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Determination of Antibiotics, Pesticides, Herbicides, Fungicides and Hormones in Water Bodies in Italy in Occurrence with European Watch List Mechanism by Using an UHPLC-MS/MS System: Method Validation, Quantification and Evaluations

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Abstract: In recent years, the quality of aquatic ecosystems has received increasing attention from European institutions. The Commission Implementing Decision (EU) 2018/840 drafted a Watch List (WL) of compounds to be monitored in Europe. In this study, we report a method based on solid phase extraction with ultra-high-performance liquid chromatography, coupled with a triple-quadrupole mass spectrometer (UHPLC-MS/MS) to analyze the whole water sample. The method was developed and validated for the determination of 12 listed compounds. The employment of solid-phase extraction by a horizon system ensures the analysis of the entire body of samples and minimizes sample manipulation. Different ng L^{-1} detection limits (from 2 to 50 ng L^{-1}), linearities (from 2 to 500 ng L^{-1}), accuracy (from 70 to 130%) and levels of precision (RSD less 20% at LOQs levels) were assessed to be satisfactory for quantification and confirmation at the levels of interest. The developed method was applied for quantitative analysis for Watch List compounds (with the exception of hormones) in surface water samples from different Italian sites during monitoring activities by the Regional Environmental Protection Agencies in the years 2019 and 2020.

Keywords: EU watch list; ultra-high-performance liquid chromatography; antibiotic determination; amoxicillin

1. Introduction

The last twenty years were characterized by increasing interest in environmental sustainability and health protection [1–6]. In this context, scientific and governmental organizations focused their attention on improving environmental protections and living conditions [7].

Regarding governmental organizations, the European Union, from the 2000s, has been making efforts aimed at protecting water bodies [8]. The most important actions were the Directive 2000/60/EC, commonly known as the Water Framework Directive (WFD), and the Directive 2010/75/EU on industrial emissions, which regulates the discharge of chemicals from industrial activities. These directives aim to achieve a good chemical status for surface and groundwater in the European Union. To reach this goal, it is important to take actions to monitor water bodies.

In 2015 there was a real innovative reform with the first Watch List (WL) investigation for new emerging substances [9]. The first WL included several substances, such as sunscreens, drugs, hormones, neonicotinoids, pesticides and antibiotics [9].

One of the main problems of the first WL was the low quantification limit (LOQ) required—17-α ethylenestradiol (EE2) (0.035 ng L^{-1}). However, several EU states have developed methods to achieve an LOQ of 0.035 ng L^{-1} for EE2, and data collected by different Member States were presented and discussed during a Joint Research Center (JRC) dissemination meeting at ISPRA [10].

WFD actions were improved and, in 2018, the European Commission reviewed several substances compared to the first Watch List [9]. For example, cinnamate and oxadiazon were eliminated and other substances widely used in everyday life were added. Among the new substances, two antibiotics (Amoxicillin and Ciprofloxacin) and one pesticide (Metaflumizone) were added in the second WL [11]. Indeed, antibiotics are becoming increasingly problematic contaminants of water sources such as surface and ground water, which are located near industrial and domestic communities.

Furthermore, during the JRC meeting, member states agreed that analyses will be performed of whole water samples. Although these substances are widely used in everyday life, their determination in water can be considered a challenge in analytical chemistry. In fact, these substances show instability/precipitation in water or several organic solvents due to their fast degradation and chemical reaction properties. Several authors performed studies on development methods for the determination of Amoxicillin, Ciprofloxacin and Metaflumizone in water; however, these research papers describe the determination of these substances at very high concentration levels (μg L^{-1}) and were performed on filtrated water samples [12–14].

Several studies reported analytical techniques that can determine antibiotics in various environmental samples using ultra-high-performance liquid chromatography (UHPLC) or capillary electrophoresis [14,15]. Usually, the analysis was carried out after a pre-concentration step, such as solid phase extraction (SPE), followed by a liquid chromatographic (LC) determination. Unfortunately, due to differences in the physical-chemical properties, such as water solubility and stability, of several substances included in WL analyses, it is not possible to perform a single extraction-procedure step.

Moreover, since the analysis of such substances must be carried out throughout Europe on a routine basis, a comprehensive, multiresidue analytical method represents a useful tool to comply with the European Decision.

In this study, we report an analytical method which is able to determine 12 compounds relevant to the 2018–2020 WL using SPE extraction procedures coupled with ultra-high-performance liquid chromatography mass spectrometry. These compounds include antibiotics, fungicide, and herbicide. The determination of three hormones, Estrone (E1), 17α-Ethynylestradiol (EE2) and 17 β-Estradiol (E2), which belong to the WL 2018–2020, was performed using a method presented in a previous paper [16].

Special attention was dedicated to Amoxicillin determination due to its degradation and epimer formation processes.

The aim of this work was to develop a precise, reproducible, and rapid ultra-high-performance liquid chromatography (UHPLC) method applicable to the determination of several analytes for Watch List 2018. The method was applied to the monitoring of 10 rivers in 10 regions of Italy.

2. Materials and Methods

2.1. Chemicals and Reagents

Amoxicillin, Ciprofloxacin, Azithromycin, Clarithromycin, Erythromycin, Metaflumizone, Imidacloprid, Methiocarb, Thiacloprid, Thiamethoxam, Clothianidin, Acetamiprid, Estrone (E1), 17α-Ethynylestradiol (EE2), and β-Estradiol (E2) were obtained from LabService Analytica. The CAS number for each compound is reported in Table 1.

Table 1. List of analyzed compounds, cas number identification and LOQ required.

Name of Substance/Group of Substances	CAS Number	Maximum Acceptable Method Detection Limit (ng/L)
17-α-ethinylestradiol (EE2)	57-63-6	0.035
17-β-estradiol (E2), Estrone (E1)	50-28-2, 53-16-7	0.4
Macrolide antibiotics (1)		19
Methiocarb	2032-65-7	2
Neonicotinoids (2)		8.3
Metaflumizone	139968-49-3	65
Amoxicillin	26787-78-0	78
Ciprofloxacin	85721-33-1	89

(1) Eritromicina (CAS 114-07-8), claritromicina (CAS 81103-11-9), azitromicina (CAS 83905-01-5). (2) Imidacloprid (CAS 105827-78-9/138261-41-3), tiacloprid (CAS 111988-49-9), tiametoxam (CAS 153719-23-4), clotianidin (CAS 210880-92-5), acetamiprid (CAS (135410-20-7/160430-64-8).

Isotopically labeled compounds, used as internal standards (IS), were chosen according to the chemical properties and retention times of the analytes, and were purchased from LabService Analytica. The purity grade of all standards was always above 94%.

Mixed stock solutions were prepared by serial dilution in acetonitrile (ACN) and stored at −20 °C in the dark to avoid possible photodegradation. To obtain a mix solution at 250 μg L^{-1} in ACN, 25 μL of each compound at 100 μg mL^{-1} was diluted with 10 mL of acetonitrile. After that, 200 μL of mix solution at 250 μg L^{-1} was diluted with 10 mL of ACN to obtain a mix solution at 5 μg L^{-1}. Calibration standard solutions were prepared from 2 to 500 ng L^{-1} in water + ACN mixture (75:25) by serial dilution from mix solution at 5 μg L^{-1}.

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 precipitation.

For Amoxicillin analyses, calibration standards were prepared differently by adding 10 μL of formic acid solution (1%) at 1 mL of the calibration standard.

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

Stock solutions were prepared in methanol and were stored at −18 °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 the literature data [17] concerning SPE extraction procedures, Empore™ SPE Disks matrix active group polystyrene-divinylbenzene (SDB-XC), diam. 47 mm, was used as a disk, coupled with a SPE-DEX 5000 Horizon Technology for extraction procedures.

LC/MS Acetonitrile grade solvents and formic acid 98% were acquired from (Merck), Milli Q water was obtained by the in-house Milli Q system.

2.2. Sample Collection and Preparation

Surface water samples were collected in 1 L PET bottles and refrigerated at 4 °C during transport.

Twenty-eight real samples were collected from different regions of Italy and shipped to the laboratory. The sampling stations and number of measurements performed for each region by the laboratory of ARPA Lombardia are reported in Tables 2 and 3, respectively. Sampling was carried out in accordance with EU directives [9].

Table 2. WL sampling stations in 2019 and 2020; RW stands for river water.

Station Code	Type	Italian Region
WL_S1	RW	Valle d'Aosta
WL_S2	RW	Piedmont
WL_S3	RW	Piedmont
WL_S4	RW	Lombardy
WL_S5	RW	Trentino Alto Adige (Trento)
WL_S6	RW	Liguria
WL_S7	RW	Tuscany
WL_S8	RW	Umbria
WL_S9	RW	Molise
WL_S10	RW	Campania
WL_S11	RW	Calabria (only in 2020)

Table 3. Number of measurements carried out by the laboratory in 2019 and 2020 for Watch List.

Italian Region	Sampling Sites	Sampling Campaign (2019 and 2020)	WL Analyses (2019 and 2020)
Campania	1	3	30
Liguria	1	3	30
Lombardy	1	3	30
Molise	1	3	30
Piedmont	2	3	45
Tuscany	1	3	30
Trentino Alto Adige (Trento)	1	3	30
Umbria	1	3	30
Valle D'Aosta	1	3	30
Calabria	1	1	15
Total	**11**	**28**	**300**

3. Results

3.1. Solid Phase Extraction Procedures

Collected samples were extracted without filtration for 5 days using SPE with SPE-DEX 5000 Horizon Technology.

To analyze the whole water sample, after references, analyses and chemical consideration of the different solubility of compounds, the authors decided to use SPE-DEX system. This was not used to concentrate compounds, but to eluate the compounds adsorbed to particulate matter.

The operating conditions for solid-phase extraction procedures using SPE-DEX 5000 Horizon Technology are reported in Table 4.

Table 4. Operating conditions for SPE procedures.

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

Using this procedure, all analytes without solubility differences were collected in water + acetonitrile 100 + 25 v/v.

3.2. LC-MS Instrumentation

Separations were performed using an ultra-high-performance liquid chromatograph (UHPLC) consisting of a binary pump EXION LC Sciex pump.

The EXION LC SCIEX system was coupled with 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 the best ionization conditions and fragmentation.

Mass spectrometry optimal parameters and transition are reported in Tables 5 and 6, respectively.

Table 5. Mass spectrometry general conditions.

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

Table 6. Analyte, m/z transitions and operating parameters.

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)
Acetamiprid-1	223.1	126.1	35	10	31	10
Acetamiprid-2	223.1	56.1	35	10	27	10
Azithromycin-1	749.5	591.3	40	10	46	12
Azithromycin-2	749.5	158.1	40	10	46	12
Clothianidin-1	250.1	168.9	20	10	19	10
Clothianidin-2	250.1	132	20	10	23	13
Clarithromycin-1	748.5	590	40	10	30	10
Clarithromycin-2	748.5	158	40	10	30	10
Metaflumizone-1	507	178	70	10	35	10
Metaflumizone-2	507	116	70	10	30	10
Methiocarb-1	226.2	169	30	10	14	10
Methiocarb-2	226.2	121	30	10	25	10
Erythromycin-1	734.5	576	60	10	30	10
Erythromycin-2	734.5	158.3	60	10	30	10
Imidacloprid-1	256.2	209	61	10	23	16
Imidaclorpid-2	256.2	175.2	61	10	23	14
Amoxicillin-1	366	208	25	10	16	10
Amoxicillin-2	366	114	25	10	16	10
Thiacloprid-1	253.1	126.1	40	10	29	10
Thiacloprid-2	253.1	186	40	10	23	10
Thiamethoxam-1	292	211	70	10	17	10
Thiamethoxam-2	292	181	70	10	30	10
Ciprofloxacin-1	332	288	27	10	34	10
Ciprofloxacin-2	332	245	27	10	31	10

The compounds were separated using a CORTEX T3 analytical column (150 mm, 4.6 mm, 5 µm). The mobile phase consisted of water + 0.02% of formic acid and acetonitrile. Elution conditions are reported in Table 7.

Table 7. Cortex T3 chromatographic separation column elution conditions.

Time (min)	Flow (mL/min)	% Water + 0.02% in Formic Acid	% Acetonitrile
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) in both positive and negative ionization modes using m/z, declustering potential and collision energy, as reported in Tables 3 and 4.

Analytes were identified both by comparing their retention times (RT) with the RT of the standards and using 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 to enhance the sensitivity.

The whole system was controlled via the Analyst software (SCIEX), while quantification of the analytes was performed with multi-quant 3.0 (SCIEX).

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

Hormones were investigated using a previously validated method, as reported in the literature [16].

3.3. Method Validation and Quality Control

The method was validated according to the acceptance criteria reported in several guidelines used in Europe. Method validation was performed in accordance with the European SANTE and UNI EN ISO and 17025 guidelines [18,19].

The linearity of the method was investigated by analyzing standard solutions in triplicate at eight concentrations, ranging from 2 to 500 ng L^{-1}. Satisfactory linearity was assumed when the determination coefficient (R^2) was higher than 0.997 based on relative responses (analyte peak area/labelled internal standard peak area), and the residuals were lower than 20%. Accuracy (expressed as percentage recovery) and precision (repeatability, expressed as relative standard deviation in percentage) were evaluated by analyzing three different surface water samples (SW) without the target analytes (previously analyzed), and fortified at several concentration levels for all compounds. The results are reported in Table 8.

The limits of quantification (LOQs) were determined both by 10 × standard deviation (S_r) of signal at the first calibration curve level and in terms of S/N, as reported in Table 9.

3.4. Analyses and Application to Environmental Samples

The developed method was applied to evaluate the concentration of the Watch List compounds.

Solid Phase Extraction, for the analyses of whole water samples, was carried out by means of an SPE-DEX 5000 Horizon Technology, as described in Section 2.2. Eluted water and organic phases were added to the internal standard and analyzed with an LC-MS/MS system, as described in Section 3.2. In brief, calibration curves were prepared for each batch analysis in the range from 2 to 500 ng L^{-1} depending on the quantified analyte. The injection volume was set at 50 µL. Separation was obtained using a Cortex T3 under elution conditions, as reported in Table 7.

Quality control solutions were analyzed at the beginning, after every ten samples and at the end of the batch analyses. The entire method was subjected to validation and quality control procedures.

Table 8. Accuracy and repeatability obtained from several levels.

Analyte	Spiked Sample Concentration ng/L	Obtained Value (ng/L)	Accuracy%	CV%
Acetamiprid	20	18.92	94.6	5.597
	50	58.30	116.6	4.77
	100	111	111.6	11.2
	250	221	88.4	5.90
Clothianidin	20	24.7	123.7	10.7
	50	46.23	92.45	9.06
	100	104.0	104.0	10.3
	250	220	88.0	6.49
Imidacloprid	20	18.69	93.47	7.98
	50	50.86	101.7	11.9
	100	97.32	97.32	4.57
	250	189	75.60	2.30
Methiocarb	20	21.11	105.6	7.56
	50	56.05	112.1	6.45
	100	101.9	101.9	6.35
	250	216.86	86.74	3.55
Thiacloprid	20	22.5	112.5	8.91
	50	54.10	108.2	5.30
	100	107.3	107.3	5.70
	250	232.30	92.92	3.41
Thiamethoxam	20	19.649	98.25	10.2
	50	51.261	102.2	9.88
	100	88.24	88.24	7.80
	250	189.0	75.61	6.70
Methiocarb	2	2.229	111.5	0.19
	50	44.71	89.42	6.42
	100	104.50	104.5	2.73
	250	224.7	96.66	81.7
Metaflumizone	50	46.487	92.97	18.3
	100	87.74	87.74	18.0
	250	193.1	77.22	13.6
Amoxicillin	100	82.87	82.87	6.66
	250	203.83	81.53	7.50
	500	448.7	89.75	6.09
Ciprofloxacin	50	50.86	96.58	11.9
	100	92.94	92.94	13.7
	250	220	88.0	6.49
Azithromycin	50	48.70	97.40	8.49
	100	82.88	82.88	6.55
	250	216.7	86.66	7.97
Clarithromycin	50	43.86	87.70	9.95
	100	80.12	80.12	6.11
	250	210	83.98	6.63
Erythromycin	50	48.288	96.58	7.19
	100	93.706	93.70	15.93
	250	221	88.55	9.00

Table 9. Obtained limit of quantification.

Analyte	LOQ Considering 10 × Standard Deviations (S_r) of Signal at First Calibration Level (ng/L)	Signal/Noise at LOQ Levels
Acetamiprid	5	227
Clothianidin	5	59
Imidacloprid	5	82
Methiocarb	2	26
Thiacloprid	5	163
Thiamethoxam	5	41
Azithromycin	10	335
Clarithromycin	10	560
Erythromycin	10	60
Metaflumizone	50	990
Amoxicillin	50	22
Ciprofloxacin	50	66

For results concerning Analyte EE2, E2 and E1, analyses were performed using a method previously validated and reported in the literature [16].

In brief, 1 L of the water sample was extracted by an SPE off-line. Then, 5 mL of reconstituted extract was injected in an SPE on-line system, coupled with high-performance liquid chromatography tandem mass spectrometry (HPLC–MS/MS) using negative electrospray ionization.

The obtained results are summarized in Tables 10 and 11.

Table 10. 2019 Watch List measurements in ng L^{-1}.

WL Sampling Site	WL_S10	WL_S6	WL_S4	WL_S3	WL_S2	WL_S7	WL_S5	WL_S8	WL_S1	WL_S9
EE2	<0.035	<0.035	<0.035		<0.035	<0.035	<0.035	<0.035	<0.035	<0.035
E2	<0.10	0.81	2.66		0.32	<0.10	<0.10	0.81	0.21	<0.10
E1	0.16	1.89	7.98		0.70	2.40	0.23	1.18	0.27	0.54
Erythromycin	<10	<10	<10		<10	38	<10	<10	<10	<10
Clarithromycin	25	38	164		<10	84	12	21	60	<10
Azithromycin	19	18	261		<10	37	28	<10	144	<10
Methiocarb	<2	<2	<2	<2		<2	<2	<2	<2	<2
Imidacloprid	<5	178	12	<5		81	<5	<5	<5	<5
Thiacloprid	<5	<5	<5	<5		29	<5	<5	<5	<5
Thiamethoxam	<5	<5	<5	<5		<5	<5	<5	<5	<5
Clothianidin	<5	<5	<5	<5		<5	<5	<5	<5	<5
Acetamiprid	<5	115	<5	<5		11	<5	<5	<5	<5
Metaflumizone	<50	<50	<50	<50		<50	<50	<50	<50	<50
Amoxicillyn	56	<50	134		<50	<50	<50	<50	65	<50
Ciprofloxacin	182	<50	<50		<50	<50	59	<50	69	<50

Table 11. 2020 Watch List measurements in ng L^{-1}.

WL Sampling Site	WL_S10	WL_S6	WL_S4	WL_S3	WL_S2	WL_S7	WL_S5	WL_S8	WL_S1	WL_S9	WL_S11
EE2	<0.035	<0.035	<0.035	<0.035	<0.035	<0.035	<0.035	<0.035	<0.035	<0.035	<0.035
E2	<0.10	<0.10	<0.10	0.5	0.44	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
E1	3.25	1.00	1.50	0.5	0.90	2.2	<0.10	0.9	<0.10	0.37	0.4
Erythromycin	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Clarithromycin	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Azithromycin	<10	<10	52	<10	<10	97	<10	<10	50	<10	221
Methiocarb	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
Imidacloprid	12	8	10	<5	7	24	<5	8	<5	7	<5
Thiacloprid	<5	<5	<5	<5	<5	10	<5	<5	<5	<5	<5
Thiamethoxam	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
Clothianidin	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
Acetamiprid	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5
Metaflumizone	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
Amoxicillyn	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
Ciprofloxacin	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50

The concentrations of the Watch List compound were below the adopted LOQ in most cases.

Clothianidin, Methiocarb, Thiamethoxam, Metaflumizone, and 17-α-ethinylestradiol were not detected in all samples collected during 2019–2020.

Acetamiprid was found in two samples in 2019, at 115 ng L^{-1} and 11 ng L^{-1}. Imidacloprid was detected in seven samples in 2020, at different concentration levels ranging from 7 to 24 ng L^{-1}. Thiacloprid was detected in only one sample in 2019 and in 2020, at 29 ng L^{-1} and 10 ng L^{-1}, respectively.

Regarding hormones, only Estrone and 17-β-estradiol were detected in different samples (in 2019 from 0.16 to 7.98 and in 2020 from 0.37 to 3.25 ng L^{-1}) and Estrone was detected at a higher level than 17-β-estradiol.

Among the antibiotics, Azithromycin and Clarithromycin were most frequently detected. Azithromycin was detected at high level in 2019 in Lombardy (261 ng L^{-1}) and in the sample collected in 2020, at a concentration of 200 ng L^{-1}, in the Calabria region.

These data can be ascribed to the use of Azithromycin as an antibiotic in COVID-19 treatment.

The observed results are in the same concentration range as described in other studies [20,21]. Erythromycin was found at lower concentration levels, probably due to the conversion and secondary reactions, as reported in the literature [22,23].

Amoxicillin and Ciprofloxacin were only detected in samples collected in 2019, because, due to the COVID-19 pandemic, WL sample collections in 2020 were only carried out in July.

4. Conclusions

In this study, an analytical method was developed and validated for the detection of several substances concerning Decision 2018/840/EU in waters. A solid-phase extraction coupled with HPLC-MS/MS was used to obtain the data by analyzing whole samples of water during Watch List activity in Italy.

The validated method was able to identify and quantify twelve compounds at concentration levels ranging from 0.16 to 261 ng L^{-1}. Method Detection limits from 0.035 to 2.24 ng/L for hormones, and from 2 to 50 ng L^{-1} for other compounds were achieved, as well as a linearity ranging from 2 to 500 ng L^{-1}, accuracy ranging from 70 to 130%, and a precision RSD of less than 20% at LOQs levels.

The developed method was used to monitor 28 analyzed samples for a total of 11 sample sites, and low-level contamination was found.

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