

Article

Study on the Stability of Antibiotics, Pesticides and Drugs in Water by Using a Straightforward Procedure Applying HPLC-Mass Spectrometric Determination for Analytical Purposes

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Abstract: The stability of analytes is a critical point in chemical analysis, especially in the field of trace levels residue analysis. Nowadays, due to advances in analytical technology and in separation sciences, the analyses of water have been improved. Unfortunately, in this context, one of the most critical issues in water analysis include compound stability from sampling station to laboratory procedures. This study was carried out to explore the stability of several compounds in water from sampling to analysis concerning analytes reported in implementing decision 2018/840—Watch List. During method development and validation, the stability of compounds was investigated to detect the best operating conditions concerning sampling, extraction and analysis. In this paper, we report a study on the stability of antibiotics, pesticides and drugs in water determined using a straight-forward procedure applying mass spectrometric detection for analytical purposes. The laboratory tests were performed in Milli-Q water and surface water by analyzing samples through direct injection, solvent mixture (Water/ACN) and solid phase extraction system from time 0 to 168 h. All the analytes of the WL are stable in aqueous solutions with the addition of at least 25% ACN even after 168 h, and the analytes have shown a matrix effect on recovery of some analytes such as Famoxadone from sampling results (recovery in surface water 72%). For all the analytes investigated, recoveries were between 70 and 130% by using SPE procedures before UHPLC-MS/MS analysis, which is in good agreement with method validation procedures.

Keywords: stability; EU Watch list; ultra-high-performance liquid chromatography; antibiotic determination



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1. Introduction

The growth of industrialization and excessive utilization of available resources by humans have resulted in the emergence of water pollution as one of the global environmental issues. Indeed, in the last years, traces of several pollutants, typically at levels of nanograms per litre, were found in environmental matrices such as water, soil and sediments [1–6].

Since the adoption of the Water Framework Directive, the EU has taken measures to tackle pollution of freshwater ecosystems (Directive 2000/60/EC) [6] through a system of structured prioritization and, in this context, one of the most important action to preserve water from pollutant was the Watch List (WL) directive. The first WL, published by Decision (EU) 2015/495, included several substances, such as sunscreens, drugs, hormones, neonicotinoids, pesticides and antibiotics [7,8], and subsequent revisions have updated the list of substances to be monitored. In June 2018, the second updated version of the EU Watch List made its appearance (Decision 2018/840) [9], and the third updated version of the EU Watch List was published in August 2020 (EU) 2020/1161 [10].

Outlined in the Watch List directive, member states must analyze surface water in order to identify and quantify several pollutants. Nowadays, due to advances in analytical technology and in separation sciences, water analyses have been improved [11,12]. Unfortunately, in this context, one of the most critical issues in water analysis includes compound stability from sampling station to laboratory procedures. For example, some of compounds reported in Watch List show instability in water depending on several physical chemical properties such as solubility, soil/liquid partition coefficient and pH [13,14].

In detail, quantification of Famoxadone and Metaflumizone in water can be considered a challenge in analytical chemistry due to their instability/precipitation, degradation byproducts and chemical reaction properties [15]. For these reasons, in order to identify and quantify the analytes reported in the Watch List, several organizations have been making efforts aimed at validating analytical methods able to determine all compounds in entire samples [16]. Some researchers have made laboratory tests to study the stability of these compounds in water with regard to sampling and analysis conditions such as stability in water, transformations and time from sampling to analysis.

In this paper, we report a study on the stability of antibiotics, pesticides and drugs in water determined using a straightforward procedure by applying mass spectrometric determination for analytical purposes. The laboratory tests were performed in Milli-Q water and surface water by analyzing samples by direct injection, solvent mixture and solid phase extraction from time 0 to 168 h.

2. Materials and Methods

2.1. Chemicals and Reagents

Sulfamethoxazole, Trimethoprim, Velanfaxine, O-Desmethylvenlafaxine, Clotrimazole, Fluconazole, Miconazole, Imazalil, Ipconazole, Metconazole, Penconazole, Prochloraz, Tetraconazole, Tebuconazole, Dimoxystrobin, Famoxadone, Metaflumizone and Ciprofloxacin were purchased from LabService Analytica (Anzola dell'Emilia, Italy).

Isotopically labeled compounds (Imidacloprid D-4, Velanfaxine D-6, Tetraconazole D-9 and Fluconazole D-4 Ciprofloxacin ^{13}C) were used as internal standards (IS), and were purchased from LabService Analytica. The purity grade of all standards was always above 94%.

For Calibration procedures, a mixed stock solution was prepared by serial dilution in acetonitrile (ACN) and stored at $-20\text{ }^{\circ}\text{C}$ in the dark to avoid possible photodegradation. In detail, to obtain a mix solution at $250\text{ }\mu\text{g/L}$ in ACN, $25\text{ }\mu\text{L}$ of each compound at $100\text{ }\mu\text{g mL}^{-1}$ was diluted with 10 mL of ACN. After that, $200\text{ }\mu\text{L}$ of mix solution at $250\text{ }\mu\text{g/L}$ was once again diluted with 10 mL of ACN to obtain a mix solution at $5\text{ }\mu\text{g L}^{-1}$. Calibration standard solutions ranging from 5 to 500 ng L^{-1} in water + ACN mixture (75:25) were prepared by serial dilution from mix solution at $5\text{ }\mu\text{g/L}$.

Intermediate mixed solutions containing all analytes and all labeled compounds were prepared weekly. Aqueous acetonitrile (75:25) working standard solutions were renewed before every analytical run to prevent precipitations.

High purity water was prepared using a Millipore Milli-Q purification system.

Stock solutions were prepared in ACN and were stored at $-18\text{ }^{\circ}\text{C}$ in amber glassware. To avoid standard degradation, calibration working solutions were prepared by serial dilutions of stock solutions in Milli-Q water before each calibration. After reviewing literature data [9] concerning SPE extraction procedures, 47 mm diameter EmporeTM SPE Disks (active group polystyrene-divinylbenzene (SDB-XC)) were used with a SPE-DEX 5000 Horizon Technology for extraction procedures.

LC/MS Acetonitrile grade solvents and formic acid 98% were acquired from (Merck).

2.2. LC-MS Instrumentation

Analyses were performed without extraction procedures except for SPE investigation studies. Separations were performed by using ultra high-performance liquid chromatograph (UHPLC) consisting of binary pump EXION LC Sciex pump, a DGU-20A 5R de-

gassing unit, a SIL-30AC autosampler equipped with a 50 μ L loop, a CTO/20AC thermostat column compartment and a CBM-20A module controller, as well as other components.

The EXION LC SCIEX system was coupled to a 6500 plus Q-Trap mass spectrometer (Sciex), equipped with a Turbo V interface by an ESI probe.

The experimental operating conditions were optimized by standard infusion to detect best ionization conditions and fragmentation.

Mass spectrometry optimal parameters and transitions used are reported in Tables 1 and 2, respectively. Two transitions were used for all analytes: one for quantification and one for qualification.

Table 1. Mass spectrometry general conditions.

Parameters	Unit	Value
Curtain Gas (CUR)	psi	30
Collision Gas	-	Medium
Ion Spray Voltage (IS)	V	4500
Temperature TEM (GS2)	$^{\circ}$ C	450
Ion Source Gas (GS1)	psi	55
Ion Source Gas (GS2)	psi	60

Table 2. Analyte, m/z transitions and operating parameters by ESI-MS_{MRM(+)}.

Analyte	Q1 Precursor Ion [M+H] ⁺ (m/z)	Q3 Product Ion (m/z)	Declustering Potential (DP)	Entrance Potential (EP)	Collision Energy (CE)	Collision Exit Potential (CXP)
Sulfamethoxazole-1	254	156	46	10	24	10
Sulfamethoxazole-2	254	92	46	10	36	9.5
Trimethoprim-1	291	230	40	10	30	10
Trimethoprim-2	291	123	40	10	30	10
Velanfaxine-1	278	260	20	10	15	10
Velanfaxine-2	278	121	20	10	35	10
O-Desmethylvenlafaxine-1	264	246	40	10	20	10
O-Desmethylvenlafaxine-2	264	107	40	10	60	9.5
Clotrimazole-1	277	242	20	10	25	9.5
Clotrimazole-2	277	163	20	10	70	9.5
Fluconazole-1	307	238	40	10	25	9.5
Fluconazole-2	307	220	40	10	22	9.5
Miconazole-1	417	161	40	10	25	9.5
Miconazole-2	417	159	40	10	25	9.5
Imazalil-1	297	159	81	10	25	9.5
Imazalil-2	297	201	81	10	25	9.5
Ipconazole-1	334	70	81	10	37	9.5
Ipconazole-2	334	125	101	10	48	9.5
Metconazole-1	320	70	100	10	50	5.0
Metconazole-2	320	125	90	10	35	5.0
Penconazole-1	284	70	81	10	37	8.0
Penconazole-2	284	159	81	10	35	15
Prochloraz-1	376	308	51	10	15	10.0
Prochloraz-2	376	70	51	10	43	5.0
Tetraconazole-1	372	159	86	10	35	8.0
Tetraconazole-2	372	70	86	10	48	12.0
Tebuconazole-1	308	70	86	10	51	8.0
Tebuconazole-2	308	125	86	10	55	6.0

Table 2. Cont.

Analyte	Q1 Precursion Ion [M+H] ⁺ (m/z)	Q3 Product Ion (m/z)	Declustering Potential (DP)	Entrance Potential (EP)	Collision Energy (CE)	Collision Exit Potential (CXP)
Dimoxystrobin-1	327	116	66	10	29	6.0
Dimoxystrobin-2	327	205	66	10	23	14.0
Famoxadone-1	392	331	46	10	13	10.0
Famoxadone-2	392	238	46	10	23	15.0
Metaflumizone-1	507	178	70	10	35	10
Metaflumizone-2	507	116	70	10	30	10.0
Ciprofloxacin-1	332	231	50	10	48	10.0
Ciprofloxacin-2	332	314	50	10	31	10.0

The compounds were separated by using a CORTEX T3 analytical column (150 mm; 4.6 mm; 5 μ m) using as mobile phase a mixture of water formic acid 0.05% + ammonium formate 5mM and methanol formic acid 0.05% + ammonium formate 5mM in gradient mode. Elution conditions are reported in Table 3.

Table 3. Elution condition.

Time (min)	Flow (mL/min)	% Water + Formic Acid 0.05% + Ammonium Formate 5 mM	% Methanol Formic Acid 0.05% + Ammonium Formate 5 mM
0.0	0.35	90	10
0.1	0.35	90	10
9.0	0.35	2	98
10.0	0.35	2	98
10.1	0.35	90	10
12.0	0.35	90	10

Analysis was performed by multiple reaction monitoring (MRM) both in positive and negative ionization mode by using m/z , declustering potential and collision energy, as reported in Tables 2 and 3.

Analytes were identified both by comparing their retention times (RT) with the RT of the standards and by qualifier ions. Two selected reaction monitoring (SRM) transitions were recorded for each compound: one for quantification and the other one for confirmation. Time-specific SRM windows were set for each retention time in order to enhance sensitivity.

The entire system was controlled via the Analyst software (SCIEX), while quantifications of the analytes were performed with Multi-quant 3.0 (SCIEX).

Quantification was based on the peak area for each compound, and baselines were adjusted manually when necessary.

2.3. Data Validation

The method was validated in terms of detection limits, accuracy and precision usually reported in literature [17]. The limits of quantification (LOQs) were estimated by 10-time standard deviation calculated at the first level of calibration curve prepared in Millipore Milli-Q. Accuracy and precision (RSD%) were calculated on a data set of six analyses conducted on water-spiked samples at LOQ. All data obtained were subject to different statistical tests. Normal distribution was evaluated by using the Shapiro–Wilk test, while outlier data were checked by the Dixon test.

Table 4 summarizes the results obtained for these performance parameters.

Table 4. Accuracy and Precision calculated at LOQ values.

Analyte	LOQ (ng/L)	Accuracy %	Precision RSD%
Trimetoprim	50	90.0	4.9
Venlafaxina	6	99.9	4.3
O-desmetilvenlafaxina	6	115.7	5.6
Clotrimazolo	10	109.7	5.7
Fluconazolo	50	97.4	5.0
Miconazolo	50	80.8	5.3
Imazalil	50	101.7	9.0
Ipconazolo	20	95.8	9.3
Metconazolo	20	95.7	11.3
Penconazolo	50	120.7	7.7
Prochloraz	50	105.1	8.3
Tetraconazolo	50	115.9	3.8
Tebuconazolo	50	116.1	5.8
Dimoxystrobin	20	90.1	9.7
Famoxadone	8	108.9	8.9
Metaflumizone	50	107.2	9.0
Ciprofloxacin	50	92.6	8.3
Sulfamethoxazole	50	93.5	9.9

For all the analytes, accuracy at LOQ Levels ranged from 70 to 130%, while precision (in term of RSD) were less than 20%.

3. Results

Test Performed

In order to investigate the chemical stability of the analyte, three different systems were spiked with an analyte mixture at a concentration of 100 ng L⁻¹ and analyzed.

In detail, the system studied are reported below:

- (i) Surface Water + analytes at 100 ng L⁻¹;
- (ii) Surface Water + ACN 25% + analytes at 100 ng L⁻¹;
- (iii) Milli Q + ACN 25% + analytes at 100 ng L⁻¹.

All samples were stored at 4 °C. The surface water used was collected from a previously analyzed river in the Lombardia region.

The result obtained are reported in Table 5 and as percentage recoveries.

The data reported in Table 5 show that analytes Clotrimazole, Miconazole, Ipconazole, Famoxadone and Metaflumizone are unstable in aqueous solution without organic solvents, while they show partial stability in the solutions obtained from aqueous solution + ACN 25%.

This trend confirms the insolubility of several substance in water as reported in literature [18].

In the first approximation, it is possible to hypothesize that low recoveries can be ascribed to the difference in solubility of the analytes in water and water with added organic solvent.

In fact, as reported in literature, several substances such as Metaflumizone, Fomaxadone and Miconazole show hydrophobic behavior.

The best stability results were obtained in the mixture Milli Q water + ACN 25% (percentage recoveries from 104 to 124%), while in surface water + ACN 25% some analytes show a decrease in stability. However, in the system surface water + ACN 25%, the analyte shows recoveries in the range from 70 to 130% in accordance with requirements for validation procedures.

Table 5. Percentage recovery of analyte at 100 ng L⁻¹ in Surface water + ACN 25%, Milli Q + ACN 25%, Surface Water + ACN 25% and Milli Q + ACN 25%.

Analyte	Recovery % Surface Water + Analyte at 100 ng L ⁻¹	Recovery % Surface Water + ACN 25% + Analyte at 100 ng L ⁻¹	Recovery % Milli Q + ACN 25% + Analyte at 100 ng L ⁻¹
Sulfamethoxazole	110 ± 6	99 ± 7	109 ± 5
Trimethoprim	73 ± 8	70 ± 6	118 ± 8
Velanfaxine	76 ± 2	78 ± 4	106 ± 3
O-Desmethylvenlafaxine	73 ± 3	75 ± 3	109 ± 5
Clotrimazole	39 ± 10	71 ± 7	106 ± 9
Fluconazole	82 ± 5	80 ± 7	110 ± 7
Miconazole	14 ± 16	110 ± 11	124 ± 8
Imazalil	60 ± 9	74 ± 3	124 ± 5
Ipconazole	49 ± 5	88 ± 5	110 ± 6
Metconazole	65 ± 9	79 ± 12	107 ± 11
Penconazole	61 ± 8	83 ± 6	118 ± 8
Prochloraz	62 ± 11	82 ± 7	111 ± 5
Tetraconazole	70 ± 8	83 ± 9	104 ± 7
Tebuconazole	71 ± 5	81 ± 7	113 ± 9
Dimoxystrobin	79 ± 8	84 ± 5	106 ± 7
Famoxadone	27 ± 12	85 ± 6	105 ± 10
Metaflumizone	6 ± 3	73 ± 4	112 ± 3
Dimoxystrobin	112 ± 4	103 ± 7	104 ± 8

This behavior would seem to highlight a possible matrix effect that decreases the recoveries of some analytes in surface water.

Furthermore, the analytes that show lowest recovery values in water with added ACN appear to have ideal behavior in the QC control sample prepared in milli-Q water.

To investigate the effects of particulate matter concerning analyte stabilities, several tests on surface water that was unfiltered, filtered and unfiltered were performed. The analytes were extracted by ultrasound in order to migrate analytes from particulate matter to solutions, and solutions were analyzed during time (0–24 h). The tests we performed are reported in detail below, and the results are reported in Table 6.

(j) Surface Water filtered + ACN 25% + analyte at 100 ng L⁻¹ analysis performed after 24 h;

(jj) Surface Water + ACN 25% + analyte a 100 ng L⁻¹ analysis performed after 24 h;

(jjj) Surface Water + ACN 25% + analyte at 100 ng L⁻¹ a after 24 h sonicated by ultrasound and analyzed;

(jjjj) Surface Water + analyte a 100 ng L⁻¹ analysis performed after 24 h.

Data reported in Table 6 highlight that, in the systems investigated, the best recoveries were obtained by system j (Surface Water filtered + ACN 25% + analyte at 100 ng L⁻¹ analysis performed after 24 h GREEN COLOR).

These data confirm that the stability of compounds is greater by the addition of ACN even after 24 h both in filtered water + ACN 25% and in unfiltered water + ACN 25%.

Furthermore, in order to underline possible effects after 24 h from sampling to analysis, different percentages for spiked samples at 100 ng L⁻¹ of “water + ACN 25% at t0” and “water + ACN 25% at t24 h” are reported in Table 7. If uncertainty values are taken into account, the differences detected cannot be considered relevant.

Table 6. Percentage recovery of analyte at 100 ng L⁻¹ after 24 h in: Surface Water filtrated + ACN 25%, Surface Water + ACN 25%, Surface Water + ACN 25% and sonicated by ultrasound and Surface Water.

Analyte	Percentage Recoveries in Surface Water filtrated + ACN 25% + Analyte at 100 ng L ⁻¹ a Time 24 h	Percentage Recoveries in Surface Water + ACN 25% + Analyte a 100 ng L ⁻¹ at Time 24 h	Percentage Recoveries in Surface Water + ACN 25% + Analyte at 100 ng L ⁻¹ a Time 24 h and Sonicated by Ultrasound	Percentage Recoveries in Surface Water + Analyte a 100 ng L ⁻¹ at Time 24 h
Sulfamethoxazole	109 ± 18	114 ± 13	96 ± 15	112 ± 6
Trimethoprim	81 ± 15	70 ± 10	55 ± 16	68 ± 4
Velanfaxine	105 ± 3	82 ± 11	62 ± 7	78 ± 1
O-Desmethylvenlafaxine	89 ± 16	88 ± 5	56 ± 6	80 ± 6
Clotrimazole	85 ± 15	75 ± 7	64 ± 14	23 ± 8
Fluconazole	87 ± 17	74 ± 9	66 ± 11	77 ± 10
Miconazole	98 ± 11	83 ± 13	67 ± 7	5 ± 2
Imazalil	91 ± 8	61 ± 8	58 ± 7	55 ± 7
Ipconazole	93 ± 17	75 ± 11	72 ± 13	49 ± 8
Metconazole	84 ± 14	69 ± 9	70 ± 12	61 ± 10
Penconazole	87 ± 15	69 ± 5	59 ± 10	63 ± 6
Prochloraz	88 ± 17	77 ± 12	66 ± 9	54 ± 8
Tetraconazole	92 ± 17	84 ± 10	68 ± 11	69 ± 8
Tebuconazole	81 ± 12	73 ± 11	55 ± 9	68 ± 7
Dimoxystrobin	79 ± 15	65 ± 9	67 ± 15	72 ± 6
Famoxadone	74 ± 8	62 ± 8	53 ± 6	8 ± 7
Metaflumizone	90 ± 17	65 ± 2	77 ± 15	3 ± 1
Ciprofloxacina	86 ± 18	94 ± 18	53 ± 18	92 ± 7

Table 7. Percentage differences about analysis performed at t0 and after 24 h.

Analyte	Percentage Recoveries in Surface Water + ACN 25% + Analyte a 100 ng L ⁻¹ at Time 0	Percentage Recoveries in Surface Water + ACN 25% + Analyte a 100 ng L ⁻¹ at Time 24 h	Percentage Differences
Sulfamethoxazole	99 ± 6	114 ± 7	-15.5
Trimethoprim	70 ± 5	70 ± 5	0.5
Velanfaxine	78 ± 7	82 ± 6	-5.6
O-Desmethylvenlafaxine	75 ± 4	88 ± 4	-16.9
Clotrimazole	71 ± 8	75 ± 7	-5.2
Fluconazole	80 ± 3	74 ± 8	7.1
Miconazole	110 ± 8	83 ± 2	24.2
Imazalil	74 ± 9	61 ± 4	17.1
Ipconazole	88 ± 9	75 ± 9	15.2
Metconazole	79 ± 10	69 ± 8	13.1
Penconazole	83 ± 8	69 ± 3	17.3
Prochloraz	82 ± 11	77 ± 13	6.5
Tetraconazole	83 ± 9	84 ± 7	-0.8
Tebuconazole	81 ± 6	73 ± 5	9.9
Dimoxystrobin	84 ± 8	65 ± 4	22.2
Famoxadone	85 ± 11	62 ± 7	27.1
Metaflumizone	73 ± 10	65 ± 6	11.0
Dimoxystrobin	103 ± 5	94 ± 9	9.1

Finally, according to EU decision, which requires samples to be analyzed in the entire water sample, quantification tests were also carried out by solid phase extraction by using SPE Horizon SPEDEX 5000 system equipped with an extraction disk in SDB and which involves the elution of the sample, the washing of the particulate collected on the filter with ACN and the reunification of the organic phase with the aqueous eluate.

In detail, operating conditions for solid phase extraction procedures using SPE-DEX 5000 Horizon Technology are reported in Table 8.

Table 8. Operating condition for SPE procedures.

Method Steps	Eluent Used	Exhaust Line or Sample Line
Condition SPE Disk	10 mL ACN	Exhaust line
Load Sample	100 mL samples	Sample line
Elute Sample Container	25 mL ACN	Sample line
Air Dry Disk Timer	30 sec by nitrogen	Sample line
Pause		
Clean System	20 mL Methanol/water 50/50	Exhaust line

By using this analytical procedure, all analytes, without solubility differences, were collected in water + ACN 100 + 25% *v/v*.

Analysis was carried out after 168 h from spiked samples, and data obtained are summarized in Table 9.

Table 9. Percentage recoveries in Surface Water + analyte a 100 ng L⁻¹ at time 168 h using SPE procedures.

Analyte	Percentage Recoveries in Surface Water + Analyte at 100 ng L ⁻¹ at Time 168 h Using SPE Procedures
Ulfamethoxazole	102 ± 7
Trimethoprim	81 ± 2
Velanfaxine	110 ± 9
O-Desmethylvenlafaxine	108 ± 2
Clotrimazole	92 ± 11
Fluconazole	110 ± 4
Miconazole	94 ± 14
Imazalil	105 ± 12
Ipconazole	95 ± 10
Metconazole	103 ± 5
Penconazole	106 ± 10
Prochloraz	111 ± 5
Tetraconazole	111 ± 8
Tebuconazole	102 ± 12
Dimoxystrobin	118 ± 4
Famoxadone	72 ± 2
Metaflumizone	79 ± 2
Ciprofloxacin	99 ± 2

For all analytes investigated, the recoveries after 168 h from spiked operations are in the range from 70 to 130%, in good accordance with requirements from guidelines in analytical chemistry.

In detail, by particulate washing with organic solvent, analytes absorbed from particulate matter can be desorbed from solid matter, eluated, collected and reunified with the aqueous solution.

Based on these considerations, it is possible to conclude that by using SPE procedure, it is possible to analyze an entire water sample according to EU guideline [19,20].

4. Conclusions

This study has highlighted some critical aspects in the analysis of several chemical compounds during Watch List analyses on water samples.

In detail, the results obtained can be summarized as follows:

- Several analytes show a matrix effect: In surface water samples, the analyte Clotrimazole, Miconazole, Ipconazole, Famoxadone and Metaflumizone, if not properly treated, are not stable in aqueous solution even within a few hours from sampling or sample preparation;
- All the analytes of the WL are stable in aqueous solutions with the addition of at least 25% ACN even after 168 h from sampling;
- The analysis of Watch List compounds can be carried out through the use of the SPEDEX Horizon 5000 system;
- For all analytes investigated, the percentage recoveries were between 70 and 130% by using SPE procedures followed by a UHPLC-MS/MS analysis, in good agreement with method validation procedures.

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S.B. and L.C. (Luisa Colzani); writing—original draft preparation, S.B., L.C. (Luisa Colzani) and D.D. project administration, P.D. All authors have read and agreed to the published version of the manuscript.

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