



On-line solid phase extraction-liquid chromatography-tandem mass spectrometry for insect repellent residue analysis in surface waters using atmospheric pressure photoionization

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ABSTRACT

Insect repellents (IRs) are a group of organic chemicals whose function is to prevent the ability of insects of landing in a surface. These compounds have been found in the environment and may pose a risk to non-target organisms. In this study, an on-line solid phase extraction – high performance liquid chromatography-tandem mass spectrometry multiresidue method was developed using an atmospheric photoionization source (SPE-HPLC-(APPI)-MS/MS). The use of the APPI as an alternative ionization technique to electrospray (ESI) and atmospheric pressure chemical ionization (APCI) allowed expanding the range of analytical techniques suitable for the analysis of IRs, so far relied in gas chromatography. High sensitivity and precision was reached with method limits of quantification between 0.2 and 4.6 ng l⁻¹ and interday and intraday precision equal or below 15%. The validated method was applied to the study of surface water samples from three European river basins with different flow regime (Adige River in Italy, Sava River in the Balkans, and Evrotas River in Greece). The results showed that two IRs (DEET and Bayrepel) were ubiquitous in the Sava and Evrotas basins, reaching concentrations as high as 105 µg l⁻¹ of Bayrepel in the Sava River, and 5 µg l⁻¹ of DEET in the Evrotas River. Densely populated areas and effluent waste waters are pointed out as the responsible for this pollution. In the alpine river Adige, only three samples showed low levels of IRs (6.01–37.8 ng l⁻¹). The concentrations measured were used to perform an environmental risk assessment based on the hazard quotients (HQs) estimation approach by using the chronic and acute eco-toxicity data available. The results revealed that despite the high frequency and eventually high concentrations of these IRs determined in the three basins, only few sites were at risk, with 1 < HQs < 3.3.

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1. Introduction

Most organisms live in an environment full of different smells and perceive through abiotic and biotic means a dynamic mix of scents called infochemicals [1]. An infochemical is a chemical that transmits information generated by one organism to a second organism in the environment. This information can then lead to a response in the receiving organism. Both the sender and the receiver benefit from this process [2]. Infochemicals play an important role in the history of life, the ability to find the habitat, the recognition of food and survival. Besides, they are the main source of communication in aquatic ecosystems, as other senses such as

vision and mechanical senses are less efficient in murky and turbulent conditions [3]. In several invertebrates and fish, it has been shown that the system for communication and detection is distorted by the presence of pollutants [4]. This distortion effect is referred to as *infochemical effect* or *infodisruption* [5]. Among the synthetic chemicals potentially causing such outcomes we find insect repellents (IR). IRs are a group of organic synthetic chemicals whose function is to prevent the ability of insects of landing in a surface and can be applied on top of clothes, skin or other surfaces and help to avoid insect bites. The most common insect bites are those of mosquitoes, flies, fleas, and spiders, and their bite can produce many adverse effects such as skin irritation, allergic reactions, infections and even cause the spread of illnesses such as west Nile fever, malaria, dengue fever or encephalitis [6]. Given their function, these compounds are an alternative to pesticides, whose purpose is to kill rather than to prevent.

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IRs have been used for many millennia as the ancient Egyptians applied castor oil extracts and the Romans used vinegar to prevent mosquito bites [6]. Today, there are new synthetic substances; research and development of these came mainly to protect the military from bites and the potential diseases associated with them. The first used IR contained essential oils derived from plants such as citronella, but had very limited duration. During World War II the M-250 was developed containing DMP (dimethyl phthalate), Indalone (Butyl 3,4-Dihydro-2,2-dimethyl-4-oxo-2H-pyran-6-carboxylate) and Rutgers 612 (2-ethyl-1,3-hexadiol), but in 1991 it was removed from production in response to an unpublished study showing that treated animals suffered shortfalls [7]. In 1953 N, N-diethyl-m-toluamide (DEET) was synthesized. This was probably the most important compound discovered regarding IRs and it still remains the most used worldwide [8]. This may be due to DEET, unlike other IRs, being effective against all types of mosquitoes, many varieties of flies, ticks, fleas and berry bugs. DEET has been used as the reference IR of application in humans and, therefore, all the new substances are compared with it [9]. Nevertheless, new compounds have been developed in order to overcome the shortcomings of DEET. New methodologies such as molecular modelling and characterization of many new repellent substances are expected to improve the length of time they are active, the number of species which they protect from, and, especially, to minimize side effects on human beings [10]. From these efforts, new substances, such as 3-[N-butyl-N-acetyl]-aminopropanoate ethyl (EBAAP, IR3535) and 2-(2-hydroxyethyl)-1-metilpropyl ester (Bayrepel), have been developed [11].

In a recent work, DEET, EBAAP, and Bayrepel were pointed out to be potential infochemicals, not only as their effectiveness relies on the disruption of chemical communication in the target species but also as they can affect not target organisms and alter their ecosystem as well [2]. Moreover, concerns appeared because DEET was found to display neurotoxicity in insects, and potentially in humans [12–15]. Recently, some studies explored the hypothesis that selected IRs are able to disturb chemical communication and cause organismic effects like drift in crustaceans and insect larvae in natural waters with different effects [2,16,17]. Nevertheless, the information on this subject is still limited.

IRs have boiling temperatures between 230 and 260 °C, which facilitates the formation of a IR layer above the applied surface [18], and partition coefficients octanol-water ($\log K_{ow}$) below 3.5, making them fairly soluble in water. This pose a potential environmental risk factor, as low $\log K_{ow}$ values generally boost their spread across the aquatic environment [19–21].

In humans, even though IRs should pose not risk when correctly applied, DEET has been reported as the source of adverse effects in half a hundred reports from the United States [22] during its extensive time usage. In regards to the risk that they pose to living organisms, studies of use and application in animals have shown that these compounds possess overall low toxicity, with 50% lethal dose (LD_{50}) in mammals in the range of the thousands of $mg\ kg^{-1}$ [23], and 50% lethal concentrations (LC_{50}) in insects in the range of $mg\ l^{-1}$ [24,25].

The analysis of this class of personal care products (PCPs) in water requires of a sample pretreatment usually consisting on solid phase extraction (SPE) [26,27], liquid–liquid extraction (LLE) [20,28] or stir bar sorptive extraction (SBSE) [19,29]. Due to their volatility, IRs have been analysed using methods based on gas chromatography with mass spectrometry detection (GC–MS) [20,30]. Nevertheless, during the last years and due to the need to create multiresidue methods, new methodologies relying on high performance liquid chromatography coupled to mass spectrometry (HPLC–MS/MS) and different ionization techniques have been developed as a feasible alternative analysis for volatile chemicals [27,31,32]. Following this trend, the aim of this work was to

develop and validate a fully-automated method based on HPLC for the analysis of 5 synthetic IRs of human use in surface water. The method relied on an on-line SPE followed by high performance liquid chromatography coupled to tandem mass spectrometry, with an atmospheric pressure photoionisation source (SPE–HPLC–APPI–MS/MS). The APPI is a relatively new ionization technique proved useful in the determination of less polar, poorly ionisable analytes. The most analysed IRs so far were DEET and Bayrepel (also known as Picaridin), both being extensively used worldwide for decades [19]. In the present study, these two IRs were analysed together with a new generation of repellents that appear to be good substitutes for DEET. Finally, an example of the applicability of the validated method for the determination of the selected IRs residues and their estimated environmental risk in river water from three European river basins is presented.

2. Experimental

2.1. Standards and reagents

DEET (97.7%), *m*-toluamide (99%), *p*-menthane-3,8-diol (PMD; 99%), *N*-octyl bicycloheptene dicarboximide (MGK-264; 99%), and piperonyl butoxide (PBO; 99%) were purchased from Sigma-Aldrich (Steinheim, Germany). Bayrepel (99%) and EBAAP (99%) were obtained from Dr. Ehrenstofer (Augsburg, Germany). Carbamazepine- d_{10} (99% D) and triphenylphosphat- d_{15} (99%D), used as surrogate standards, were obtained from Sigma-Aldrich. Table 1 lists some physicochemical information about the target IRs.

The HPLC-quality solvents methanol (MeOH), acetonitrile (ACN), acetone, toluene, and water were provided by Merck (Darmstadt, Germany).

Individual stock solutions were prepared in MeOH at $100\ mg\ l^{-1}$. Mix standard solutions were prepared at $1\ mg\ l^{-1}$ and subsequently diluted as needed.

2.2. Sampling site

Three European rivers were selected for this study: the Evrotas River in Greece, the Sava River in the Balkans, and the Adige River in Italy. These three basins were selected as representatives of different characteristics, such as flow regime. Fig. 1 shows the three river basins in their geographic context.

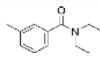

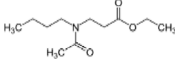
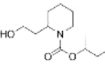
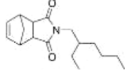

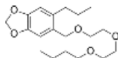
The Evrotas River is the most important river in the Laconia region in Greece. It has 82 km longitude and its basin encompasses 2418 km^2 . This river is a characteristic Mediterranean river, alternating between a somewhat constant flow of water and partial droughts during the warmest months of the year. The river flows across a rurally developed area and it is the target of hydric over-exploitation and pollution. Along its basin, there is one wastewater treatment plant (WWTP) [33].

The Sava River, tributary of the Danube River, is one of the longest rivers in Europe, with 945 km longitude and a basin area of 97,713 km^2 . This river is extended over Slovenia, Croatia, Bosnia-Herzegovina, and Serbia, and supports a population of approximately 8.2 million people (42% of the total population of these 4 countries). The high course of the river is mainly affected by hydromorphologic pressures, in the middle course it is impacted by rural activities and eutrophication problems, whereas in the lower course urban and industrial areas exert pressure on its waters [33].

The Adige River is the second longest river of Italy, with 410 km of longitude and a basin of 12,000 km^2 . This river originates from glacial ice in the Alps and its flow is greatly dependant on the season, with higher flows during the summer season due to the melting of ice. The higher course of the river is impacted by tourism,

Table 1

Name, abbreviation, structure, CAS number, molecular weight (MW), structure, partition coefficient octanol-water (Log Kow) and solubility of the target compounds.

Name	Abbreviation	CAS number	MW (g mol ⁻¹)	Structure	Log Kow ^a	Solubility (mg l ⁻¹) ^b
<i>N,N</i> -diethyl- <i>m</i> -toluamida	DEET	134-62-3	191.27		2.20	666
<i>m</i> -Toluamide	<i>m</i> -toluamide	618-47-3	135.06		1.18	8613
Ethyl 3-[acetyl(butyl)amino]propanoate	EBAAP	52304-36-6	215.15		1.51	1867
Hydroxyethyl isobutyl piperidine carboxylate	Bayrepel	119515-38-7	229.16		1.55	2886
<i>N</i> -octyl bicycloheptene dicarboximide	MGK-264	113-48-4	275.18		3.70	11.78
<i>p</i> -Menthane-3,8-diol	PMD	42822-86-6	172.15		2.29	670.7
Piperonyl butoxide	PBO	51-03-6	338.21		4.29	0.638

^a Log Kow estimated with KOWWIN v1.67.^b Solubility in water at 25 °C estimated with WSKOW v 1.41.**Fig. 1.** Geographical location of the three studied rivers.

whereas its high and middle courses house several dams greatly altering its hydrology [33].

2.3. Sample collection

Thirty-three surface water samples were collected: 8 samples from the Evrotas River, 13 samples from the Sava and 12 from the Adige. Sampling was performed in June 2014 in the Evrotas River, in September 2014 in the Sava, and in May 2015 in the Adige.

The sample name and sampling location and description are shown in Table A1 of the Supporting Information.

2.4. Sample preparation

The sample treatment is described in Gago-Ferrero et al. [34]. Upon arrival to the laboratory, the waters were filtrated using 0.45 μm nylon membrane filters (Whatman; Maidstone, UK). An aliquot of 25 ml of the filtered water was then spiked with the surrogate standards solution to achieve a concentration of 50 ng l⁻¹. The following on-line analysis was carried out with 5 ml of that solution.

2.5. Sample extraction

Samples were loaded using an automatic injector and were transferred into a Transcend chromatograph (Thermo Fisher Scientific; Waltham, Massachusetts, US) for on-column SPE. The SPE column HyperSep Retain PEP (EQUAN 5) from Thermo Fisher Scientific was used.

The analytes were loaded using 100% water as mobile phase during a period of 3.42 min and a flow rate of 1.25 ml min⁻¹, after which they were eluted into the chromatographic system using the mixture 90% MeOH:10% water and a flow rate of 0.3 ml min⁻¹ during the next 2 min.

2.6. HPLC-MS/MS determination

Separation of the analytes was achieved using a Purospher® Star RP-18 (125 × 2.0 mm; 5 μm) C18 column (Merck) LC analytical column. Eluent A consisted of water and B of MeOH. The gradient was as follows: it started with a 75% of B, increased to 80% at min. 3.42; at min. 3.83 it reached 90% of B and achieved 100% of B at min. 5.83; this proportion was maintained until the min. 8.67, and then

Table 2
SRM transitions, S-Lens, and collision energy (CE) for the target IRs.

Compound	SRM Transitions (<i>m/z</i>)	S-Lens (V)	CE (V)
DEET	192 → 91	58	29
	192 → 119		15
<i>m</i> -Toluamide	136 → 91	48	21
	136 → 77		24
EBAAP	216 → 77	52	28
	216 → 143		15
Bayrepel	230 → 130	47	16
	230 → 112		18
MGK-264	276 → 210	68	14
	276 → 98		23
PBO	356 → 177	49	16
	356 → 149		33
PMD	173 → 155	73	5
	173 → 110		8
Carbamazepine- <i>d</i> ₁₀	247 → 204	68	23
	247 → 204		40
Triphenylphosphate- <i>d</i> ₁₅	342 → 222	77	27
	342 → 81		36

returned to the initial conditions up to min. 9.5. The analyses were performed at room temperature and at a flow rate of 0.3 ml min⁻¹.

Analytes were detected by APPI-MS/MS using a combined APCI/APPI ion source (Thermo Fisher Scientific) in positive mode operated under selected reaction monitoring (SRM) in a TSQ Vantage (Thermo Fisher Scientific) triple quadrupole mass spectrometer. The source and MS/MS experimental parameters were as follows: discharge current: 4 V; vaporisation temperature: 350 °C; sheath gas pressure: 0.5 bar; auxiliary gas pressure: 20 bar; capillary temperature: 300 °C, and collision pressure: 1 mTorr. The optimisation of the SRM transitions for each compound was carried out by the flow injection analysis of 500 ng ml⁻¹ individual standard solutions. Table 2 lists the SRM transitions and the collision energies applied for the selected IRs.

2.7. QA/QC

In order to ensure the reliability of the analyses, the following considerations were taken: (1) to avoid cross-contamination gloves were used during the whole process, solvent bottles were exclusively employed for these analyses, and all glass laboratory material was washed with HPLC-grade water and organic solvents and heated at 350 °C overnight; (2) reagent blanks were obtained by conducting the same treatment as the samples by substituting them with HPLC grade water; (3) quality control solutions spiked at known concentrations of the target analytes were used through the analysis; (4) quantification was performed by internal standard calibration based on peak areas.

3. Results and discussion

3.1. Method optimisation

3.1.1. SPE separation and purification

For the on-line SPE three SPE columns were tested using the same loading and unloading conditions (see Section 2.5): EQUAN 5, HyperSep Retain-CX (EQUAN 6) and HyperSep Retain-AX (EQUAN 8) all them provided by Thermo Fisher Scientific. EQUAN-5 is composed of polystyrene divinylbenzene material modified with urea groups that allows the retention of analytes with a wide range of polarities. EQUAN-6 is stuffed of a polymer that retains basic compounds, whereas EQUAN-8 consists of a polymeric phase that interacts with acidic compounds.

Hence, the selection criterion for the column was based on an efficient retention and a quick elution of the selected IRs from it. Figure A1 shows the reconstructed ion chromatograms for a sample

containing 50 ng l⁻¹ of each IR for the three extraction column in absence of the analytical column. EQUAN 5 was the only one among the tested SPE columns able to retain and to elute all the target analytes and surrogate standards. On the other hand, EQUAN 8 was unable to quickly unload all analytes, being unable of retaining the standard TPhP-*d*₁₅ but allowing a better separation of EBAAP from an impurity. Similarly, EQUAN 6, presented overall better retention and elution than the other columns for *m*-toluamide, Bayrepel, DEET, and carbamazepine-*d*₁₀. Nevertheless, after all the tests performed, EQUAN 5 offered the best retention efficiency and quickest elution for all the studied compounds, and thus, it was selected for further analyses. In all cases, the signal of MGK-264 was composed by two peaks, corresponding to the endo- and exo-enantiomers produced in the synthesis process [35].

3.1.2. Optimisation of the HPLC-APPI-MS/MS

For the chromatographic separation of the target analytes, isocratic and non-isocratic gradients varying the proportions water and MeOH were tested. The gradients had increasing proportions of MeOH, from 60% to 100% and varying initial conditions. MeOH was substituted with ACN and similar assays were performed. As no significant differences were observed, MeOH was finally selected as organic phase because it is a slightly protonic solvent which enhances APPI ionization [36].

Whenever APPI sources are used, it is common to add doping agents (dopant-assisted-APPI) in order to enhance the signal of the analytes during the detection process [32,36]. These substances are used at high concentrations leading to improve in 10–100-folds the ionization performance. The process begins with the ionization of the dopant agent, which acts as an intermediary to ionize the molecules of the analytes. After defining the extraction and separation best conditions, the optimization of the dopant agent was completed by performing three analyses: (1) without doping agent, (2) with toluene, and (3) with acetone. The doping agents were added to the system using an external pump containing a loaded syringe of 2.5 ml at a flow rate of 0.03 ml min⁻¹. The syringe was connected to the chromatographic system after the analytical column using a T-junction. A sample containing a mix of the target compounds and the surrogate standards at 50 ng l⁻¹ was used in all tests. Figure A2 shows the reconstructed ion chromatograms for the three experiments. As expected, the addition of doping agents increased the signal in most cases. When comparing the intensity of the signal, acetone was found to be the best doping agent, achieving signal increments between 10 and 90% with respect to the test without doping agents. In comparison, toluene offered no significant increase for most of the target

Table 3

SPE-HPLC-APPI-MS/MS method performance. ILOD: instrumental limits of detection; ILOQ: instrumental limits of quantification; MLOD: method limits of detection; MLOQ: method limits of quantification; %RSD: relative standard deviation.

Compound	Retention time (min)	Calibration Range (ng l ⁻¹)	r ²	ILOD (pg)	ILOQ (pg)	Precision (%RSD)		MLOD (ng l ⁻¹)	MLOQ (ng l ⁻¹)	ME (%)
						Intraday	Interday			
DEET	5.83	1–500	0.999	0.5	1	5	12	0.1	0.2	10
<i>m</i> -Toluamide	5.26	3–500	0.998	1.0	3.5	6	14	0.2	0.7	9
Bayrepel	5.92	1–500	0.996	1.5	4.5	7	9	0.3	0.9	12
MGK 264	7.12–7.41	1–500	0.999	1.0	3	7	12	0.1	0.4	13
PBO	7.67	3–500	0.999	7.0	23	13	15	1.4	4.6	14

compounds, increases of over 90% for the surrogate standards and around 30% for *m*-toluamide. Nevertheless, toluene also enhanced the background signals in the *m*-toluamide transitions, hindering its identification. The ionization of PMD was not successfully under any condition, probably due to its structural lack of easily photoionisable groups. A different problem arose with EBAAP; *n*-butyl benzenesulfonamida (*n*-BBS), a common compound used as a plasticiser, had similar ionised molecular mass ($[M-H]^+ = 214 \text{ g mol}^{-1}$), and was found to leak from the capillaries of the instrument. Its signal was quite intense, interfering with the EBAAP's $[M-H]^+$ ion signal, and thus preventing the quantitative analysis. However, and despite the signal enhancement provided by acetone, its use was discarded because the unequivocal identification of the compounds was reliable without its addition, and because of the lack of suitable automatic equipment to perform the addition of the dopant during the analysis of large batches of samples.

3.2. Method performance

The performance of the developed method is presented in Table 3. PMD and EBAAP could not be analysed by the developed method due to the issues described in Section 3.1.2. Wide calibration ranges were obtained; 1–500 ng l⁻¹ for DEET, Bayrepel, and MGK 264 and between 3 and 500 ng l⁻¹ for *m*-toluamide and PBO, always achieving good linearity ($r^2 > 0.999$). Intraday and interday precision, expressed as relative standard deviations (%RSD) were in the range 6–15%. The instrumental limits of detection (ILOD) were in the range 0.5–7 pg, whereas the instrumental limits of quantification (ILOQ) ranged from 9 to 15 pg. The method limits of detection (MLOD) and limits of quantification (MLOQ) were in the ranges 0.1–1.4 ng l⁻¹ and 0.2–4.6 ng l⁻¹, respectively. The IUPAC guidelines [37] were followed to calculate the ILOD, ILOQ, MLOD, and MLOQ. Additionally, matrix effects were estimated by comparing the slope of the calibration curves in HPLC grade water and in surface waters free of the target analytes (matrix matched standards). A small increase in the signal (14%) of the target compounds was observed as a consequence of matrix effects.

Fig. 2 presents the reconstructed ion chromatogram of a 50 ng l⁻¹ mixture standard solution.

The developed methodology showed some advantages. For instance, compared with methodologies reliant on GC–MS, it achieves better (35 ng ml⁻¹) [29] or similar MLODs and MLOQs to those found in the literature (0.2–13 ng l⁻¹) [19,26]. The fact that the described methodology performs the purification of the samples on-line is advantageous, reducing the analysis time and solvents' volume and potential mass losses derived of the different steps of off-line SPE procedures. In the same line, Wang et al. [31,32] developed a methodology for the analysis of DEET, among other micropollutants, using SPE-HPLC-APPI-MS/MS, achieving MLODs in the range 0.3–15 ng l⁻¹. In comparison, our methodology represents a step forward, as it expands the number of IRs analysed by HPLC–MS/MS and greatly reduces the chromatographic run time, as the retention times for DEET in Wang et al. was 14 min and in our method it was 5.83 min.

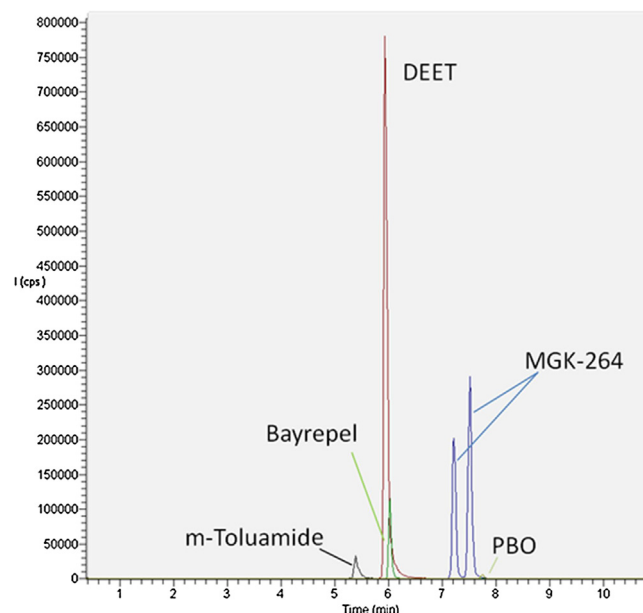


Fig. 2. Reconstructed ion chromatogram of a standards solution (50 ng l⁻¹) recorded by the validated method. I (cps): signal intensity.

3.3. Application of the method to the analysis of river waters

The concentrations of IRs determined in the water samples from Adige, Evrotas and Sava rivers are presented in Fig. 3 and Table A2 of the Supporting Information. Mean concentrations, and detection frequencies are showed in Table 4. Results indicated that the Adige River had the overall lowest concentrations and detection frequencies (25%). This might be due to its nature as a glacial originated river and a lower use of IRs along its basin. However, both the Evrotas and Sava rivers had higher concentrations and 100% of the samples contained IRs. IRs' maximum and minimum concentration in the Evrotas River were 91.15 and 4955 ng l⁻¹, respectively, whereas the concentrations in the Sava River were somewhat lower, between 3.410 and 105.4 ng l⁻¹. *m*-toluamide and MGK 264 were not detected in any sample and PBO was measured in only one water sample from the Evrotas River (19.33 ng l⁻¹). Both MGK-264 and PBO have high Log K_{ow} values, 3.70 and 4.29, respectively, that could favour their adsorption on sediments and suspended particulate matter. DEET has been widely used for the last 50 years and our results show that this trend is maintained through the countries these three rivers flow. In the Evrotas, DEET presented high concentrations (above 400 ng l⁻¹) in all samples but one. USkollini7 and USkollono22 are in a background reference site for drought periods but both points showed pretty different concentrations (2,005 and 59.22 ng l⁻¹). This important difference might be attributed to the sampling date. USkollini7 is located in the uppermost course of the river in a scarcely populated area. During the summer, it is usual that the river practically dries and thus notoriously increasing the effects of pollution discharges, for instance by WWTPs effluents, as

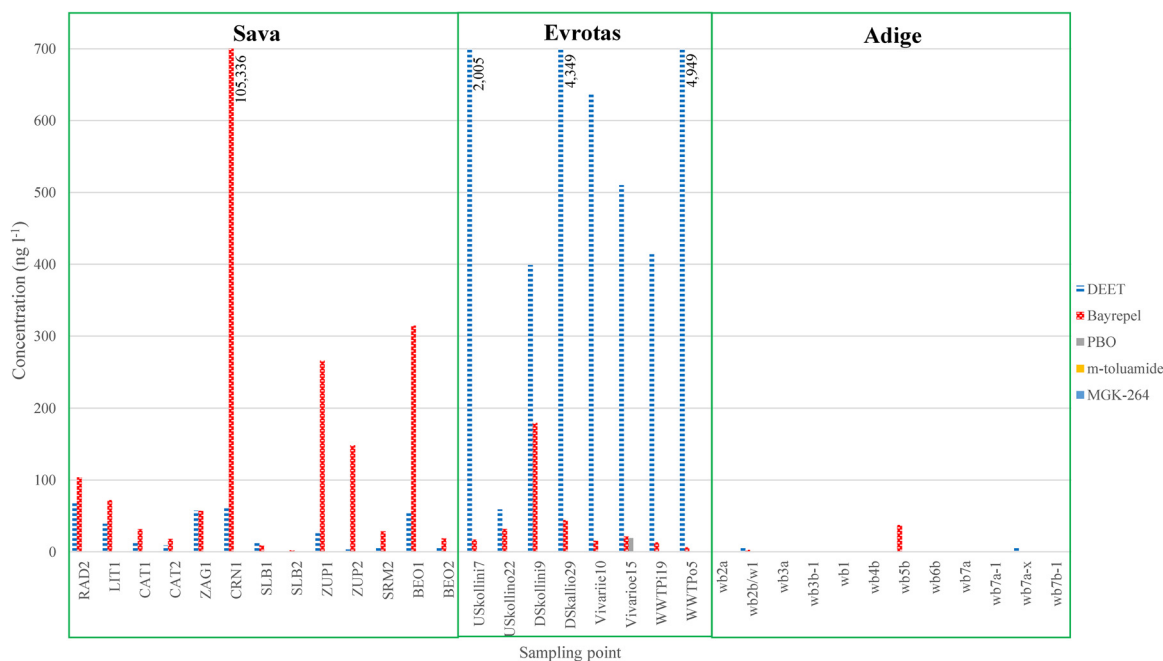


Fig. 3. IRs concentrations in the samples from the rivers Sava, Evrotas, and Adige.

Table 4
Mean concentration, standard deviation (SD) and frequency of detection (%) of target IRs in the three river basins studied. n.d.: not detected; n.a.: not applicable.

		DEET	<i>m</i> -Toluamide	Bayrepel	MGK-264	PBO
Sava	Mean concentration (ng l ⁻¹)	12.34	n.d.	57.28	n.d.	n.d.
	SD (ng l ⁻¹)	25.14	n.a.	n.a.	n.a.	n.a.
	Frequency (%)	100	0	100	0	0
Evrotas	Mean concentration (ng l ⁻¹)	573.24	n.d.	19.38	n.d.	19.33
	SD (ng l ⁻¹)	n.a.	n.a.	57.14	n.a.	n.a.
	Frequency (%)	100	0	100	0	13
Adige	Mean concentration (ng l ⁻¹)	6.41	n.d.	20.15	n.d.	n.d.
	SD (ng l ⁻¹)	0.56	n.a.	24.37	n.a.	n.a.
	Frequency (%)	17	0	17	0	0

a consequence of the lack of dilution. USKollini22 is located some kilometres downstream and the water input from tributaries would be sufficient as to provide the river with enough water to dilute the pollutants transported by the river. A concentration increase was observed in DSKollini9 and DSKallio29, a more populated area that relies on extensive agricultural uses. As the Evrotas lack proper WWTPs for most of its course, high concentrations after leaving the area (4349 ng l⁻¹) may be caused by direct release of untreated wastewaters into the river. The Vivariie10 and Vivarioe15 are reference pollution areas, nevertheless had high concentrations of DEET. Despite that, the increasing amount of water due to tributaries water inputs may explain the reduction in the concentration experienced with respect to DSKallio29. WWTPi19 and WWTPo5 area receives the effluent wastewaters from the city of Sparta. Due to the hydrophilic nature of IRs, conventional wastewater treatments might not be enough to remove these compounds from the wastewaters before their release to the environment [38].

Concentrations of DEET and Bayrepel were found along the Sava river basin. This may be related to the fact that the region has been affected by a high population of mosquitoes [39]. RAD2, located in a reference area in the uppermost course of the river has concentration levels of DEET and Bayrepel higher than most of the rest of the river. Overall concentrations of Bayrepel are higher than those of DEET, with really high concentrations in CRN1 (105,336 ng l⁻¹), ZUP1 (266.3 ng l⁻¹), ZUP2 (148.5 ng l⁻¹) and BEO1 (314.7 ng l⁻¹). Both ZUP1 and BEO1 are sites located before a highly populated urban area, whereas CRN1 is located after an urban centre. The

three locations are navigable stretches of the river, and CRN1 and ZUP1 are close to oil refinery facilities. Nevertheless, a similar pattern is reproduced along the basin, with concentrations peaking before urban centres, but decreasing between 38 and 93% after leaving the urban centres behind, with the highest reduction observed between BEO1 and BEO2. These results are in agreement with previous data showing that densely populated areas and WWTPs are well-known sources of IRs' pollution [39], as they are not completely removed. However, other factors such as water dilution effect should be taken into consideration, especially along the lower course of the river. On the other hand, Bayrepel, a relatively new IRs in comparison, has proven to be ubiquitous in both, the Sava (2.23–105,34 ng l⁻¹) and Evrotas rivers (6.48–179.6 ng l⁻¹).

Only samples A8 and A10 from the Adige contained DEET, 6.8 and 6.01 ng l⁻¹, respectively). All analysed samples were from the high and middle course of the river. This sampling area is impacted by tourism as there are several ski and holiday resorts, especially populated during the winter. The absence of IRs in most samples might be explained by the overall cooler temperatures of the water in the alpine region. Similar concentrations to those observed in this study have been reported in surface waters from Germany, which were in the range 3–40 ng l⁻¹ [19]. In the USA, concentrations of DEET measured were between 49 and 97 ng l⁻¹ [40,41]. In our survey it appears that DEET is more extensively used in the Evrotas basin (Greece) than Bayrepel, whereas the opposite is observed in the countries surrounding the Sava River. A similar scenario was observed in Germany, where decreased concentrations of DEET

and higher concentrations of Bayrepel have been reported since the introduction of DEET [42]. In 2008 Terzić et al. [39] analysed wastewaters from Croatia, Bosnia and Herzegovina, and Serbia concluding that DEET was still the main IRs used in the region, with only 3% of the samples containing Bayrepel. Our results for these two IRs in the Sava basin suggested that the substitution process has continued taking place. The occurrence of these compounds in remote locations, such as those of the upper Adige River, or the higher courses of the Sava and Evrotas Rivers, may be caused by their use during touristic and other recreational activities, a link that has been previously reported [25]. Additionally, the land use and occurrence of DEET has been linked to urban nucleus and WWTP effluent discharges as the main source of this compound, followed by farmlands, mixed use land (urban centres and farmlands), and local population extent [25].

3.4. Environmental risk assessment of IRs

In order to gain some insight on their potential ecological risk, an environmental risk assessment was performed. An estimation based on hazard quotients (HQs) calculation following the European Medicines Agency (EMA) guidelines, as described in a previous study [43], was carried out. Toxicity studies have been usually oriented to characterise the potential harmful effects that IRs may pose to humans, resulting in low usage risk despite the reported adverse effects observed in children [13,22]. No-observed-adverse-effects concentrations (NOEC) were used when available, and predicted no-effect concentrations (PNEC) were calculated using the available half maximal effective concentration (EC_{50}) and LC_{50} data taken from the literature [12,38,44,45]. Despite the lack of data regarding chronic toxicity for these compounds; estimates exist for generic green algae and fish [38]. ECOSAR™, software developed by the US Environmental Protection Agency (EPA), was used to estimate ecotoxicity data for Bayrepel. This modelling tool provides toxicity data based on mathematical relationships between $\log K_{ow}$ and corresponding measured toxicity values [46].

Table A3 shows the estimated HQs for the determined IRs concentrations based on chronic and acute toxicity data. Overall, DEET and Bayrepel do not pose risk to the selected organisms. In the Sava and Adige rivers, HQs, were between 10^{-4} and 10^{-2} . However, some sampling areas are at risk; this is the case of CRN1 where HQs > 1 were estimated for Bayrepel in daphnids (HQ = 3), algae (HQ = 3.3), and fish (HQ = 1.2). In Evrotas River sampling sites DSkallio29 and WWTPo5 were found to be at risk for *Pseudokirchneriella subcapitata*, with HQ values of 1.1 and 1.2, respectively.

As regards chronic (long-term) toxicity, HQs calculated in Evrotas River were above 1; as well as already observed for acute toxicity, DSkallio29 and WWTPo5 were also at risk for *Daphnia magna*, with HQs of 1.2 and 1.3, respectively.

4. Conclusions

A fully-automated analytical method was developed and validated for the analysis of IRs in water samples using on-line SPE followed by liquid chromatography separation and tandem-mass spectrometry detection thanks to the use of APPI as ionization technique. The described methodology is fast, selective, and sensitive, allowing the detection of environmental concentrations of IRs. The developed method was applied to the analysis of usually used and new developed IRs in water samples from three European rivers with different features. Three compounds were identified, with high concentrations of DEET and Bayrepel (Bayrepel in Sava River). The espacial distribution of IRs was unequal along the basins, likely as a consequence of the differences in the human presence and economical and tourism activities in the surrounding areas. Bayrepel

and DEET were frequently observed in Sava and Evrotas. Adige River was the less polluted of the studied rivers likely due to the lack of urban areas in most stretches and to the mostly low temperatures. According to the estimated HQs, the reported concentrations posed environmental risk for DEET in two locations in the Evrotas River, and in one in the Sava. In view of these results, further research is needed to fully characterise the impact of these infochemical compounds in the environment, and the potential harmful effects that newly developed IRs may pose to the aquatic ecosystems.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.chroma.2018.02.027>.

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