2max). If the calculated C value

(C0) is smaller than the critical C value (Ccritical), the variance group is considered statistically homogenous and the LSR model is considered homoscedastic. $\sum_i^k S_i^2$ is the sum of the variance group.

$$C_0 = \frac{S_{max}^2}{\sum_i^k S_i^2} \quad \text{S1}$$

According to Cochran's C test with 5% significance, the LSR models for all compounds were considered homoscedastic (S4).

The significance of the regression was determined based on ANOVA's F-test, which was applied to the ratio of the regression mean square (MSR) and the mean square error (MSE).[1] If the calculated F-value is greater than the critical F-value, the MSR is statistically greater than the MSE, indicating that the variability of A/A_{IS} (dependent variable) depends on concentration (independent variable), demonstrating that the least-square regression model is statistically significant. The linear LSR models built for all compounds were considered statistically significant. The ANOVA table for all LSR models can be found in the supplementary information (Table S5).

Table S4: Cochran's C test for homogeneity of the variances of the LSR points.

Compound	Number of points	C_0	$C_{Critical\ (\alpha = 0.05)}$
Acetaminophen	5	0.670	0.684
Caffeine	6	0.383	0.616
Diclofenac	6	0.478	0.616
Sulfathiazole	6	0.466	0.616

The R^2 value is used to measure how well the variability in the independent variable can explain the variance in the dependent variable, or how well the LSR model fits the data. R^2 ranges from 0 to 1, with a value closer to 1 indicating a better fit. A desired value of R^2 is 0.99 or above.[1] The R^2 values for the LSR models developed to determine all compounds were higher than 0.99. The R^2 values for all LSR models can be found in the supplementary information (Table S5).

When the appropriate LSR model is employed, all residuals of the dependent variable ($A/A_{IS\ observed} - A/A_{IS\ expected}$) should be uniform in size. Therefore, residuals can also be used as an indicator of how appropriate the LSR models are.[2] It is common to evaluate the standardized residuals (residuals divided by the standard deviation (σ) of residuals). If the LSR model is appropriate, the standardized residual should be within the range of -3σ to 3σ. For the LSR models, plots of the dependent variable's standardized residuals versus the independent variable (concentration) were analyzed (Figures S4 to S7) and all standardized residuals were found between -3σ to 3σ, suggesting that all LSR models are appropriate, according to Miller and Miller.[2]

The instrumental working range was established as the interval between the instrumental LQ for each compound and 100,000 ng L^{-1}, whereas the working range of the method was established as the interval between the method's LQ for each compound and 100,000 ng L^{-1}.

Table S5: ANOVA table LSR models.

	Variation source	DF	SS	MS	$F_{crit\ v=13}$	F_{cal}	R^2
Acetaminophen	**Regression**	1	7.7356	7.7356	4.667	20680	0.9993
	Error	13	0.0049	0.0004			
	Total	14	7.7405				

	Variation source	DF	SS	MS	$F_{crit\ v=16}$	F_{cal}	R^2
Caffeine	**Regression**	1	5.6928	5.6928	4.494	4372	0.9963
	Error	16	0.0208	0.0013			
	Total	17	5.7137				

	Variation source	DF	SS	MS	$F_{crit\ v=16}$	F_{cal}	R^2
Diclofenac	**Regression**	1	2.2118	2.2118	4.494	3860	0.9959
	Error	16	0.0092	0.000572938			
	Total	17	2.2209				

	Variation source	DF	SS	MS	$F_{crit\ v=15}$	F_{cal}	R^2
Sulfathiazole	**Regression**	1	5.7629	5.7629	4.543	5946	0.9975
	Error	15	0.0145	0.0010			
	Total	16	5.7775				

[a]DF = Degrees of freedom, [b]SS = Sum Square, [c]MS = Mean Square, [d]F_{crit} = Critical 95% confidence, [e]Fcal = F calculated.

Matrix Effect

Matrix Effect (ME) was calculated based on Student's t-test for the solvent curve sensitivity (b_{sol}) and matrix-matched curve sensitivity (b_{mat}), as shown by Equations S2 to S4.

$$t_{cal} = \frac{b_{sol} - b_{mat}}{S_{b_{sol} - b_{mat}}} \quad \text{S2}$$

$$S^2_{b_{sol} - b_{mat}} = S^2_{P,y/x}\left[\frac{1}{(n_{sol} - 1)S^2_{x_{sol}}} + \frac{1}{(n_{mat} - 1)S^2_{x_{mat}}}\right] \quad \text{S3}$$

$$S^2_{P,y/x} = \frac{(n_{sol} - 2)S^2_{y/x_{sol}} + (n_{mat} - 2)S^2_{y/x_{mat}}}{n_{sol} + n_{mat} - 4} \quad \text{S4}$$

t_{cal} = Calculated t
b_{sol} = Solvent curve sensitivity estimate using n_{sol} points
b_{mat} = Matrix-matched curve sensitivity estimate using n_{mat} points
$S_{b_{sol} - b_{mat}}$ = Standard deviation estimate of the difference between the solvent curve sensitivity and the matrix-matched curve sensitivity
$S^2_{y/x_{sol}}$ = Solvent curve mean square sum of the residual.
$S^2_{y/x_{mat}}$ = Matrix-matched curve mean square sum of the residual
$S^2_{x_{sol}}$ = Concentration variance of the points in the solvent curve
$S^2_{x_{sol}}$ = Concentration variance of the points in the matrix matched curve.
n_{sol} = Number of points on the solvent curve
n_{sol} = Number of points on the matrix-matched curve

If the solvent curve sensitivity is statistically equal to the matrix-matched curve sensitivity, it is considered that there is no matrix effect. On the other hand, if the mentioned curve sensitivities are not statistically equal, it is considered that there is matrix effect. Based on the hypothesis test of Equation S5, the existence or non-

existence of a matrix effect was determined. For each compound and matrix, the calculated and critical t values were compared considering $n_{sol} + n_{mat} - 4$ degrees of freedom and a significance level of 5%. For calculated t < critical t, H_0 was accepted, and it was considered that there is no matrix effect. On the other hand, for calculated t > critical t, H_0 was rejected, and it was considered that there is a matrix effect.

$$H_0: b_{sol} = b_{mat}$$
$$H_1: b_{sol} \neq b_{mat} \quad \text{S5}$$

The matrix effect caused significate response suppression for all compounds, as calculated t was always higher than critical t. (Table S6).

Table S6: Student's t-test results for matrix effect evaluation on River water.

Compound	Number of points	Critical t	Calculated t
Acetaminophen	5	2.447	7.866
Caffeine	6	2.306	7.790
Diclofenac	6	2.306	5.910
Sulfathiazole	6	2.306	5.910

Although the response of all studied compounds was strongly affected by the matrix effect, the use of internal standards was effective in correcting the influence of signal suppression on the determination of compound concentrations in the samples (Table S7).

Table S7: Student's t-test results for matrix effect evaluation of internal standard spiked River water samples.

Compound	Number of points	Critical t	Calculated t
Acetaminophen	5	2.447	1.347
Caffeine	6	2.306	0.149
Diclofenac	6	2.306	1.407
Sulfathiazole	6	2.306	0.338

Matrix-matched and solvent curves of all studied compounds with and without the use of internal standards are presented in Figures S9 to S12.

Solid Phase Extraction Recovery

Three levels of recovery (with three independent fortified samples for each level) were tested (Table S8). Recoveries were acceptable (acceptable range according to INMETRO[3]: 40% to 120%) for all compounds ranging from 40.2% to 92.9%. The method's accuracy was assessed through extraction recoveries.

Table S8: Mean of compound recoveries for SPE on three levels of concentration. ACT = acetaminophen, CAF = caffeine, DIC = diclofenac SUF = sulfathiazole.

Fortification level/(ng L^{-1})	ACT	CAF	DIC	SUF
80	57.9%	71.3%	40.2%	92.9%
200	56.1%	64.7%	53.6%	92.6%
280	57.7%	48.3%	50.0%	83.1%

The method's precision was obtained from the coefficient of variation of the extraction recoveries (Table S9), which was also satisfactory (acceptable range according to INMETRO[3]: 30% or smaller).

Table S9 Coefficient of variation (CV) of compound's recoveries for SPE extraction on three levels of concentration. ACT = acetaminophen, CAF = caffeine, DIC = diclofenac SUF = sulfathiazole.

Fortification level/(ng L^{-1})	ACT	CAF	DIC	SUF
80	11.0%	16.1%	9.1%	2.0%
200	5.2%	5.0%	4.6%	4.6%
280	1.0%	22.9%	12.2%	10.7%

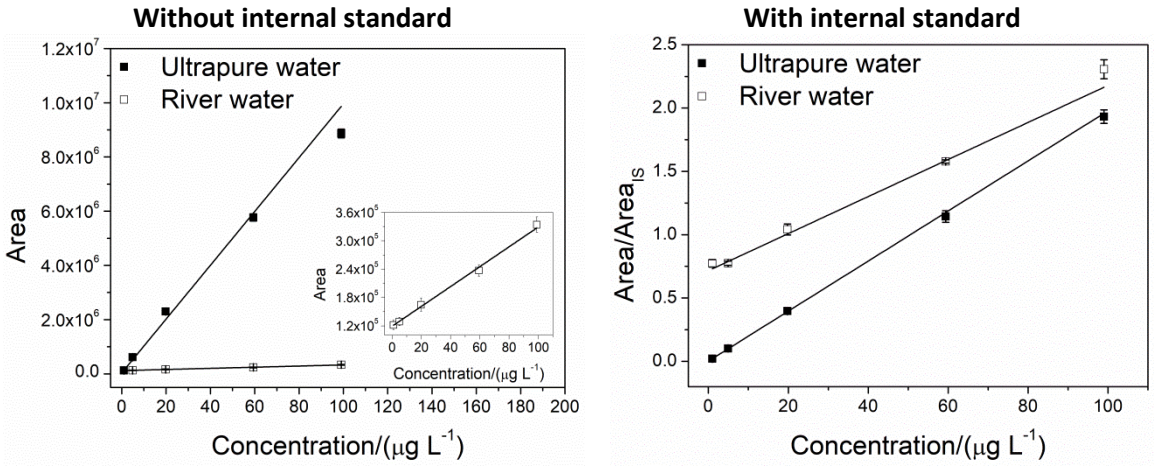

Figure S9: Matrix-matched and solvent curves for acetaminophen with and without internal standard correction.

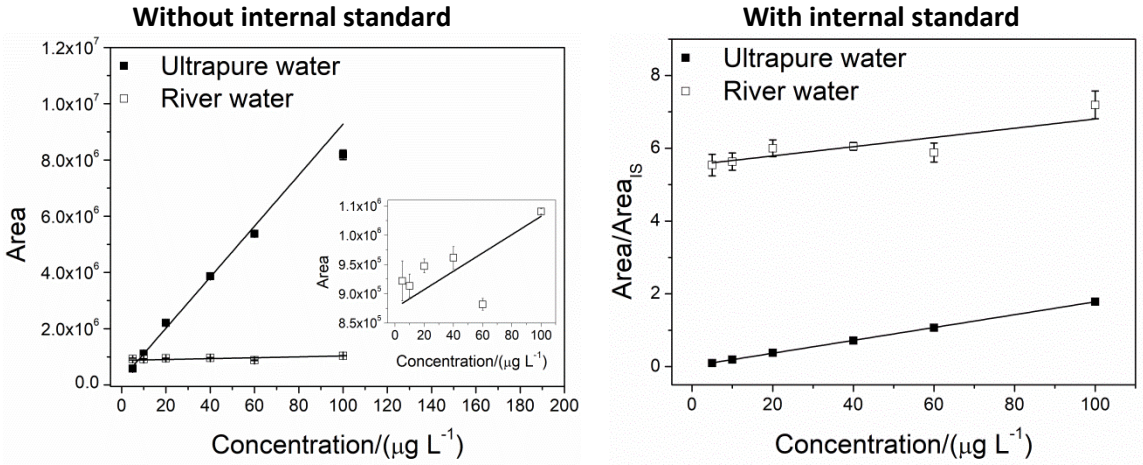

Figure S10: Matrix-matched and solvent curves for caffeine with and without internal standard correction.

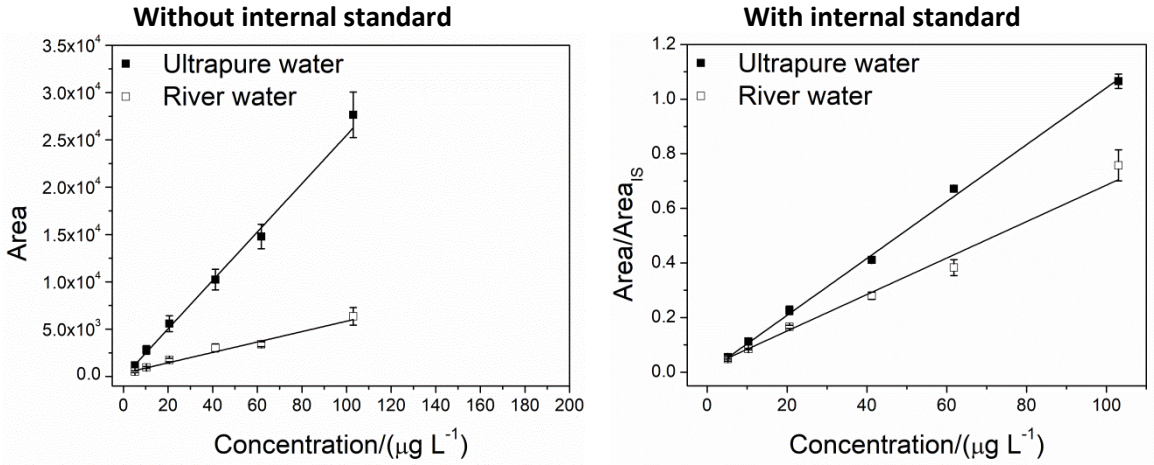

Figure S11: Matrix-matched and solvent curves for diclofenac with and without internal standard correction.

Without internal standard **With internal standard**

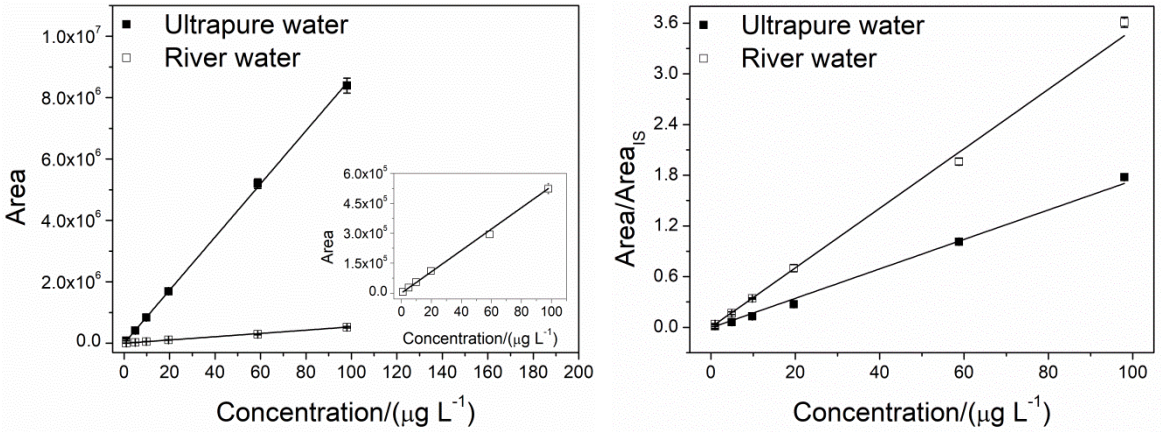

Figure S12: Matrix-matched and solvent curves for sulfathiazole with and without internal standard correction.

Table S10: Occurrence (ng L^{-1}) of acetaminophen (ACT), caffeine (CAF), diclofenac and sulfathiazole (SUF) in Brazilian creeks and rivers.

Reference	ACT	CAF	DIC	SUF
Correa et al. 2021[4]	11.1 - 204.8	-	23.3 - 561	-
Veras et al. 2019[5]	3.0 - 42	-	19 - 193	-
Campanha et al. 2015[6]	< 3.0 - 30421	< 0.04 - 129585	< 0.04 - 385.6	-
Carvalho et al. 2022[7]	-	< 1.0 - 4015.4	-	-
Ide et al. 2017[8]	-	< 8.2 - 27000	-	-
Martini et al. 2021[9]	-	5.5 - 69600	-	-
Quadra et al. 2021[10]	-	280 -1763	-	-
Roveri et al. 2022[11]	1.06 - 22.24	9.00 - 560.00	0.76 - 3.93	-
Santos et al. 2022[12]	0.2 - 0.2	6.7 - 815.4	-	-
Sousa et al. 2018[13]	27.9 - 20961	-	4.88 - 364	-
Souza et al. 2021[14]	-	< 5.0 - 16466.4	-	-
Sposito et al. 2018[15]	-	< 19.8 - 1040	-	-
Thomas et al. 2014[16]	-	-	63 - 785	-
Sabino et al.2020[17]	-	-	-	-
Americo-Pinheiro et al. 2017[18]	-	-	120 - 5500	-
In this work	**157 - 7449**	**60 - 122520**	**62 - 176**	**34 - 40**

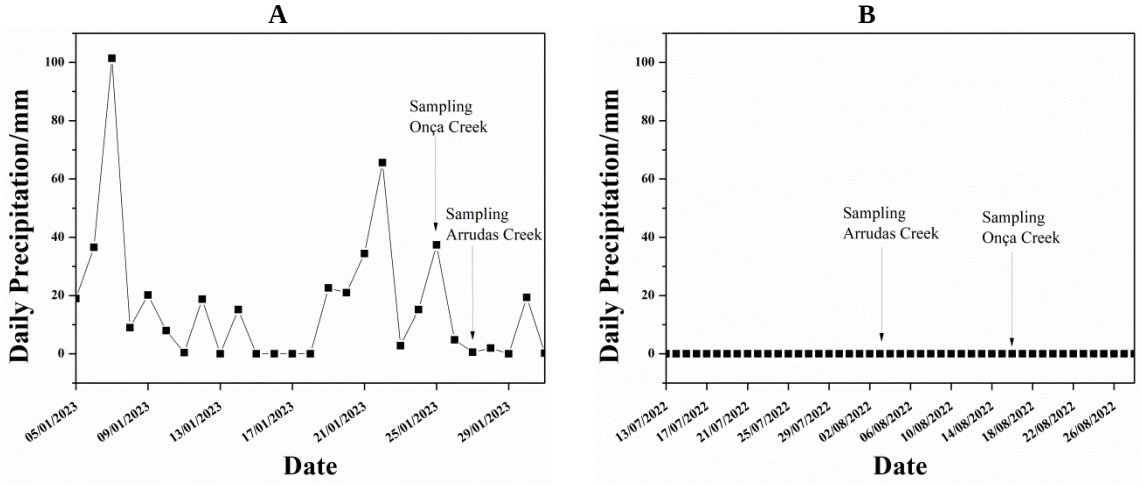

Figure S13: Daily rainfall data during the sampling periods collected by the CIIAGRO[19] meteorological station located in Belo Horizonte. The dates of the sampling conducted at Arrudas and Onça Creek are highlighted in the figures. A) Rainy season. B) Dry season.

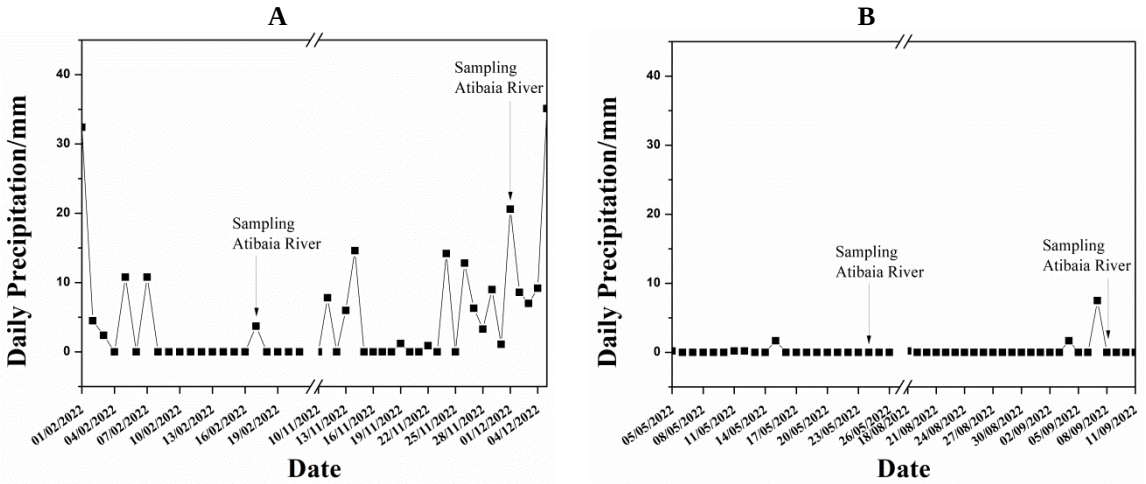

Figure S14: Daily rainfall data during the sampling periods collected by the CIIAGRO[19] meteorological station located in Campinas. The dates of the sampling conducted at the Atibaia River are highlighted in the figures. A) Rainy season. B) Dry season.

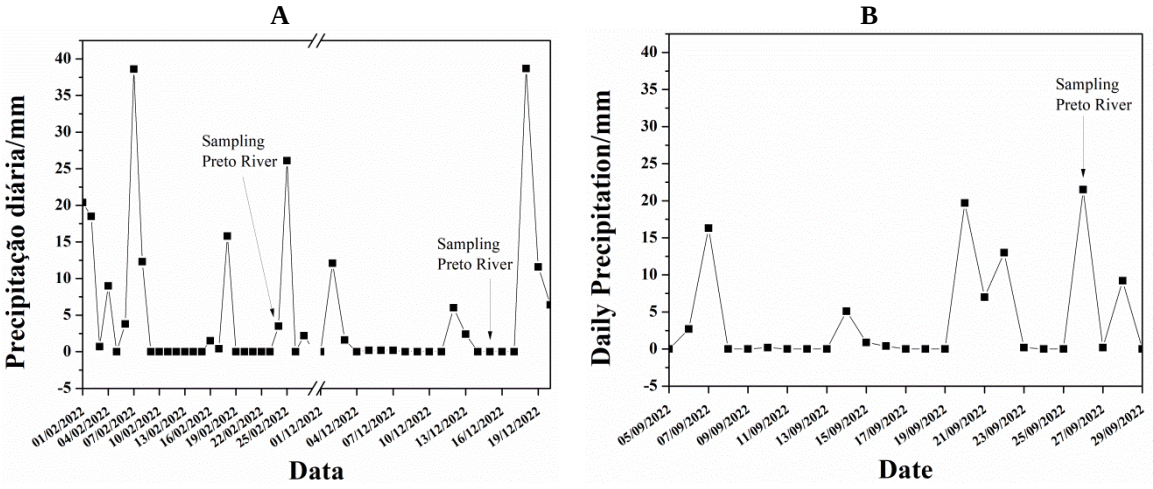

Figure S15: Daily rainfall data during the sampling periods collected by the CIIAGRO[19] meteorological station located in Mirassol (a neighboring city of São José do Rio Preto). The dates of the collections conducted at the Preto River are highlighted in the figures. A) Rainy season. B) Dry season.

References

1 J. N. Miller and J. C. Miller, in *Statistics and Chemometrics for Analytical Chemistry*, Prentice Hall/Pearson, Edinburgh, Sixth Edit., 2010, pp. 141–141.

2 J. N. Miller and J. C. Miller, in *Statistics and Chemometrics for Analytical Chemistry*, Prentice Hall/Pearson, Edinburgh, Sixth Edit., 2010, pp. 149–149.

3 INMETRO, in *DOQ-CGCRE-008: Orientação Sobre Validação de Métodos* , INMETRO, 2020, vol. Revisão 9, pp. 21–30.

4 J. M. M. Corrêa, A. L. Sanson, C. F. Machado, S. F. Aquino and R. J. C. F. Afonso, *Environ Sci Pollut Res Int*, 2021, **28**, 30242–30254.

5 T. B. Veras, A. L. R. de Paiva, M. M. M. B. Duarte, D. C. Napoleão and J. J. da S. P. Cabral, *Chemosphere*, 2019, **222**, 961–969.

6 M. B. Campanha, A. T. Awan, D. N. R. de Sousa, G. M. Grosseli, A. A. Mozeto and P. S. Fadini, *Environ Sci Pollut Res Int*, 2015, **22**, 7936–7947.

7 A. C. C. de Carvalho, B. F. da Silva, A. A. Machado, M. A. da S. Santarossa and W. da S. Paganini, *Engenharia Sanitaria e Ambiental*, 2022, **27**, 845–852.

8 A. H. Ide, R. A. Osawa, L. O. Marcante, J. da C. Pereira and J. C. R. de Azevedo, *Clean (Weinh)*, 2017, **45**, 1700334.

9 G. de A. Martini, C. C. Montagner, W. Viveiros, G. A. Quinaglia, D. D. França, N. C. G. Munin, M. Lopes-Ferreira, S. O. Rogero and J. R. Rogero, *Environmental Science and Pollution Research*, 2021, **28**, 20313–20329.

10 G. R. Quadra, Z. Li, P. S. A. Silva, N. Barros, F. Roland and A. Sobek, *Arch Environ Contam Toxicol*, 2021, **81**, 142–154.

11 V. Roveri, L. L. Guimarães, W. Toma and A. T. Correia, *Environmental Science and Pollution Research*, 2022, **29**, 89712–89726.

12 V. S. Santos, J. S. X. Anjos, J. F. de Medeiros and C. C. Montagner, *Environ Monit Assess*, 2022, **194**, 637.

13 D. N. R. de Sousa, A. A. Mozeto, R. L. Carneiro and P. S. Fadini, *Environmental Science and Pollution Research*, 2018, **25**, 4607–4620.

14 R. C. de Souza, A. A. Godoy, F. Kummrow, T. L. dos Santos, C. J. Brandão and E. Pinto, *Environmental Science and Pollution Research*, 2021, **28**, 20751–20761.

15 J. C. V. Sposito, C. C. Montagner, M. Casado, L. Navarro-Martín, J. C. J. Solórzano, B. Piña and A. B. Grisolia, *Chemosphere*, 2018, **209**, 696–704.

16 K. V. Thomas, F. M. A. da Silva, K. H. Langford, A. D. L. de Souza, L. Nizzeto and A. V. Waichman, *J Am Water Resour Assoc*, 2014, **50**, 302–308.

17 J. A. Sabino, A. L. de Sá Salomão, P. M. de Oliveira Muniz Cunha, R. Coutinho and M. Marques, *Ecotoxicology*, 2021, **30**, 130–141.

18 J. H. P. Américo-Pinheiro, W. D. Isique, N. H. Torres, A. A. Machado, S. L. de Carvalho, W. V. Valério and L. F. R. Ferreira, *Engenharia Sanitaria e Ambiental*, 2017, **22**, 429–435.

19 CIIAGRO, CIIAGRO - Centro Integrado de Informações Agrometeorológicas, http://www.ciiagro.org.br/ema/.