EXTERNAL MEMORANDUM

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cc: Project Central File W0275.23008 – PACOG 2023 Watershed Planning

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Date: December 5, 2023

Subject: Sampling and Analysis Plan (version 3)

Table 1. Version history.

Version	Date	Revisions/ Notes
1	March 7, 2022	Original Sampling and Analysis Plan (SAP) submittal to CDPHE.
2	July 31, 2023	Update to reflect support of selected 9-Element Watershed Plan for St. Charles River Outlet Watershed and incorporate previous comments from CDPHE following submittal of the SAP on March 7, 2023.
3	TBD	Update SAP to incorporate CDPHE comments dated August 22, 2023.

Table 2. Approval signatures.

Approved by:	Signature	Approval Date
CDPHE Restoration and Protection Unit - Watershed Section		
PACOG – Executive Director		

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Table 3. Members and contacts.

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OBJECTIVE AND PURPOSE

The objective of the sampling and analysis plan (SAP) is to provide the Pueblo Area Council of Governments (PACOG) guidance on the direct stream sampling of the St. Charles River Outlet Watershed.

The purpose of direct stream sampling the St. Charles River is to characterize the St. Charles River Outlet Watershed as a basis for developing effective management strategies to meet water quality goals.

WATER QUALITY AND LOAD REDUCTION WITHIN THE ST. CHARLES RIVER OUTLET WATERSHED

WATER QUALITY

In seeking to establish the suitable load reduction targets and other objectives (such as recreational, economic, ecological, etc.) within the St. Charles River Outlet Watershed, PACOG aims to explore the following questions through the sampling and analysis of the watershed.

1. What is the water quality status of the St. Charles River Outlet Watershed?
2. What are the causes of water quality impairment within the St. Charles River Outlet Watershed?
3. What are the indicators associated with Nonpoint Sources (NPS) of pollution?
4. What are the indicators associated with Point Sources (PS) of Pollution?
5. How much of the water quality impairment (pollutant loading) is due to PSs of pollution within the St. Charles River Outlet Watershed?
6. How much of the water quality impairment (pollutant loading) is due to NPSs of pollution within the St. Charles River Outlet Watershed?
7. How are the NPSs of pollution affecting water quality, aquatic life, recreational use, and human health?
8. Are there locations that exhibit higher pollutant levels than other locations?
9. Are onsite wastewater treatment systems (OWTS) and Agricultural Runoff contributing to chronic or acute pollution levels, pollution hotspots?
10. Are there other potential NPS of pollution identified during the sampling events?
11. What level of reduction in NPS pollutant loading is necessary to achieve attainment water quality status for the St. Charles River Outlet Watershed?
12. What measures or best management practices (BMPs) may be proposed/implemented to reduce such NPS pollutant loading?

DECISION CRITERIA

In the pursuit of defining appropriate load reduction objectives and other goals, PACOG plans to employ the subsequent decision criteria. These criteria will assist in evaluating the strategies necessary to attain the desired water quality in the St. Charles River Outlet Watershed.

Table 4. Decision Criteria

Decision Criteria	Description
Water Quality Standards	Assess the specific water quality standards set by regulatory bodies, such as the CDPHE, for the St. Charles River Outlet Watershed. Determine the acceptable pollutant levels for parameters such as dissolved oxygen, nutrients, sediment, and other pollutants.

Decision Criteria	Description
Environmental Impacts	Evaluate the potential environmental impacts of current pollutant levels on aquatic life, ecosystems, and human health. Consider the adverse effects of pollutants on the St. Charles River Outlet Watershed health and the surrounding environment.
Feasibility	Assess the feasibility of implementing measures to reduce pollutant loading to meet the required water quality standards. Consider available technologies, best management practices, and their effectiveness in reducing pollutants.
Compliance	Consider compliance with federal, state, and local regulations regarding water quality standards and pollutant limits. Determine the legal requirements and permissible pollutant levels for the river to achieve attainment status.
Cost	Evaluate the costs associated with implementing measures to reduce pollutant loading against the anticipated benefits. Consider the economic implications of different approaches to achieve attainment status.
Stakeholder Input	Consider input from stakeholders, including environmental groups, local communities, industries, and governmental agencies, regarding their perspectives on pollutant reduction strategies. Assess potential social, economic, and cultural impacts of the chosen measures.
Long-term Monitoring	Establish a plan for ongoing monitoring of water quality parameters to assess the effectiveness of implemented measures. Incorporate adaptive management strategies to adjust based on monitoring data and changing conditions.

OUTCOMES

The direct outcomes associated with completing the sampling and analysis activities presented in this document include the following:

1. Determine the baseline pollutant loading within the St. Charles River Outlet Watershed.
2. Identify sources of NPS of pollutant loading within the St. Charles River Outlet Watershed.
3. Identify appropriate best management practices targeting the NPS of pollution within the St. Charles River Outlet Watershed.
4. Collaborate with PACOG stakeholders to determine the most appropriate best management practices, using the decision criteria presented in Table 4. Decision Criteria , and establish pollutant loading reduction goals.
5. Implement best management practices.
6. Monitor and assess how pollutant loading changes over time with the implementation of best management practices.
7. Determine if best management practices are effective in meeting the pollutant reduction goals and re-strategize, as necessary.
8. Achieve desired pollutant load reductions within the St. Charles River Outlet Watershed.

The overall outcomes associated with achieving the desired pollutant load reduction within the St. Charles River Outlet Watershed include the following.

Table 5. Overall outcomes of pollution reduction within the St. Charles River Outlet Watershed.

Outcomes	Description
Improved Water Quality	Better water clarity, reduced pollutant loading, and improved aquatic habitat.
Environmental Health and Biodiversity	Restoration and preservation of the St. Charles River Outlet Watershed ecosystem. Promoting healthier aquatic life, and other wildlife dependent on the river ecosystem.

Outcomes	Description
Compliance and Regulatory Achievements	Meeting water quality standards and achieving attainment demonstrates compliance with environmental regulations and successful efforts in maintaining or restoring St. Charles River Outlet Watershed health.
Public Health Benefits	Providing safer water resources for recreational activities, drinking water, and agricultural uses thereby reducing health risks associated with contaminated water.
Economic Benefits	Enhancing recreational opportunities, property values, and supporting industries reliant on clean water resources such as agriculture.
Stakeholder Satisfaction and Engagement	Increase fostering collaborative efforts for continued environmental stewardship
Long-term Sustainability	Implementing strategies and management practices to reduce pollutant loading contributes to the long-term sustainability of the river ecosystem. Ongoing monitoring and adaptive management further support sustainable management practices and continual improvement in water quality.

SAP ROLES AND RESPONSIBILITIES

The roles and responsibilities associated with the sampling and analysis of the St. Charles River Outlet Watershed are as listed in Table 6. Roles and responsibilities.

Table 6. Roles and responsibilities

Role	Responsibility
Professional Engineer	Coordination of environmental data collection and upload data to CDPHE Water Quality Exchange (WQX) and the Ambient Water Quality Management System (AWQMS) websites. Coordinate with jurisdiction personnel to ensure consistent timing of sample collections.
Environmental Regulatory Compliance Specialist	Collection and transportation of direct water quality samples to Colorado Analytical Laboratory and the Center for Environmental Mass Spectrometry at the University of Colorado.
Analyze analytes and pollutants	Col-Analytical will determine the presence of analytes found in the St. Charles River samples1.
Analyze analytes and pollutants	UC-CEMS will determine the presence of analytes found in the St. Charles River samples.

ST. CHARLES RIVER OUTLET WATERSHED INFORMATION

BACKGROUND

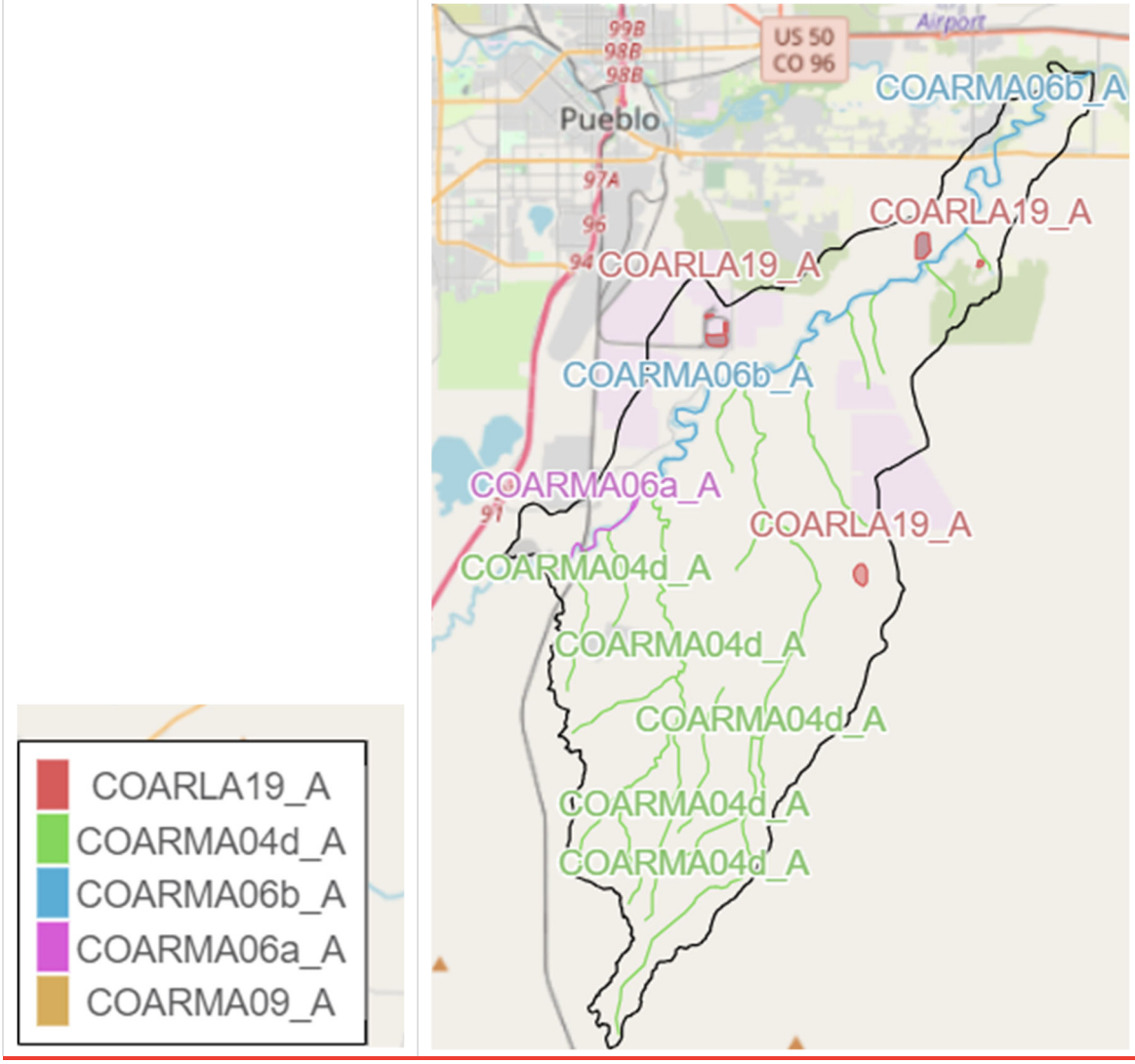
Historically, the St. Charles River Outlet Watershed was an area primarily used for agriculture and cattle grazing. The St. Charles Mesa, directly west of the St. Charles River Outlet Watershed, is recharged by the Bessemer ditch. The Bessemer Ditch has supplied irrigation water to farms and gardens within the St. Charles Mesa area since it was established, over one hundred years ago. The headwaters of the St. Charles River Outlet are southwest of the St. Charles Mesa.

[1] See Table 16. NPS Pollutants of concern and respective analysis entity.

Table 7. St. Charles River Outlet Watershed relevant description.

Type	Value
Stream Segment Identification:	COARMA06b_A
Stream Segment Description:	Mainstem of the Saint Charles River from the confluence with Edson Arroyo to the confluence with the Arkansas River.
Hydrological Unit Code (HUC):	110200021201
HUC Name:	Outlet St. Charles River (referred to as St. Charles River Outlet Watershed)
HUC Area, square miles:	58.04

Figure 1. Stream segments of the St. Charles River Outlet Watershed.



In the State of Colorado, there are several identified sources of NPS pollution. Such NPS of pollution are listed in the Colorado's Nonpoint Source Program: 2022 Annual Report (Table 8. Colorado's Non-Point Source Program: 2022 Annual Report - NPS of pollution.).

Table 8. Colorado's Non-Point Source Program: 2022 Annual Report - NPS of pollution.

NPS of pollution
Abandoned Mine Lands
Agriculture
Atmospheric Deposition
Forestry
Hydromodification and Habitat Alteration
Urbanization

In 2022, PACOG stakeholders identified two major NPS pollution of concern for the St. Charles River Outlet Watershed including the following.

- Fertilizers, herbicides, insecticides, and salts from irrigation practices and other agricultural processes.
- Bacteria and nutrients from livestock, pet wastes and faulty septic systems (Onsite Water Treatment Systems, OWTS).

The PACOG St. Charles River Outlet Watershed direct stream sampling intends to determine if and how these NPS of pollution affect the water quality of the Watershed and develop the watershed inventory.

WATERBODY CONDITIONS

PROTECTED USES IN COLORADO

The Colorado Water Quality Commission has adopted five categories of classified waterbody uses (Table 9. Colorado protected uses). The protected uses associated with the St. Charles River Outlet Watershed are also listed in Table 9. Colorado protected uses.

Table 9. Colorado protected uses

Colorado Protected Uses	St. Charles River Outlet Watershed protected uses
Agriculture	✓
Aquatic Life	✓
Domestic Water Supply	✓
Recreation	✓
Wetlands	Not applicable.

REGULATION 63 – STREAM CLASSIFICATIONS AND WATER QUALITY STANDARDS

The Colorado Water Quality Commission has implemented the stream classifications and water quality standards for the St. Charles River Outlet presented in Figure 2. 5 CCR 1002-32 Regulation 32 Stream classifications and water quality standards.

Figure 2. 5 CCR 1002-32 Regulation 32 Stream classifications and water quality standards.

6b. Mainstem of the Saint Charles River from the confluence with Edson Arroyo to the confluence with the Arkansas River.

COARMA06B	Classifications
Designation	Agriculture
UP	Aq Life Warm 2
	Recreation E
	Water Supply
Qualifiers:	
Other:	

*Selenium(acute) = See selenium assessment location at 32.6(4).
*Selenium(chronic) = See selenium assessment location at 32.6(4).
*Uranium(acute) = See 32.5(3) for details.
*Uranium(chronic) = See 32.5(3) for details.
*Temperature =
DM=32.6 and MWAT=WS-II from 3/1-11/30
DM=WS-II and MWAT=WS-II from 12/1-2/29

Physical and Biological	DM	MWAT
Temperature °C	varies*	varies*
	acute	chronic
D.O. (mg/L)	---	5.0
pH	6.5 - 9.0	---
chlorophyll a (mg/m^2)	---	TVS
E. Coli (per 100 mL)	---	126
Inorganic (mg/L)		
	acute	chronic
Ammonia	TVS	TVS
Boron	---	0.75
Chloride	---	250
Chlorine	0.019	0.011
Cyanide	0.005	---
Nitrate	10	---
Nitrite	---	0.05
Phosphorus	---	---
Sulfate	---	WS
Sulfide	---	0.002

Metals (ug/L)	acute	chronic
Arsenic	340	---
Arsenic(T)	---	0.02-10 [A]
Cadmium	TVS	TVS
Cadmium(T)	5.0	---
Chromium III	---	TVS
Chromium III(T)	50	---
Chromium VI	TVS	TVS
Copper	TVS	TVS
Iron	---	WS
Iron(T)	---	1000
Lead	TVS	TVS
Lead(T)	50	---
Manganese	TVS	TVS/WS
Mercury(T)	---	0.01
Molybdenum(T)	---	150
Nickel	TVS	TVS
Nickel(T)	---	100
Selenium	173*	50*
Silver	TVS	TVS
Uranium	varies*	varies*
Zinc	TVS	TVS

All metals are dissolved unless otherwise noted.
T = total recoverable
t = total
tr = trout

D.O. = dissolved oxygen
DM = daily maximum
MWAT = maximum weekly average temperature
See 32.6 for further details on applied standards.

INTEGRATED WATER QUALITY MONIORING AND ASSESSMENT REPORT (305(B) REPORT)

As required by Section 303(d) of the Clean Water Act, the State of Colorado has prepared the 2022 Integrated Water Quality Monitoring and Assessment Report in which Colorado reported lists of waterbodies that do not meet water quality standards. In the 2022 Integrated Water Quality Monitoring and Assessment Report, the Saint Charles River received designations as presented in Table 10. Relevant St. Charles River Outlet Watershed 2022 Integrated Water Quality Monitoring and Assessment Report designation.

Table 10. Relevant St. Charles River Outlet Watershed 2022 Integrated Water Quality Monitoring and Assessment Report designation.

Stream Segment	IR Category	Aquatic Life Tier	Recreational Tier	Miles	Aquatic Life Use	Recreational Use	Agricultural Use	Water Supply Use
COARMA06b_A	303(d) list	Class 2 Warm Water Aquatic Life	Existing Use	15.5	Fully supporting	Fully supporting	Fully supporting	Not supported

STATE IMPAIRED WATER LISTING 303(D)

As required by the Section 303(d) of the Clean Water Act, the State of Colorado, within the Colorado Code of Regulations (CCR), has established 5 CCR 1002-93 (Regulation No. 93 – Colorado's Section 303(D) List of Impaired Waters and Monitoring and Evaluation List, Regulation #93). This regulation establishes the following.

- Water quality limited segments requiring Total Maximum Daily Loads (TMDLs) (5 CCR 93.2 (1)).

- Colorado's Monitoring and Evaluation List (5 CCR 93.2 (2)).
- Waterbodies where at least one classified use is not being supported but a TMDL is not needed because either a TMDL or a 4b plan has already been developed (5 CCR 93.2 (3)).

The listed impaired portion of the St Charles River is the mainstem from the confluence with Edson Arroyo to the confluence with the Arkansas River and has the following Regulation #93 designation.

Table 11. St. Charles River Outlet Watershed impairment listing (303(d)).

HUC	Waterbody Identification	Affected Use	Pollutant	EPA Category	Cycle First Listed
110200 021201	COARMA06b_A	Water Supply Use	Chromium VI (Total)	5.-303(d) List – Impaired without a TMDL completed	2024
			Manganese (dissolved)		2016

DIRECT STREAM SAMPLING

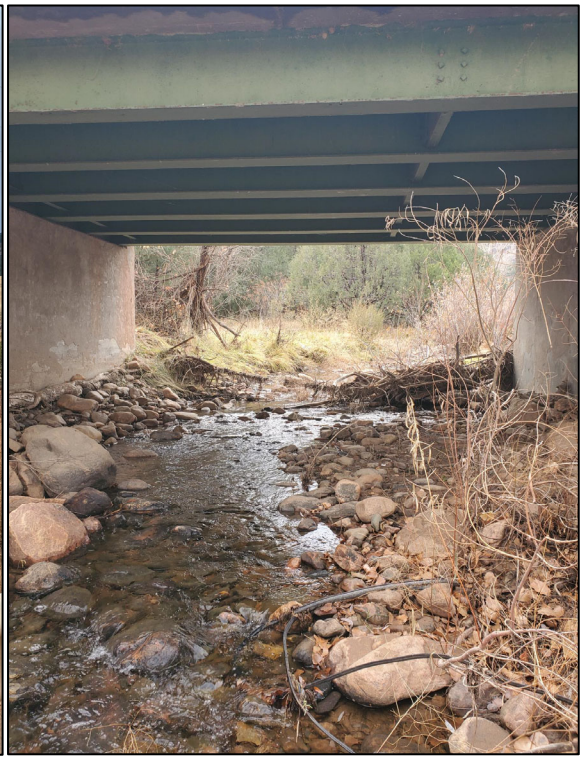
DIRECT STREAM SAMPLING LOCATIONS

To understand the St. Charles River Outlet Watershed pollutant loading, PACOG has identified the following locations (Table 12. PACOG St. Charles River Outlet direct stream sampling locations. The PACOG St. Charles River Outlet direct stream sampling locations are presented in Figures 3-7.

Table 12. PACOG St. Charles River Outlet direct stream sampling locations.

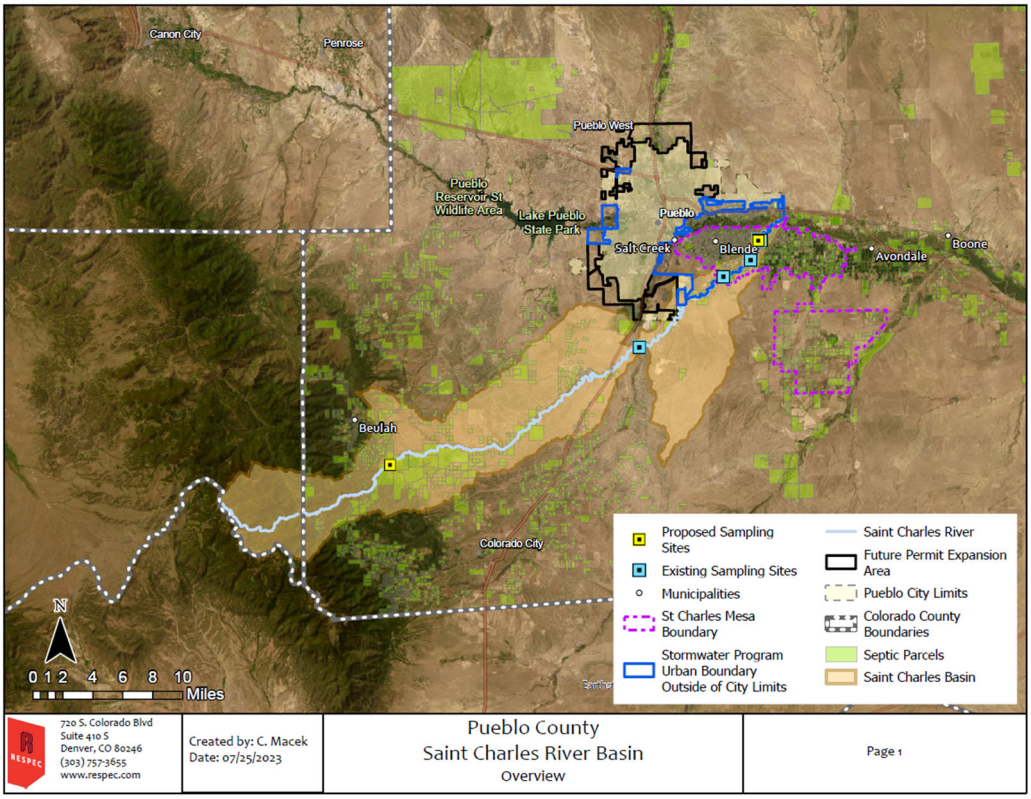
Name	Description	Latitude/ Longitude
Upper (Beulah)	South of Beulah, County Road 230 S crossing of the St. Charles River.	38.03110813389672, -104.9438017935073
Lower (Santa Fe)	Santa Fe Drive crossing of the St. Charles River (St. Charles River at Business Route 50)	38.24479680321068, -104.48747369550742

Figures 3 and 4. PACOG St. Charles River Outlet direct stream sampling location - Upper (Beulah)



Figures 5 and 6. St PACOG St. Charles River Outlet direct stream sampling location - Lower (Santa Fe)

Figure 7. PACOG St. Charles River Outlet direct stream sampling locations.



EXISTING WATER QUALITY MONITORING ON THE ST. CHARLES RIVER OUTLET WATERSHED

It is anticipated that PACOG may compare sampling results from this study to historical and ongoing (existing) monitoring performed and/or data managed by entities such as the US. Geological Survey (USGS), CDPHE – Water Quality Exchange (WQX), and the Colorado Department of Agriculture (CDA). The following monitoring locations are as presented in Table 13. Existing monitoring sites on the St. Charles River.

Table 13. Existing monitoring sites on the St. Charles River.

Site Description	Site Identification	Latitude	Longitude	Entity
St. Charles River at Business Route 50	21COL001_WQX-7503	38.2455170000	-104.4895150000	CDPHE-WQX
St. Charles River at S Road	21COL001_WQX-7503A	38.2266480000	-104.4995180000	CDPHE-WQX
St. Charles River South of Baxter	000086	38.245442	-104.48946	CDPHE-WQX
St. Charles River at Vineland, CO.	07108900	38.24555835	-104.4863597	USGS
St. Charles River above 27th Lane Bridge	21COL001_WQX-7289 C	38.2107400000	-104.5330610000	CDPHE-WQX

SCHEDULE

DIRECT STREAM SAMPLING SCHEDULE

In 2024-2025, PACOG intends to perform direct stream sampling on the St. Charles River Outlet according to the schedule presented in Table 14. PACOG 2024 – 2025 St. Charles River Outlet direct stream sampling collection schedule.

Table 14. PACOG 2024 – 2025 St. Charles River Outlet direct stream sampling collection schedule.

Quarter	Months	Number of Sampling Events	Flow Regime	Media
1st	January - March	1	Winter – Low flow	Surface Water
2nd	April – June	1	Spring - Runoff	Surface Water
3rd	July - September	1	Summer - Base flow	Surface Water
4th	October - December	1	Fall – Low Flow	Surface Water

DIRECT STREAM PROJECT SCHEDULE

PACOG intends to follow the St. Charles River Outlet direct stream sampling schedule presented in Table 15. PACOG St. Charles River Outlet direct stream sampling project schedule.

Table 15. PACOG St. Charles River Outlet direct stream sampling project schedule.

Activity	Q1 2024	Q2 2024	Q3 2024	Q4 2024	Q1 2025	Q2 2025	Q3 2025	Q4 2025	Q1 2026	Q2 2026
Direct Sampling Events	■	■	■	■	■	■	■	■		
Submittal of WQ Samples to Laboratories	■	■	■	■	■	■	■	■		
Data Verification and Validation		■	■	■	■	■	■	■	■	
Upload Data to WQX and AWQMS databases			■	■	■	■	■	■	■	■

PARAMETERS

The NPS pollutants of concern and parameters PACOG intends to study during the 2024-2025 direct sampling of the St. Charles River Outlet are presented in Table 16. NPS Pollutants of concern and respective analysis entity.

Table 16. NPS Pollutants of concern and respective analysis entity.

RESPEC In-Field	COL - Analytical	UC-CEMS		
		Pollutants Screening Method [2]		Low-Level Method[2]
Conductivity	Aluminum	1, 7-dimethylxanthine	Imazapyr	
Dissolved Oxygen	Ammonia	10-Hydroxy-carbamazepine	Imidacloprid	
Flow	Arsenic	4-methyl-benzotriazole	Iopromide	
pH	Beryllium	5-methyl-benzotriazole	Iprodione	
Temperature	Cadmium	Acetaminophen	Isoproturon	
	Calcium	Acetamiprid	Isoxaben	
	Chromium VI	Acetochlor	Isoxaflutole	2,4-D
	Copper	Alachlor	Lamotrigine Glucuronide	Atenolol
	E. Coli	Albuterol	Malathion	Atrazine
	Hardness	Amphetamine	Melamine	Bupropion
	Iron	Atorvastatin	Meprobamate	Carbamazepine
	Lead	Azithromycin	Metalaxyl	Clarithromycin
	Magnesium	Azoxystrobin	Metformin	Cotinine
	Manganese	Benzothiazole	Methadone	DEET
	Molybdenum	Benzotriazole	Methidathion	Dextrorphan
	Nickel	Bromuconazole	Methiocarb	Diazinon
	Nitrate	Buprofezin	Methiocarb sulfone	Diltiazem
	Nitrite	Caffeine	Methomyl	Diphenhydramine
	Nitrogen	Carbaryl	Metolachlor	Diuron
	Orthophosphate	Carbendazim	Metribuzin	EDDP
	Phosphorus	Carbofuran	Miconazole	Erythromycin
	Selenium	Cannabidiol	Naproxen	Fexofenadine
	Silver	Chlorpyrifos methyl	Nicosulfuron	Fluridone
	Sulfate	Cimetidine	Oxycodone	Gemfibrozil
	Uranium	Codeine	Oxyfluorfen	Imazamox
	Zinc	Cyproconazole	Parathion-methyl	Lamotrigine
		Cyromazine	Pendimethalin	Metoprolol
		Deethylatrazine	Phosmet	Penoxsulam
		Dehydronifedipine	Piperonyl butoxide	Propranolol
		Deisopropylatrazine	Profenofos	Sulfamethoxazole
		Desmethyl-tramadol	Prometon	Topramezone
		Desmethyl-venlafaxine	Propazine	Tramadol
		Dextromethorphan	Propiconazole	Triclopyr
		Diazepam	Propoxur	Triclosan
		Dichlorvos	Prosulfuron	Trimethoprim
		Diclofenac	Ranitidine	Venlafaxine
			Simazine	

[2] Presence of such analytes/pollutants indicates evidence of NPS pollution from Agricultural Runoff and/or OWTS failure (See Appendix A).

RESPEC In-Field	COL - Analytical	UC-CEMS		
		Difenoconazole	Spinosyn A	
		Diflubenzuron	Spinosyn D	
		Dihydrocodeine	Sucralose	
		Dimethenamide	Sulfadimethoxine	
		Dimethoate	Tebuconazole	
		Dimethomorph	Tebufenozide	
		Erythrohydrobupropion	Terbuthylazine	
		Erythromycin Anhydrate	THC	
		Ethoprop	Thiabendazole	
		Fentanyl	Thiacloprid	
		Flufenacet	Thiophanate-methyl	
		Fluoxetine	Tributyl phosphate	
		Fluroxypyr	Triclocarban	
		Gabapentin	Triflumizole	
		Hydrocodone	Triphenyl phosphate	
		Hydroxyatrazine	Tris(2-chloroethyl) phosphate	
		Ibuprofen	Warfarin	

EQUIPMENT

The following sections detail the field equipment that will be necessary to execute this SAP and calibration of equipment, as applicable, to ensure collection of defensible data.

EQUIPMENT LIST

The following field equipment is needed to complete the direct stream sampling.

Table 17. Equipment list.

Equipment		
1. Multi-sensor sonde and handheld device with GPS receiver. 2. 47 mm Swinnex filter holder. 3. 47 mm and 0.45 µM pore size cellulose acetate membrane filters. 4. 47 mm and #28 pore size glass fiber "roughing" pre-filters.	5. Sterile plastic syringe. 6. Coolers and cubed ice preservative. 7. Dry ice, if applicable. 8. Bucket/rope. 9. Plastic forceps. 10. Calibration cups or sleeves 11. Nitrile gloves. 12. Indelible markers and pencils.	13. Bottle Labels. 14. De-ionized laboratory water (D.I.). 15. Field notebooks or electronic forms stored on handhelds. 16. Laboratory chain-of-custody (COC) forms. 17. Personal Protection Equipment. 18. Sample containers.

FIELD INSTRUMENT CALIBRATION

All monitoring equipment used in the field will be maintained according to the manufacturer's recommendations. Meters should be calibrated before use each day, and per instructions in the operations manual. Personnel using field instruments are expected to read and be thoroughly familiar with all procedures detailed in SOPs and instruction manuals for all field instruments.

SAMPLING PROCEDURES

SURFACE WATER SAMPLE COLLECTION

Stream samples are collected as "grab" samples. The grab sample is collected by filling each sample bottle directly from the stream. Alternatively, a sampling container could be used to collect a large enough volume of water to fill all sample bottles. The grab sample should be collected from the main channel thalweg (the line of fastest flow in the stream channel and often the deepest), just below the water surface. If stream conditions are unsafe for the sampler to wade into the thalweg, the grab sample may be made from the stream bank where active flow occurs or where stream flow is directed along the bank, or from a bridge using a thoroughly rinsed bucket.

SURFACE WATER FIELD MEASUREMENTS

Field measurements for pH, temperature, dissolved oxygen, flow, and specific conductance will be made at the same time when water chemistry samples are collected. These measurements can be made in situ (directly from the stream), or from a discrete sample collected in a container (bucket). These measurements shall be recorded using the field equipment identified in this SAP.

SAMPLE CONTAINERS AND PRESERVATION

To determine the pollutant loadings within the St. Charles River Watershed, personnel will collect water quality samples to be analyzed by the selected laboratories using the collection techniques in the following tables. Most samples will be placed in a cooler and stored on cubed ice to 4 degrees Celsius immediately after collection for transport to the appropriate laboratory or other sub-contracted laboratories.

Table 18. RESPEC In-field assessment.

Analyte/ Parameter/ Pollutant	Upper (Beulah)	Lower (Santa Fe)	Analysis Method
Conductivity	Yes	Yes	Probe
Dissolved Oxygen	Yes	Yes	Probe
Flow	Yes	Yes	Probe
pH	Yes	Yes	Probe
Temperature	Yes	Yes	Probe

Table 19. UC - CEMS sample containers and preservation.

Analyte/ Parameter/ Pollutant	Upper (Beulah)	Lower (Santa Fe)	Analysis Method	Bottle Type/ Size	Field Preservative	Holding Time	Laboratory
Low Level and Screening Method[3]	Yes	Yes	EPA Method 1694	Amber Glass/ Sufficient Volume	Cool to 4°C	48 Hours	UC - CEMS

[3] See Table 16. NPS Pollutants of concern and respective analysis entity.

Table 20. Col – Analytical sample containers and preservation.

Analyte/ Parameter/ Pollutant	Upper (Beulah)	Lower (Santa Fe)	Analysis Method	Bottle Type/ Size	Field Preservative	Holding Time	Laboratory
Aluminum	Yes	Yes	EPA 200.7	Amber Glass/ Sufficient Volume	Cool to 4°C	14 DAYS	COL-ANALYTICAL
Ammonia	Yes	Yes	SM 4500-NH3-G	Plastic or Glass/ Sufficient Volume	Cool to 4°C	28 DAYS	COL-ANALYTICAL
Arsenic	Yes	Yes	EPA 200.7	Amber Glass/ Sufficient Volume	Cool to 4°C	14 DAYS	COL-ANALYTICAL
Beryllium	Yes	No	EPA 200.7	Amber Glass/ Sufficient Volume	Cool to 4°C	14 DAYS	COL-ANALYTICAL
Cadmium	Yes	No	EPA 200.7	Amber Glass/ Sufficient Volume	Cool to 4°C	14 DAYS	COL-ANALYTICAL
Calcium	No	No	EPA 200.7	Amber Glass/ Sufficient Volume	Cool to 4°C	14 DAYS	COL-ANALYTICAL
Chromium VI	Yes	Yes	EPA 200.7	Amber Glass/ Sufficient Volume	Cool to 4°C	14 DAYS	COL-ANALYTICAL
Copper	Yes	Yes	EPA 200.7	Amber Glass/ Sufficient Volume	Cool to 4°C	14 DAYS	COL-ANALYTICAL
E. coli	Yes	Yes	Colilert	IDEXX bottles/ 120 ml	Cool to 4°C	6 hours	COL-ANALYTICAL
Hardness	Yes	No	EPA 130.1	Amber Glass/ Sufficient Volume	4°C, HN03 to pH < 2.	14 DAYS	TBD
Iron	Yes	Yes	EPA 200.7	Amber Glass/ Sufficient Volume	Cool to 4°C	14 DAYS	COL-ANALYTICAL
Lead	Yes	Yes	EPA 200.7	Amber Glass/ Sufficient Volume	Cool to 4°C	14 DAYS	COL-ANALYTICAL
Magnesium	No	No	EPA 200.7	Amber Glass/ Sufficient Volume	Cool to 4°C	14 DAYS	COL-ANALYTICAL
Manganese	Yes	Yes	EPA 200.7	Amber Glass/ Sufficient Volume	Cool to 4°C	14 DAYS	COL-ANALYTICAL
Molybdenum	Yes	No	EPA 200.7	Amber Glass/ Sufficient Volume	Cool to 4°C	14 DAYS	COL-ANALYTICAL
Nickel	Yes	Yes	EPA 200.7	Amber Glass/ Sufficient Volume	Cool to 4°C	14 DAYS	COL-ANALYTICAL
Nitrate	Yes	Yes	EPA 300.0	Glass or Polyethylene/ Sufficient Volume	Cool to 4°C	48 HOURS	COL-ANALYTICAL
Nitrite	Yes	Yes	EPA 300.0	Glass or Polyethylene/ Sufficient Volume	Cool to 4°C	48 HOURS	COL-ANALYTICAL
Nitrogen	Yes	Yes	EPA 300.0	Glass or Polyethylene/ Sufficient Volume	H 2SO4 to a pH <2 and cooled to 4°C	28 DAYS	COL-ANALYTICAL

Analyte/ Parameter/ Pollutant	Upper (Beulah)	Lower (Santa Fe)	Analysis Method	Bottle Type/ Size	Field Preservative	Holding Time	Laboratory
Orthophosphate	Yes	Yes	EPA 300.0	Glass or Polyethylene/ Sufficient Volume	Cool to 4°C	48 HOURS	COL-ANALYTICAL
Phosphorus	Yes	Yes	EPA 365.1	Plastic or Glass/ Sufficient Volume	H 2SO4 to a pH <2 and cooled to 4°C	28 DAYS	COL-ANALYTICAL
Selenium	Yes	Yes	EPA 200.7	Amber Glass/ Sufficient Volume	Cool to 4°C	14 DAYS	COL-ANALYTICAL
Silver	Yes	Yes	EPA 200.7	Amber Glass/ Sufficient Volume	Cool to 4°C	14 DAYS	COL-ANALYTICAL
Sulfate	Yes	Yes	EPA 300.0	Glass or Polyethylene/ Sufficient Volume	Cool to 4°C	28 DAYS	COL-ANALYTICAL
Uranium	Yes	No	TBD	Amber Glass/ Sufficient Volume	Cool to 4°C	14 DAYS	TBD
Zinc	Yes	No	EPA 200.7	Amber Glass/ Sufficient Volume	Cool to 4°C	14 DAYS	COL-ANALYTICAL

SAMPLE DOCUMENTATION AND HANDLING

The following sections describe the documentation of field activities and documentation and handling of samples.

FIELD DOCUMENTATION

Field notebooks, including daily field forms and photographs will be used to document field activities.

FIELD LOG NOTEBOOK AND FIELD FORMS

Personnel shall document all monitoring activities using standard field log notebooks, which contain pre-printed field log forms on Rite-in-the-Rain waterproof paper. Each sampling event will have its own log entry, with all pertinent data requested on the field log form provided. Each log entry will include at least the following; sample date and sample ID number, site number and description, sample collector's name, site latitude and longitude and associated GPS documenting data, start/sample/end times, how and where the sample was collected, whether samples were collected directly into the sample container or poured out of a bucket, all field measurements and how the measurements were taken (e.g. directly out of the stream, out of a bucket), sample filtering information, observations and comments, and summary of QA activity, if any.

All documentation will be done at the time of sampling using the preprinted and formatted "Monitoring Field Log" notebooks. Only field personnel may be in custody of the notebooks during field activities. Field log entries must be dated, legible, preferably made in black indelible ink, and contain accurate documentation. Corrections to erroneous data will be made by crossing through the entry and entering the correct information. The person making the correction must initial and date where the error occurred.

PHOTOGRAPHS

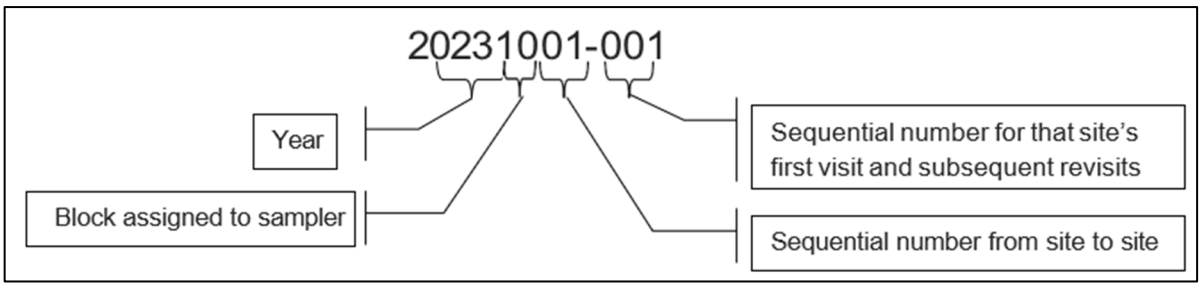
Photographs shall be taken at each new site and include an upstream, downstream and benchmark snapshot. Photographs shall be downloaded, re-titled to identify the location identification, waterbody, and snapshot location (e.g., upstream); and stored.

SAMPLE LABELING

Every sample will have a unique barcode identification number. Each sample shall have a barcode generated, printed on weatherproof address labels, and affixed to the exterior of each bottle set prior to a given sample trip. Each "set" shares the same barcode.

This unique barcode identification number is an eleven-digit number that is bracketed by (*) asterisks. The * character is the start and stop reading character for the barcode reader. The first four numbers of the barcode are the four-digit fiscal year. The fifth number denotes the block assigned to the sampler or specific program. The remaining six numbers in the bar code are sequential numbers based on sites and site revisits within a year. Each sampler shall be responsible for making sure that each number used in their block is unique. See example below for further details.

Figure 8. Direct sample bottle labeling example.



CHAIN OF CUSTODY

All samples will be submitted along with an official Chain-of-Custody. The chain of custody form shall be completed according to the instructions for completing the form. All requested information shall be provided. Samples are to be immediately placed in a cooler, preserved with cubed ice, and delivered to the appropriate laboratory, see Table 9. Sample holding times shall be accounted for when a schedule is projected, and samples delivered to meet all holding times. If samples are delivered on a Friday, samples should be to the lab no later than the time specified by the appropriate laboratory to ensure proper relinquishing of samples to laboratory staff.

LABORATORIES

PACOG intends to use the laboratories presented in Table 3. Members and contacts. to analyzing the direct surface water samples. See Tables 19 and 20 to review the sample collection and laboratory analysis methodology.

QUALITY ASSURANCE AND QUALITY CONTROL

Quality assurance (QA) is a set of operating principles that, if strictly followed during sample collection and analysis, will produce data of known and defensible quality. This will ensure that the accuracy of the data can be stated with a high level of confidence.

Assuring the quality of surface water data is accomplished by following standard operating procedures (e.g., observing proper sample collection techniques, proper maintenance and calibration of field meters), collecting quality control (QC) samples, reviewing and analyzing QA/QC data, and making appropriate adjustments to surface water quality data collection procedures on the basis of the results of QA/QC procedures. QA/QC procedures may be divided into three categories:

- Field procedures quality control.
- Data quality control.
- Laboratory quality control.

FIELD QUALITY CONTROL

Standard operating procedures will be utilized as a primary tool to ensure field procedure quality control. Staff performing field activities for the Program will receive the training necessary to ensure that all SOPs are fully and properly used when completing field-monitoring activity. Each project-specific SOP will describe and or reference all specific quality assurance/quality control methods to be followed. At a minimum, the following water chemistry quality control samples will be taken:

- Field duplicates
- Field blanks ("Trip blanks")

FIELD DUPLICATES

Field duplicates will be field sample replicates and will be used to determine field precision. Duplicate samples, including duplicate field measurements, are a set of similar samples collected from the same site, at about the same time, and analyzed in the same manner. Duplicate samples may be equated to "fraternal twins" in that they originate from one source, but each sample may contain a slightly different chemical composition. Duplicate sample results must be compared to assure reasonable agreement. In general, the acceptable results from duplicates are a 30% difference for cations, anions, and nutrients. For total and dissolved metals, particularly when concentrations are near detection levels, a difference up to 50% may be allowed, based on best professional judgment by the RESPEC Project Manager.

Duplicate samples shall be taken and analyzed from a minimum of 10% of the total number of samples collected during the implementation of this SAP.

TRIP BLANKS

Trip blanks, or also known as field blanks, help to ensure that sampling equipment, sampling containers, and de- ionized rinse water is effectively cleaned and/or free from contaminants that may be introduced into a sample via the equipment or rinse water. Field or Trip blanks, also referred to as equipment blanks, are blank solutions (solutions of D.I. water) that are processed through the equipment used for collecting and processing an environmental sample. Four types of surface water quality sampling equipment have blank samples taken from them:

- DI water container
- Sample container
- Filter apparatus
- Sample collection device (bucket)

All results from equipment blank samples shall be at or near the minimum reporting level (or non-detect level). Any detection of contaminants in equipment blanks shall be addressed by the RESPEC Project Manager and may entail modified cleaning or decontamination procedures.

Blanks shall be taken and analyzed once per field visit which results in <15 routine samples. Since the intent is to ensure that equipment decontamination procedures are followed to exact specifications, the trip blank shall be collected even if the field visit trip only includes one routine sample.

For field trips that result in ≥15 routine samples then one trip blank shall be collected at the start of the visit and a second trip blank shall be collected at the end of the week to ensure that equipment decontamination procedures are followed to exact specifications during trips of heavy usage.

DATA QUALITY CONTROL

Data quality control procedures and measures are grouped into four categories to be reviewed:

- Steps for measuring compliance with WQCD procedures.
- Laboratory issues.

- Bias and errors.
- Additional considerations.

All QC data shall be reviewed following completion of this SAP. If all data-acceptance criteria in the SAP are met, then the analytical data are acceptable.

LABORATORY QUALITY CONTROL

PACOG will utilize the laboratories listed in Table 3. Members and contacts. as the primary sources of analytical services for water samples during the implementation of this SAP. The following items will be reviewed, at a minimum, to verify laboratory QA/QC:

- Verifying QA/QC with Laboratory personnel.
- Method Detection Limits and Method Reporting Limits.
- PQL issues.
- Duplicates and blanks.
- Contamination issues.
- Post-sample submittal filtering and preservation.

If analytical services are provided by a laboratory other than the laboratories listed in Table 3. Members and contacts., the same steps will be taken, as outlined above, to verify acceptable laboratory quality control.

DATABASE MANAGEMENT

Water chemistry samples are collected along with field data and visual observations per instructions in the Surface Water Field Measurements and Sample Containers and Preservation sections of this SAP. Field measurements are recorded onto a Microsoft Excel workbook upon completion of the sample trip. Sample sets are delivered to the appropriate laboratory for laboratory analysis. When samples are collected in remote locations, occasionally microbiological samples may be delivered to sub- contracted laboratories to facilitate quicker analysis. Field data and observations are downloaded into a Microsoft Excel workbook by the RESPEC representative. The laboratory returns water chemistry data via Microsoft Excel CSV spreadsheets known collectively as "laboratory extracts."

Field data and observations along with microbiological data received from off-site laboratories are transferred into a format to be joined with the water chemistry data by the RESPEC staff. Field, chemistry, and microbiological data coalesce and are analyzed for quality control before the data is uploaded to the WQX AWQMS database. Once in the WQX, the data will be available to all interested parties through the EPA's Water Quality Portal.

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APPENDIX A - UC CEMS REFERENCE PUBLICATIONS

9

APPLICATION OF LC–MS/MS AND LC–TOF-MS FOR THE IDENTIFICATION OF PESTICIDE RESIDUES AND THEIR METABOLITES IN ENVIRONMENTAL SAMPLES

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9.1 INTRODUCTION AND OCCURRENCE OF PESTICIDES IN ENVIRONMENTAL SAMPLES

The presence of pesticides in environmental samples (mainly in water, soil, and food) has been a known issue for several decades. Hundreds of papers and reviews have been published reporting the presence of these contaminants in several environmental compartments. The origin of this type of contamination is mainly via agricultural practices (pesticides applied to soil and crops), via industrial waste (pesticide manufacturers), or through domestic practices. The end result is always the presence of trace concentrations of pesticides (ranging from ng/l to μg/l) in environmental matrices, such as water, soil, sediment, and food commodities. It is well known that the presence of pesticide residues in food can affect human health. Different organizations have set stringent regulatory controls on pesticide use in order to minimize exposure of the general population to pesticide residues in food. For example, in the European Union (EU), a new regulation, 396/2005/EC, of the European Parliament and Council (Commission Regulation (EC) 396/2005) has come into force for pesticides at low levels in food products in order to meet health concerns (EU Pesticides Database, 2014). For fruits and vegetables intended for production of baby food, a maximum residue limit (MRL) of 0.01 mg/kg is applicable for all pesticides, and compounds without a stated regulation have the lowest MRLs at 0.01 mg/kg as well (Commission Directive 2003/13/EC). In water, similar regulations are in place for the presence of pesticide residues in drinking water, with the limit of 0.1 μg/l being the maximum permitted concentration for each individual pesticide.

These low MRLs have encouraged the development of more sensitive analytical methods to meet the requirements in complex samples. Therefore, sensitive and reliable confirmatory methods are required to monitor pesticide residues in water and food. With the advance of mass spectrometric techniques, new instrumental methodologies are constantly being sought for better detection and monitoring. One of the problems for multiresidue methods by conventional liquid chromatography–mass spectrometry (LC–MS) is the decision of which pesticides should be measured. Currently, more than 1000 pesticides are used worldwide, both legally and illegally, on food products and in the treatment of soil and crops. Most of these pesticides have MRLs for both food and water to protect the consumer. The MRL concentrations have to be monitored as part of the quality control of food, especially fruits and vegetables; thus, large-scale multiresidue methods with hundreds of pesticides are needed for quality control. However, the ability to monitor hundreds of pesticides in a single analysis is a challenging problem both for chromatography and mass spectrometry (MS).

One of the most popular methods for the identification of selected target pesticide residues is liquid chromatography–tandem mass spectrometry (LC–MS/MS). This technique is most suited for relatively small (from a few compounds to hundreds) groups of pesticides. Nevertheless, some pioneering reviews (Ferrer and Thurman, 2003; Picó, 2006; Botitsi et al., 2011) on pesticides in food and water pointed out on the unique ability of accurate mass to identify both target compounds and nontargets by liquid chromatography–time-of-flight mass spectrometry (LC–TOF-MS), thus offering a possible solution to this conundrum. Therefore, LC–TOF-MS

Mass Spectrometry for the Analysis of Pesticide Residues and Their Metabolites, First Edition. Edited by Despina Tsipi, Helen Botitsi and Anastasios Economou.

is a relatively new and valuable technique for the control of pesticides to ensure food and water safety. In this sense, time-of-flight (TOF) techniques can record accurate full spectrum throughout the acquisition range and have resulted in an excellent tool for the unequivocal target and nontarget identification and confirmation of pesticide residues in vegetable and fruits, as shown in the recent past (Thurman et al., 2005a; Williamson and Bartlett, 2007).

This chapter gives an overview of the different analytical techniques used in LC–MS for the detection of pesticide residues in environmental samples, with a specific focus on tandem mass spectrometry (MS/MS) and TOF techniques, and the applications that have been recently generated in the environmental field. This chapter gives several examples of pesticide analysis that exemplify the unique features of these techniques for the identification of target and nontarget or unknown compounds.

9.2 STATE-OF-THE-ART TECHNIQUES FOR THE IDENTIFICATION OF PESTICIDES AND THEIR DEGRADATION PRODUCTS

There is no doubt that LC–MS has been the universal method of choice when analyzing pesticides and their degradation products in environmental samples for at least 20 years now. The most popular technique for the identification and confirmation of pesticides is MS/MS (LC–MS/MS), using either collision cells or linear traps to obtain information on fragment ions. This technique is more focused to target analysis where the analyst is looking at a specific group of analytes; some may vary from few analytes within a family (3–6) to large multiresidue methods (>100). However, sensitivity usually becomes an issue when targeting a large number of compounds. This is the reason why time-of-flight mass spectrometry (TOF-MS) techniques have become popular in the last few years, since it gives full-spectrum data at all times. A large number of compounds (virtually no limit) can be analyzed in a single run while obtaining valuable accurate mass information for each compound that ionizes under atmospheric pressure conditions (i.e., electrospray). Furthermore, high-resolution techniques with additional structural information on fragment ions are needed, and this has made these techniques become more and more popular. Therefore, extra information on metabolites or degradation products can be achieved by exploring the accurate mass spectra of unknown peaks in the chromatogram. These techniques provide a high degree of confidence for identification of target analytes and aid to the structural elucidation of degradation products and unknown compounds, which are also present in environmental samples. Furthermore, the possibility of creating universal accurate mass databases with TOF analyses for sets of compounds has broadened the range of applications as well, going from target to nontarget identification. In the next section, we will discuss both techniques of detection, a targeted approach using LC–MS/MS techniques and a nontargeted tactic for the discovery and identification of relevant compounds using LC–TOF-MS.

9.2.1 LC–MS/MS for the Analysis of Target Compounds

LC–MS/MS with triple quadrupole in multiple reaction monitoring (MRM) mode has become so far the most widely used technique for monitoring and quantitation of pesticides in food, as reported extensively in the literature (Klein and Alder, 2003; Hetherton et al., 2004; Jansson et al., 2004; Wang et al., 2005; Alder et al., 2006; Hernández et al., 2006; Kovalczuk et al., 2006; Leandro et al., 2006; Botitsi et al., 2007; Hiemstra and de Kok, 2007; Pozo et al., 2007). The advantages of MS/MS are based on high sensitivity, reduction of sample treatment steps, and reliable quantitation and confirmation at the low-level concentrations required (Pozo et al., 2007). The simplicity of methodologies using triple quadrupole as a detection technique, together with the low limits of detection (LODs) achieved and the MS/MS capability, makes this technique a valuable and unique tool for routine monitoring programs established in regulatory official laboratories. The ease of use is often an essential tool for this type of regulatory agencies, which lack the high-skilled personnel required for more sophisticated techniques.

To develop a triple quadrupole MS/MS method, one needs to first generate MRM transitions for each compound. An optimized MRM transition includes a precursor ion, a product ion, and an optimized collision energy. The first step consists of selecting a proper precursor ion, which usually consists of the protonated or deprotonated molecule. The second step is to generate product ions at different collision energies and then choosing a couple of fragments. Each pair of precursor and a fragment ion is considered a transition. According to EU identification criteria (Andre et al., 2001; Commission Directive 2002/657/EC, 2002), it is enough to achieve identification of a certain compound using two MRM transitions and their relative ion abundance ratio, provided the retention time matches. This application of identification criteria is essential to ensure the unequivocal identification of target analytes in environmental samples. Usually, the transition with the higher abundance is used for quantitation, while the other transition is used as a confirmatory one. The instrument is then set up to monitor as many transitions as possible for a wide range of pesticide compounds. Some instruments require the use of retention time windows for a multianalyte approach, whereas other instruments will schedule the different transitions by using time-dependent algorithms.

Another recent advance in LC–MS is the development of stationary column phases, such as the ones containing smaller

particle sizes (1.7 and 1.8 μm) (Nguyen et al., 2006), which have allowed improved peak resolution and, therefore, increased sensitivity in chromatographic separations. The van Deemter equation indicates that as the particle size decreases to less than 2.5 μm, there is a significant gain in efficiency and that efficiency does not diminish at increased flow rates or linear velocities (Swartz, 2005). This is especially useful when the number of compounds is high since it allows baseline separation and detection of all the compounds present in a complex sample. The only requirement when coupling MS/MS is to achieve rapid data acquisition, so the improved resolution is not degraded (Pozo et al., 2007).

A previous work by our group (Ferrer et al., 2007) evaluated an MS/MS methodology to not only screen but also to quantitate and confirm 100 pesticides in a single analysis using a combination of the new 1.8 μm LC columns (for maximum peak resolution) and time segments with 100 transitions per segment in order to have both a quantifier ion and a qualifier ion, which satisfies the EU specifications for unequivocal identification and confirmation by MS (Commission Directive 2002/657/EC, 2002; Hernández et al., 2004). A validation study was carried out using matrix-matched samples of vegetables for quantitation and as alternative to compensate effects of suppression or enhancement of signal due to the matrix (Ferrer et al., 2007). Table 9.1 summarizes the most relevant MS settings such as fragmentor voltage and collision energies used for each one of the 100 compounds that were investigated, as well as all the MRM transitions selected for screening, quantitation, and confirmation. The first transition shown was used for quantitation (calibration curves and reproducibility), and the second transition was used for confirmatory purposes and to calculate LODs as well. LODs are shown for all the pesticides studied as well.

Confirmation of positive identifications in real samples requires the additional second MRM transition and the evaluation of ion ratios between the two monitored transitions as compared to a reference standard. The confirmation criteria using MS/MS cover a range of maximum permitted tolerances for relative ion intensity, expressed as a percentage of the intensity of the most intense transition (Andre et al., 2001; Commission Directive 2002/657/EC, 2002). For example, Figure 9.1 shows the ion ratios for benalaxyl in solvent and in an extract of green pepper spiked with the pesticide mix at 50 μg/kg (500 pg on column). The *m/z* 148 ion was used for quantitation, and the *m/z* 294 ion was used as the qualifier ion, with a window set at ±25% for an ion ratio of 45. As shown in Figure 9.1 in the two ion profiles, benalaxyl was easily identified in this complex matrix due to the selectivity of the MRM transitions and instrument sensitivity. Confirmation of the identity of the pesticides in real samples is usually based on ion ratio for the transitions monitored (Commission Directive 2002/657/EC; European Commission SANCO/12571/2013).

9.2.2 LC–TOF-MS and LC–QTOF-MS for the Analysis of Target and Nontarget Compounds

LC–MS employing accurate mass measurement has been proven as a successful technique for quantitative analysis of target compounds and rapid qualitative analysis of *unknown* environmental mixtures. One of the main reasons that TOF has become so popular in the last few years is the fact that accurate mass measurements are specific and universal for any kind of analyte and do not depend on the type, brand, or specific instrumentation used. The degree of fragmentation may vary depending on the instrument, but the specific accurate mass value and/or accurate isotope information will be consistent for a given analyte, no matter what type of ionization, collision-induced dissociation, and MS/MS fragmentation are used. Accurate mass determination allows obtaining unique information for a given molecule, plus additional information from isotopic patterns, mass defect, and specific fragment ions (Ferrer and Thurman, 2009).

One of the initial weaknesses of LC–TOF-MS and liquid chromatography–quadrupole time-of-flight mass spectrometry (LC–QTOF-MS) was the lack of quantitative results. However, recent breakthroughs in instrument design now make LC–TOF-MS a successful quantitative tool (Williamson and Bartlett, 2007) with mass accuracies that are in the 1–2 ppm range for several types of instruments when used in environmental analyses (Nuñez et al., 2004; Ferrer et al., 2005a; Ferrer et al., 2005b; Kaufmann and Butcher, 2006; Ojanpera et al., 2006; Sancho et al., 2006; Grimalt et al., 2007). These changes relate to extending the linear dynamic range of the instrument by using analog-to-digital converter (ADC) rather than time-to-digital converter (TDC) (Fjeldsted, 2003). Furthermore, as mentioned earlier, innovations in chromatographic particle chemistry (from 5 to 3.5 or 1.8 μm packing as well as new bonding chemistries) have improved the baseline separation of pesticides (Hernández et al., 2004).

In general, official routine laboratories analyze a certain number of target compounds (ranging from 10 up to less than 300 different compounds) (Leandro et al., 2006; Mezcua et al., 2006) depending on the legal requirements for positive identifications and the nature of the methodology used in the respective labs. The literature has hundreds of papers reporting diverse LC–MS methodologies for the analysis of all the different classes of pesticide compounds. Several review papers have tried to compile all the existent information regarding mass spectrometric data (including fragment ions) using different instrumentations (ion trap, triple quad, TOF, QTOF), but unfortunately, in every case, singular information is obtained depending on the method of detection used (Soler and Pico, 2007). For example, when using tandem mass spectrometric techniques, the instrument parameters (especially the fragmentor voltage and collision energy) play an important role on the number of fragments and relative intensities

TABLE 9.1 Retention times t_R, MRM transitions, and MS operating parameters selected for the analysis of 100 pesticides[a]

Compound	t_R (min)	Fragmentor voltage (V)	MRM transitions (*m/z*)	Collision energy (eV)	LODs[b] (µg/kg)
Acetamiprid	12.2	80	223>126	15	0.3
			223>56	15	
Acetochlor	23.1	120	270>224	10	0.8
			270>148	10	
Alachlor	23.1	80	270>238	10	0.8
			270>162	15	
Aldicarb	14.3	80	116>89	5	2
			116>70	5	
Aldicarb sulfone	7.9	80	223>76	5	5
			223>148	5	
Aldicarb sulfoxide	6.1	80	207>89	5	2
			207>132	5	
Atrazine	17.5	120	216>174	15	0.4
			216>132	20	
Azoxystrobin	21.3	120	404>372	10	0.3
			404>344	15	
Benalaxyl	24.4	120	326>148	10	0.5
			326>294	5	
Bendiocarb	16.5	80	224>109	10	1
			224>167	5	
Bensulfuron-methyl	19	120	411>149	20	0.4
			411>182	15	
Bromoxynil	17.9	120	278>199	30	40
			278>223	30	
Bromuconazole	21.5+22.5	80	376>159	20	1
			376>70	20	
Buprofezin	26.6	120	306>201	10	0.7
			306>116	15	
Butylate	27.7	120	218>57	10	5
			218>156	10	
Carbaryl	17.4	80	202>145	5	10
			202>117	10	
Carbendazim	7.1	80	192>160	15	0.5
			192>132	20	
Carbetamide	13.9	80	237>118	10	0.5
			237>192	5	
Carbofuran	16.6	120	222>165	10	0.9
			222>123	15	
Chlorfenvinphos	23.7	120	359>155	10	2
			359>127	15	
Chlorotoluron	16.8	120	213>72	20	0.3
			213>140	20	
Chlorpyrifos-methyl	25.9	80	322>125	15	10
			322>290	15	
Cyanazine	15.3	120	241>214	15	2
			241>174	15	
Cyproconazole	20.3	120	292>70	15	0.5
			292>125	15	
Cyromazine	3.4	120	167>85	25	10
			167>125	20	
Deethylatrazine	11.2	120	188>146	15	1
			188>104	20	
Deethylterbuthylazine	15.4	120	202>146	15	0.8
			202>110	20	

Table 9.1 (*Continued*)

Compound	t_R (min)	Fragmentor voltage (V)	MRM transitions (*m/z*)	Collision energy (eV)	LODs[b] (μg/kg)
Deisopropylatrazine	8.7	120	174>96	15	4
			174>132	15	
Diazinon	25.3	160	305>169	20	0.3
			305>153	20	
Dichlorvos	15.4	120	221>109	15	5
			221>145	15	
Difenoconazole	24.7+24.9	160	406>251	20	0.3
			406>337	15	
Difenoxuron	18	120	287>72	20	0.6
			287>123	15	
Diflubenzuron	22.3	80	311>158	10	6
			311>141	15	
Dimethenamide	21.2	120	276>244	10	0.4
			276>168	15	
Dimethoate	11.8	80	230>199	5	0.7
			230>171	10	
Dimethomorph	19.2+19.6	120	388>301	20	0.6
			388>165	25	
Diuron	17.1	120	233>72	20	0.8
			233>160	20	
Ethiofencarb	17.9	80	226>107	5	0.7
			226>164	5	
Fenamiphos	20.8	120	304>217	20	0.6
			304>234	15	
Fenuron	11.2	120	165>72	15	1.5
			165>120	15	
Flufenacet	23.0	80	364>152	10	0.5
			364>194	5	
Flufenoxuron	27.6	80	489>158	10	5
			489>141	15	
Fluometuron	17.9	120	233>72	20	1
			233>160	20	
Fluroxypyr	14.9	80	255>209	10	10
			255>181	15	
Hexaflumuron	25.1	120	461>158	10	7
			461>141	20	
Hydroxyatrazine	8.1	120	198>156	15	4
			198>86	20	
Imazalil	18.5	160	297>159	20	10
			297>255	20	
Imazapyr	9.2	160	262>217	15	0.7
			262>234	15	
Imazaquin	15.4	160	312>199	25	0.6
			312>267	20	
Imidacloprid	11.4	80	256>175	10	4
			256>209	10	
Ioxynil	19.6	120	372>118	30	20
			372>245	30	
Iprodione	22.6	120	330>245	10	12
			330>288	10	
Irgarol 1051	19.2	120	254>198	15	0.8
			254>156	20	
Irgarol metabolite	13.6	120	214>158	15	1.2
			214>110	20	

(*continued*)

TABLE 9.1 (*Continued*)

Compound	t_R (min)	Fragmentor voltage (V)	MRM transitions (*m/z*)	Collision energy (eV)	LODs[b] (μg/kg)
Isofenphos	26.4	80	346>217	20	1
			346>245	10	
Isoproturon	17.7	120	207>72	15	1.3
			207>165	15	
Lenacil	15.5	80	235>153	10	8
			235>136	15	
Linuron	20.7	120	249>160	20	1
			249>182	15	
Lufenuron	26.8	80	511>158	10	3
			511>141	20	
Malathion	22.7	80	331>99	10	0.8
			331>127	5	
Mebendazole	14.8	120	296>264	20	0.6
			296>105	25	
Metalaxyl	17.7	120	280>192	15	1
			280>220	10	
Metamitron	10.6	120	203>175	15	0.9
			203>104	20	
Methidathion	20.8	80	303>85	10	0.7
			303>145	5	
Methiocarb	20.4	80	226>121	10	0.8
			226>169	5	
Methiocarb sulfone	13.2	80	258>122	5	30
			258>217	10	
Methomyl	8.6	80	163>88	5	0.8
			163>106	5	
Metolachlor	23.2	120	284>252	10	0.4
			284>176	15	
Metolcarb	15.3	80	166>109	5	2
			166>91	10	
Metribuzin	15.9	120	215>187	15	1
			215>131	20	
Molinate	22.2	120	188>126	10	2
			188>83	15	
Monuron	14.9	120	199>72	15	1.5
			199>126	15	
Nicosulfuron	13.7	120	411>182	15	0.8
			411>213	10	
Nitenpyram	11.0	120	271>225	10	0.7
			271>99	15	
Oxadixyl	14.9	80	279>219	10	5
			279>102	10	
Parathion-ethyl	24.6	120	292>236	10	5
			292>264	5	
Pendimethalin	28.5	80	282>212	5	4
			282>194	10	
Phosmet	21.2	80	318>160	5	6
			318>133	5	
Prochloraz	23.2	80	376>308	10	5
			376>266	10	
Profenofos	26.6	120	373>303	15	5
			373>345	10	
Promecarb	20.9	80	208>109	10	0.7
			208>151	5	

Table 9.1 (***Continued***)

Compound	t_R (min)	Fragmentor voltage (V)	MRM transitions (*m/z*)	Collision energy (eV)	LODs[b] (μg/kg)
Prometon	14.0	120	226>142	20	2
			226>184	20	
Prometryn	18.3	120	242>158	20	0.9
			242>200	20	
Propachlor	19.1	80	212>170	10	1
			212>152	15	
Propanil	19.8	120	218>127	20	0.8
			218>162	15	
Propiconazole	23.7+24.0	120	342>159	20	0.7
			342>69	20	
Prosulfocarb	27.1	120	252>91	15	0.6
			252>128	10	
Simazine	14.9	120	202>132	20	0.7
			202>124	20	
Spiromesifen	30.1	80	371>273	5	7
			371>255	20	
Sulfosulfuron	18.4	120	471>211	10	0.8
			471>261	15	
Teflubenzuron	25.6	80	381>158	10	9
			381>141	15	
Terbuthylazine	20.5	120	230>174	15	0.3
			230>132	20	
Terbutryn	18.6	120	242>186	15	1
			242>71	20	
Thiabendazole	7.8	120	202>175	30	6
			202>131	30	
Thiacloprid	14.0	120	253>126	15	2
			253>186	10	
Thiocyclam	6.3	120	182>137	10	50
			182>73	20	
Triazophos	22.9	120	314>162	20	0.6
			314>286	10	
Triclocarban	25.2	120	315>162	15	2
			315>128	15	
Trifloxystrobin	26.1	120	409>186	15	0.4
			409>206	10	
Triflumizole	24.9	80	346>278	5	3
			346>73	10	

[a] Reproduced with permission from Ferrer et al. (2007).
[b] LODs were calculated for all 100 compounds spiked in a green pepper matrix sample.

obtained, as mentioned in the last section. For this reason, many attempts to exploit MS/MS fragmentation mass libraries have failed due to the differences in instrumentation and operating conditions. However, this is not the case of TOF techniques, since accurate mass measurements are specific and universal for every target analyte and do not depend on the instrumentation used. In this way, a number of publications regarding the use of accurate mass databases of pesticides have been reported (Ferrer et al., 2006; Thurman et al., 2006a). Accurate mass determination allows obtaining specific information for a given molecule plus an additional confirmation if more fragments are present in the spectra.

A study from our group (Ferrer and Thurman, 2007; Ferrer et al., 2007) described an LC–TOF-MS multiresidue method for 101 commonly used pesticides, including complete information on accurate masses for the protonated molecules and fragment ions, retention times on a C_8 reversed-phase column, LODs, and calibration curves. The potential of TOF-MS for the quantitative analyses of pesticides in food and water samples at concentrations in the low μg/l range was fully evaluated. The proposed method for vegetable and fruit samples consisted of a sample treatment step using an extraction with acetonitrile followed by quantitative analyses by LC–TOF-MS. Water samples were

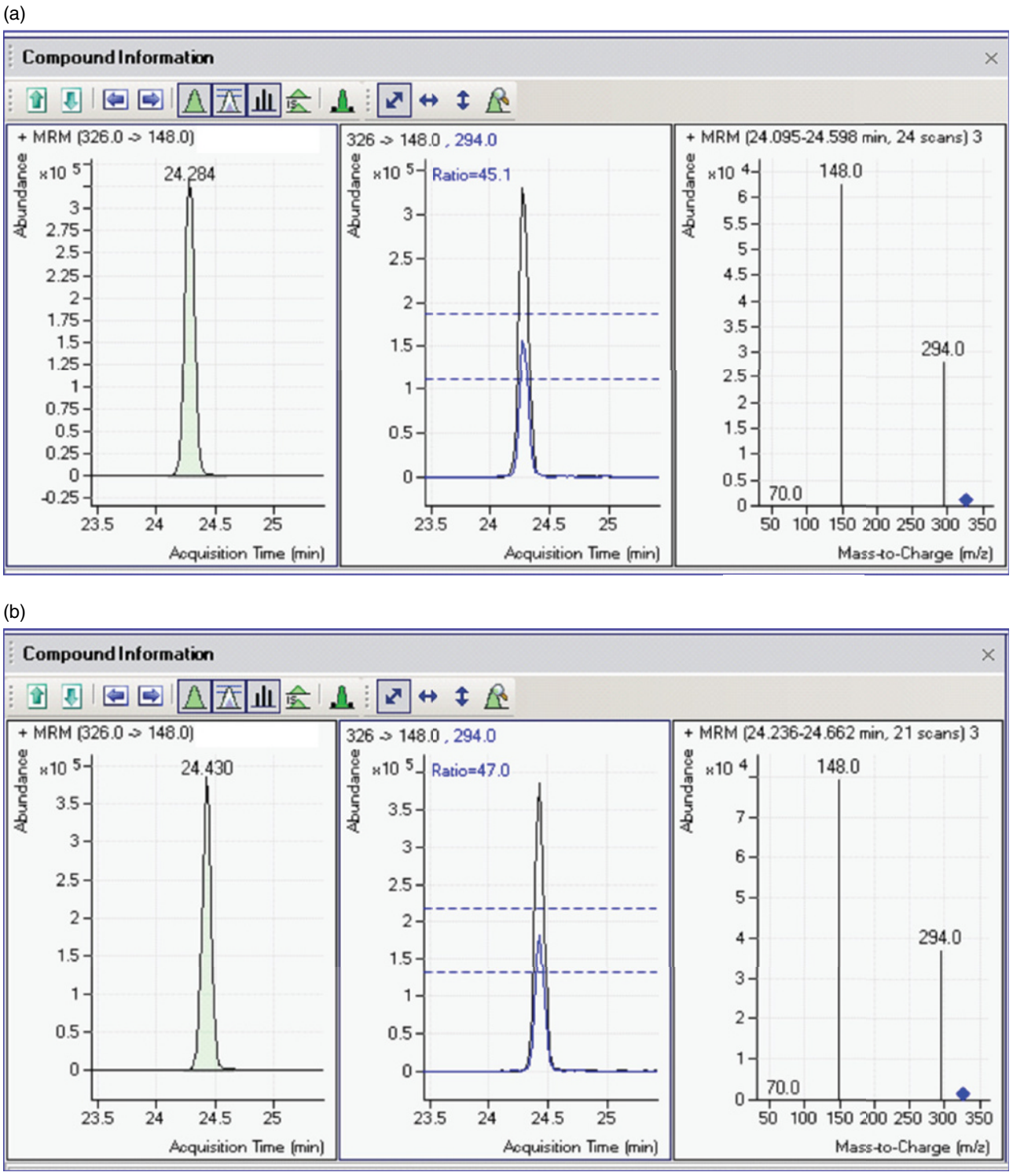


FIGURE 9.1 Ion ratios for benalaxyl in (a) solvent and (b) green pepper matrix. Concentration: 50 µg/kg. (Reproduced with permission from Ferrer et al. (2007).)

also evaluated and analyzed. The sample treatment applied to water samples was based on solid-phase extraction (SPE) using C_{18} cartridges. The method developed was sensitive for the detection of 101 pesticides in food samples down to 0.01 mg/kg; many MRLs of the EU Reg. 396/2005 and its amendments have been established at this concentration

level. This method works well for accurate mass instruments since it is not instrument specific. Thus, it is highly useful for identification of at least 101 pesticides in food and water matrices. The method was successfully applied to real environmental samples including food commodities and surface water samples.

Table 9.2 compiles the chemical formulas and exact accurate masses obtained by TOF-MS, as well as the retention times of 101 pesticides in a C_8 column. Of all the pesticides, 76 presented an $[M+H]^+$ peak as a base peak in the spectrum (base peak ions are marked in bold in Table 9.2). Surprisingly, 25 pesticides did not present the protonated molecule as a main base peak in the spectrum in spite of the low fragmentor voltage used; in all these cases, the larger ion was a fragment ion. Only one compound (aldicarb) presented a sodium adduct as a base peak, and in only one case (cartap), both the protonated molecule and the sodium adduct were absent; only two fragments showed up in the spectrum in this particular case. Some of the most common degradation products in environmental samples were also included in this study (e.g., degradation products for atrazine, aldicarb, etc.) for more complete and detailed information.

The accurate mass analysis of the protonated molecule together with that of additional characteristic fragment ion(s) (including characteristic isotopic signals and retention times) enables the unambiguous identification and confirmation of the studied pesticides at low concentration levels. This fits the requirements of the EU according to the identification point system (Commission Directive 2002/657/EC, 2002; Hernández et al., 2004). Another important tool that has made TOF one of the key methodologies for identification of compounds is the existence of accurate mass databases, as published extensively. An individual scientist can apply these universal databases to each specific problem and then often get a correct identification on the analyte of interest (Ferrer et al., 2006; Thurman et al., 2006a; Polettini et al., 2008). Other tools, which are available with TOF instrumentation and will be discussed in this chapter, include the use of molecular features, accurate mass filters and isotopic mass defect, diagnostic ions, and mass profiling to distinguish between control samples and positive samples. Examples will be given for each one of these accurate mass tools in the next section.

9.3 USE OF ACCURATE MASS TOOLS FOR THE IDENTIFICTION OF PESTICIDE RESIDUES AND THEIR METABOLITES IN FOOD AND WATER SAMPLES

With TOF-MS techniques, the possibility of identification of target and nontarget compounds based on accurate mass covers a wide range of tools (Ferrer and Thurman, 2009). All these tools are based on the capability of precise accurate mass (<3 ppm) that the actual TOF instruments are offering for the analysis of environmental samples. Such precision was not available just 10 years ago. Thus, a whole variety of MS tools that use different aspects of accurate mass have been and are being developed in recent years and at present. We will review many of these accurate mass tools and present some examples for the unequivocal identification of pesticides and their degradation products and metabolites in environmental samples.

9.3.1 Molecular Features

A molecular feature extraction (MFE) software consists of compiling accurate mass ions, excluding background noise, and plotting the extracted ion chromatograms of the most intense peaks found in a chromatogram. Thus, a molecular feature is defined as a discrete molecular entity defined by a combination of retention time, mass, and response in an LC–MS analysis. In general, MFE operates on raw mass spectral data generating lists of chemically qualified molecular features (while background is removed, interferences are resolved, and isotopic clusters and molecular adducts are recognized). The screening criteria usually consist of ±5 ppm accurate mass window, ±0.2 min retention time window, and a minimum of 10,000 counts (signal to noise of ~10:1). The ions are grouped by entities that include common adducts (sodium, ammonia, etc.) and isotope clusters.

As an example, Figure 9.2 shows the MFE for an extract of a pepper sample spiked with a mix of pesticides. As it can be seen in this figure, a total of 4235 ion features were found in the chromatogram, corresponding to matrix components in the pepper. One can generate as many empirical formulas as wanted, and from there, one can try to elucidate the chemical structure. But the most common approach is to compare the data obtained from a known database to try to match as many compounds as possible. This approach will be explained in the next section. In this particular experiment and in order to measure the complexity of the pepper matrix, an accurate mass extraction of all ions above the baseline of 10,000 counts was carried out. This analysis of the pesticide-spiked pepper matrix used MFE-based software. The program grouped all related adducts, proton, sodium, and ammonium and their related isotopic patterns into individual extracted ions and displayed them as chromatographic peaks (Fig. 9.2). When the pepper matrix was extracted, it contained thousands of individual molecular features with ion intensities of 10,000 counts or more. The 10,000 count rule of thumb is a value that yields a valid accurate mass and isotopic pattern for the A+1 and A+2 isotopes of all ions formed, which is needed for formula generation and testing by accurate mass. Figure 9.2 shows the overlaying of these 4235 molecular features as well as the total ion chromatogram for the pepper matrix spiked with a mix of pesticides.

TABLE 9.2 LC–TOF-MS accurate masses for the protonated molecules $[M+H]^+$ and the main fragment ions (q1, q2, q3) and retention times (t_R) for all the compounds studied (fragmentor voltage 190V)[a, b]

Compound	t_R (min)	Elemental composition[c]	Accurate mass $[M+H]^+$ *m/z*	q 1 *m/z*	q 2 *m/z*	q 3 *m/z*
Acetamiprid	16.8	$C_{10}H_{11}N_4Cl$	**223.0745**	126.0105		
Acetochlor	26.1	$C_{14}H_{20}NO_2Cl$	270.1255	224.0837	**148.1121**	133.0886
Alachlor	26.1	$C_{14}H_{20}NO_2Cl$	270.1255	238.0993	**162.1277**	
Aldicarb	18.7	$C_7H_{14}N_2O_2S$	213.0668[d]	116.0528	**89.0419**	70.0651
Aldicarb sulfone	11.8	$C_7H_{14}N_2O_4S$	**223.0747**	148.0427	166.0532	86.0600
Aldicarb sulfoxide	6.5	$C_7H_{14}N_2O_3S$	207.0798	132.0478	**89.0419**	
Atrazine	21.4	$C_8H_{14}N_5Cl$	**216.1010**	174.0541	146.0228	
Azoxystrobin	24.3	$C_{22}H_{17}N_3O_5$	**404.1241**	372.0979		
Benalaxyl	26.8	$C_{20}H_{23}NO_3$	**326.1751**	294.1489	208.1332	148.0757
Bendiocarb	20.8	$C_{11}H_{13}NO_4$	224.0917	**167.0703**	109.0284	
Bensultap	21.4	$C_{17}H_{21}NO_4S_4$	**432.0426**	290.0338		
Bromoxynil	21.7	$C_7H_3NOBr_2$	**275.8654**			
Bromuconazole	24.0 + 24.8	$C_{13}H_{12}N_3OCl_2Br$	**375.9614**	158.9763		
Buprofezin	27.4	$C_{16}H_{23}N_3OS$	**306.1635**	201.1056		
Butylate	29.7	$C_{11}H_{23}NOS$	**218.1573**	162.0947		
Captan	24.4	$C_9H_8NO_2SCl_3$	299.9414	**263.9647**	235.9693	
Carbaryl	21.3	$C_{12}H_{11}NO_2$	202.0863	**145.0648**		
Carbendazim	7	$C_9H_9N_3O_2$	**192.0768**	160.0505		
Carbofuran	20.8	$C_{12}H_{15}NO_3$	222.1125	**165.0910**	123.0446	
Cartap	3.1	$C_7H_{15}N_3O_2S_2$		150.0406	**104.9827**	
Chlorfenvinphos	26.5	$C_{12}H_{14}O_4PCl_3$	**358.9768**	204.9373	155.0468	98.9842
Chlorpyrifos-methyl	28.2	$C_7H_7NO_3PSCl_3$	**321.9023**	124.9821		
Cyanazine	19.6	$C_9H_{13}N_6Cl$	**241.0963**	214.0854		
Cyproconazole	23.6	$C_{15}H_{18}N_3OCl$	**292.1211**	125.0153	70.04	
Cyromazine	2.9	$C_6H_{10}N_6$	**167.1040**	108.0556		
DEET	21.3	$C_{12}H_{17}NO$	**192.1383**	119.0491	91.0542	
Deethylatrazine	15.9	$C_6H_{10}N_5Cl$	**188.0697**	146.0228		
Desethyl-terbuthylazine	19.6	$C_7H_{12}N_5Cl$	**202.0854**	146.0228		
Deisopropylatrazine	13	$C_5H_8N_5Cl$	**174.0541**	146.0228		
Diazinon	27.8	$C_{12}H_{21}N_2O_3PS$	**305.1083**	169.0794	153.1022	
Dichlorvos	20	$C_4H_7O_4PCl_2$	**220.9532**	127.0155	109.0049	
Difenoconazole	26.4 + 26.6	$C_{19}H_{17}N_3O_3Cl_2$	**406.0720**	337.0393	251.0025	
Difenoxuron	21.6	$C_{16}H_{18}N_2O_3$	**287.1390**	123.0441		
Diflubenzuron	25.2	$C_{14}H_9N_2O_2F_2Cl$	311.0393	**158.0412**	141.0146	
Dimethenamide	24.3	$C_{12}H_{18}NO_2SCl$	**276.0820**	244.0557	168.0841	
Dimethoate	16.6	$C_5H_{12}NO_3PS_2$	230.0069	198.9647	170.9698	**124.9821**
Dimethomorph	22.5 + 22.8	$C_{21}H_{22}NO_4Cl$	**388.1310**	301.0626		
Diuron	21.7	$C_9H_{10}N_2OCl_2$	**233.0243**	72.0444		
Ethiofencarb	21.8	$C_{11}H_{15}NO_2S$	226.0896	164.0706	**107.0491**	
Fenamiphos	24.1	$C_{13}H_{22}NO_3PS$	**304.1131**	276.0818		
Fenuron	15.7	$C_9H_{12}N_2O$	**165.1022**	72.0444		

Flufenacet	26.1	$C_{14}H_{13}N_3O_2F_4S$	364.0737	**194.0976**	152.0506	
Flufenoxuron	29.5	$C_{21}H_{11}N_2O_3F_6Cl$	**489.0435**	158.0412		
Fluoroacetamide	3.1	C_2H_4NOF	**78.0350**			
Fluroxypyr	19.2	$C_7H_5N_2O_3FCl_2$	**254.9734**	208.9679	180.9730	
Hexaflumuron	27.5	$C_{16}H_8N_2O_3F_6Cl_2$	**460.9889**	158.0412		
Hydroxyatrazine	12.1	$C_8H_{15}N_5O$	**198.1349**	156.0880		
Imazalil	18.1	$C_{14}H_{14}N_2OCl_2$	**297.0556**	255.0086	158.9763	
Imazapyr	13.7	$C_{13}H_{15}N_3O_3$	**262.1186**	234.1237		
Imazaquin	19	$C_{17}H_{17}N_3O_3$	**312.1343**	284.1394	266.1288	
Imidacloprid	16	$C_9H_{10}N_5O_2Cl$	**256.0596**	209.0588	175.0978	
Ioxynil	23	$C_7H_3NOI_2$	**371.8377**			
Iprodione	25.6	$C_{13}H_{13}N_3O_3Cl_2$	**330.0407**	244.9879		
Irgarol 1051	21.2	$C_{11}H_{19}N_5S$	**254.1434**	198.0808		
Irgarol metabolite	17	$C_8H_{15}N_5S$	**214.1121**	158.0495		
Isoproturon	21.6	$C_{12}H_{18}N_2O$	**207.1492**	165.1022	72.0444	
Lenacil	19.6	$C_{13}H_{18}N_2O_2$	235.1441	**153.0659**		
Lufenuron	28.9	$C_{17}H_8N_2O_3F_8Cl_2$	**510.9857**	158.0412		
Malathion	26	$C_{10}H_{19}O_6PS_2$	**331.0433**	285.0015	127.0390	124.9821
Mebendazole	18.4	$C_{16}H_{13}N_3O_3$	**296.1030**	264.0768		
Metalaxyl	21.5	$C_{15}H_{21}NO_4$	**280.1543**	248.1281	220.1332	192.1383
Metamitron	15.2	$C_{10}H_{10}N_4O$	**203.0927**	175.0978		
Methidathion	24.1	$C_6H_{12}N_2O_4PS_3$	302.9691	145.0066	**85.0396**	
Methiocarb	23.7	$C_{11}H_{15}NO_2S$	226.0896	**169.0682**	122.0726	121.0648
Methiocarb sulfone	17.7	$C_{11}H_{15}NO_4S$	**258.0795**	201.058	122.0726	
Methomyl	12.6	$C_5H_{10}N_2O_2S$	163.0536	106.0321	**88.0215**	72.9981
Metolachlor	25.9	$C_{15}H_{22}NO_2Cl$	**284.1412**	252.115		
Metolcarb	19.7	$C_9H_{11}NO_2$	166.0863	**109.0648**	94.0413	
Metribuzin	20.1	$C_8H_{14}N_4OS$	**215.0961**	187.1012		
Molinate	24.8	$C_9H_{17}NOS$	**188.1104**	126.0913		
Monuron	19.2	$C_9H_{11}N_2OCl$	**199.0633**	72.0444		
Nicosulfuron	18.1	$C_{15}H_{18}N_6O_6S$	**411.1081**	213.0328	182.0560	
Nitenpyram	12.1	$C_{11}H_{15}N_4O_2Cl$	**271.0956**	225.1027	196.0636	99.0917
Oxadixyl	19.1	$C_{14}H_{18}N_2O_4$	279.1339	**219.1128**	133.0886	132.0808
Parathion-ethyl	27.3	$C_{10}H_{14}NO_5PS$	**292.0403**	264.0090	235.9777	
Pendimethalin	30.2	$C_{13}H_{19}N_3O_4$	282.1448	**212.0666**	194.0560	
Phosmet	24.3	$C_{11}H_{12}NO_4PS_2$	318.0018	**160.0393**		
Prochloraz	23	$C_{15}H_{16}N_3O_2Cl_3$	376.0381	**308.0006**	265.9537	
Profenofos	28.6	$C_{11}H_{15}O_3PSClBr$	**372.9424**	344.9111	302.8642	
Promecarb	24.4	$C_{12}H_{17}NO_2$	208.1332	**151.1117**	109.0653	
Prometon	16.6	$C_{10}H_{19}N_5O$	**226.1662**	184.1193	142.0723	
Prometryn	20.3	$C_{10}H_{19}N_5S$	**242.1434**	200.0964	158.0495	
Propachlor	22.8	$C_{11}H_{14}NOCl$	**212.0837**	170.0367		
Propanil	23.3	$C_9H_9NOCl_2$	**218.0134**	161.9872	127.0183	
Propiconazole	25.9 + 26.1	$C_{15}H_{17}N_3O_2Cl_2$	**342.0771**	158.9763		

(*continued*)

TABLE 9.2 (*Continued*)

Compound	t_R (min)	Elemental composition[c]	Accurate mass $[M+H]^+$ *m/z*	q 1 *m/z*	q 2 *m/z*	q 3 *m/z*
Prosulfocarb	29	$C_{14}H_{21}NOS$	**252.1417**	128.107	91.0542	
Simazine	19.1	$C_7H_{12}N_5Cl$	**202.0854**	132.0323		
Spinosad A	20.7	$C_{41}H_{65}NO_{10}$	**732.4681**	544.3633		
Spinosad D	21.4	$C_{42}H_{67}NO_{10}$	**746.4838**	558.3789		
Spiromesifen	30.7	$C_{23}H_{30}O_4$	**371.2217**	255.138		
Spiroxamine	19.7	$C_{18}H_{35}NO_2$	**298.2741**	144.1383	100.1121	
Teflubenzuron	27.9	$C_{14}H_6N_2O_2F_4Cl_2$	**380.9815**	158.0412		
Terbuthylazine	23.8	$C_9H_{16}N_5Cl$	**230.1167**	174.0541	146.0228	
Terbutryn	20.4	$C_{10}H_{19}N_5S$	**242.1434**	186.0808		
Thiabendazole	8.8	$C_{10}H_7N_3S$	**202.0433**	175.0324		
Thiacloprid	18.3	$C_{10}H_9N_4SCl$	**253.0309**	126.0105		
Thiocyclam	4.5	$C_5H_{11}NS_3$	182.0126	**136.9548**		
Thiosultap	3.2	$C_5H_{13}NO_6S_4$	**311.9698**	232.013		
Triclocarban	27.5	$C_{13}H_9N_2OCl_3$	**314.9853**	161.9872	127.0183	
Triflumizole	25.9	$C_{15}H_{15}N_3OF_3Cl$	346.0929	**278.0554**		
Trifluralin	30.6	$C_{13}H_{17}N_3O_4F_3$	**336.1166**			

In bold is the base peak ion observed in the spectrum at 190 V.

[a] Reproduced with permission from Ferrer and Thurman (2007).

[b] Base peak ions in the spectra are marked in bold.

[c] Elemental compositions correspond to the neutral molecule.

[d] Ion corresponding to the sodium adduct [M+Na]+.

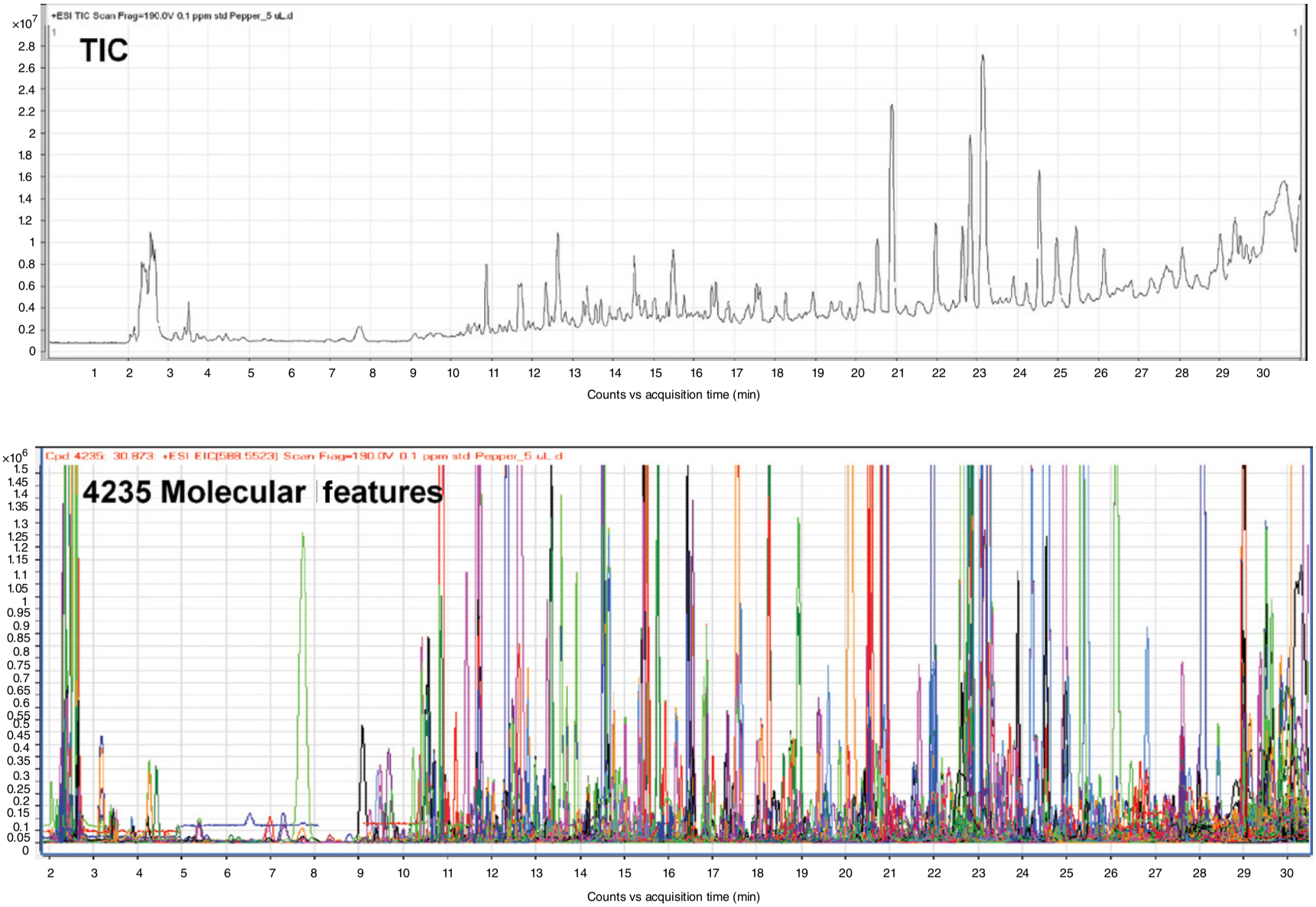


FIGURE 9.2 (Top) Total ion chromatogram (TIC) of a pesticide-spiked pepper matrix. (Bottom) Extracted molecular features for the spiked pepper matrix showing 4235 molecular features. (Reproduced with permission from Thurman et al. (2013a).)

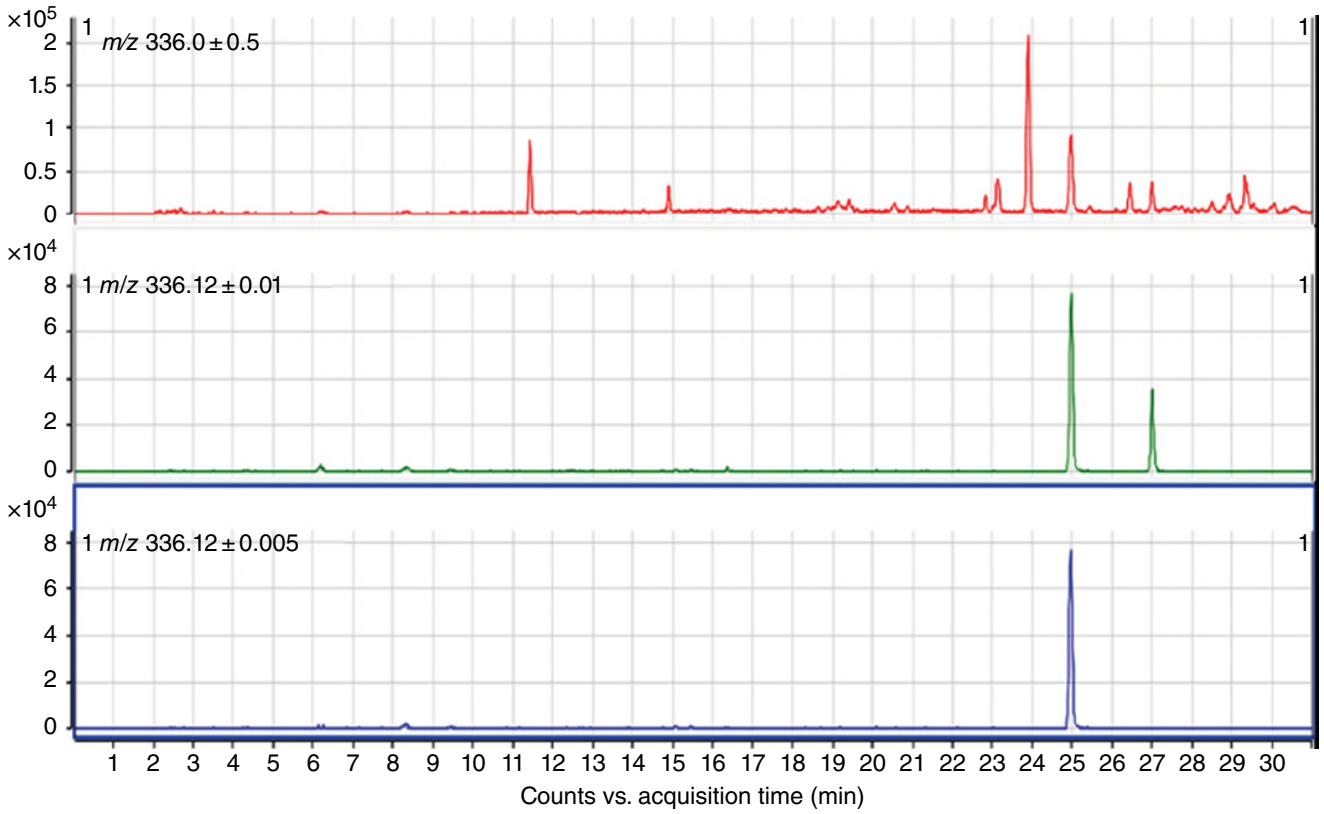


FIGURE 9.3 Extracted ion chromatogram at *m/z* 336 with windows of extraction of ±0.5, ±0.01, and ±0.005. The extracted ions decrease from approximately 10 features to one feature by narrowing the window of extraction. The features decreased from 10 to 1 because the mass defect of the red pepper is larger than 0.12±0.005. (Reproduced with permission from Thurman et al. (2013a).)

The complexity of the spiked pepper sample reminds one of the proverbial search for *a needle in a haystack*. The value of the high resolution of the mass spectrometer and the measurement of accurate mass lies in the ability to search through the *haystack*, the pepper matrix, in order to find the specific ions for the individual pesticides. In this case, though, the search is done one accurate mass at a time. For example, the sodium adduct of kresoxim-methyl has an accurate mass of *m/z* 336.1210; thus, the extraction of the ion will focus on using a smaller extraction window of *m/z* 336. The value of narrowing the extraction window is shown in Figure 9.3. Here, the *m/z* 336 ion is extracted with three window sizes from ±0.5, 0.1, and 0.01 mass units, u. The number of molecular features narrows from 4235 total features to approximately 10, then 2, and finally only 1 at the narrowest extraction window of 0.005 mass units. The final single peak is the sodium adduct of kresoxim-methyl (Thurman et al., 2013a).

Strengths of the MFE software include rapid screening of hundreds of compounds at sensitive levels compared to a manual approach and the ease of use of a database for any accurate mass spectrometer instrumentation capable of routine sub-5 ppm mass accuracy.

9.3.2 Accurate Mass Filters and Isotopic Mass Defect

The majority of the 4235 molecular features shown in Figure 9.2 represent the pepper matrix, the exceptions being the individual spiked pesticides and their molecular adducts. If we examine the mass defects of the matrix versus the mass defect of the spiked pesticides, they are easily separated using the high resolving power of the mass spectrometer. The mass defect refers to the difference between the nominal mass and the accurate mass of the compound (Thurman and Ferrer, 2010). Because of the structure of many pesticides, which contain elements such as sulfur, phosphorus, and the halogens, the mass defect is shifted closer to the nominal mass (Thurman and Ferrer, 2010) than the majority of the matrix of most vegetables, which are rich in hydrogen relative to the pesticides. Typically, at a mass of *m/z* 336, for example, the majority of ions in the pepper matrix have a mass defect from 0.15 to 0.25. This shift is taken advantage of with the high resolution of the mass spectrometer, which results in the reduction of interfering ions as shown in Figure 9.3. Thus, the value of high-resolution MS for the analysis of isobaric pesticides lies in the ability to separate the *m/z* 336.1210 ion from the possible interfering ions of the matrix.

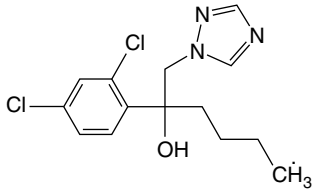
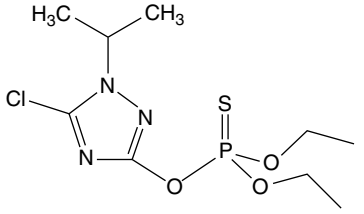
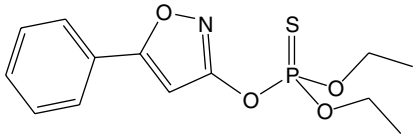
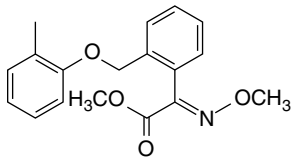
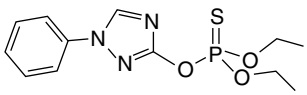
Table 9.3 shows the elemental compositions and structures of five isobaric compounds that differ by 0.0120–0.0700 mass units. None of the compounds are isomeric (with the same accurate mass); however, they will require a maximum mass spectrometric resolving power of approximately 26,000 in order to have complete separation by accurate mass with high resolution based on their closest mass differences, as shown in the following calculations. A resolving power of 26,000 is based on the smallest mass difference of 0.012 and a nominal mass of 314 (i.e., 314 divided by 0.012 yields resolving power at 50% separation at half height of 26,167 for what is called full width at half maximum (FWHM)). For complete baseline separation of the two closest masses, it requires twice FWHM or approximately 50,000 resolving power (Thurman et al., 2006b). This calculation presumes that the two compounds with the nearest mass difference should coelute and be of equal intensity. The instrument used in this study was operating at a mass resolving power of 26,500 at a mass of *m/z* 300, which should be adequate to separate these five pesticides at FWHM.

A closer look at the mass spectrum for the coelution of isazophos and kresoxim-methyl at 25 min (Fig. 9.4) shows that there are two $[M+Na]^+$ *m/z* 336 ions at *m/z* 336.0312 and 336.1210 with isotopic signatures at *m/z* 337.0339 and 337.1243 and *m/z* 338.0284 and 338.1274. These isotopic signatures at A+1 and A+2 show that chlorine is present in the first $[M+Na]^+$ ion at *m/z* 336.0312 (corresponding to isazophos; Table 9.3) and not present in the $[M+Na]^+$ ion at *m/z* 336.1210 (corresponding to kresoxim-methyl; Table 9.3), which fits the hypothesis of coelution for isazophos and kresoxim-methyl. Thus, it is possible to distinguish isazophos and kresoxim-methyl as their corresponding sodium adducts by high-resolution MS. If kresoxim-methyl had formed a $[M+H]^+$ at *m/z* 314, theoretically, they would have also separated.

9.3.3 Diagnostic Ion Approach

Depending on the family of pesticides studied (triazines, phenylureas, organophosphates, etc.), a trend is observed for fragmentation ions present in their respective spectra (Ferrer

TABLE 9.3 List of five isobaric pesticides and accurate masses of main adduct ions[a]

Name	Elemental composition	Ret. time (min)	$[M+H]^+$	$[M+Na]^+$	Chemical structure
Hexaconazole	$C_{14}H_{17}Cl_2N_3O$	23.4	314.0821	—	
Isazophos	$C_9H_{17}ClN_3O_3PS$	25.0	314.0490	336.0309	
Isoxathion	$C_{13}H_{16}NO_4PS$	26.5	314.0610	336.0430	
Kresoxim-methyl	$C_{18}H_{19}NO_4$	25.0	314.1387	336.1206	
Triazophos	$C_{12}H_{16}N_3O_3PS$	23.9	314.0723	336.0542	

[a]Reproduced with permission from Thurman et al. (2013a).

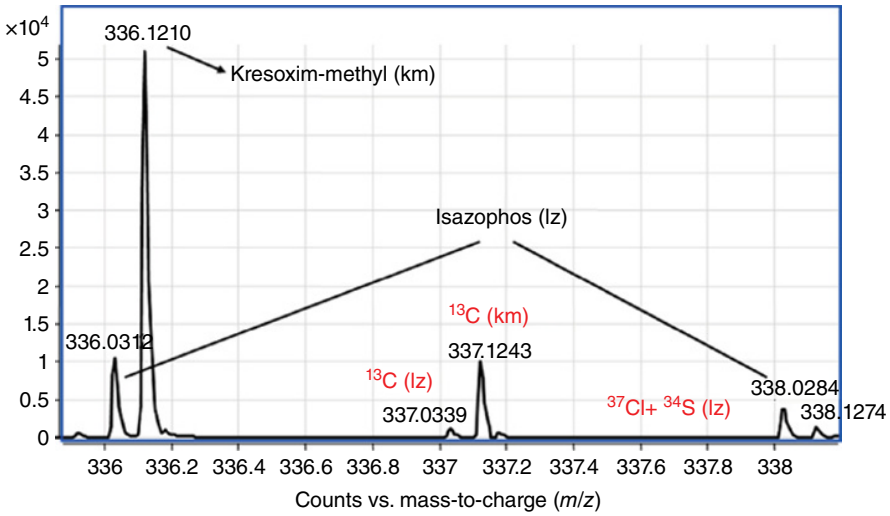


FIGURE 9.4 Accurate mass spectrum of the *m/z* 336 ions of kresoxim-methyl and isazophos, which coelute at 25.0 min. (Reproduced with permission from Thurman et al. (2013a).)

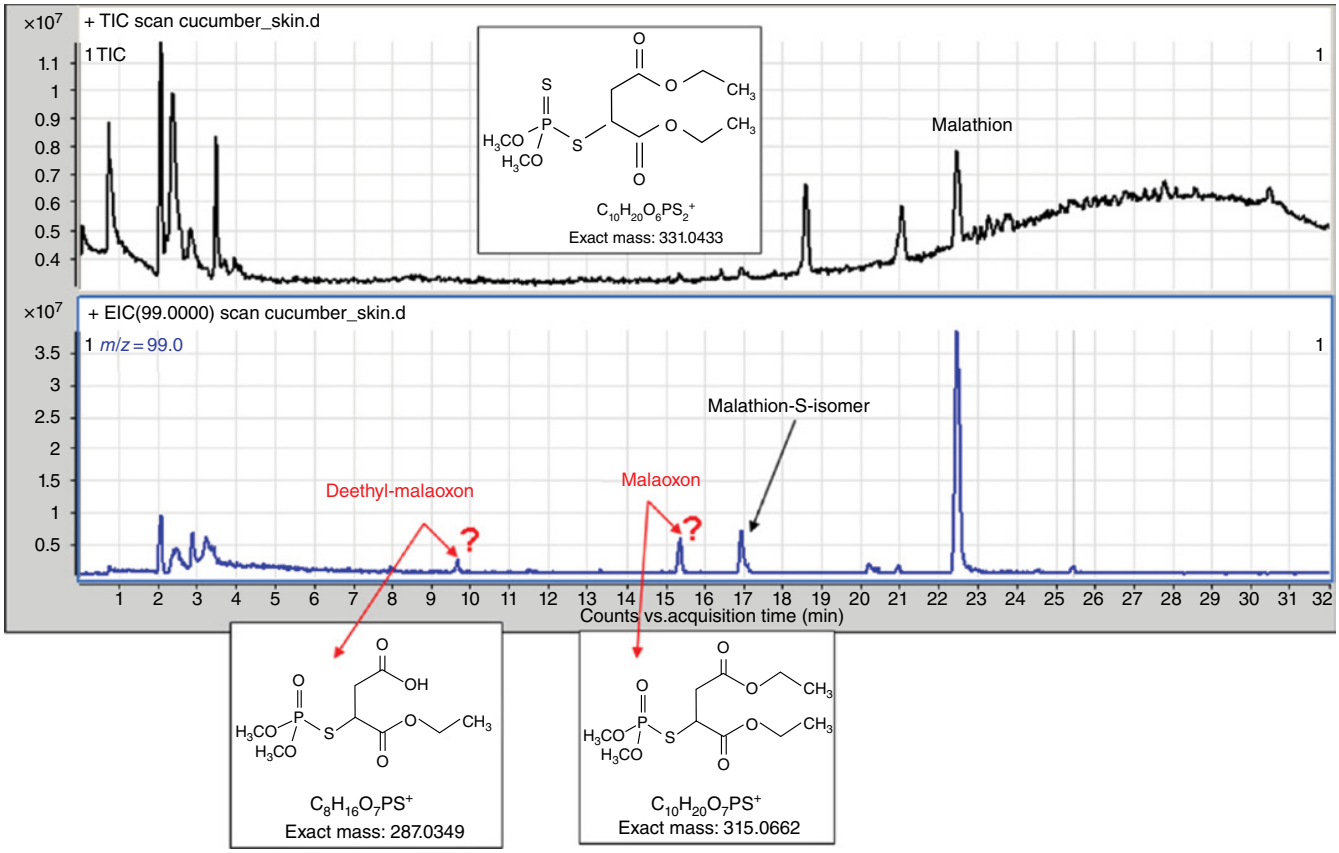


FIGURE 9.5 (Top) Total ion chromatogram of a cucumber sample. (Bottom) Extracted ion chromatogram for *m/z* 99.

and Thurman, 2007). For example, several organophosphate pesticides show the diagnostic ions at *m/z* 124.9821 and 99.0077 corresponding to the elemental formula of $C_2H_6O_2PS$ and $C_4H_2O_3$, respectively. Figure 9.5 shows an extracted chromatogram for the ion at *m/z* 99 after the analysis of a cucumber sample. Malathion was positively identified by its accurate mass as shown in the upper chromatogram. However, two more peaks appeared at the extracted mass of 99 (see lower chromatogram) corresponding to two possible malathion metabolites. One of them was identified as

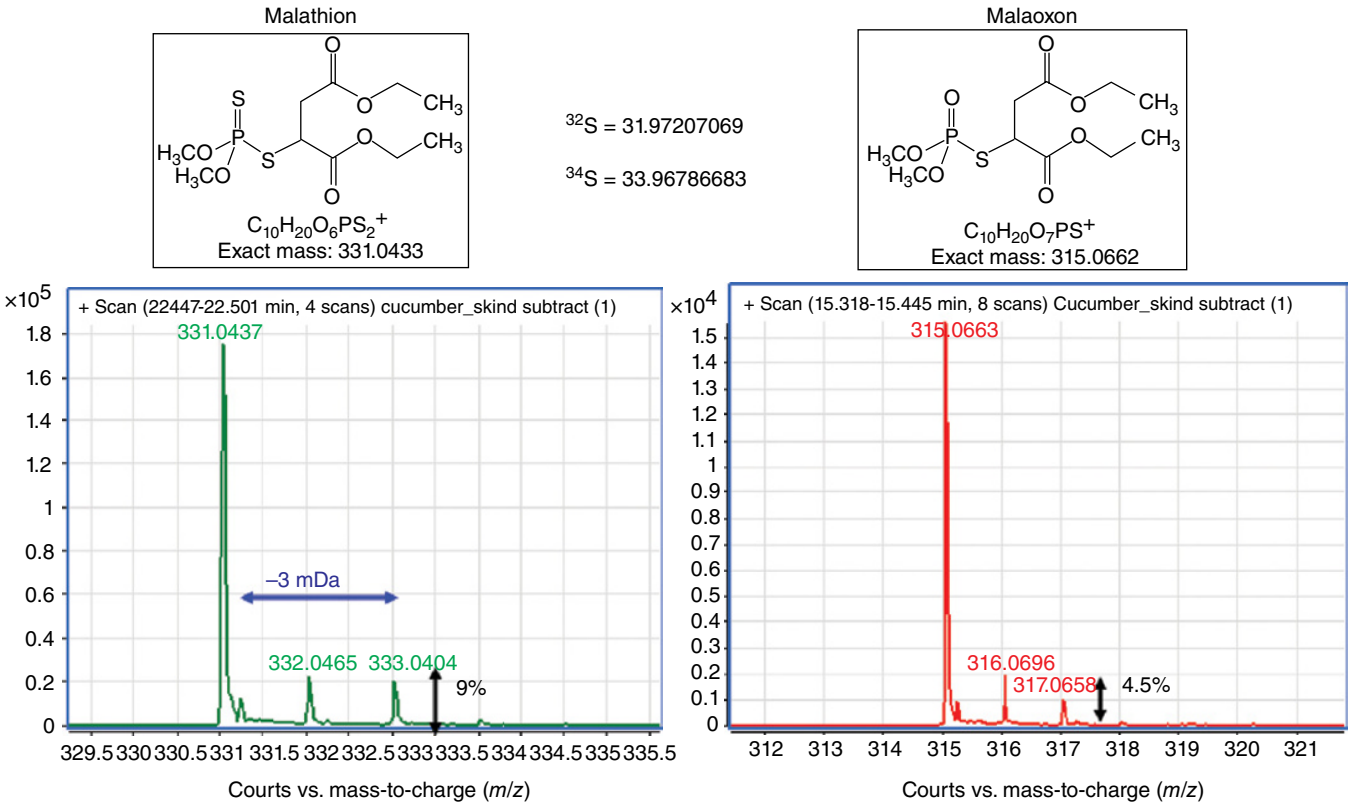


FIGURE 9.6 Accurate mass spectra of malathion and malaoxon.

molecular formula $C_{10}H_{20}O_7PS$, which is one less sulfur atom and one more oxygen atom, thus suggesting the identity as an oxon degradation product, also known as malaoxon. Similarly, the second peak at 9.7 min was 28 mass units smaller, thus suggesting the de-ethylation of malaoxon and thus being identified as deethyl-malaoxon metabolite. By taking a closer look at the spectra generated by malathion and malaoxon (Fig. 9.6), one can see the effect of the isotopic mass defect due to the sulfur atom. Both compounds show a sulfur isotopic signature for the ^{34}S isotope. The mass defect is 3 mDa due to the difference between the ^{32}S and the ^{34}S isotope accurate mass. Also shown in the figure are the isotopic signature of 9% corresponding to the existence of two sulfur atoms in the malathion chemical structure and the isotopic signature of 4.5% corresponding to only one sulfur atom in the malaoxon structure. This exemplifies the usefulness of the diagnostic ions, isotopic mass defect, and isotopic pattern recognition when applied to degradation products of pesticides in food or water samples.

9.3.4 Accurate Mass Databases

For many years, the use of reverse-search methods for gas chromatography–mass spectrometry (GC–MS) has made it possible to search large National Institute of Standards and Testing (NIST) pesticide libraries in minutes (Wylie, 2006) and has made screening quite simple for pesticides amenable to GC–MS. Unfortunately, similar reverse-search methods have not been available for LC–MS for two reasons. First, the single quadrupole and triple quadrupole mass spectrometers do not operate in full-scan mode for pesticide screening because of a lack of sensitivity (Ferrer and Thurman, 2009). Secondly, although libraries for LC–MS three-dimensional ion trap have been built, they have not been popular due to difficulties in reproducibility of fragmentation and the need for authentic standard analysis for each instrument (Baumann et al., 2000; Gergov et al., 2004; Josephs and Sanders, 2004). Thus, the only approach that uses full-spectrum information is LC–TOF-MS, which is both sensitive and accurate (Ferrer and Thurman, 2005) but uses only the accurate mass of the $[M+H]^+$ ion. The combination of accurate mass and sensitivity is needed for screening of compounds by their empirical formula.

The pioneer efforts to search data using an accurate mass database were made by several authors, such as Thurman et al. (Thurman et al., 2005a), Bobeldijk et al. (Bobeldijk et al., 2001), and Ojanperä et al. (Pelander et al., 2003). For example, Thurman et al. (Thurman et al., 2005a) used an approach of TOF, ion trap, and the Merck Index database to identify pesticides in food and also degradation products, without the initial use of primary standards (Thurman et al., 2005b). Bobeldijk et al. also used the Merck Index, the NIST

library, and their own database to screen water pollutants (Bobeldijk et al., 2001). The methods in these pioneering examples relied on manually searching the databases, compound by compound. Later on, several papers extended this approach and were published. For example, Ojanperä et al. (Pelander et al., 2003, Laks et al., 2004) used mass accuracy of 30 ppm and database analysis to identify approximately 600 drugs in blood and urine without the use of primary standards, using only the protonated molecule. Other automated databases for pesticide analysis in food samples used a similar approach as well (Ferrer et al., 2006; Thurman et al., 2006a).

In spite of the progress that has been made, the ability to do true library analysis is still a problem to be solved for LC–MS and for rapid analysis of environmental samples. The problems to be overcome include reproducible spectra and ion ratios, routine programs for rapid screening of samples rather than manual checking of data, and some estimate of the probability of the correct identification. Variation in fragmentation intensity is not critical with the use of accurate mass since the accurate mass of the fragment ion gives its molecular formula. In fact, accurate mass measurements are specific and universal for every target analyte regardless the instrumentation used. Usually, unambiguous identification is accomplished by means of accurate mass measurements from (de)protonated molecules, fragment ions, and isotope intensity/signature matching. Thus, the accurate mass database approach is a screening tool, and it is powerful and fast because only the molecular formula is needed.

Here, we will describe two different approaches on the use of databases. The first one uses an automated molecular feature process as described in a previous section and then a commercial or homemade database based on a *csv* file. The second approach is called *reversed database search* in which a total ion chromatogram is searched for ions included in such database. Databases usually contain information of the monoisotopic exact mass of the $[M+H]^+$, at least one product ion, and retention time of the compound.

Following the first approach (as described before), a compilation of ions was gathered through an MFE (see Fig. 9.7) for a pear extract. The next step was to match these accurate masses with any compounds included in a commercial database (called *pesticides database* from Agilent Inc., which includes more than 1600 compounds). As shown in Figure 9.7, one of the hits obtained was for phosmet, a common organophosphate pesticide. In the insert, a detailed mass spectrum is plotted. Note that this molecule presented both the sodium adduct at *m/z* 339.9841 and the protonated molecule at *m/z* 318.0021. The MFE was able to group both ions as part of the same identity, as explained in a previous section. This approach is fast to run since only the molecular features found by the software are matched with each individual compound in the database.

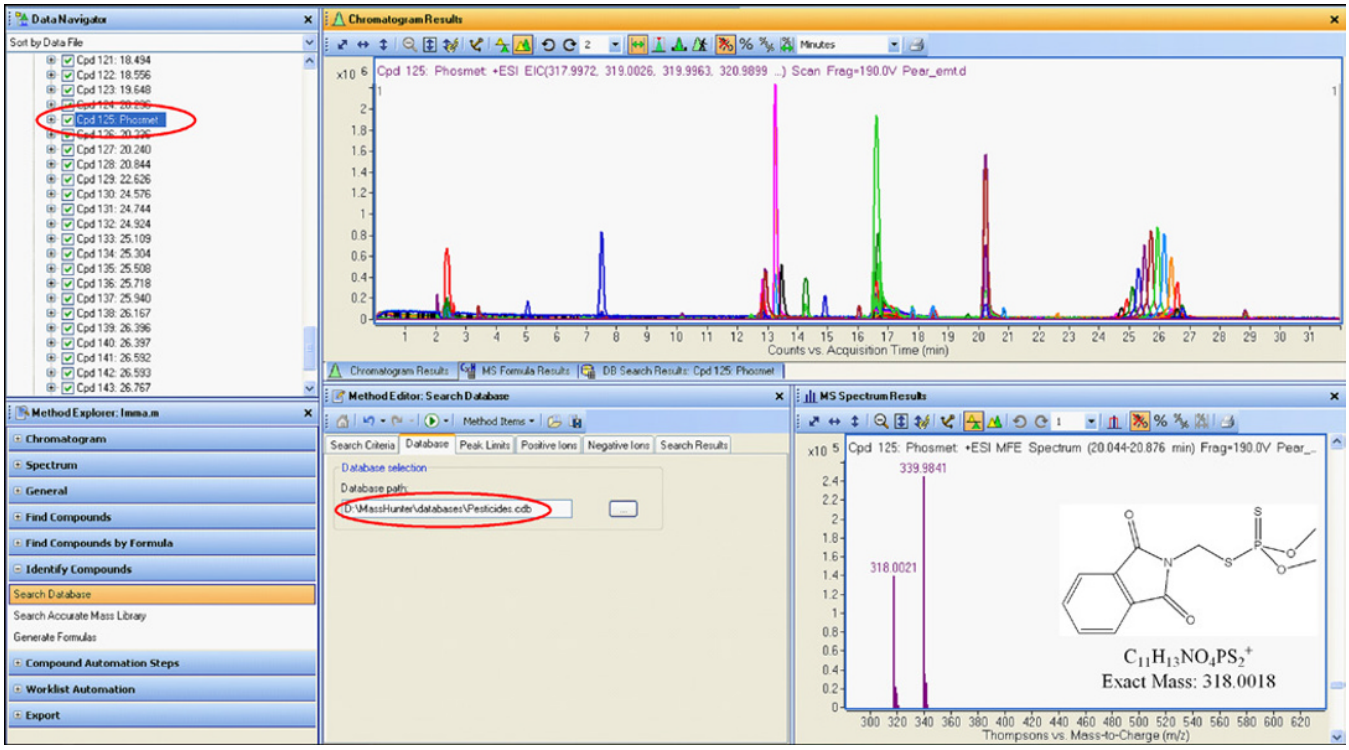


FIGURE 9.7 Molecular feature extraction and database search for a pear sample. Mass spectrum for phosmet is shown in the inset.

The second approach, also known as *reversed database search*, consists of running the database to see if any positive hits are found. This approach is usually slower than the last one as each one of the database entries has to be verified against each of the extracted ion chromatograms for a given accurate mass. However, simpler databases with a smaller number of compounds will be faster as fewer ions have to be extracted. This automatic screening method requires a thorough full optimization of the accurate mass window used and retention time (always optional) tolerances, which play an important role on the selectivity, accuracy, and successfulness of the whole procedure.

The pesticides database was run on a sample of apple extract, and the presence of pyrimethanil, a known fungicide applied to fruits, was verified. Figure 9.8 depicts an excerpt of the automated generated report of this particular database search. It is important to note the high score obtained for this particular hit. This score is a combination of mass accuracy, isotope intensity, and isotope spacing. Also, as shown in the figure, a good mass accuracy (with an error below 2 ppm) was obtained for this identification, thus confirming the presence of this compound in the sample. In this case, no standard had been analyzed by this instrument when this finding was made, so a pure standard was purchased and analyzed and verified this positive identification in the sample of apple extract.

9.3.5 Accurate Mass Profiling

Mass profiling is basically a comparison of ions that are present in control samples (blanks) versus the ions that are present in positive samples. Sometimes, a recursive extraction of the data set is necessary in order to remove small differences that are not significant statistically. In this way, features that are unique to positive samples (those containing new peaks) are illustrated in the mass profiler graphs obtained, which show intensity of ions versus retention time. Another possibility using mass profiling is the capability of generating principal component analysis (PCA) plots. These plots usually exemplify the different results obtained grouped under diverse experimental conditions.

A dissipation study that involved the application of imidacloprid (an insecticide) to onion plants was recently carried out in our laboratory (Thurman, et al. 2013b). The main objective was the identification of plant metabolites after a systemic application of the pesticide. Extracts of soil and

Target compound screening report

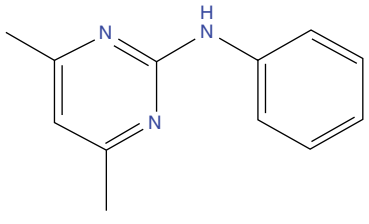
Date file	Apple skin extract.d	Sample name	
Sample type	Sample	Position	P1-C4
Instrument name	Instrument 1	User name	
Acq method		Acquired time	2/25/2010 6:45:54 pm
IRM calibration status	Success	Da method	Pesticide Database.m
Comment			

Compound table

Label	Tgt name	Tgt score	Mass error (ppm)	Tgt formula	Obs.RT	Ref.mass	Obs.mass
Cpd 2: Clofencet	Clofencet	47.46	0.73	C13H11ClN2O3	2.105	278.0458	278.046
Cpd 1: Anilazine	Anilazine	55.09	2.07	C9H5C13N4	2.105	273.958	273.9585
Cpd 3: Demeton-O-methyl-oxon	DEmeton-O-methyl-oxon	81.37	−0.18	C6H15O4PS	2.176	214.0429	214.0428
Cpd 4: Benzoximate	Benzoximate	42.11	3.9	C18H18ClNO5	2.412	363.0874	363.0888
Cpd 5: Senkikkine	Senkikkine	95.37	−3.05	C19H27NO6	3.649	365.1838	365.1827
Cpd 6: Seneciphylline-N-oxide	Senciphylline-N-oxide	68.13	0.81	C18H23NO6	13.486	349.1525	349.1528
Cpd 7: Terbuchlor	Terbuchlor	41.88	4.22	C18H28ClNO2			
Cpd 8: Pyrimethanil	pyrimethanil	99.72	0.56	C12H13N3			
Cpd 11: Dinoterb	Dinoterb	47.17	1.36	C10H12N2O5			
Cpd 10: Dinoseb	Dinoseb	47.17	1.36	C10H12N2O5			
Cpd 9: Dinoprop	Dinoprop	47.17	1.36	C10H12N2O5			
Cpd 12: Cinerin 1	Cinerin I	89.6	−3.11	C20H28O3			
Cpd 13: Milbemectin A4	Milbemectin A4	96.35	−0.48	C32H46O7			
Cpd 14: Dodemorph	Dodemorph	97.96	0.53	C18H35NO			
Cpd 15: Diofenolan	Diofenolan	95.25	−2.67	C18H20O4			
Cpd 16: Calciferol	Calciferol	63.72	−3.83	C28H44O			

---End of report---

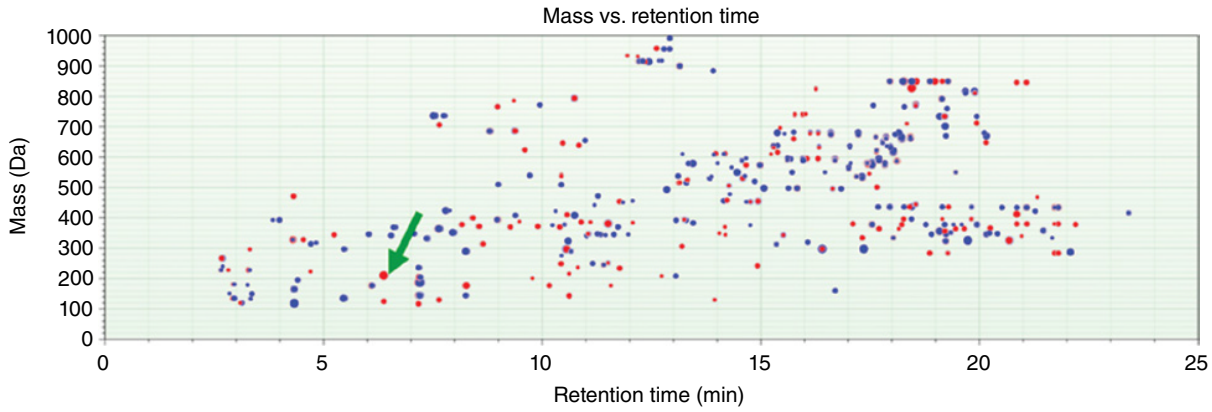
Pyrimethanil



$C_{12}H_{14}N_3^+$

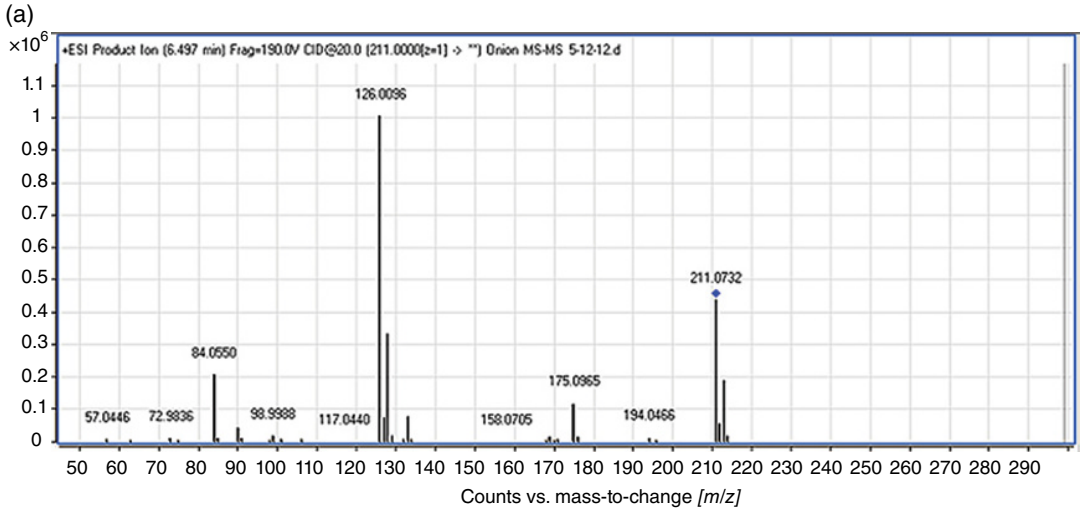
Exact mass: 200.1182

FIGURE 9.8 Database automated report for an apple extract.



	Feature summary									Experiment(3)			
	ID	RT	SD	Mass	SD	Abundance	RSD	Freq.	Mark	RT	Mass	Abundance	RSD
19	19	17.657	0.001	298.0186	0.0003	22940380	1.10	3				0	
20	20	6.378	0.007	210.0667	0.0001	22010370	1.10	3		6.378	210.0667	44020740	0.04
21	21	18.157	0.002	675.3111	0.0004	21588940	0.36	6		18.156	675.3113	28663160	0.03
22	22	18.220	0.002	666.2873	0.0007	21056570	0.20	6		18.219	666.2878	24956140	0.01

FIGURE 9.9 Mass profiler plot for an extract of onion plants compared to control samples.



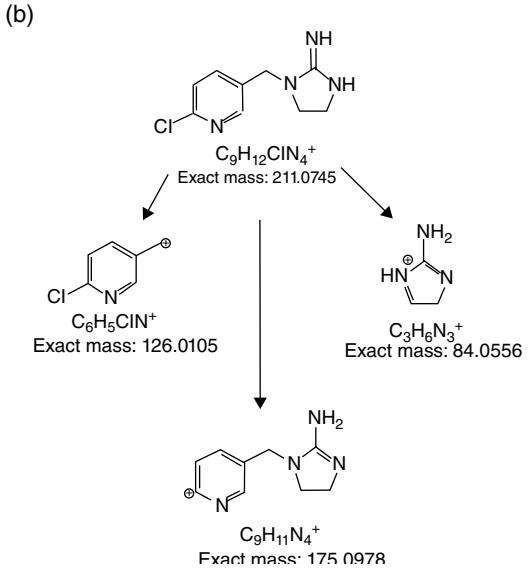


FIGURE 9.10 (a) MS/MS accurate mass spectrum at the collision energy of 20eV for the metabolite of imidacloprid at *m/z* 211 and (b) putative structures for the fragment ions and fragmentation pathway.

plants were analyzed by LC–QTOF-MS. Although imidacloprid metabolism had been studied in a number of crops, it had not been studied in detail in onions (Mandic et al., 2005); thus, to show and develop a robust method for its determination along with its metabolites was crucial to our study of its fate in onions. We chose UHPLC–QTOF-MS because of the power of the method to determine accurate mass and the formulas of various degradation products. Furthermore, we were able to apply MS/MS analysis with accurate mass to determine new or hypothesized metabolites of imidacloprid.

Chromatograms of both control samples (onions not treated with imidacloprid) and positive samples (onions treated with imidacloprid) were compared using the Mass Profiler software (Agilent Inc., Palo Alto, CA, USA). The software first analyzes all groups of ions (known also as features) in the chromatogram of both samples and compiles this into a database. Three replicates of each sample are taken and averaged. Next, the software compares the two samples looking for features that are unique to the positive samples (Fig. 9.9). The comparison resulted in 112 different features (in red or dark gray) that were unique to the samples treated with imidacloprid. Differences in the wilting of the plant (i.e., browning of plant) could also be a cause for different molecular features. Ions at the same retention time, for example, 15.7 min, are usually the same fragment ions of a feature. One of the features that showed up as unique in the positive sample was the *m/z* 211.0732 ion, which was the major difference between the control and dosed onion plants. This metabolite is called guanidine metabolite of imidacloprid and is a major one in plant metabolism, as previously reported in the literature (Casida, 2011). Further, MS/MS experiments were carried out to unequivocally identify this guanidine metabolite, as seen in Figure 9.10a. The main fragments obtained by MS/MS were at masses *m/z* 175.0965, 126.0096, and 84.0550. Using the accurate mass information and molecular formula generation, all three fragments were confirmed as shown in the fragmentation pathway drawn in Figure 9.10b, thus confirming the identity of this important metabolite in the onion plant.

9.4 CONCLUSIONS

Pesticide residues in environmental samples are successfully analyzed by advanced LC–MS techniques. The most popular methodology involves the use of selected or multiple ion transitions using MS/MS for the analysis of specific target pesticides. However, in just the last decade, a whole revolution involving the use of TOF accurate mass techniques has occurred among the environmental field. These techniques offer a wide range of accurate mass tools that can be used for the identification of nontarget pesticides, their metabolites, or degradation products and other unknown compounds present in the samples. Some of these tools include the use of MFE, isotopic mass defect filters, diagnostic ion approaches, accurate mass database searches, and mass profiling capabilities. The use of any of these individual tools or a combination of several of them leads to a successful identification of pesticide contaminants and related compounds in environmental samples.

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25

Occurrence of Transformation Products of Emerging Contaminants in Water Resources of the United States

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25.1 Introduction: Emerging Contaminants

The identification of emerging contaminants (ECs) in water samples has been the focus of many water agencies and water treatment facilities around the world. Specifically, in the United States, the Environmental Protection Agency (EPA) has guided and released new regulations [1] in order to narrow the contaminant candidate list (CCL3) to possible toxic emerging compounds of interest. Most recently, a new candidate list called "The Third Unregulated Contaminant Monitoring Rule (UCMR 3)" from EPA was launched in May 2012 [2]. The Unregulated Contaminant Monitoring Rule (UCMR) provides EPA and other interested parties with scientifically valid data on the occurrence of contaminants in drinking water. These data serve as a primary source of occurrence and exposure information that the agency uses to develop regulatory decisions. UCMR 3 monitoring will take place from 2013 to 2015, and includes monitoring for 28 chemicals and 2 viruses. Regulatory water agencies will be required to report concentrations for these contaminants in the near future. No pharmaceuticals are included in this recent list, only hormones. But in the meantime, a trend to try and detect as many compounds as possible in environmental water sources has become the challenge.

Pharmaceuticals found in water samples, due to human discharge (via direct or indirect sources), are by far the most extensive range of ECs reported to date. In the last 10 years, pharmaceuticals have been extensively detected in surface water in Europe [3–5] and in the United States [6]. The results of the reconnaissance by the U.S. Geological Survey showed

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that 80% of all surface water had detectable concentrations of pharmaceutical compounds. Approximately 82 compounds were detected, including steroids, antibiotics, analgesics, heart medications, and other compounds [6]. Typically the concentrations are in the sub-microgram-per-liter range.

It has been over a decade since the first U.S. survey of water samples [6] was carried out. This paper is still the most cited paper in the history of pharmaceuticals in water samples. Since then, many other papers and reviews have reported identifications of several of the ECs of concern. In general, there is a trend in the literature to only report and measure already known and published ECs. Only a few studies have reported newly identified and discovered pharmaceutical compounds and their transformation products (TPs) [7–9]. It is important to mention that sometimes TPs or metabolites exceed the concentrations of the parent compounds, becoming then more environmentally relevant than the starting active ingredients.

The majority of the pharmaceuticals identified in environmental samples have been detected using liquid chromatography-mass spectrometry (LC/MS). Most specifically, the advent of time-of-flight techniques (TOF) applied to environmental analyses has just begun in the last few years [10]. Applications range from routine analytical methods that analyze a few target compounds to more extensive methods that include a variety of analytes, including also non-target and unknown identification. Due to the high complexity of some environmental samples (i.e., wastewater, sludge samples, soil samples), high-resolution techniques with additional structural information on fragment ions are needed. These techniques provide a high degree of confidence for identification of target analytes and aid the structural elucidation of TPs and unknown compounds, which are usually present in environmental samples. The possibility of creating universal accurate mass databases with time-of-flight analyses for sets of compounds has broadened the range of applications as well, going from target to non-target identification.

This chapter gives an overview of the different tools used in LC/MS, with a specific focus on time-of-flight techniques, and the applications that have recently generated in the environmental field. This manuscript gives several examples of EC analysis that exemplify the unique features of time-of-flight for the identification of non-target and unknown compounds. LC/MS employing accurate mass measurement has been proved as a successful technique for both quantitative analysis of target compounds and rapid qualitative analysis of "unknown" environmental mixtures.

25.2 State-of-the-Art Techniques for the Identification of Emerging Contaminants and Their Transformation Products

There is no doubt that LC-MS has been the universal method of choice when analyzing ECs and TPs in environmental water samples for at least 20 years now. The most popular technique for identification and confirmation of pharmaceuticals is tandem mass spectrometry (LC/MS-MS) using either collision cells or linear traps to obtain information on fragment ions. This technique is more focused toward target analysis, where the analyst is looking at a specific group of analytes that may vary from a few analytes within a family (3–4) to large multiresidue methods (>100). However, sensitivity usually becomes an issue when targeting a large number of compounds. This is the reason why time-of-flight mass spectrometry techniques have become popular in the last few years, since they give full-spectra data at all times. A large number of compounds (virtually no limit) can be analyzed in a single run, while obtaining valuable accurate mass information for each compound that ionizes. Furthermore,

extra information on metabolites or TPs can be achieved by exploring the accurate mass spectra of unknown peaks in the chromatogram. In this section we will discuss both techniques of detection, a targeted approach using LC/MS-MS techniques and a non-targeted tactic for the discovery and identification of relevant compounds using LC/TOF-MS.

25.2.1 Liquid Chromatography/Tandem mass Spectrometry (LC/MS-MS) for the Analysis of Target Compounds. EPA Method 1694

LC/MS-MS using linear traps and triple quadrupoles seems to be the preferred method for routine analysis of pharmaceutical compounds in environmental samples. Overall, hundreds of papers have been published reporting findings of pharmaceuticals in non-treated and treated waters [11]. However, in spite of the numerous papers reporting analysis of pharmaceuticals, no analytical methodology seems to be the preferred one as a standardized methodology for these types of compounds until recently. EPA Method 1694 [12] was published in December 2007 as a guiding and screening method for those scientists analyzing pharmaceuticals in environmental samples. The standard EPA protocol uses solid-phase extraction (SPE) for water samples followed by the analysis of extracts by tandem mass spectrometry using a single transition for each compound, with retention time guidelines for identification.

We applied EPA Method 1694 in our lab for the analysis of pharmaceuticals in wastewater, surface water and drinking water samples [13]. The implementation for this method consisted of the analysis of 70 analytes (of 75 total analytes in the original method) and 18 labeled internal standards (of 20 total), which are a mixture of pharmaceuticals and personal care products that are analyzed by LC/MS-MS. In our work we addressed some of the analytical issues that were not covered in the original method, such as degradation of some compounds in solvent mixtures and assignment of a second transition for multiple reaction monitoring transitions (MRM) for additional mass spectrometry quality assurance. The main goal of this work was to show the usefulness of the EPA method for generic screening and monitoring of pharmaceuticals in water and wastewater.

The method was applied to the analysis of several drinking water, surface water and wastewater samples from several locations in Colorado, USA. Surprisingly, only 8 out of the 70 compounds were consistently found in environmental water samples: caffeine, carbamazepine, clarithromycin, diltiazem, diphenhydramine, erythromycin, sulfamethoxazole and trimethoprim, which were confirmed with two MRM transitions. The results for the concentrations found are shown in Table 25.1. These samples are representative of several inputs of wastewater contamination. One drinking water sample was also analyzed, and gave a positive hit for carbamazepine, a common antiepileptic and anti-depressant prescribed drug. Since then, we have refined our target methods using triple quadrupole mass spectrometry for a subset of 25–30 compounds that are regularly found in surface and wastewater samples. From these 30 compounds, usually 18–20 analytes are always found in surface and groundwater impacted by wastewater sources.

It is the view of the authors that confirmation of positive identifications in real samples requires the additional second MRM transition and the evaluation of ion ratios between the two monitored transitions as compared to a reference standard [14]. Confirmation of the identity of target analytes in real samples is usually based on ion ratio statistics for the transitions monitored. Thus, the confirmation criteria using tandem mass spectrometry cover a range of maximum permitted tolerances according to relative ion intensity, expressed as a percentage of the intensity of the most intense transition [13,14].

Table 25.1 *Analysis of representative surface water, wastewater and drinking water from different locations in Colorado showing concentrations for several pharmaceuticals. Samples were analyzed for all 70 pharmaceuticals; only 8 compounds were consistently found. Units: ng/L. Reproduced with permission from [13] Copyright (2010) Elsevier Ltd.*

Samples	Caffeine	Carbamazepine	Clarithromycin	Diltiazem	Diphenhydramine	Erythromycin	Sulfamethoxazole	Trimethoprim
Wastewater Location 1	12	5	10	n.d.	27	n.d.	30	45
Wastewater Location 2	n.d.	15	40	10	n.d.	21	15	5
Wastewater Location 3	n.d.	14	172	153	70	1200	53	429
Surface Water Location 4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Surface Water Location 5	n.d.	21	n.d.	n.d.	15	52	n.d.	n.d.
Surface Water Location 6	10	n.d.	n.d.	n.d.	n.d.	n.d.	5	n.d.
Surface Water Location 7	580	147	5	36	8	7	210	105
Drinking Water Location 8	n.d.	5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d. = not detected (below LOD).

25.2.2 Liquid Chromatography/Time-of-Flight/Mass Spectrometry (LC/TOF-MS) for the Analysis of Non-target Compounds

Recently, LC/TOF-MS has been used for the unequivocal confirmation of contaminants (including pharmaceuticals, pesticides and surfactants) in a variety of samples, such as water and sediments [10] by accurate mass measurement of protonated molecules. In this sense, several authors have reported accurate mass confirmation of pharmaceuticals in surface and wastewater samples [15–19] as well as sediment and sludge [20] using time-of-flight techniques. Detection of drugs in urine is also one of the topics that have been recently covered by LC/TOF-MS techniques [21–24]. In many of these studies time-of-flight techniques were successfully used for the unequivocal identification of TPs of known contaminants, as well as unknown compounds [25–28]. It is worth mentioning also several applications of time-of-flight mass analysis for the identification and confirmation of metabolites or TPs of pesticides and pharmaceuticals in environmental samples [29–39].

One of the main reasons that TOF has become so popular in the last few years is because accurate mass measurements are specific and universal for any kind of analyte and do not depend on the type, brand, or specific instrumentation used. The degree of fragmentation may vary depending on the instrument used, but the specific accurate mass value and/or accurate isotope information will be consistent for a given analyte, no matter what type of ionization, collision induced dissociation and MS-MS fragmentation is used. Accurate mass determination allows one to obtain unique information for a given molecule, plus additional information from isotopic patterns, mass defect and specific fragment ions [10].

Sometimes, a single stage time-of-flight mass analyzer (TOF/MS) generates valuable information by imparting enough energy into the $[M + H]^+$ ions in the source region to cause fragmentation [39]. Time-of-flight mass analysis generates increased resolving power of signals on the m/z axis in comparison to quadrupole mass spectrometers. Furthermore, this enhanced resolving power benefits analyses involving complex environmental matrices by separating isobaric interferences from the contaminant signals of interest. The improved resolution also facilitates the measurement of accurate masses within 3 ppm, which are accepted for the verification of elemental compositions. Elemental compositions of contaminants and their fragment ions clearly constitute higher order identifications than those obtained by nominal mass measurements.

25.2.3 Liquid Chromatography/Quadrupole-Time-of-Flight/Mass Spectrometry (LC/Q-TOF-MS) for Structural Elucidation of Unknown Compounds and Transformation Products

Most published methods only include information on the exact mass of the protonated or deprotonated molecule, a few report just one fragment ion per compound. To our knowledge, no studies also include accurate mass information of more than one fragment ion obtained by MS-MS for a large number of compounds (>80). Only recently, an extensive accurate mass library was developed and commercialized by Broecker *et al.* [40] for more than 2500 compounds. Another study by our group compiled information on a 100 pharmaceutical compounds, including detailed data on fragment ions obtained by a Q-TOF-MS instrument [41]. We also included a total of 16 different metabolites for the most environmentally relevant pharmaceuticals. Accurate mass information for each compound was obtained and compiled in an extensive table.

Accurate mass measurements of fragment ions become particularly important in the structure elucidation of non-targets and unknowns. In this sense, the Q-TOF MS/MS is unique

among TOF instruments in its ability to give accurate mass measurements (1 to 2 millimass units) of the fragment ions that are ejected from the collision chamber. This is very useful when trying to elucidate the identity of unknown or non-target compounds, the more fragment accurate mass information one can get from time-of-flight mass techniques the better understanding for the structural elucidation of a certain compound. The same reasoning applies to the elucidation of possible TPs. When knowing what the starting compound is, the information about fragment ions and their accurate masses will play an important role in deciphering the chemical structure of the metabolite or TP.

Another important tool that has made TOF one of the key methodologies for identification of compounds is the existence of accurate mass databases, as published extensively. An individual scientist can apply these universal databases to each particular problem and then often get an identification of the analyte of interest [42–44]. Other tools, that are available with TOF instrumentation, and will be discussed in this chapter, include the use of molecular features, accurate mass filters and the isotopic mass defect, and the use of mass profiling to distinguish between control samples and positive samples. Examples will be given for each one of these accurate mass tools.

25.3 Use of Accurate Mass Tools for the Identification of Emerging Contaminants

25.3.1 Molecular Features

For many years, the use of reverse-search methods for gas chromatography/mass spectrometry (GC/MS) has made it possible to search large National Institute for Standards and Testing (NIST) pesticide libraries in minutes [45] and has made screening quite simple for pesticides amenable to GC/MS. Unfortunately similar reverse-search methods have not been available for LC/MS for two reasons. First, the single quadrupole and triple quadrupole mass spectrometers do not operate in full scan mode for pesticide screening because of a lack of sensitivity [46]. Secondly, although libraries for LC/MS three-dimensional ion trap have been made, they have not been popular due to difficulties in reproducibility of fragmentation and the need for authentic standard analysis for each instrument [47–49]. So, the only approach that uses full spectrum information is LC/TOF-MS, which is both sensitive and accurate [50], but uses only the accurate mass of the $[M + H]^+$ ion. The combination of accurate mass and sensitivity is needed for screening of compounds by their empirical formula.

The MFE software compiles accurate mass ions, excludes background noise, and plots extracted ion chromatograms of the most intense peaks found in a chromatogram. So a molecular feature is defined as a discrete molecular entity defined by combination of retention time, mass and response in an LC/MS analysis. In general, MFE operates on raw mass spectral data generating lists of chemically qualified molecular features (background is removed, interferences are resolved, isotopic cluster and molecular adducts are recognized). The screening criteria usually consist of ± 5 ppm accurate mass window, ± 0.2 min retention time window, and a minimum 1000 counts (signal to noise of $\sim$10:1). The ions are grouped by entities that include common adducts (sodium, ammonia, etc.) and isotope clusters.

As an example, Figure 25.1 shows the total ion current chromatogram (a) and the molecular feature extraction (b) for a surface water sample taken in the South Platte River (Colorado, USA). As can be seen in this figure, a total of 1498 ion features were found in the chromatogram. One can generate as many empirical formulae as wanted and from there one

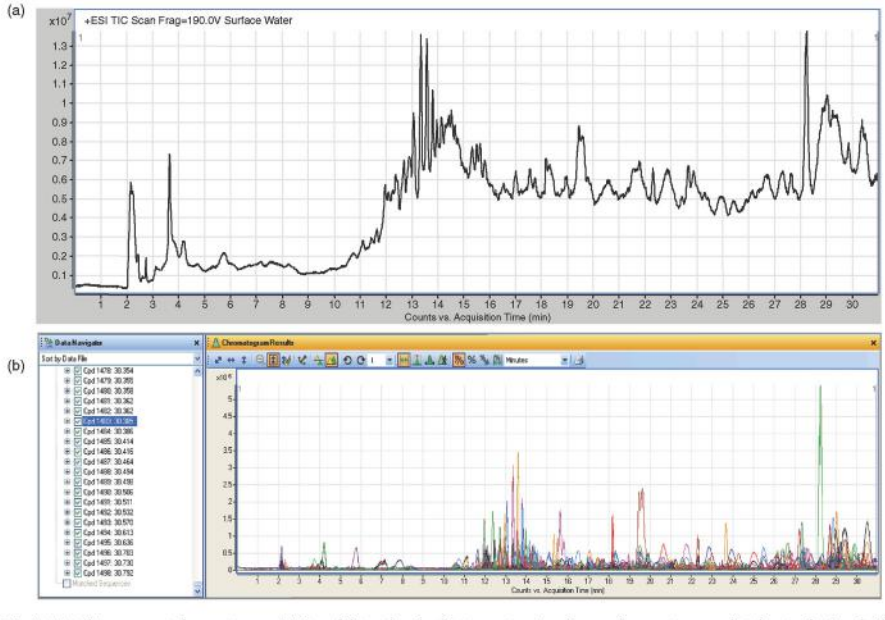


Figure 25.1 *(a) Total ion current chromatogram (TIC) and (b) molecular feature extraction for a surface water sample taken in the South Platte River (Colorado, USA).*

can try to elucidate the chemical structure. But, the most common approach is to compare the data obtained to a known database to try to match as many compounds as possible. This approach is explained in the next section.

Strengths of the MFE include rapid screening of 100 compounds at sensitive levels compared to a manual approach and the ease of use of the database for any accurate mass spectrometer instrumentation capable of routine sub 5-ppm mass accuracy.

25.3.2 Accurate Mass Databases

The pioneer efforts to search data using an accurate mass database were made by several authors, such as Thurman *et al.* [28], Bodeldijk *et al.* [51], Ojanpera *et al.* [24]. For example, Thurman *et al.* [28] used an approach of TOF, ion trap, and the Merck Index database to identify pesticides in food and also TPs, without the initial use of primary standards [29]. Bobeldijk *et al.* also used the Merck Index, the NIST library, and their own database to screen water pollutants [51]. The methods in these examples rely on manually searching the databases, compound by compound. Recently, several papers [24,52] have extended this approach, using mass accuracy of 30 ppm and database analysis to identify ~600 drugs in blood and urine without the use of primary standards, using only the protonated molecule.

In spite of the progress that has been made, the ability to do true library analysis is still a problem to be solved for LC/MS and for rapid analysis of environmental samples. The problems to be overcome include reproducible spectra and ion ratios, routine programs for rapid screening of samples rather than manual checking of data, and some estimate of the probability of the correct identification. Variation in fragmentation intensity is not critical with the use of accurate mass, since the accurate mass of the fragment ion gives its molecular formula. In fact, accurate mass measurements are specific and universal for every target analyte, regardless of the instrumentation used. Usually, unambiguous identification is accomplished by means of accurate mass measurements from (de)protonated molecules, fragment ions, and isotope intensity/signature matching. Thus, the accurate mass database approach is a screening tool, and it is powerful and fast because only the molecular formula is needed.

Here we will describe two different approaches to the use of databases. The first uses an automated molecular feature database, as described in the last section, and then a commercial or homemade database based on a csv file. The second approach is called "reversed database search" in which a total ion chromatogram is searched for ions included in such a database. Databases usually contain information of the monoisotopic exact mass of the MH^+, at least one product ion, and retention time of the compound. The advantages and limitations of both approaches are discussed, as well as the reliability (match probability) of a database search using accurate mass.

Following the first approach (as described in the last section) a compilation of ions was gathered through a molecular feature extraction (see Figure 25.1) for one of the surface water samples collected in the South Platte River. The next step was to match these accurate masses with any compounds included in a commercial database (called "forensics database" from Agilent Inc.). As shown in Figure 25.2, one of the hits obtained was for gabapentin, a common anti-epileptic pharmaceutical. In the insert, a detailed mass spectrum is plotted. Each one of the fragments was elucidated from the parent chemical structure (see Figure 25.3). The fact that this molecule gave such a rich CID spectrum allowed the detailed fragmentation elucidation by using accurate mass, as seen in Figure 25.3. The protonated molecule loses water, followed by ammonia, and then an additional water loss occurs, followed by a loss of C_4H_4 to give the smallest fragment. This example shows how the use of

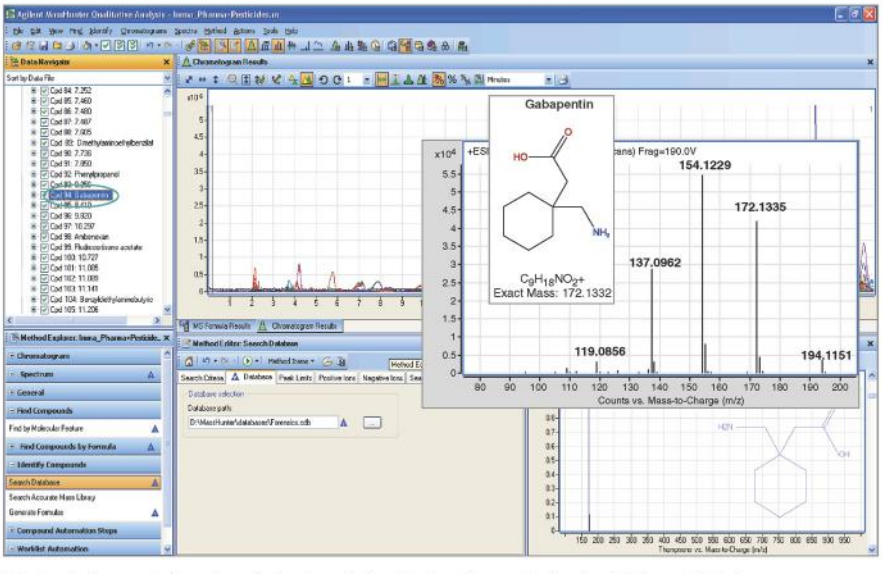


Figure 25.2 *Results from applying a forensic database (Agilent Inc.) to the sample described in Figure 25.1. Inset spectrum corresponds to gabapentin.*

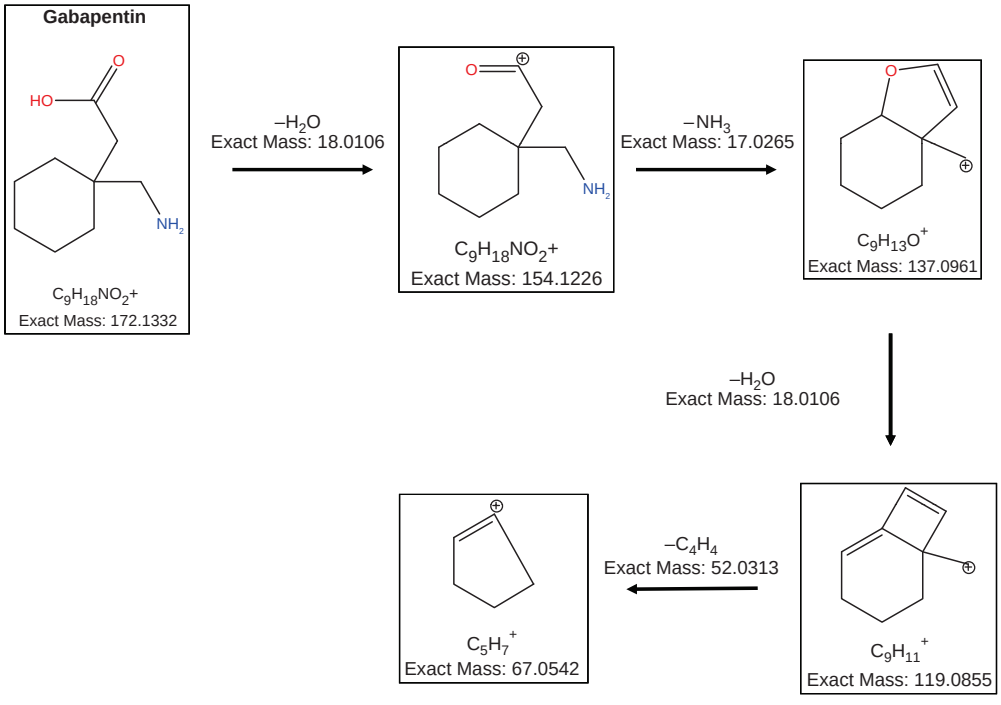


Figure 25.3 *Fragmentation pathway for gabapentin.*

accurate fragmentation information can be used as a complementary set of data to provide unambiguous confirmation of the new finding. This is an example of a successful hit using this approach as, when this experiment was carried out, no commercial standard was available at our lab. Later, we purchased the standard and confirmed, both by retention time and accurate mass, as well as fragmentation pattern, the accuracy of the finding.

The second approach, also known as "reversed database search," consists of running the database to see if any positive hits are found. This approach is usually slower than the last one as each one of the database entries has to be verified against each of the extracted ion chromatograms for a given accurate mass. This automatic screening method requires a thorough full optimization of the accurate-mass window used and retention time (always optional) tolerances, which play an important role in the selectivity, accuracy, and successfulness of the whole procedure. Using this approach, and by running the same database as mentioned before, we verified the presence of one of the metabolites of dextromethorphan, also known as dextrorphan, in a surface water sample impacted by a wastewater source.

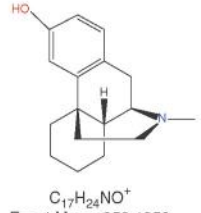
Figure 25.4 depicts an excerpt of the automated generated report of a database search. It is important to note the high score obtained for this particular hit. This score is a combination of mass accuracy, isotope intensity and isotope matching. Also, as shown in the figure a good mass accuracy (with an error below 2 ppm) was obtained for this identification, thus confirming the presence of this compound in the sample. Again, in this case, no standard had been analyzed by this instrument when this finding was made, so a pure standard was purchased, analyzed, and we verified this positive identification in a water sample.

Sample Type	Sample	P1-C2
Instrument Name	Instrument 1	
Acq Method	Multiresidue_Pesticide s.m	1/20/2010 12:12:15 PM
IRM Calibration Status	Success	Forensic_search_1.m
Comment		

Compound Table

Label	Tgt Name	Tgt Score	Mass Error (ppm)	Tgt Formula	Obs. RT	Ref. Mass	Obs. Mass
Cpd 313: 5-Butyl-5-phenyl-hydantoin	5-Butyl-5-phenyl-hydantoin	82.57	-1.47	C13H16N2O2	13.428	232.1212	232.1208
Cpd 317: Oxazidione	Oxazidione	47.19	-1.12	C20H19NO3	13.475	321.1365	321.1361
Cpd 316: Pyriproxifen	Pyriproxifen	47.19	-1.12	C20H19NO3	13.475	321.1365	321.1361
Cpd 320: Dextrorphan (Levorphanol)	Dextrorphan (Levorphanol)	96.73	1.59	C17H23NO	13.487	257.178	257.1784
Cpd 319: Pirandamine	Pirandamine	96.73	1.59	C17H23NO	13.487	257.178	257.1784
Cpd 318: Racemorphan	Racemorphan	96.73	1.59	C17H23NO	13.487	257.178	257.1784
Cpd 321: Vinblastin	Vinblastin	68.85	-0.57	C46H58N4O9	13.546	810.4204	810.4199
Cpd 323: Chlormadinone	Chlormadinone	46.35	1.83	C21H27ClO3	13.569	362.1649	362.1655
Cpd 322: Cismadinone	Cismadinone	46.35	1.83	C21H27ClO3	13.569	362.1649	362.1655
Cpd 326: Pipratecol	Pipratecol	47.2	-1.08	C19H24N2O4	13.581	344.1736	344.1732
Cpd 325: Formoterol	Formoterol	47.2	-1.08	C19H24N2O4	13.581	344.1736	344.1732
Cpd 324: Tolamolol		47.2	-1.08	C19H24N2O4	13.581	344.1736	344.1732

Dextromethorphan Metabolite (Dextrorphan)



$C_{17}H_{24}NO^+$
Exact Mass: 258.1852

Figure 25.4 *Example of a generated automated report with Mass Hunter using a forensic database.*

25.3.3 Accurate Mass Filters and Isotopic Mass Defect

Chlorine appears in many pesticides and pharmaceutical products that are important to environmental analysis. Because chlorine contains two isotopes, Cl^{35} and Cl^{37}, there is a distinctive A + 2 isotope pattern that is generated by a single chlorine atom in a molecule. Furthermore, there is an isotopic mass defect that occurs with chlorine-37 that makes the identification of chlorine in a molecule relatively easy [53]. More than one chlorine atom in a molecule generates an A + 2 and A + 4 isotopic pattern, which is characteristic and commonly shown in all mass spectrometry books as a key to compound identification of chlorinated compounds [54]. Using this rule, a chlorine mass filter was developed by our group [55]. The chlorine mass-filter is used to screen both LC/TOF-MS and LC/QTOF-MS data files in order to discover compounds that contain chlorine. The chlorine filter uses MassHunter software to generate formulae for chlorine-containing compounds.

An example is given for a wastewater sample. The initial identification of lamotrigine, an anti-depressant pharmaceutical not previously reported in water samples, was accomplished using the mass-defect filter that looked for chlorinated analytes in the extract of a wastewater sample after LC/TOF-MS analysis in MS-only mode. The mass defect filter essentially looks at the accurate mass of the monoisotopic mass of an analyte and the A + 2 isotopic mass. Both the intensity and the accurate mass are used to detect chlorinated compounds using the mass defect filter. In the case of lamotrigine, the mass defect filter detected a peak at 13.7 min with a mass of *m/z* 256.0153 and an A + 2 isotope with a mass of *m/z* 258.0122 and an intensity of 66% (see Figure 25.5). The mass defect filter showed that the A + 2 peak had a relative isotopic mass defect of −0.0030 u, indicating a chlorinated compound with two chlorine atoms [29,56]. The second step after the mass defect filter was to determine the molecular formula of the unknown chlorinated compound. The best fit for the ion formula was $C_9H_8Cl_2N_5$ with a match of 99 out of 100 based on MassHunter Software, which evaluates the accurate mass of the A ion, the isotope intensity matching, and isotope spacing (also called the isotopic mass defect) or accurate mass of the isotopes. The neutral formula, $C_9H_7Cl_2N_5$, was then run through the Merck Index database for a formula match and gave lamotrigine as its only formula. When the formula was put through a much larger database, ChemSpider, the match was for 65 compounds; however, there were only 13 patented structures and only 1 compound was listed in a Wikipedia-available article and that was lamotrigine. A quick read showed that this compound is the number three most used bipolar medication in the US at this time; thus, it was given the most likelihood of a correct identification. Later, a standard was purchased and the identification was verified [7].

Also, a metabolite of lamotrigine was discovered using the same procedure as described above. A second peak of much less intensity and earlier retention time (9.9 min) had been detected in the 256 *m/z* extracted ion chromatograms of several wastewater samples containing lamotrigine. The spectrum of this peak revealed a much larger ion at 432.0472 *m/z*, thus an MS-MS experiment was carried out to confirm that the 256 ion formed indeed from the 432 ion. A literature search for the empirical formula $C_{15}H_{15}Cl_2N_5O_6$ (at 432.0472 *m/z*) revealed that this was a potential glucuronide metabolite of lamotrigine [57,58]. The finding was verified by analyzing a pure standard of 2-*N*-glucuronide lamotrigine. A second MS-MS experiment was performed to fragment the ion at *m/z* 256, hence simulating a pseudo MS^3 experiment, and a spectrum that matched that of lamotrigine was obtained, thus totally confirming the identification of the 2-*N*-glucuronide metabolite (see Figure 25.6).

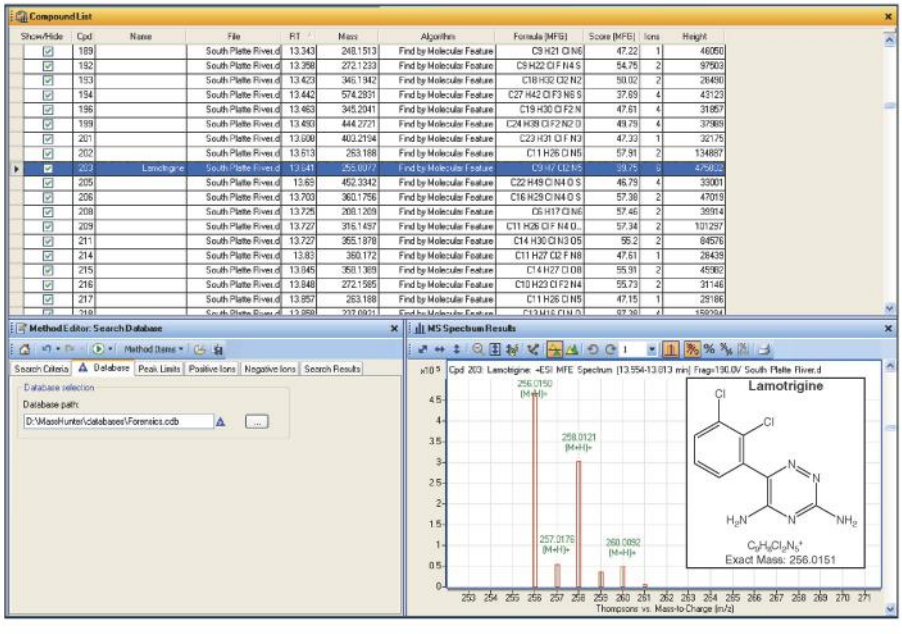


Figure 25.5 *Results for a chlorine mass filter used for identification of chlorinated species. In the insert the accurate mass spectrum for lamotrigine is shown.*

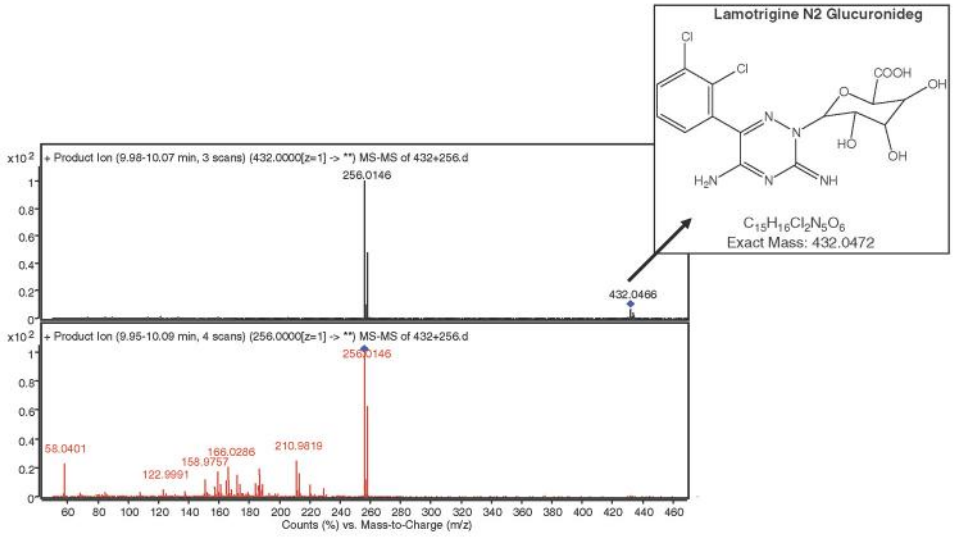


Figure 25.6 *MS-MS accurate mass spectrum for the N2-glucuronide metabolite of lamotrigine.*

The combination of mass accuracy, database matching, and identifying a fragment ion shows the power of using the chlorine mass-filter to find and identify trace chlorinated substituents in water samples impacted by wastewater. This approach works really well for complex water matrices by identifying specific chlorinated compounds, which in turn could be potential metabolites from known target analytes.

25.3.4 Accurate Mass Profiling

Urine metabolic profiling combined with LC/QTOF-MS was used to find and identify the metabolites of dextromethorphan, a common over-the-counter (OTC) cough suppressant [8]. Chromatograms of both blank urine and urine taken 4 h after ingestion of dextromethorphan were compared using Mass Profiler software. The software first analyzes all groups of ions (known as features) in the chromatogram of both samples and compiles this into a database. Three replicates of each sample are taken and averaged. Next the software compares the two samples, looking for features (plotted as gray dots) that are unique to the dextromethorphan urine (Figure 25.7a). The

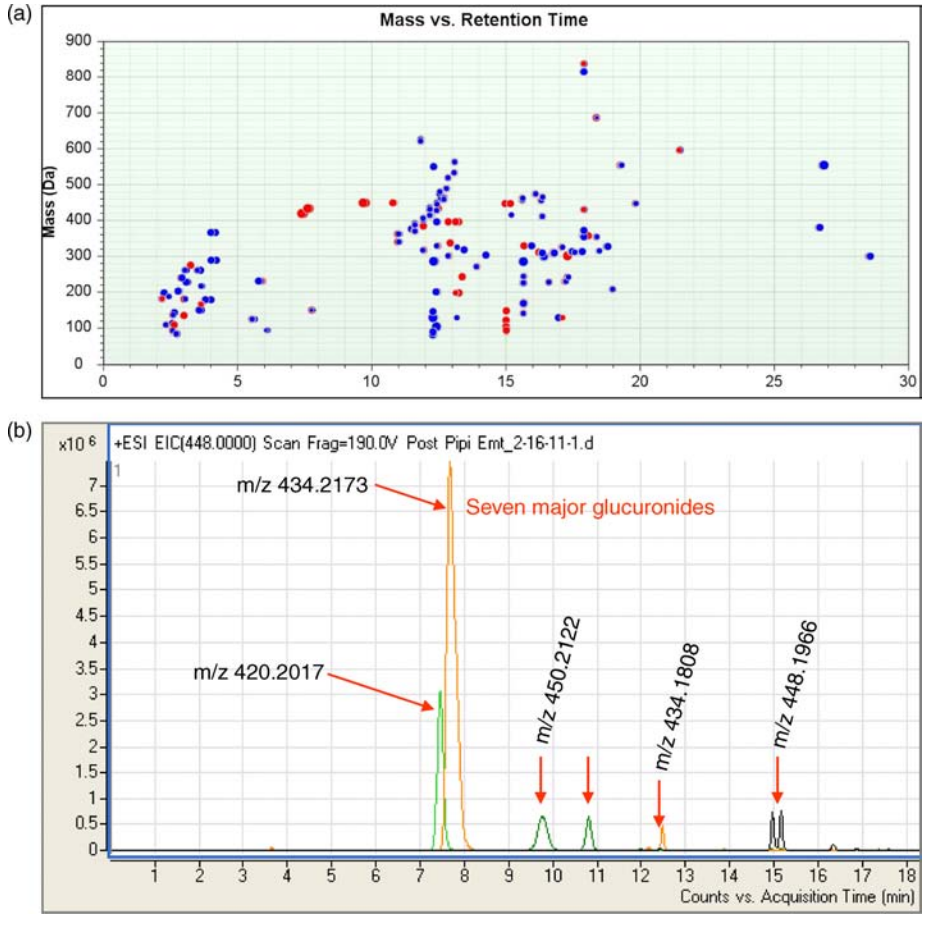
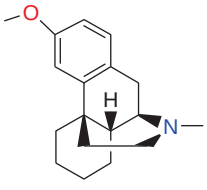
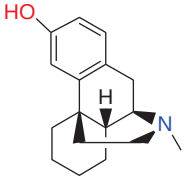
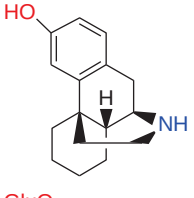
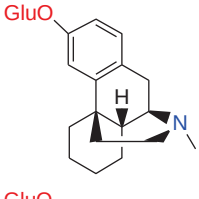
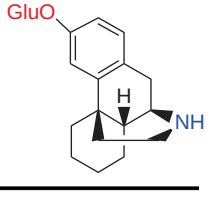


Figure 25.7 *(a) Mass profiler plot of a urine sample 4 h after taking a 10 mg dose of dextromethorphan. (b) Extracted ion chromatograms of seven major glucuronide metabolites.*

Table 25.2 *Compounds, formulae, exact mass, measured mass, error, and structures of dextromethorphan and its metabolites. Reproduced with permission from [8] Copyright (2012) Elsevier Ltd.*

Compound Name	Formulae (MH^+)	Exact mass MH^+ (*m/z*)	Measured mass MH^+ (*m/z*)	Error (ppm)	Structure of the compound
Dextromethorphan	$C_{18}H_{26}NO$	272.2009	272.2010	0.4	
Dextrorphan	$C_{17}H_{24}NO$	258.1852	258.1854	0.8	
N-demethyldextrorphan	$C_{16}H_{22}NO$	244.1696	244.1699	1.2	
Dextrorphan Glucuronide	$C_{23}H_{32}NO_7$	434.2173	434.2175	0.5	
N-demethyldextrorphan Glucuronide	$C_{22}H_{30}NO_7$	420.2017	420.2016	0.2	

comparison resulted in 27 features that were unique to this sample, and 136 individual ions. Ions at the same retention time, for example, 15.1 min, were the same fragment ions of a feature, based on MS/MS analysis discussed in [8]. Figure 25.7b shows a chromatogram with 7 major metabolites identified.

Table 25.2 shows the structure and accurate masses of dextromethorphan and its four major metabolites, which are reported in the pharmaceutical literature [59–62]. The metabolites are dextrorphan and *N*-demethyldextrorphan and glucuronides of each of these two compounds. The calculated exact masses for each of these compounds were extracted from

the total ion chromatogram of the positive urine sample and compared to the measured masses (data shown in Table 25.2). The measured masses for the protonated molecule of each compound varied from 0.1 to 0.3 mmu, which is 1 ppm mass accuracy or less for all targeted compounds.

25.4 Occurrence of Transformation Products in Environmental Waters in the US

Our lab has analyzed several hundred samples for pharmaceuticals in the last 2–3 years from different locations in the US and comprising different types of water samples (drinking, groundwater, surface water, lake water and wastewater) by LC/TOF-MS and LC/Q-TOF-MS. The majority of drinking and groundwater samples contain few or no pharmaceuticals, with the exceptions of those cases where groundwater comes from production wells along main rivers or reservoirs. The main detections of pharmaceuticals usually occur in surface water samples and wastewater samples. Interestingly, from all the pharmaceuticals analyzed, the same set of compounds occurs in the majority of water samples [41]. Table 25.3 shows the most representative data from surface water samples collected during 2011 in the U.S.

After analyzing a large number of samples we have come up with some findings (new compounds detected and new metabolites) that are worth mentioning here and this is why these compounds were included in previous data sets [41]. Identities of compounds were based on retention time and accurate mass of the protonated/deprotonated molecules and their fragment ions. MS-MS acquisition was performed on those cases where a new compound or metabolite was discovered. For example, a new finding was the anti-convulsant (also used as anti-depressant) lamotrigine and its *N*2-glucuronide found in wastewater, surface water and even groundwater samples [20]. To date no other environmental reports of this pharmaceutical and/or metabolite have been reported in the literature. This compound is frequently detected in water samples (see Table 25.3) and at high concentrations, suggesting that it is replacing the "older" anti-convulsant drugs (carbamazepine, citalopram, fluoxetine, etc.) prescribed for human intake. Other findings include metabolites of already well-known drugs, such as bupropion, carbamazepine and venlafaxine, to mention a few. These are important findings as the metabolite concentrations often exceed the parent compound concentration. Figure 25.8 shows an example of a common detected drug (metoprolol) and its newly identified acid metabolite in a surface water sample. The MS-MS experiments at 30 V revealed the most important fragments of this metabolite (as shown in the inset spectrum).

Finally, we have summarized the most important findings in Table 25.3, which represents the study of a sub-selected and representative set of approximately 100 surface water samples (downstream of effluent discharge) analyzed by LC/Q-TOF-MS. The percent of detections for each compound found in the water samples is depicted by a percentile number. From this table we can conclude that about 36 different pharmaceuticals are commonly detected in waters impacted by wastewater sources. Some of the pharmaceuticals not included in this table have never been detected, or detections were lower than 10%. Compounds such as carbamazepine, bupropion, lamotrigine, diphenhydramine, gemfibrozil, metoprolol, propanolol, sulfamethoxazole, thiabendazole, trimethoprim, venlafaxine, and their respective metabolites, are the most common pharmaceuticals detected in water

Table 25.3 *Detections (%) of the most commonly found pharmaceuticals in water samples (surface water downstream from effluent discharge). Reproduced with permission from [41] Copyright (2012) Elsevier Ltd.*

Compound	Detections in water samples (%)	Average concentration (ng/L)
1,7-Dimethylxanthine	10	110
10,11-Dihydroxy-carbamazepine	45	80
10-Hydroxy-carbamazepine	85	255
Atenolol	74	166
Bupropion	68	140
Caffeine	70	220
Carbamazepine	95	350
Cetirizine	82	70
Citalopram	79	85
Clarithromycin	75	46
Cotinine	22	40
Demethyl-dextrorphan	65	10
Des-venlafaxine	78	84
Dextrorphan	75	50
Diltiazem	69	47
Diphenhydramine	80	57
Erythrohydrobupropion	78	180
Erythromycin	55	137
Erythromycin Anhydrate	35	62
Fluoxetine	25	65
Gabapentin	44	54
Gemfibrozil	74	95
Hydroxy-bupropion	75	150
Ibuprofen	20	21
Lamotrigine	97	455
Metoprolol	91	237
Metoprolol acid	85	74
2*N*-glucuronide lamotrigine	68	95
Naproxen	64	22
Nor-citalopram	66	74
Propanolol	88	53
Sulfamethoxazole	95	320
Thiabendazole	75	188
Triclocarban	64	96
Trimethoprim	76	264
Venlafaxine	78	310

samples. Also shown in this table is the average concentration found for each one of the compounds analyzed. The highest concentrations were those corresponding to the detections of anti-convulsants, anti-depressants, psychiatric drugs and beta-blockers in water samples. More environmental studies would be needed to understand the fate and transport of these type of pharmaceuticals in the environment, especially those of new appearance.

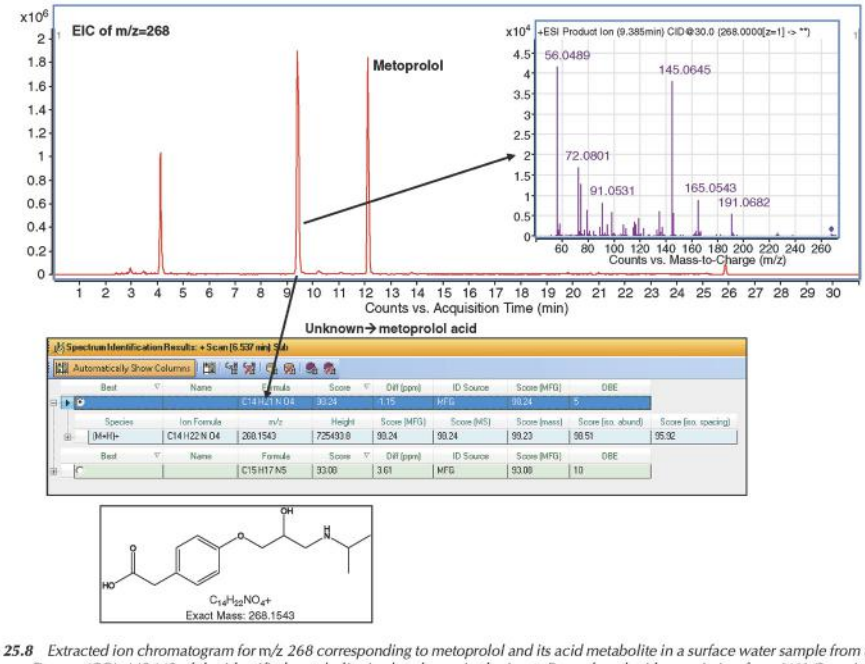


Figure 25.8 *Extracted ion chromatogram for m/z 268 corresponding to metoprolol and its acid metabolite in a surface water sample from the Platte River near Denver (CO). MS-MS of the identified metabolite is also shown in the inset. Reproduced with permission from [41] Copyright (2012) Elsevier Ltd.*

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Analysis of 70 Environmental Protection Agency priority pharmaceuticals in water by EPA Method 1694

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ABSTRACT

The U.S. Environmental Protection Agency (EPA) Method 1694 for the determination of pharmaceuticals in water recently brought a new challenge for treatment utilities, where pharmaceuticals have been reported in the drinking water of 41-million Americans. This proposed methodology, designed to address this important issue, consists of solid-phase extraction (SPE) followed by liquid chromatography–mass spectrometry (LC/MS–MS) using triple quadrupole. Under the guidelines of Method 1694, a multi-residue method was developed, validated, and applied to wastewater, surface water and drinking water samples for the analysis of 70 pharmaceuticals. Four distinct chromatographic gradients and LC conditions were used according to the polarity and extraction of the different pharmaceuticals. Positive and negative ion electrospray were used with two MRM transitions (a quantifier and a qualifier ion for each compound), which adds extra confirmation not included in the original Method 1694. Finally, we verify, for the first time, EPA Method 1694 on water samples collected in several locations in Colorado, where positive identifications for several pharmaceuticals were found. This study is a valuable indicator of the potential of LC/MS–MS for routine quantitative multi-residue analysis of pharmaceuticals in drinking water and wastewater samples and will make monitoring studies much easier to develop for water utilities across the US, who are currently seeking guidance on analytical methods for pharmaceuticals in their water supplies.

1. Introduction

The analytical challenge of measuring emerging contaminants in the environment has been a major research focus of scientists for the last 20 years. Water quality is a critical issue especially for sustainable socioeconomic development. Anthropogenic activities are one of the main causes for water quality damage and, consequently, social concern calls for quality control action. Even after water treatment, it has been demonstrated in many studies that organic contaminants escape conventional wastewater treatment processes and they end up in aquatic systems. Pharmaceuticals and personal care products are an important group of contaminants that have been targeted, especially in the last decade [1–5]. There are several methods addressing the analysis of these analytes, mainly using tandem mass spectrometry techniques [6–16]. In this sense, LC/MS–MS using ion-trap and triple quadrupole seems to be the preferred method for routine analysis of pharmaceutical compounds in environmental samples. Some other reports include the use of accurate mass techniques such as liquid chromatography–time of flight-mass spectrometry (LC/TOF-MS) for the analysis of these types of compounds and related degradation products [11,17,18].

By reviewing the papers in the literature one can easily list the pros and cons of different methodologies and applications for diverse families of pharmaceutical compounds. Overall, hundreds of papers have been published reporting findings of pharmaceuticals in non-treated and treated waters [12]. However, in spite of the numerous papers reported for analysis of pharmaceuticals, no analytical methodology seems to be the preferred one as a standardized methodology for these types of compounds until recently. EPA Method 1694 [1] was published in December 2007 as a guiding and screening method for those scientists analyzing pharmaceuticals in environmental samples. The standard EPA protocol uses solid-phase extraction (SPE) for water samples followed by the analysis of extracts by tandem mass spectrometry using a single transition for each compound, with retention time guidelines for identification. The EPA method is under review at this time by the general scientific public for use by water utilities.

We have applied EPA Method 1694 in our lab for the analysis of pharmaceuticals in wastewater, surface water and drinking water samples. The implementation for this method consists of the analysis of 70 analytes (of 75 total analytes in the original method) and

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Table 1
EPA Pharmaceutical compounds studied in this work.

Group 1 (Positive ESI)		
Acetaminophen	1,7-Dimethylxanthine	Roxithromycin
Ampicillin	Diphenhydramine	Sarafloxacin
Azithromycin	Enrofloxacin	Sulfachloropyridazine
Caffeine	Erythromycin	Sulfadiazine
Carbadox	Erythromycin anhydrate	Sulfadimethoxine
Carbamazepine	Flumequine	Sulfamerazine
Cefotaxime	Fluoxetine	Sulfamethazine
Ciprofloxacin	Lincomycin	Sulfamethizole
Clarithromycin	Lomefloxacin	Sulfamethoxazole
Cloxacillin	Miconazole	Sulfanilamide
Codeine	Norfloxacin	Sulfathiazole
Cotinine	Ofloxacin	Thiabendazole
Dehydronifedipine	Oxacillin	Trimethoprim
Digoxigenin	Oxolinic acid	Tylosin
Digoxin	Penicillin G	Virginiamycin
Diltiazem	Penicillin V	

Group 2 (Positive ESI)
Anhydrochlorotetracycline
Anhydrotetracycline
Chlorotetracycline
Demeclocycline
Doxycycline
4-Epianhydrochlorotetracycline (EACTC)
4-Epianhydrotetracycline (EATC)
4-Epichlortetracycline (ECTC)
4-Epioxytetracycline (EOTC)
4-Epitetracycline (ETC)
Isochlortetracycline
Meclocycline
Minocycline
Tetracycline (TC)

Group 3 (Negative ESI)
Gemfibrozil
Ibuprofen
Naproxen
Triclocarban
Triclosan
Warfarin

Group 4 (Positive ESI)
Albuterol
Cimetidine
Metformin
Ranitidine

Labeled standards		
$^{13}C_2$-^{15}N-Acetaminophen	$^{13}C_2$-Erythromycin	$^{13}C_3$-Ibuprofen
$^{13}C_3$-Atrazine	Fluoxetine-d_6	^{13}C-Naproxen-d_3
$^{13}C_3$-Caffeine	$^{13}C_6$-Sulfamethazine	$^{13}C_6$-Triclocarban
Carbamazepine-d_{10}	$^{13}C_6$-Sulfamethoxazole	$^{13}C_{12}$-Triclosan
$^{13}C_3$-^{15}N-Ciprofloxacin	$^{13}C_3$-Trimethoprim	Warfarin-d_5
Cotinine-d_3	Gemfibrozil-d_6	$^{13}C_6$-2,4,5-Trichlorophenoxyacetic acid

18 labeled internal standards (of 20 total), which are a mixture of pharmaceuticals and personal care products that are analyzed by LC/MS–MS. Table 1 shows the list of pharmaceuticals studied here (note that some compounds and internal standards in EPA method could not be commercially obtained at this time). In our paper we address some of the analytical issues that were not covered in the original method, such as degradation of some compounds in solvent mixtures and assignment of a second transition for multiple reaction monitoring transitions for additional mass spectrometry quality assurance.

The main goal of this work was to improve and polish the EPA Method 1694 by (i) addressing some issues related with mass spectrometric ions and transitions assigned to some compounds in the original method, (ii) providing additional confirmation, by adding a second MRM transition for 66 of the 70 analytes analyzed, and (iii) providing an approach that simplifies sample treatment, chromatography, and solid-phase extraction for the targeted pharmaceuticals in environmental water samples. The general goal is to remove some complexity of the analysis and simplify without losing on data quality and, ultimately, show the usefulness of the EPA method for generic screening and monitoring of pharmaceuticals in water and wastewater.

2. Experimental

2.1. Chemicals and reagents

Pharmaceutical analytical standards were purchased from Sigma–Aldrich (St. Louis, MO, USA), Cole Parmer (Vernon Hills, IL, USA) and Cerilliant (Round Rock, TX, USA). Labeled internal standards were obtained from Cambridge Isotope Laboratories (Andover, MA, USA). Individual pharmaceutical stock solutions (approximately 1000 μg/mL) were prepared in pure acetonitrile or methanol depending on the solubility of each individual compound, and stored at −18 °C. From these solutions, working standard solutions were prepared by dilution with acetonitrile and water. HPLC-grade acetonitrile and methanol were obtained from Burdick and Jackson (Muskegon, MI, USA). Formic acid, ammonium acetate and acetic acid were obtained from Sigma–Aldrich (St. Louis, MO, USA). A Milli-Q-Plus ultra-pure water system from Millipore (Milford, MA, USA) was used throughout the study to obtain the HPLC-grade water used during the analyses.

2.2. Sample collection and preparation

Drinking water samples were taken from the tap at the Center for Environmental Mass Spectrometry (Boulder, CO). Wastewater samples were collected from several wastewater treatment plants in Denver, Boulder and Estes Park (CO, USA). Surface water samples were collected from several locations including rivers and reservoirs in Colorado. Water samples were extracted with Oasis HLB cartridges (500 mg, 6 mL) obtained from Waters (Milford, MA, USA). SPE cartridges were conditioned with 4 mL of methanol followed by 6 mL of HPLC-grade water. A volume of 200 mL of water sample was pre-concentrated through the cartridge, afterwards the cartridges were air dried for 3 min and finally analytes were eluted with 5 mL of methanol. The sample extracts were evaporated to a final volume of 0.5 mL. "Blank" surface water extracts were used to prepare the matrix-matched standards for validation purposes. The water extracts were spiked with the mix of pharmaceuticals at different concentrations (ranging from 0.01 to 100 ng/mL or ppb) and subsequently analyzed by LC/MS–MS.

2.3. LC/MS–MS instrumentation

The separation of the analytes was carried out using an HPLC system (consisting of vacuum degasser, autosampler and a binary pump) (Agilent Series 1290, Agilent Technologies, Santa Clara, CA, USA) equipped with a reversed phase C_{18} analytical column of 100 mm × 2.1 mm and 3.5 μm particle size (Agilent Zorbax Eclipse Plus) and a HILIC (Hydrophilic Interaction Chromatography) analytical column of 100 mm × 2.1 mm and 3.5 μm particle size (Agilent Zorbax HILIC Plus). This HPLC system was connected to a triple quadrupole mass spectrometer Model 6460 Agilent (Agilent Technologies, Santa Clara, CA, USA) equipped with electrospray Jet Stream technology operating in positive and negative ion mode, using the following operation parameters: capillary voltage: 4000 V; nebulizer pressure: 35 psig; drying gas: 8 L/min; gas

Table 2
Chromatographic conditions for the separation of the four groups of compounds from EPA Method 1694.

LC Conditions	Group 1	Group 2	Group 3	Group 4
Column	Agilent Zorbax Eclipse Plus C_{18}, 100 mm × 2.1 mm (3.5 μm)	Agilent Zorbax Eclipse Plus C_{18}, 100 mm × 2.1 mm (3.5 μm)	Agilent Zorbax Eclipse Plus C_{18}, 100 mm × 2.1 mm (3.5 μm)	Agilent Zorbax Rx-SiL, 100 mm × 2.1 mm (3.5 μm)
Mobile phase	10% ACN 90% H_2O with 0.1% HCOOH	10% ACN 90% H_2O with 0.1% HCOOH	40% MeOH and ACN (1:1) 60% H_2O with 0.1% ammonium acetate + 0.1% acetic acid	98% ACN 10% H_2O with 10 mM ammonium acetate, pH 6.7
Flow-rate	0.2–0.3 mL/min	0.2 mL/min	0.2 mL/min	0.25 mL/min
Gradient	t_0 = 10% ACN, 0.2 mL/min t_5 = 10% ACN, 0.2 mL/min t_6 = 10% ACN, 0.3 mL/min t_{24} = 60% ACN, 0.3 mL/min t_{30} = 100% ACN	t_0 = 10% ACN t_{10} = 10% ACN t_{30} = 100% ACN	t_0 = 40% ACN/MeOH $t_{12.5}$ = 100% ACN/MeOH	t_0 = 98% ACN t_5 = 98% ACN t_{12} = 70% ACN
Column temp.	25 °C	25 °C	25 °C	25 °C
Injection vol.	15 μL	15 μL	15 μL	15 μL

temperature: 250 °C; sheath gas flow: 10 L/min; sheath gas temperature: 300 °C; nozzle voltage: 0 V in positive and 1500 V in negative ion mode; dwell time: 10 ms. The fragmentor voltages and collision energies were optimized for each compound and ranged from 50 to 130 V and from 0 to 45 eV, respectively. The data recorded was processed with MassHunter software (Agilent Technologies).

The analytes were subdivided in 4 groups (according to EPA protocol for sample extraction) and different LC conditions for the chromatographic separation of each group were used as reported in Table 2. Depending on each group of compounds a different analytical column and specific gradient was used following EPA guidelines. The column temperature was maintained at 25 °C and the injected sample volume was 15 μL for all the groups.

3. Results and discussion

3.1. Improvements to mass spectrometry

The first effort of this method development project was to determine the proper ions and transitions for all pharmaceuticals studied in EPA Method 1694. This was carried out by analyzing each individual standard in solvent by itself. The first ion mass assignment deals with four of the five compounds that make up the penicillin family: cloxacillin, oxacillin, penicillin G, and penicillin V. These four compounds and ampicillin have the general chemical structure shown in Fig. 1. The selected precursor ions in the original EPA Method 1694 show that the ions selected are 32 mass units higher for three of the four compounds and 33 mass units higher for cloxacillin. The reason for these mass assignments, we found, was due to methanolysis, which occurs when a standard of the penicillin family is made up in methanol and allowed to stand at room temperature for periods of time. This phenomena was first reported by Bruno et al. [19] when developing a method to analyze penicillins in aqueous environmental samples. When the addition of methanol occurs there is an increase in mass of 32 mass units from the protonated molecule, as shown in Fig. 2 for oxacillin. Furthermore, hydrolysis may also happen with the addition of water and an increase in mass of 18 mass units, which was found for oxacillin, penicillin G, and penicillin V. In these cases, hydrolysis occurs at the strained four-member ring of the penicillin structure. Fig. 3 shows the chromatogram for two small isomers of oxacillin hydrolysis, the parent compound oxacillin, and the methyl ester of oxacillin. These degradation products were confirmed by accurate mass analysis (results not shown here).

When these compounds are analyzed by electrospray they will fragment with the loss of either methanol or water to return to their original mass (i.e., oxacillin methyl ester at m/z 434 fragments with the loss of methanol to m/z 402, and the hydrolysis product will fragment from m/z 420 to m/z 402). After this loss, the protonated molecule will fragment to the characteristic diagnostic ion of the penicillin family, m/z 160, which is shown in Fig. 1. A similar fragmentation pathway occurs for the hydrolysis product with a loss of water back to the original penicillin structure, which then also fragments to the diagnostic ion of m/z 160. These compounds will have different retention times, with the hydrolysis product(s) typically eluting first, followed by the parent compound and/or the methyl ester (Fig. 3).

Ampicillin did not show either hydrolysis or methanolysis and this is probably due to the amine group blocking access to the four-membered ring and preventing hydrolysis or methanolysis (see structures in Fig. 1). Thus, the original EPA method is using the precursor ions of the methyl ester degradation products rather than the parent compound on four of the penicillin family members. If these compounds are to be monitored in the environment, then it is necessary to look for the parent and its degradates in order to have a complete picture of their occurrence in water and wastewater samples. Given that water is the matrix being analyzed, it is most likely that over time these penicillins would be transformed into their hydrolysis products rather than their methyl esters, although it may be possible for methyl esters to form during isolation by solid-phase extraction and their elution by methanol. So it is important to take into account all these ion mass assignments for correct identification of penicillins.

The other antibiotic that had a different mass was virginamcyin at m/z 508 (original EPA Method) rather than m/z 526. In this case, the anhydrate of virginamycin was being monitored rather than the parent compound. Thus, it may be necessary again to monitor both of these compounds for correct identification in water samples.

Another compound, digoxin, was measured as its sodium adduct at m/z 803 in Method 1694 rather than m/z 781, the protonated molecule. We found that the sodium adduct forms exclusively under electrospray ionization and will not fragment, which is a common occurrence in mass spectrometry because of the lack of a proton that is typically involved in a fragmentation reaction. It is sometimes possible to fragment a sodium adduct via a charge remote fragmentation and retention of the charge with sodium, but this was not the case for this compound. Therefore, digoxin could only be quantified using its sodium adduct since a second characteristic transition was not detected.

Sulfanilamide, was measured as its ammonium adduct by the EPA method at m/z 190, rather than its protonated molecule at m/z 173. We found that the protonated molecule was formed in our instrument rather than the ammonium adduct. This should

Table 3
MRM transitions and MS operating parameters selected for the analysis of the pharmaceutical compounds in Groups 1–4. The labeled standards are shown in italics.

Compound	Fragmentor voltage	MRM transitions (*m*/*z*)	Collision energy (eV)
Group 1			
Acetaminophen	90	152 > 110 152 > 65	15 35
$^{13}C_2$-^{15}N-Acetaminophen	*90*	*155 > 111* *155 > 93*	*15* *25*
Ampicillin	70	350 > 160 350 > 106	10 15
$^{13}C_3$-Atrazine	*120*	*219 > 177* *219 > 98*	*15* *25*
Azithromycin	130	749.5 > 591.4 749.5 > 158	30 35
Caffeine	110	195 > 138 195 > 110	15 25
$^{13}C_3$-Caffeine	*110*	*198 > 140* *198 > 112*	*15* *25*
Carbadox	80	263 > 231 263 > 130	5 35
Carbamazepine	110	237 > 194 237 > 179	15 35
Carbamazepine-d_{10}	*110*	*247 > 204* *247 > 202*	*15* *35*
Cefotaxime	90	456 > 396 456 > 324	5 5
Ciprofloxacin	110	332 > 314 332 > 231	20 35
$^{13}C_3$-^{15}N-Ciprofloxacin	*110*	*336 > 318* *336 > 235*	*15* *35*
Clarithromycin	110	748.5 > 158 748.5 > 590	25 15
Cloxacillin	90	436 > 160 436 > 277	15 15
Cloxacillin methyl ester	70	469 > 437 469 > 160	35 15
Codeine	130	300 > 215 300 > 165	25 35
Cotinine	90	177 > 98 177 > 80	25 25
Cotinine-d_3	*90*	*180 > 80* *180 > 101*	*25* *25*
Dehydronifedipine	130	345 > 284 345 > 268	25 25
Digoxigenin	90	391 > 355 391 > 337	15 15
Digoxin	No response, Na adduct		
Diltiazem	130	415 > 178 415 > 150	25 25
1,7-Dimethylxanthine	90	181 > 124 181 > 96	15 25
Diphenhydramine	70	256 > 167 256 > 152	15 35
Enrofloxacin	130	360 > 316 360 > 342	15 15
Erythromycin	90	734.5 > 158 734.5 > 576	35 15
$^{13}C_2$-Erythromycin	*90*	*736.5 > 160* *736.5 > 578*	*25* *15*
Erythromycin anhydrate	90	716.5 > 158 716.5 > 116	25 25
Flumequine	90	262 > 174	35

Table 3 (*Continued*)

Compound	Fragmentor voltage	MRM transitions (*m*/*z*)	Collision energy (eV)
		262 > 244	15
Fluoxetine	90	310 > 148	0
		310 > 44	10
Fluoxetine-d_6	*90*	*316 > 154*	*0*
		316 > 44	*10*
Lincomycin	110	407 > 126	25
		407 > 359	15
Lomefloxacin	130	352 > 308	15
		352 > 265	25
Miconazole	90	415 > 159	35
		415 > 69	25
Norfloxacin	70	320 > 302	15
		320 > 276	15
Ofloxacin	110	362 > 318	15
		362 > 261	25
Oxacillin	70	402 > 160	15
		402 > 243	5
Oxacillin methyl ester	90	434 > 160	15
		434 > 144	35
Oxolinic acid	90	262 > 244	15
		262 > 216	25
Penicillin G	90	335 > 160	5
		335 > 176	5
Penicillin G methyl ester	110	367 > 217	15
		367 > 160	15
Penicillin V	70	351 > 160	5
		351 > 114	25
Penicillin V methyl ester	70	383 > 160	15
		383 > 114	25
Roxithromycin	130	837.5 > 679	15
		837.5 > 158	35
Sarafloxacin	130	386 > 299	25
		386 > 368	25
Sulfachloropyridazine	90	285 > 156	10
		285 > 92	25
Sulfadiazine	110	251 > 156	15
		251 > 92	25
Sulfadimethoxine	80	311 > 156	20
		311 > 92	35
Sulfamerazine	110	265 > 156	15
		265 > 92	25
Sulfamethazine	90	279 > 156	15
		279 > 186	15
$^{13}C_6$-Sulfamethazine	*90*	*285 > 186*	*25*
		285 > 162	*25*
Sulfamethizole	80	271 > 156	10
		271 > 92	25
Sulfamethoxazole	110	254 > 156	15
		254 > 92	25
$^{13}C_6$-Sulfamethoxazole	*110*	*260 > 162*	*15*
		260 > 98	*25*
Sulfanilamide	70	173 > 156	5
		173 > 92	15
Sulfathiazole	108	256 > 156	10
		256 > 92	20
Thiabendazole	130	202 > 175	25
		202 > 131	35
Trimethoprim	110	291 > 230	25
		291 > 261	25

Table 3 (*Continued*)

Compound	Fragmentor voltage	MRM transitions (*m*/*z*)	Collision energy (eV)
$^{13}C_3$-Trimethoprim	*110*	*294 > 233* *294 > 264*	*25* *25*
Tylosin	110	916.5 > 174 916.5 > 772	35 35
Virginiamycin	110	526 > 508 526 > 355	5 15
Group 2			
Anhydrochlortetracycline	122	461 > 444 461 > 410	15 15
Anhydrotetracycline	90	427 > 410 427 > 154	15 25
Chlorotetracycline	110	479 > 462 479 > 197	15 35
Demeclocycline	130	465 > 430 465 > 448	25 15
Doxycycline	110	445 > 428 445 > 154	15 25
4-Epianhydrochlortetracycline	134	461 > 444 461 > 426	15 15
4-Epianhydrotetracycline	90	427 > 410 427 > 105	15 35
4-Epichlortetracycline	134	479 > 462 479 > 197	15 15
4-Epioxytetracycline	130	461 > 444 461 > 426	15 15
4-Epitetracycline (ETC)	110	445 > 410 445 > 427	15 5
Isochlortetracycline	138	479 > 462 479 > 252	15 45
Meclocycline	110	477 > 460	15
Minocycline	90	458 > 441	15
Tetracycline (TC)	110	445 > 410 445 > 427	15 5
Group 3			
Gemfibrozil	70	249 > 121	5
Gemfibrozil-d_6	*70*	*255 > 121*	*5*
Ibuprofen	50	205 > 161	0
$^{13}C_3$-Ibuprofen	*50*	*208 > 163*	*0*
Naproxen	50	229 > 169 229 > 170	25 5
^{13}C-Naproxen-d_3	*50*	*233 > 169* *233 > 170*	*25* *5*
Triclocarban	100	313 > 160 313 > 126	10 25
$^{13}C_6$-Triclocarban	*90*	*319 > 160* *319 > 132*	*5* *25*
Triclosan	75	287 > 35 289 > 37	5 5
$^{13}C_{12}$-Triclosan	*75*	*299 > 35*	*5*
Warfarin	125	307 > 117 307 > 161	35 15
Warfarin-d_5	*90*	*312 > 161* *312 > 255*	*15* *25*
$^{13}C_6$-2,4,5-Trichlorophenoxyacetic acid	*110*	*259 > 201* *259 > 165*	*5* *25*

Table 3 (*Continued*)

Compound	Fragmentor voltage	MRM transitions (*m*/*z*)	Collision energy (eV)
Group 4			
Albuterol (salbutamol)	90	240 > 148	15
		240 > 166	5
Cimetidine	100	253 > 159	10
		253 > 95	25
Metformin	80	130 > 60	10
		130 > 71	25
Ranitidine	110	315 > 176	15
		315 > 130	25

be noted for those doing LC/MS–MS of this compound for correct selection of the precursor ion, depending on the instrument being used.

Two compounds, metformin and the methyl ester of cloxacillin, were one mass unit too high, so the EPA method was measuring the ^{13}C isotope of the parent compound rather than the parent compound. Furthermore, they were also using the ^{13}C for the fragment ion, which is a much less sensitive transition. This should be corrected for best LC/MS–MS performance.

Finally, the compounds, azithromycin, diphenhydramine, roxithromycin, and tylosin, were more than 0.5 mass units off in their mass assignment. The fragment ion for diphenhydramine was also off by one mass unit; thus, this compound was being measured as its ^{13}C isotope rather than by its monoisotopic mass. All of our mass assignments were confirmed by accurate mass and were calculated as exact mass with a proton, ammonium, or sodium ion added, as appropriate (results not shown here). In summary, these were the inaccuracies in mass spectrometry that were

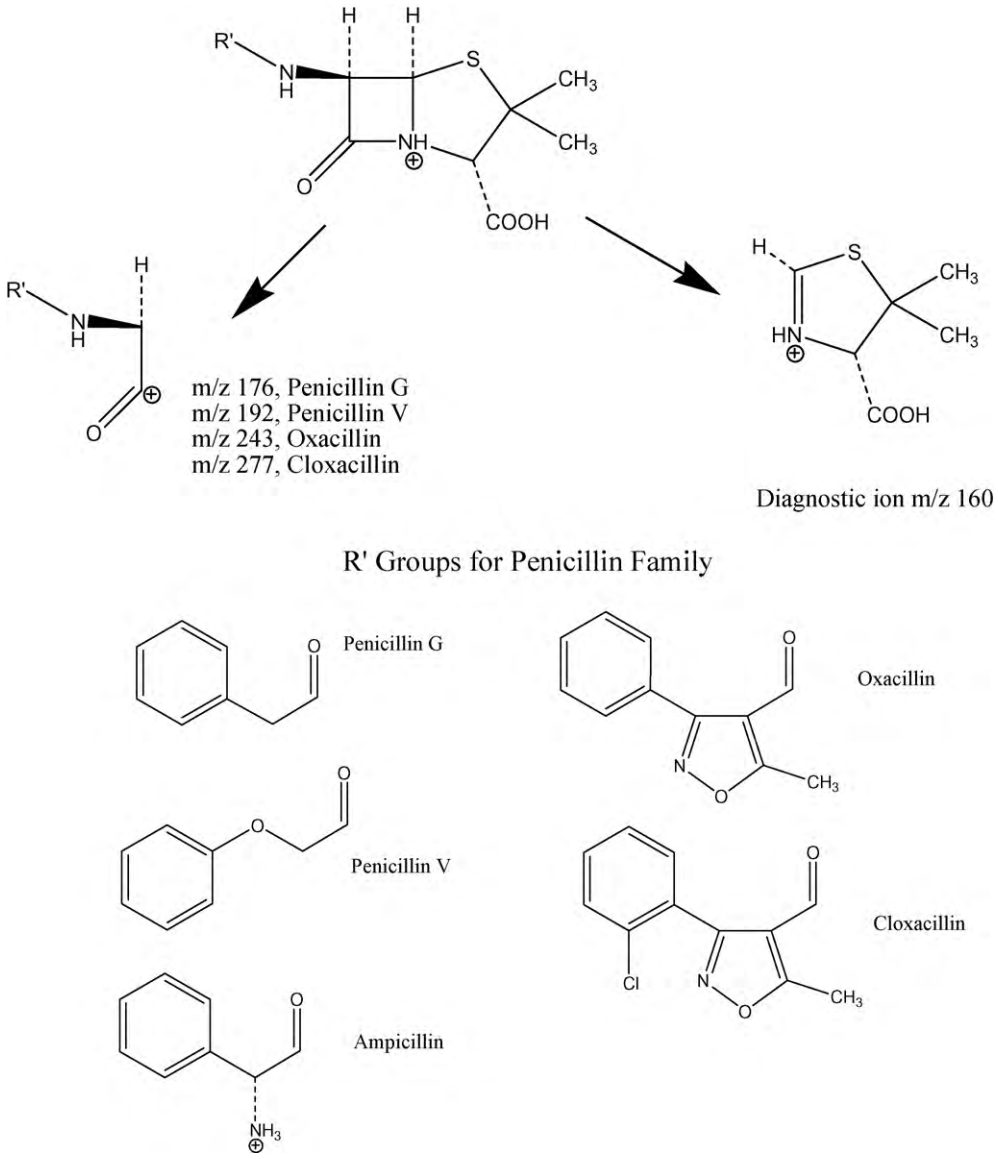

Fig. 1. Fragmentation patterns for penicillin compounds showing their corresponding diagnostic ions.

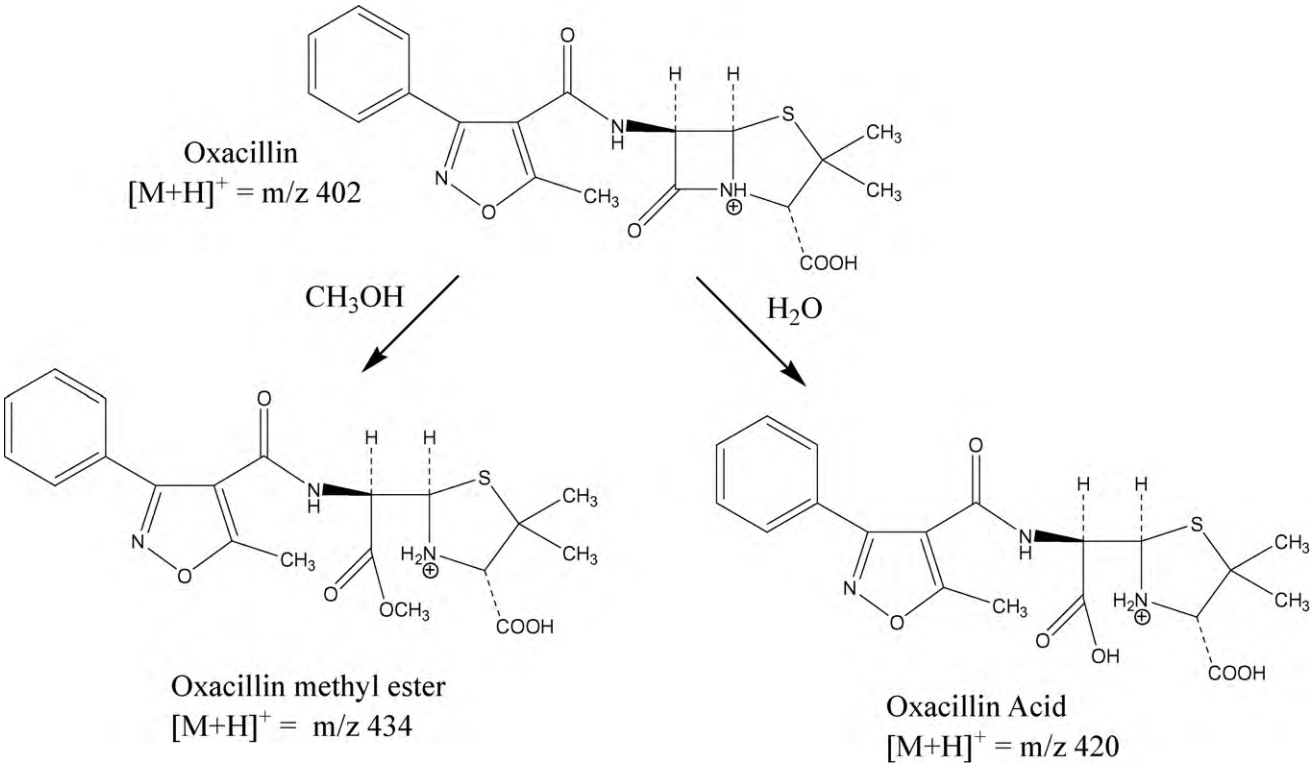

Fig. 2. Methanolysis and hydrolysis degradation products of oxacillin.

found during the set up of EPA Method 1694 for standard operation.

3.2. Optimization of LC/MS–MS conditions: ions and transitions

To select the optimum experimental conditions and product ions for the target compounds, individual solutions of each analyte were made up at a concentration of 1 μg/mL in methanol and water (1:9) and 10 μL was directly injected into the LC/MS–MS system. An automated procedure (Optimizer software, Agilent) was used to get the optimum fragmentor voltage and collision energies for each analyte. Full-scan spectra were acquired first to optimize the collision induced dissociation (CID) fragmentation applied at the source in order to get the maximum sensitivity for the precursor ion. Afterwards, MS–MS spectra under product ion mode of operation are acquired to obtain the product ions. Once the product ions (two or more) are automatically selected for every analyte, an MRM experiment is carried out to select the optimum collision energy for each specific transition. Collision energies varied between 0 and 45 eV.

Table 3 summarizes the optimized values for the fragmentor voltage and collision energies used for each one of the pharmaceutical compounds investigated, as well as all the MRM transitions selected for screening, quantitation and confirmation. Internal standards are also listed in this table with the corresponding MRM transitions and optimized values. The first transition shown was used for quantitation (calibration curves and reproducibility) and the second transition was used for confirmatory purposes and to calculate limits of detection as explained later in the text. A total of 4 compounds (meclocycline, minocycline, gemfibrozil and ibupro-

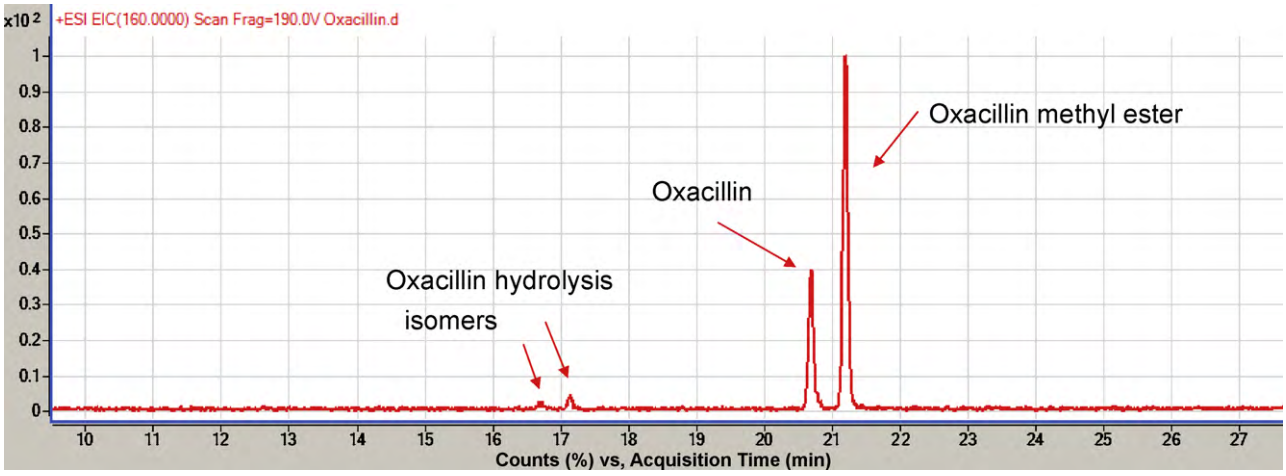

Fig. 3. Chromatogram of oxacillin and corresponding hydrolysis and methanolysis degradation products.

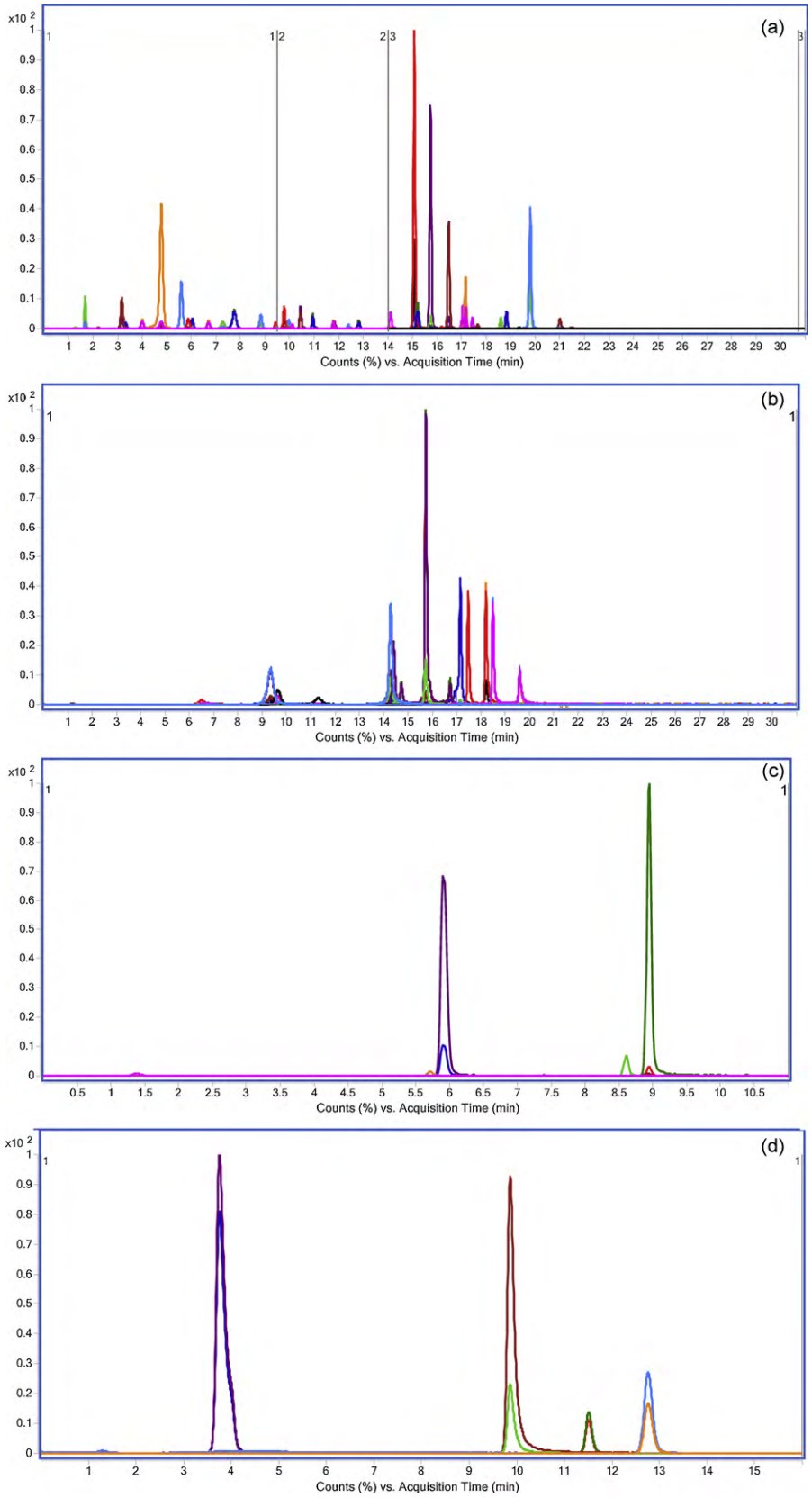

Fig. 4. (a) MRM extracted chromatogram for pharmaceuticals in Group 1 (positive ion). Three time segments were used in this chromatographic separation; (b) MRM extracted chromatogram for pharmaceuticals in Group 2 (positive ion); (c) MRM extracted chromatogram for pharmaceuticals in Group 3 (negative ion); (d) MRM extracted chromatogram for pharmaceuticals in Group 4 (positive ion). See Table 3 for compound identification.

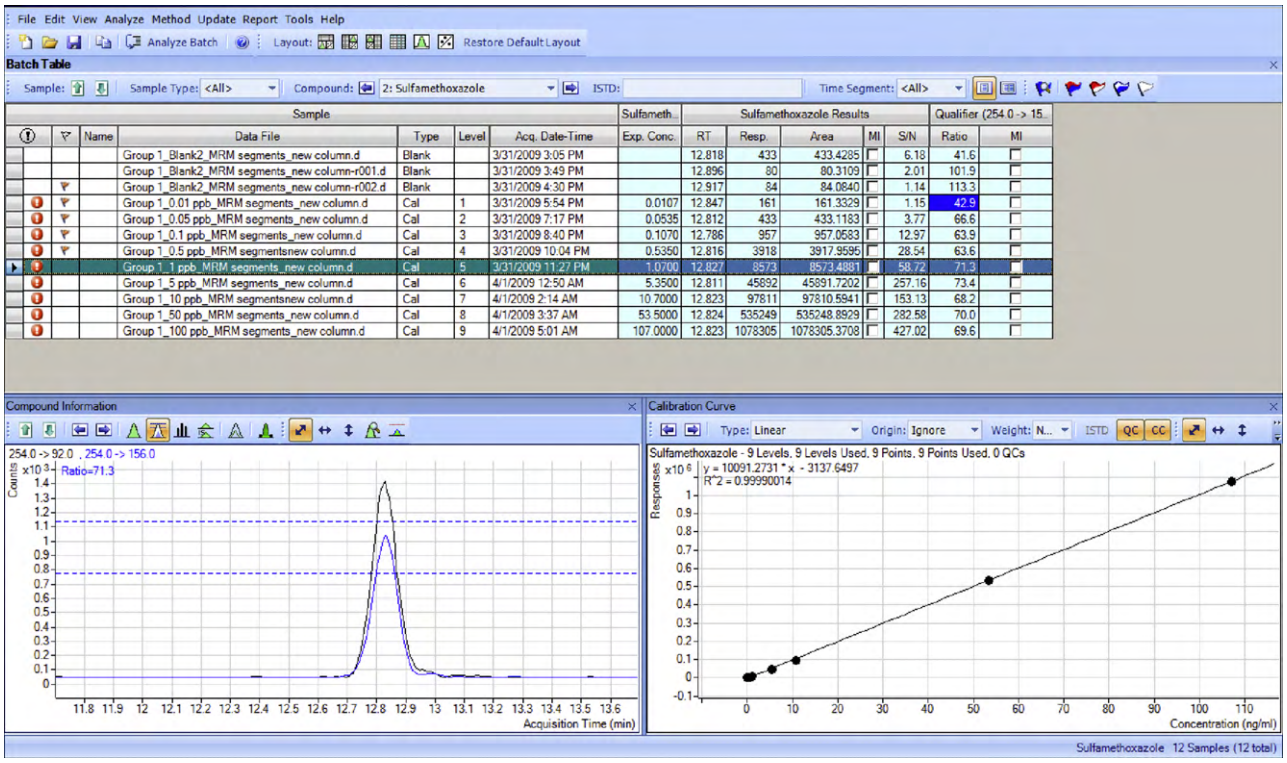

Fig. 5. External calibration curve for sulfamethoxazole in a wastewater matrix using a nine point curve from 0.1 to 100 μg/L (ppb) using a linear fit with no origin treatment.

fen) presented only one fragment ion, so in these 4 cases a single MRM transition was monitored. A dwell time of 10 ms was used for every MRM transition. Compounds in Groups 1, 2 and 4 were detected under positive ionization, whereas compounds in Group 3, which includes mainly carboxylic compounds, were detected in negative ionization. However, most of these compounds can also be analyzed in positive ionization [20] and, in fact, we found that warfarin was more sensitive under positive conditions rather than negative.

The inclusion of the MRM transitions for the methyl esters of the penicillin family of compounds is necessary for correct screening of these compounds in water samples, as commented in the previous section. Furthermore, the addition of a second transition for the majority of compounds confirms the identity of the analytes and it must be used as a complementary data set for correct identification and confirmation of pharmaceuticals in environmental water samples [21].

3.3. Chromatographic separations

EPA Method 1694 guidelines establishes that any chromatographic gradient may be applied for the separation of the pharmaceutical compounds as long as the last eluting peak has a later retention time than the standard EPA method. Under these guidelines we developed 4 different chromatographic methods for each group of compounds (see Table 2). This was carried out by analyzing mixes of standards in solvent. For pharmaceuticals included in Groups 1, 2 and 3, a reversed phase gradient using a C_{18} analytical column with 3.5 μm particles was used. This column enabled elution of compounds in much narrower peaks than using a 5 μm particle one, resulting in better chromatographic resolution and increased peak height. The typical peak width was 5–10 s at base, thus permitting very good separation of all compounds in only 30 min. For Groups 1 and 2, a common slow gradient using acetonitrile and water with 0.1% formic acid worked well for all the compounds studied. For Group 1, which included 46 pharmaceuticals, three different MS–MS time segments were recorded in the chromatographic run in order to gain sensitivity. In this way, a total of about 30 transitions were monitored in each segment. The time segment changes were set up at 9.5 and 14 min. For Group 3, which includes some of the most hydrophobic compounds the gradient used started at 40% of methanol:acetonitrile (1:1) following EPA guidelines. For compounds included in Group 4, which are the most polar analytes, a HILIC column was used for the separation. In this case, a gradient starting at 98% of acetonitrile and gradually decreasing in organic composition (see Table 2) was used for the separation of the four compounds in this group.

Fig. 4a–d show the chromatograms corresponding to a 10 μg/L standard for all the pharmaceuticals studied. Extracted ion chromatograms are overlaid for each one of the target analytes according to their respective MRM transitions. All compounds were well separated under the conditions used in this methodology. It is worth mentioning that EPA Method 1694 reconstitutes the sample extracts with 75% organic solvent and their chromatographic gradient conditions start at 5–10% organic solvent. Unfortunately there were no chromatogram figures in the original report, but one would assume that peak shape, especially for those polar analytes, would be compromised under these conditions. In our method reported here we use the same exact composition for sample extracts and gradient conditions, thus clearly showing accurate and optimized chromatographic peak shape for all the compounds.

3.4. Optimization of SPE procedure

Optimization of the solid-phase extraction (SPE) procedure was performed with the aim of reaching acceptable recoveries for the widest group of compounds in a single extraction step rather than the multiple extraction procedure outlined by the original EPA Method. Thus, the SPE procedure for this multi-analyte method was executed with a single extraction step using a low sample volume. For recovery studies, environmental water sam-

ples were spiked with a known amount of pharmaceuticals and processed through the cartridges. Areas obtained after chromatographic analyses were then compared to the areas corresponding to the analyses of blank matrixes of the same type spiked directly with the same amount of pharmaceutical compounds. Table 4 shows the recoveries of extraction obtained for the compounds studied. In general, acceptable recoveries were obtained for the majority of compounds, which is in agreement with the EPA Method. Comparison at neutral pH and at acidic and alkaline conditions was tested according to the EPA method. Recoveries were not better, in general, after pH adjustment due to the incompatibility of the compounds and hydrolysis reactions of several analytes, which was especially true for the penicillin family that is highly susceptible to hydrolysis as commented earlier. Tetracyclines were not recovered under the conditions used here; they involve addition of a complexing agent, such as EDTA, which requires a separated SPE method [22].

Initial recovery experiments were carried out in spiked de-ionized water, surface water, drinking water, and wastewater. Each matrix presents a different set of circumstances that must be addressed. De-ionized water, because of the low ionic strength, often gave the highest recoveries but do not reflect real water samples. Likewise drinking water, which contains adjuvants or treatment substances such as alum, organic coagulants, metal ions, and chlorine gave varying results. Finally, wastewater samples have higher concentrations of suspended solids that also may affect recovery of pharmaceuticals. In general, recoveries from wastewater were between 10 and 15% lower than reagent water samples, probably due to strong matrix effects and competition of interferents for specific sites in the sorbent. After testing these various matrices we determined that surface water gave the most reproducible recoveries by SPE and the recoveries reported in Table 4 were carried out with this matrix.

The accuracy and precision of our final extraction method was determined from spike and recovery experiments for the analytes shown in Table 4 at a concentration of 0.5 μg/L. The absolute recoveries of the pharmaceuticals varied from 10 to 123% and they were similar to the ones reported by EPA. Attempts were made to improve recoveries but it became apparent that the EPA Method 1694 included a group of compounds that chemically cannot be recovered at 100% because of a variety of problems. Furthermore, the recoveries shown in Table 4 are for surface water samples, which are more difficult matrices than reagent water. Thus, our experience is that of all analytes studied, approximately 50 of these compounds may be recovered from environmental water samples with a generic SPE procedure. The use of labeled standards is a necessity for good recovery data. At the moment 20 labeled standards are included in Method 1694.

3.5. Method validation

3.5.1. Identification and quantitation

A total of 4 identification points as required by the EU [21] were obtained for each analyte by monitoring two transitions or fragment ions for each precursor ion in the MRM mode. The most intense transition served for quantitation purposes, whereas the second transition was monitored for confirmation of the analyte. Quantitation was performed using calibration with matrix-matched standards to prevent slight variations in the signal for some analytes and possible enhancement or suppression of the signal, especially from wastewater samples as compared to pure solvent [16]. For quantitation and confirmation purposes the peak area and peak ion ratios of both transitions (quantifier and qualifier, respectively) were measured using the automated Mass Hunter quantitation software. Using this approach, samples can be quantified automatically using batches, which include the files of the

Table 4
Extraction recoveries from surface water samples, instrumental LODs and correlation coefficients (R^2) for the analysis of the pharmaceutical compounds in Groups 1–4.

Compound	%Recovery	LODs Jet Stream 6460 (μg/L)	R^2
Acetaminophen	103	0.1	0.994
Albuterol	94	0.05	0.998
Ampicillin	10	0.6	0.996
Anhydrochlortetracycline	n.d.	5.0	0.994
Anhydrotetracycline	n.d.	1.0	0.996
Azithromycin	52	5.0	0.994
Caffeine	97	0.5	0.999
Carbadox	92	0.3	0.997
Carbamazepine	107	0.06	0.999
Cefotaxime	11	2.0	0.998
Chlorotetracycline	n.d.	0.5	0.999
Cimetidine	56	0.02	0.998
Ciprofloxacin	54	0.5	0.995
Clarithromycin	49	0.1	0.996
Cloxacillin	80	3.0	0.994
Codeine	96	0.3	0.992
Cotinine	106	0.05	0.999
Dehydronifedipine	105	0.03	0.999
Demeclocycline	n.d.	4.0	0.998
Digoxigenin	100	0.4	0.991
Diltiazem	69	0.05	0.998
1,7-Dimethylxanthine	102	0.6	0.999
Diphenhydramine	92	0.05	0.996
Doxycycline	n.d.	1.0	0.998
Enrofloxacin	26	0.3	0.998
4-Epianhydrochlortetracycline	n.d.	5.0	0.997
4-Epianhydrotetracycline	n.d.	0.5	0.996
4-Epichlortetracycline	n.d.	1.0	0.996
4-Epioxytetracycline	n.d.	5.0	0.995
4-Epitetracycline	n.d.	1.0	0.998
Erythromycin	49	0.3	0.998
Erythromycin anhydrate	55	0.3	0.995
Flumequine	77	0.05	0.999
Fluoxetine	52	0.2	0.999
Gemfibrozil	95	0.1	0.997
Ibuprofen	105	5.0	0.996
Isochlortetracycline	n.d.	5.0	0.993
Lincomycin	33	0.05	0.998
Lomefloxacin	34	0.4	0.994
Metformin	22	0.05	0.996
Miconazole	123	0.5	0.993
Minocycline	n.d.	20.0	0.993
Naproxen	105	1.0	0.999
Norfloxacin	60	1.0	0.997
Ofloxacin	36	0.4	0.997
Oxacillin	98	1.0	0.999
Oxolinic acid	100	0.03	0.999
Penicillin G	79	1.0	0.999
Penicillin V	86	1.0	0.998
Ranitidine	105	0.08	0.996
Roxithromycin	40	0.5	0.998
Sarafloxacin	53	0.5	0.998
Sulfachloropyridazine	105	0.2	0.999
Sulfadiazine	93	0.5	0.997
Sulfadimethoxine	99	0.05	0.997
Sulfamerazine	99	0.1	0.999
Sulfamethazine	88	0.3	0.999
Sulfamethizole	107	0.3	0.999
Sulfamethoxazole	105	0.2	0.997
Sulfanilamide	118	4.0	0.997
Sulfathiazole	45	0.4	0.995
Tetracycline	n.d.	0.8	0.998
Thiabendazole	23	0.05	0.999
Triclocarban	103	0.1	0.993
Triclosan	75	1.0	0.995
Trimethoprim	104	0.5	0.996
Tylosin	33	6.0	0.999
Virginiamycin	52	0.4	0.999
Warfarin	113	0.1	0.997

n.d. = not determined.

Table 5
Analysis of representative surface water, wastewater and drinking water from different locations in Colorado showing concentrations for several pharmaceuticals. Samples were analyzed for all 70 pharmaceuticals; only 8 compounds were consistently found. Units: ng/L.

Samples	Caffeine	Carbamazepine	Clarithromycin	Diltiazem	Diphenhydramine	Erythromycin	Sulfamethoxazole	Trimethoprim
Wastewater Location 1	12	5	10	n.d.	27	n.d.	30	45
Wastewater Location 2	n.d.	15	40	10	n.d.	21	15	5
Wastewater Location 3	n.d.	14	172	153	70	1200	53	429
Surface water Location 4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Surface water Location 5	n.d.	21	n.d.	n.d.	15	52	n.d.	n.d.
Surface water Location 6	10	n.d.	n.d.	n.d.	n.d.	n.d.	5	n.d.
Surface water Location 7	580	147	5	36	8	7	210	105
Drinking water Location 8	n.d.	5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d. = not detected (below LOD).

calibration standards selected. An example is shown in Fig. 5 for sulfamethoxazole in a wastewater sample matrix. As it can be observed in this figure, a series of values (retention times, peak areas and calculated concentrations) are obtained as well as information on ion ratios and calibration curve data. Values in white denote values between the expected ion ratio ranges, whereas values in dark gray represent outlier values.

3.5.2. Analytical performance

Measurements on accuracy, intra-day and inter-day precision were carried out for all the analytes studied. The accuracy and intra-day precision of the developed method was assessed at two different concentration levels in spiked water extracts. At each level, the analysis was performed in five replicates. Accuracy, expressed as the mean from the five measurements ranged from 88 to 105% (percentage between the calculated and the theoretical concentrations for each analyte). Intra-day precision was calculated as the percent relative standard deviation (RSD) from the five measurements and ranged between 2 and 5%. Inter-day precision was measured by analyzing spiked water extracts in five consecutive days and ranged from 4 to 11% RSD.

3.5.3. Linearity and limits of detection

To confirm the suitability of the method for analysis of real samples, matrix-matched standards were analyzed in a wastewater matrix from an effluent site, at nine concentrations (0.01, 0.05, 0.1,

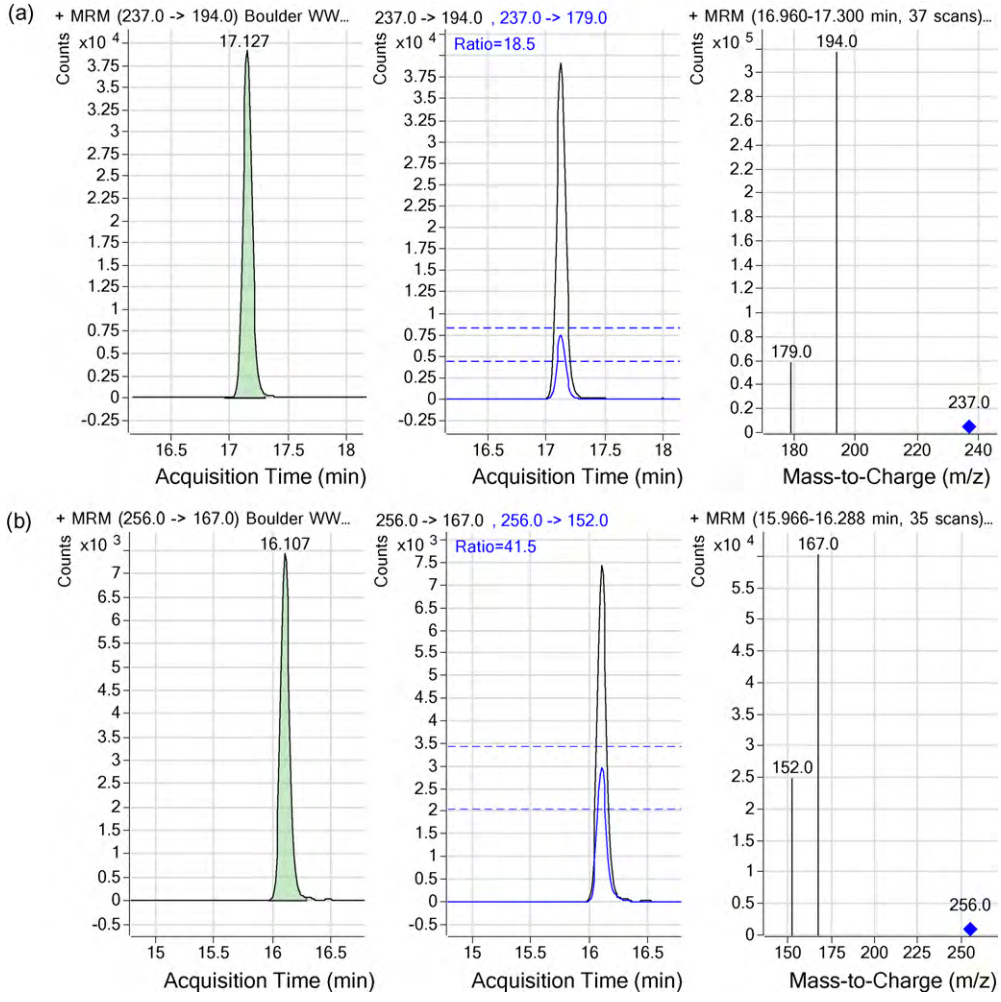

Fig. 6. MRM chromatograms of a wastewater sample for (a) carbamazepine and (b) diphenhydramine using 2 transitions. Ion ratios are also shown.

0.5, 1, 5, 10, 50, and 100 μg/L or ppb). For the calibration curves only the area of the quantifying transition was taken into account. Table 4 shows the correlation coefficients obtained for all the analytes studied. In general, matrix-matched calibration curves were linear between the concentrations studied with correlation coefficients higher than 0.99 for all the compounds analyzed.

The instrumental limits of detection (LODs) were estimated from the injection of wastewater spiked samples at concentration levels corresponding to a signal-to-noise ratio of about 3. In this case, both transitions were taken into account: the presence of both ions, the quantitation and the confirmatory ion was required to establish a detectable concentration. The LODs obtained for all the standards spiked in a wastewater matrix are included in Table 4. These limits of detection are higher and more realistic than others reported for only one transition [1] since it takes into account not only the main transition but it also confirms the compound with the second MRM transition. The best LODs were obtained for albuterol (0.02 μg/L or 0.3 pg on column) and the highest LODs were for minocycline (above 20 μg/L or 300 pg on column).

3.5.4. Matrix effects

Matrix effects are common in surface and wastewater samples due to the presence of natural organic matter in such samples [16]. Matrix effects typically mean suppression, however they also mean matrix interferences that are present in the sample and hence, they have an effect on the ionization and/or detection of the compounds. On the other hand, when using tandem mass spectrometric techniques, the spectral interferences are further minimized due to the higher selectivity of the two MRM transitions [6,11]. The use of LC/MS–MS with two MRM monitored transitions is a highly selective technique that discriminates most of the interferences present in the matrix. The results obtained showed ion suppression effects for 60% of the analytes in the range 10–30%. The use of internal standards as surrogates in the water samples before extraction accounted for these matrix effects for all of the compounds and guided to a better quantitation of the analytes detected in water samples.

3.6. Application to environmental samples

The methodology developed in this work was applied to the analysis of environmental water samples. Several surface water, wastewater and drinking water samples from different locations in Colorado were analyzed for the presence of all 70 pharmaceuticals. Only 8 out of the 70 compounds were consistently found in environmental water samples: caffeine, carbamazepine, clarithromycin, diltiazem, diphenhydramine, erythromycin, sulfamethoxazole and trimethoprim, which were confirmed with two MRM transitions. The results for the concentrations found are shown in Table 5. These samples are representative of several inputs of wastewater contamination. One drinking water sample was also analyzed, and gave a positive hit for carbamazepine, a common antiepileptic and antidepressant prescribed drug. Fig. 6 shows the ion ratios qualifying for carbamazepine and diphenhydramine in a wastewater extract. As shown in Fig. 6 in the two ion profiles, both pharmaceuticals were easily identified at low ng/L levels in this complex matrix due to the selectivity of the MRM transitions and instrument sensitivity.

It is our view that confirmation of positive identifications in real samples requires the additional second MRM transition and the evaluation of ion ratios between the two monitored transitions as compared to a reference standard [21]. Confirmation of the identity of target analytes in real samples is usually based on ion ratio statistics for the transitions monitored. In this way, the confirmation criteria using tandem mass spectrometry cover a range of maximum permitted tolerances according to relative ion intensity, expressed as a percentage of the intensity of the most intense transition [21]. Fig. 6 shows also the ion ratios for carbamazepine and diphenhydramine in a wastewater sample which were in accordance with theoretical accepted ion ratios between the qualifiers and the quantifiers ions (±30 and ±25, respectively). This gives an even greater assurance of correct analysis than prescribed by EPA Method 1694.

4. Conclusions

The results of this study show that liquid chromatography tandem mass spectrometry (LC/MS–MS) is a robust, sensitive, and reliable methodology for the study of pharmaceuticals in water samples, using high throughput procedures as outlined in EPA Method 1694. We have improved the method by additional ion mass assignments for the penicillin family and a second MRM transition for 66 compounds. The additions shown in this paper allow the method to be used successfully for the monitoring of pharmaceuticals in wastewater, surface water and drinking water. Further examination of some analytes is required for the best analytical results, especially those compounds not having an isotopic standard. Also more work is required for some analytes regarding solid-phase extraction and optimization of recovery. The mass spectrometric additions of this paper will be highly useful to those researchers in the field following EPA Method 1694 and it will speed final implementation and its validation by water utilities and their laboratories.

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Widespread occurrence of neuro-active pharmaceuticals and metabolites in 24 Minnesota rivers and wastewaters

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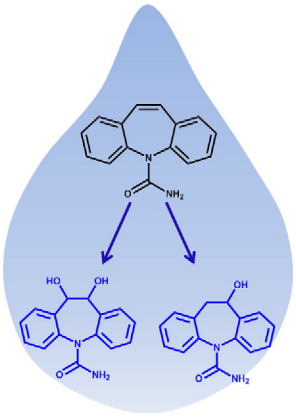
HIGHLIGHTS

- Neuro-active pharmaceuticals and their associated major metabolites were measured.
- We evaluated wastewater and receiving rivers from 24 locations across Minnesota.
- We utilized liquid chromatography time-of-flight mass spectrometry.
- Metabolites generally are found at higher concentrations than parent compounds

GRAPHICAL ABSTRACT



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ABSTRACT

Concentrations of 17 neuro-active pharmaceuticals and their major metabolites (bupropion, hydroxy-bupropion, erythro-hydrobupropion, threo-hydrobupropion, carbamazepine, 10,11,-dihydro-10,11,-dihydroxycarbamazepine, 10-hydroxy-carbamazepine, citalopram, *N*-desmethyl-citalopram, fluoxetine, norfluoxetine, gabapentin, lamotrigine, 2-N-glucuronide-lamotrigine, oxcarbazepine, venlafaxine and O-desmethyl-venlafaxine), were measured in treated wastewater and receiving surface waters from 24 locations across Minnesota, USA. The analysis of upstream and downstream sampling sites indicated that the wastewater treatment plants were the major source of the neuro-active pharmaceuticals and associated metabolites in surface waters of Minnesota. Concentrations of parent compound and the associated metabolite varied substantially between treatment plants (concentrations ± standard deviation of the parent compound relative to its major metabolite) as illustrated by the following examples; bupropion and hydrobupropion 700 ± 1000 ng L^{-1}, 2100 ± 1700 ng L^{-1}, carbamazepine and 10-hydroxy-carbamazepine 480 ± 380 ng L^{-1}, 360 ± 400 ng L^{-1}, venlafaxine and *O*-desmethyl-venlafaxine 1400 ± 1300 ng L^{-1}, 1800 ± 2300 ng L^{-1}. Metabolites of the neuro-active compounds were commonly found at higher or comparable concentrations to the parent compounds in wastewater effluent and the receiving surface water. Neuro-active pharmaceuticals and associated metabolites were detected only sporadically in samples upstream from the effluent outfall. Metabolite to parent ratios were used to evaluate transformation, and we determined that ratios in wastewater were much lower than those reported in urine, indicating that the metabolites are relatively more labile than the parent compounds in the treatment plants and in receiving waters. The widespread occurrence of neuro-active pharmaceuticals and metabolites in Minnesota effluents and surface waters indicate that this is likely a global environmental issue, and further understanding of the environmental fate and impacts of these compounds is warranted.

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1. Introduction

Prescription medication is an important class of emerging contaminants, and detection of these compounds in surface water and drinking water has been widely reported (Heberer, 2002; Kolpin et al., 2002; Benotti et al., 2008; Focazio et al., 2008; Kasprzyk-Hordern et al., 2009; Vazquez-Roig et al., 2012; López-Serna et al., 2013). It has been estimated that 8% of the U.S. population has been prescribed medication for depression and other neurological illnesses (Stagnitti, 2002). Prescribed neuro-active pharmaceuticals include anti-depressants, anti-seizure compounds, and mood-stabilizers, and these compounds (e.g. bupropion, carbamazepine, fluoxetine, and venlafaxine) have been identified at nanogram per liter concentrations in surface waters (Brooks et al., 2005; Miao et al., 2005; Leclercq et al., 2009; Metcalfe et al., 2010; Schultz et al., 2010). Importantly, these compounds are primarily excreted in urine and feces as biologically active Phase I and Phase II metabolites (Holčapek et al., 2008). Phase I metabolites are the result of in-vivo biochemical oxidation, reduction, and hydrolysis reactions that increase their aqueous solubility and facilitate elimination from the body (Holčapek et al., 2008). Phase II metabolites (often termed conjugated metabolites) are the result of biochemical reactions that add a molecule (i.e. glucuronic acid) to the parent compound (Holčapek et al., 2008). Phase II metabolites deconjugate back to the parent compound in wastewater (D'Ascenzo et al., 2003) and are infrequently identified in surface waters (Ferrer and Thurman, 2010).

Assessment of the environmental relevance of neuro-active pharmaceuticals and associated metabolites depends upon knowledge of environmental concentrations and subsequent ecological impacts. Reported concentrations and wastewater treatment plant efficiency in removing neuro-active pharmaceuticals and their associated metabolites were observed to vary considerably. For example, carbamazepine concentrations in three treatment plants in France varied between 112 and 258 ng L^{-1} (Leclercq et al., 2009); whereas carbamazepine concentrations as high as 4600 ng L^{-1} were reported in a treatment plant in Wales (Kasprzyk-Hordern et al., 2009). Carbamazepeine was not removed by a conventional activated sludge wastewater treatment plant, although it was removed (73% reduction) in a wastewater treatment plant with a prolonged hydraulic retention time (78 days, Leclercq et al., 2009), and metabolites of carbamezepine were shown to be relatively more labile (Leclercq et al., 2009). Concentrations of venlafaxine were shown to be relatively consistent in wastewater influent and effluent (300 ± 100 ng L^{-1}), whereas fluctuations in wastewater effluent of its primary metabolite *O*-desmethyl-venlafaxine were much greater (1000 ± 800 ng L^{-1}, Rua-Gomez and Püttmann, 2012). Neuro-active pharmaceuticals are chemically transformed in wastewater treatment plants to metabolites that may retain their bio-active properties, although few studies have directly quantified potential adverse effects on aquatic organisms. Nanogram per liter concentrations of parent neuro-active compounds (bupropion, fluoxetine, sertraline, venlafaxine) have been shown to alter behavior (i.e. diminished predator avoidance) in fathead minnows (*Pimephales promelas*, Painter et al., 2009)) and amphipods (*Echinogammarus marinus*, Guler and Ford, 2010), and increase mortality and alter tissue morphology in fathead minnows (Schultz et al., 2011). The bio-activity of neuro-active metabolites has been demonstrated in-vivo by the medical community (Howell et al., 1993; DeVane, 1999; Haas et al., 2004). Metabolites of neuro-active compounds have been shown to accumulate in the brain tissue of white suckers *Catostomus commersoni*, Schultz et al., 2010) and brook trout with evidence for negative biological effects (Lajeunesse et al., 2011).

Several studies have evaluated concentrations of neuro-active pharmaceuticals and their metabolites in wastewater effluent (Tixier et al., 2003; Miao et al., 2005; Kasprzyk-Hordern et al., 2009; Leclercq et al., 2009; Schultz et al., 2010; Rua-Gomez and Püttmann, 2012), although these studies were confined to a limited number of treatment plants (between one and three) and investigated only a few of the commonly prescribed neuro-active pharmaceuticals. To understand potential environmental risks of neuro-active pharmaceuticals in wastewater effluent it is important to evaluate both the parent compound and major metabolites (Celiz et al., 2009; Boxall et al., 2012). Unfortunately, *a posteriori* prediction of which neuro-active metabolites should be monitored can be constrained because environmental mass spectrometry traditionally relies on the use of known standards for targeted analyses.

The primary purpose of this study was to identify neuro-active pharmaceutical compounds and their associated metabolites that were consistently found in a broad spectrum of wastewater effluents on a regional scale. This study relied on the use of liquid chromatography, time-of-flight mass spectrometry with accurate mass (LC–TOF-MS) to identify metabolites of neuro-active pharmaceuticals that are at substantial concentrations in wastewater effluent and warrant further evaluation. After identification of important metabolites, their existence was confirmed using authentic standards, when available. This study evaluates 17 neuro-active pharmaceuticals and associated metabolites in effluent from 24 municipal treatment plants and their corresponding receiving waters (upstream and downstream) from around the state of Minnesota. Consequently, results from this study provide a benchmark for future studies evaluating their relevance as potential environmental contaminants of concern.

2. Methods

2.1. Sample collection

Twenty-four municipal wastewater treatment plants were selected from around the State of Minnesota (Table 1, Fig. 2) due to their diverse wastewater influent characteristics, different treatment processes, the existence of previously conducted studies, and variability of the receiving waters (Lee et al., 2011). The size of the treatment plants varied from 0.01 $m^3\ s^{-1}$ to 7.2 $m^3\ s^{-1}$ and served populations from several thousand to nearly 2 million people (Table 1 and Fig. 2). Water quality samples were collected from the wastewater effluent, the receiving water upstream from the effluent outfall, and from a downstream location in the fall of 2010. This study was part of a larger study focusing on wastewater contributions to surface waters (Lee et al., 2011), and not on treatment efficacy, consequently influent samples were not collected. Grab wastewater samples were collected directly from each WWTP effluent discharge channel and integrated width- and depth-sampling techniques were used to collect surface water samples (U.S. Geological Survey, 2006). All samples collected were transported to the laboratory at 4 °C, and analyzed within 10 days of collection. The fraction of wastewater effluent (f_{ww}) in the receiving stream (Table 2) was estimated by dividing the reported effluent discharge by the sum of the effluent discharge and measured upstream flow on the day of the sampling using U.S. Geological Survey protocols (Rantz et al., 1982a; Rantz et al., 1982b).

2.2. Analytical

Parent neuro-active pharmaceutical compounds (bupropion, hydroxy-bupropion, carbamazepine, fluoxetine, and venlafaxine) were initially measured using high-performance liquid chromatography/tandem mass spectrometry (LC/MS/MS) as previously described (Ferrer and Thurman, 2010, 2012) and in the Supplemental Materials (SM). Briefly, each water sample was spiked with the labeled internal standard d_{10}-carbamazepine (Cambridge Isotope Laboratories, Andover, MA.), concentrated using solid phase extraction (SPE), target compounds eluted with methanol, evaporated to 0.5 mL, and injected (20 μL) onto a high performance liquid chromatography system (HPLC) connected to a triple quadrupole mass spectrometer (Model 6460; Agilent Technologies, Santa Clara, CA).

Table 1
Concentrations in ng L^{-1} of neuro-active compounds and associated metabolites in wastewater effluent.

Treatment Plant #	Name of facility	population served	Effluent discharge $m^3 s^{-1}$	Treatment	HRT (h)	threo/erythro-hydrobupropion	hydroxybupropion	Bupropion	DiOH-CBZ	10-OH-CBZ	Oxcarbazepine	Carbamazepine	N-desmethylcitalopram	Citalopram	Fluoxetine	Gabopentin	Gluc-LMG	lamotrigine	DMV	Venlafaxine
1	Metropolitan	1,800,000	7.22	AS	11.6	4100	700	1100	160	640	210	600	120	290	47	1800	<100	940	3200	2400
3	Rochester	100,000	0.62	AS	24	3300	48	1600	100	240	210	540	200	200	76	1800	<100	570	2500	1900
4	Grand Rapids	12,000	0.36	AS	10.4	<10	<10	<10	25	<10	<10	130	<10	21	<10	<10	<100	370	51	80
6	Hutchinson	13,900	0.11	AS	68	3400	380	68	100	350	<10	420	<10	<10	15	88	<100	680	1400	1100
7	Marshall	13,000	0.19	AS	8.7	1300	370	200	200	470	130	380	18	67	18	800	<100	610	860	520
9	Fairmont	10,889	0.05	AS	7.5	2700	1000	970	180	190	<10	530	130	340	30	1900	<100	1000	3300	2800
10	Melrose	3,400	0.11	AS	NI	450	<10	28	<10	80	<10	84	16	36	16	220	<100	56	220	120
12	Lake City	5,300	0.03	AS	9.4	2600	790	1100	110	500	23	250	170	520	46	3100	<100	260	3100	2000
14	Ely	3,900	0.02	AS	NI	1900	400	400	97	550	88	360	100	270	63	480	<100	240	2500	3000
16	Eveleth	3,900	0.01	AS	20	4200	<10	920	190	1900	480	370	130	320	52	1100	<100	570	2600	2200
17	Spring Valley	2,561	0.01	AS	110	2000	<10	180	250	230	80	960	190	170	30	<10	<100	720	680	930
19	Sauk Center	4,111	0.02	AS	30	3400	300	550	150	970	290	1300	100	200	36	2500	<100	1200	2800	2300
20	Hinckley	1,438	0.02	AS	NI	1900	520	2600	140	310	<10	530	90	280	31	1600	<100	530	900	1300
21	Zimmerman	5,000	0.02	AS	43	5700	2000	700	19	420	<10	71	81	180	27	940	<100	900	5700	2500
22	Lester Prairie	1,774	0.01	AS	7.7	5400	2000	520	260	250	200	1100	32	130	21	1200	<100	360	10000	5500
	AS average value ± standard deviation					*2800 ± 1600*	*570 ± 650*	*570 ± 470*	*130 ± 77*	*470 ± 460*	*110 ± 140*	*510 ± 370*	*92 ± 66*	*200 ± 140*	*34 ± 20*	*1200 ± 940*	--	*600 ± 320*	*2700 ± 2600*	*1900 ± 1400*
2	Duluth	111,203	1.37	TF	9	200	130	44[a]	<10	490	<10	300	27	86	19	1600	<100	160	250	230
5	Austin	23,000	0.12	TF	12.9	3500	53	4300	63	93	23	380	90	222	28	1600	455	420	1600	1300
8	Worthington	11,283	0.09	TF	NI	730	<10	260	42	240	<10	430	92	66	23	<10	<100	460	22	600
11	Litchfield	7,500	0.07	TF	NI	950	<10	77	180	180	<10	490	58	88	15	84	<100	310	310	680
13	Luverne	4,617	0.02	TF	19	1500	<10	240	100	210	<10	340	140	210	31	180	<100	660	410	610
18	Pelican Rapids	2,476	0.03	TF	9	840	260	780	<10	79	<10	200	18	36	11	1100	<100	54	700	520
	TF average value ± standard deviation					*1300 ± 1200*	*73 ± 100*	*950 ± 1700*	*64 ± 68*	*210 ± 150*	*<10*	*360 ± 100*	*71 ± 46*	*120 ± 80*	*21 ± 8*	*760 ± 760*	--	*340 ± 220*	*560 ± 580*	*670 ± 360*
15	East Grand Forks	8,000	0.05	Pond	NI	20	14	<10	400	<10	<10	1500	10	27	<10	430	<100	1000	<10	<10
23	Williams	865	0.001	Pond	86	20	<10	<10	39	240	<10	38	<10	<10	<10	160	<100	390	1200	750
24	Lynd	410	NA	Pond	NA	20b	85	<10	28	<10	<10	87	<10	<10	<10	1100	<100	93	20[b]	12
	Pond average value ± standard deviation					*20*	*35 ± 43*	*<10*	*160 ± 210*	*79*	*<10*	*530 ± 820*	*<10*	*<10*	*<10*	*560 ± 480*	--	*490 ± 460*	*620 ± 880*	*260 ± 430*

Treatment plant #s correspond to rank by design flow; AS = activated sludge; TF = trickling filter; Pond = stabilization pond; HRT = WWTP hydraulic retention time < corresponds to below method detection limits; NA = Not applicable; NI = no information made available.

[a] Quantified from LC–TOF-MS, parent not detected LC/MS/MS.

[b] Detected but parent ion not detected LC–TOF-MS; DiOH-CBZ = 10,11,-dihydro-10,11,-dihydroxycarbamazepine; 10-OH-CBZ = 10-hydroxy-carbamazepine; Gluc-LMG = 2-N-glucuronide-lamotrigine; DMV = *O*-desmethyl-venlafaxine.

Table 2
Downstream sampling site parameters and total metabolite:parent ratio.

							WWTP	DS	WWTP	DS	WWTP	DS	WWTP	DS
WWTP	Treatment[a]	Receiving Stream	Sampling date	f_{ww}[b]	Median hydraulic transit time to downstream sampling site[c] (min)	Distance from WWTP to downstream sampling site (m)	metabolite:Bupropion	metabolite:Bupropion	metabolite:Carbamazepine	metabolite:Carbamazepine	metabolite:Citalopram	metabolite:Citalopram	metabolite:Venlafaxine	metabolite:Venlafaxine
St. Paul	AS	Mississippi River	9/24/2010	0.07	NM	1500	4.2	2.6	1.3	2.6	0.4	0.9	1.3	1.2
Rochester	AS	Zumbro River	9/22/2010	0.27	170	2000	2.2	2.9	0.6	0.8	1.0	0.3	1.3	1.0
Grand Rapids	AS	Mississippi River	9/30/2010	0.05	NM	480	2.4	ND	0.2	ND	0.0	0.6	0.6	0.0
Hutchinson	AS	South Fork of the Crow River	9/14/2010	0.16	73	710	55	2.8	1.1	0.6	0.7	0.7	1.3	1.3
Marshall	AS	Redwood River	10/7/2010	0.19	120	2100	7.9	2.9	1.8	2.8	0.3	0.7	1.6	1.8
Fairmont	AS	Center Creek	9/9/2010	0.84	220	380	3.9	4.2	0.7	0.6	0.4	1.1	1.2	1.1
Melrose	AS	Sauk River	9/17/2010	0.10	99	1100	16	0.1	1.1	0.1	0.5	0.7	1.8	29.7
Lake City	AS	Mississippi River	9/23/2010	NA	NM	140	3.2	8.5	2.4	2.9	0.3	0.5	1.5	1.3
Ely	AS	Shagawa Lake	9/28/2010	NA	NM	21	5.9	ND	1.8	ND	0.4	ND	0.8	ND
Eveleth	AS	Elbow Creek	9/29/2010	0.93	7	44	4.6	4.4	5.6	8.8	0.4	0.5	1.2	1.2
Spring Valley	AS	Spring Valley Creek	9/21/2010	0.08	51	270	11	15.1	0.5	0.9	1.1	1.5	0.7	0.5
Sauk Center	AS	Sauk River	9/16/2010	0.03	120	1000	6.7	ND	0.9	0.0	0.5	0.6	1.2	1.1
Hinckley	AS	GrindstoneRiver	9/2/2010	0.04	300	2200	9.6	1.2	0.9	0.0	0.3	0.4	0.7	0.8
Zimmerman	AS	Tibbits Brook	9/3/2010	0.53	6	30	11	8.5	6.2	3.7	0.4	0.4	2.3	1.3
Lester Prarie	AS	South Fork of the Crow River	9/15/2010	0.00	NM	2500	14	1.0	0.4	ND	0.3	ND	1.9	2.8
		Activated sludge average					*11*	*4.5*	*1.7*	*2.0*	*0.5*	*0.7*	*1.3*	*3.2*
Duluth	TF	Lake Superior	10/1/2010	0.13	NM	310	8.3	8.2	1.6	0.7	0.3	ND	1.1	1.2
Austin	TF	Cedar River	9/8/2010	0.12	48	530	1.8	1.1	0.4	0.6	0.4	0.5	1.2	1.2
Worthington	TF	Okabena Creek	9/9/2010	0.98	NM	2700	3.8	6.6	0.7	0.5	1.4	2.1	0.0	0.1
Litchfield	TF	Jewitts Creek	10/8/2010	0.18	29	380	13	6.4	0.7	2.4	0.7	1.5	0.5	0.5
Luverne	TF	Rock River	10/6/2010	0.02	11	140	7.1	ND	0.9	ND	0.7	1.1	0.7	0.9
Pelican Rapids	TF	Pelican River	10/19/2010	0.01	26	450	2.4	ND	0.4	ND	0.5	ND	1.3	ND
		Trickling filter average					*6.2*	*5.6*	*0.8*	*1.1*	*0.7*	*1.3*	*0.8*	*0.8*
East Grand Forks	Pond	Red River of the North	10/21/2010	0.0004	NM	620	3.6	0.2	7.2	ND	ND	ND	1.0	D
Williams	Pond	Williams Creek	10/20/2010	0.06	10	12	4.4	ND	0.3	0.5	0.4	ND	1.7	2.2
Lynd	Pond	Red wood River	11/23/2010	NA	NM	300	13	ND	0.3	ND	1.0	ND	1.7	ND
		Stabilization pond average					*7.0*	*0.2*	*2.6*	*0.5*	*0.7*	*NA*	*1.4*	*2.2*

[a] AS = activated sludge; TF = trickling filter; Pond = stabilization pond; WWTP = wastewater treatment plant effluent.
[b] fww = fraction of wastewater (effluent discharge/(effluent discharge + upstream discharge).
[c] Median hydraulic transit times were determined by dye studies (Lee et al., 2011); DS = downstream, NM = not measured, ND = parent not detected, D = only metabolite detected.

Extracts previously analyzed by LC/MS/MS were subsequently injected (within 1 day) onto an HPLC system connected to a time-of-flight mass spectrometer model 6520 Agilent (Agilent Technologies, Santa Clara, CA). The initial identification of neuro-active metabolites in water samples was performed by evaluation of the accurate mass (±0.0030 m/z) of prominent peaks, diagnostic ions extracted from the full scan LC–TOF-MS chromatogram, and fragment ions indicative of neuro-active metabolites (bupropion, hydroxy-bupropion, erythro-bupropion, threo-hydrobupropion, carbamazepine), 10,11,-dihydro-10,11,-dihydroxycarbamazepine (DiOH-CBZ), 10-hydroxy-carbamazepine (10-OH-CBZ), citalopram, N-desmethylcitalopram (DMCit), gabapentin, lamotrigine, 2-N-glucuronide-lamotrigine (Gluc-LMG), oxcarbazepine, venlafaxine and O-desmethyl-venlafaxine (DMV). Diagnostic ions for each compound are shown in SM Fig. 1. Following identification of possible metabolites, authentic standards were used to verify retention times and mass spectra; bupropion, carbamazepine, DiOH-CBZ, 10-OH-CBZ, citalopram, and gabapentin were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA); identification of fluoxetine, lamotrigine, oxcarbazepine, and DMV (purchased from Sigma Aldrich (St. Louis, MO) was verified by LC–TOF-MS analysis. Authentic standards were not readily available for the metabolites threo-hydrobupropion, erythro-bupropion, DMCit, and Gluc-LMG.

Reported concentrations were based on LC/MS/MS results for compounds where standards and analytical techniques were previously established (bupropion, hydroxy-bupropion, carbamazepine, fluoxetine, and venlafaxine) and quantified by a seven point calibration curve using authentic standards and the internal standard d_{10}-carbamazepine. Metabolites and compounds not initially identified by LC–MS/MS were quantified using methods that slightly varied based on availability of authentic standards (SM Table 1). Concentrations of DiOH-CBZ, 10-OH-CBZ, oxcarbazepine, and DMV were calculated relative to parent compound concentrations determined by LC–MS/MS, using LC–TOF-MS diagnostic ions areas for the metabolite and parent compound relative to the internal standard d_{10}-carbamazepine, accounting for differences in ion response based on analysis of individual standards in an uncontaminated surface water sample to compensate for effects of matrix suppression. Relative

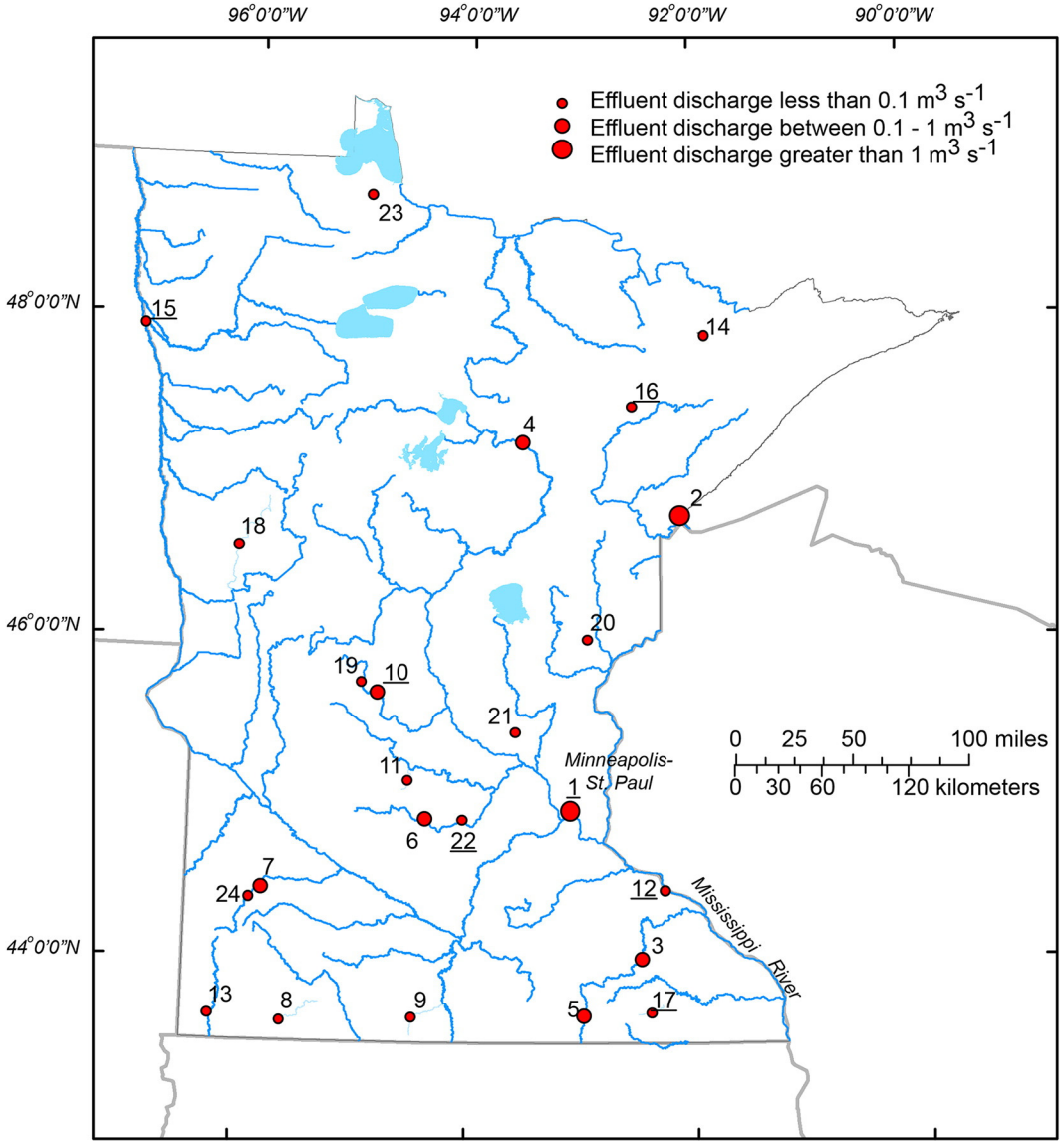

Fig. 1. Location of sampling sites (Lee et al., 2011), Minnesota, USA; underlined sites indicate where more than 3 neuro-active compounds and associated metabolites were detected in the sample collected upstream from the effluent outfall; base from the Minnesota Department of Natural Resources, 1:24,000, Universal Transverse Mercator Projection, Zone 15.

concentrations of erythro-hydrobupropion and threo-hydrobupropion were determined by a single point calibration comparing LC–TOF-MS diagnostic ion areas to the buproprion concentration determined using LC–MS/MS. Because standards for erythro-hydrobupropion and threo-hydrobupropion were not available, we assumed that the response factor for each of the metabolite ion and parent ion was similar. Concentrations of citalopram, DMCit, lamotrigine, and Gluc-LMG, were determined by a single point calibration comparing LC–TOF-MS diagnostic ion areas relative to the internal standard d_{10}-carbamazepine, accounting for differences in ion response based on analysis of individual standards in an uncontaminated surface water sample. The method detection limit was defined as the lowest concentration of the chemical that yielded minimum ion signal-to-noise ratios of 3:1 for both the quantitation and the confirmatory ions.

To evaluate the effectiveness of the LC–MS/MS and LC–TOF-MS methods, water samples (n = 2, LC–MS/MS; n = 1, LC–TOF-MS) were spiked with authentic standards (40 ng L^{-1} LC–MS/MS, 80 ng L^{-1} LC–TOF-MS), and the concentrations were determined; recovery ranged from 71-108%, mean 96% for LC–MS/MS, 65-116%, mean 81% for LC–TOF-MS (SM Table 1). Analytical variability for neuro-active compounds (bupropion, carbamazepine, fluoxetine, lamotrigine, and venlafaxine) was evaluated by replicate analyses consisting of one sample analyzed in duplicate, and one sample analyzed in triplicate and averaged 3% (SM Table 1). Analytical variability for neuro-active metabolites and compounds not quantified by LC–MS/MS (erythro-hydrobuprion, threo-hydrobupropion, 10-OH-CBZ, DiOH-CBZ, oxcarbazepine, citalopram, DMCit, Gluc-LMG, DMV) was evaluated by analyzing one sample in duplicate and averaged 16% (SM Table 1). Quality assurance quality control consisted of 7 field-blank samples and 2 field-duplicate pairs. Targeted compounds were not detected in any of the blank samples. LC–TOF-MS is a useful tool for establishing the presence of neuro-active pharmaceuticals and associated metabolites (Ferrer and Thurman, 2012), and one of the goals of this study was to demonstrate the utility of this technique. Equipped with information on compound occurrence, standards and labeled standards (e.g. deuterated and/or ^{13}C labeled structural analogs) can then be used to refine and/or expand contaminants evaluated by LC–MS/MS, which is more sensitive with respect to absolute compound concentration. For those compounds determined solely by LC–TOF-MS (citalopram, DMCit, gabapentin, and lamotrigine), reported concentrations are semi-quantitative.

The LC–TOF-MS measured concentrations of carbamazepine, oxcarbazepine, 10-OH-CBZ, and DiOH-CBZ were summed for each of the WWTP effluents (ng L^{-1}), this value multiplied by the measured wastewater effluent discharge (m^3 s^{-1}), and then divided

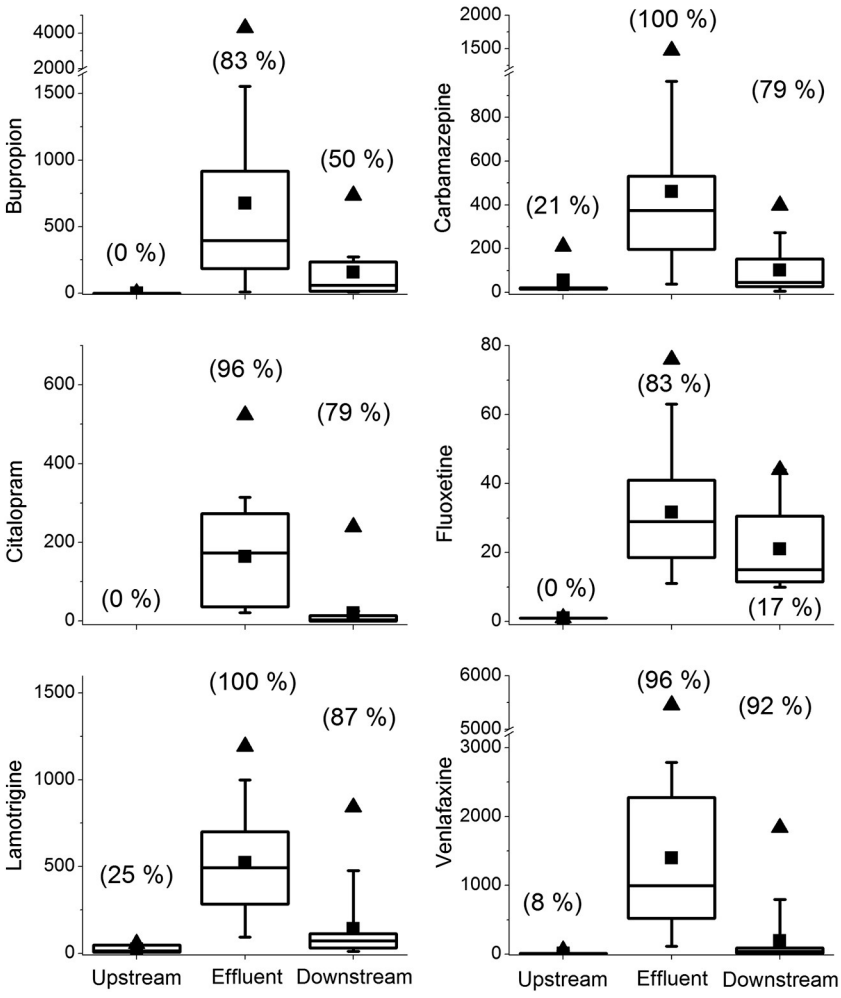

Fig. 2. Parent neuro-active compounds separated by sampling location relative to wastewater effluent outfall, all concentrations in ng L^{-1}, box diagram corresponds to quartile ranges, whiskers represent 10–90% values, mean value represented by black square, maximum value represented by black triangle, values in parentheses correspond to percent detection in sampled waters associated with treatment plants receiving municipal wastewater (n = 24); not shown gabapentin (detected 8% of upstream samples, detected in 88% of effluent samples, detected in 54% of downstream samples).

by the estimated human population being served by the respective wastewater treatment plants to give a per capita load of carbamazepine compounds from each of the WWTPs.

3. Results and discussion

3.1. Occurrence in wastewater treatment plants

Neuro-active pharmaceuticals and associated metabolites were evaluated at 24 different wastewater facilities across a broad geographic region (Fig. 1) that serve nearly 40% of the Minnesota's population. The evaluated wastewater treatment facilities represent a broad wastewater spectrum encompassing different demographics (e.g. small towns vs. large metropolitan areas) and varying types of treatment (Table 1). Treatment processes at each of the evaluated sites were grouped into three classes; activated sludge (n = 15), trickling filter (n = 6), stabilization ponds (n = 3), and the quartile ranges, median, mean concentration ± standard deviation evaluated for each group (Table 1). The seven neuro-active pharmaceutical compounds, bupropion, carbamazepine, citalopram, fluoxetine, gabapentin, lamotrigine and venlafaxine, were frequently detected at tens of ng/L to μg/L concentrations in the 24 municipal wastewater effluents and receiving waters (Fig. 2, Table 1). Fig. 2 shows that concentrations were higher in wastewater relative to downstream receiving water. The targeted neuro-active pharmaceuticals were frequently detected in wastewater (83–100% detection, n = 24), less frequently detected in downstream receiving waters (17–92%), and much less frequently detected in urface upstream from the effluent outfall (0–25%). Concentrations varied considerably between treatment plants, but in general concentrations (reported as the mean ± standard deviation, n = 24) were the highest for venlafaxine and its major metabolite DMV (1400 ± 1300 ng L^{-1}, 1800 ± 2300 ng L^{-1}) and for bupropion and threo/erythro-hydrobupropion (700 ± 1000 ng L^{-1}, 2100 ± 1700 ng L^{-1}). Other consistently identified neuro-active pharmaceuticals include gabapentin (1000 ± 900 ng L^{-1}), lamotrigine (520 ± 320 ng L^{-1}), carbamazepine and its major metabolites 10-OH-CBZ and DiOH-CBZ (480 ±380 ng L^{-1}, 360 ± 400 ng L^{-1}, 120 ± 100 ng L^{-1}). Citalopram and its major metabolite DMCit (160 ± 130 ng L^{-1}, 80 ± 60 ng L^{-1}) and fluoxetine (28 ± 18 ng L^{-1}) were also routinely detected in wastewater effluent, although generally at relatively lower concentrations. Norfluoxetine (the primary metabolite of fluoxetine) was not identified above detection limits (10 ng L^{-1}) and does not appear to occur at concentrations similar to fluoxetine in wastewater effluent. The Phase II metabolite of lamotrigine (Gluc-LMG) was only identified in one wastewater effluent, although we have observed it at substantial concentrations in other studies (Ferrer and Thurman, 2010). Other Phase II metabolites (conjugated metabolites) were not found at similar concentrations as the parent compounds and Phase I metabolites discussed above.

Pharmaceutical compounds are only partially removed by wastewater treatment processes, primarily due to biodegradation by the microbial community and sorption to solid material (Kwon and Armbrust, 2006; Miège et al., 2008; Hörsing et al., 2011). In general, the concentrations of parent neuro-active compounds and associated metabolites (Table 1) were lower in wastewater effluent relying primarily on trickling filter processes as compared to activated sludge processes, although the results were not significant ($p > 0.05$). There was no linear correlation ($r^2 = 0.2$, n = 17) between the hydraulic retention time of each wastewater treatment plant and the summed concentration of targeted neuro-active compounds and their metabolites. Because carbamazepine compounds are relatively unaffected by treatment processes (Zhang et al., 2008), a per capita load of carbamazepine compounds from each of the WWTPs was determined to evaluate if potential differences in demographics influenced the concentrations of carbamazepine compounds (Fig. 3). The per capita load ± standard deviation of carbamazepine compounds from the 23 municipal domestic WWTPs with continuous discharge was 0.57 ± 0.35 mg day^{-1}(one of the evaluated WWTPs discharged effluent seasonally). Somewhat surprisingly, this per capita load was relatively consistent and potential demographic differences (i.e. large metropolitan areas vs. small towns) were not apparent. Influent concentrations of neuro-active compounds were not measured, thus, we turned to the use of metabolite:parent compound ratios to give us clues about the persistence of neuro-active pharmaceuticals in treatment plants and surface waters.

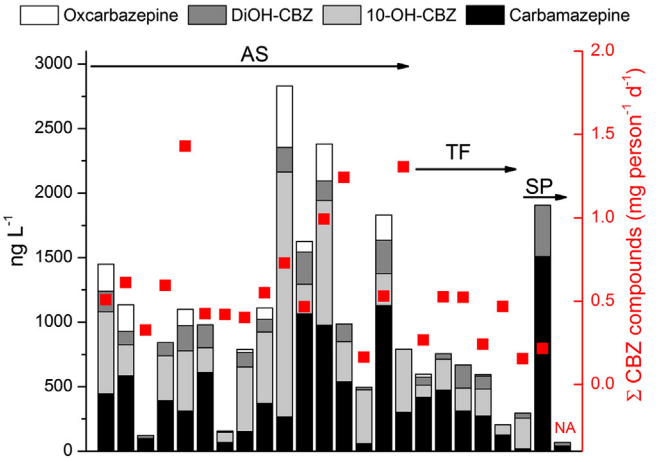

Fig. 3. Carbamazepine compound concentrations grouped by treatment type; AS = activated sludge, TF = trickling filter, SP = stabilization pond, arranged in decreasing order of wastewater effluent discharge; red square indicates (CBZ) carbamazepine compound mass in evaluated wastewater treatment plant effluents per capita. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Metabolite to parent ratios

The relative importance of major metabolites as compared to the parent compound was assessed by evaluating the ratio between the sum of concentrations of major metabolites divided by the measured concentration of the parent compound (Fig. 4, Table 2). Fig. 4 shows that for bupropion, carbamazepine, citalopram, and venlafaxine the median metabolite:parent compound ratio ranged from 0.4 to 6; consequently, metabolites represent a substantial amount of the total mass of neuro-active compounds in surface waters. Mean metabolite:parent compound ratios were higher for WWTPs utilizing activated-sludge processes as compared to trickling-filter processes; only 3 WWTPs utilizing stabilization ponds were evaluated and therefore limit generalized observations. The bupropion metabolites were generally found at greater concentrations than the parent compounds in all of the wastewater effluents evaluated, and the summed concentrations of bupropion and associated metabolites was higher in effluents utilizing activated sludge. For the carbamazepine metabolites, results indicate that 10-OH-CBZ is generally the dominant metabolite, followed by DiOH-CBZ (Fig. 3). At WWTPs using activated-sludge processes, carbamazepine metabolites were generally detected at higher concentrations than in trickling filters and stabilization ponds. Citalopram generally was found at higher concentrations than its primary metabolite DMCit, and mean metabolite:parent ratios were similar between treatment processes. Concentrations of the metabolite DMV generally exceeded concentrations of its parent compound venlafaxine in effluents using activated-sludge processes, whereas, in effluents using trickling filters, DMV was comparable to concentrations of venlafaxine.

Metabolites of neuro-active pharmaceuticals were observed at concentrations that generally exceeded or were similar to parent compound concentrations in effluents and surface waters downstream

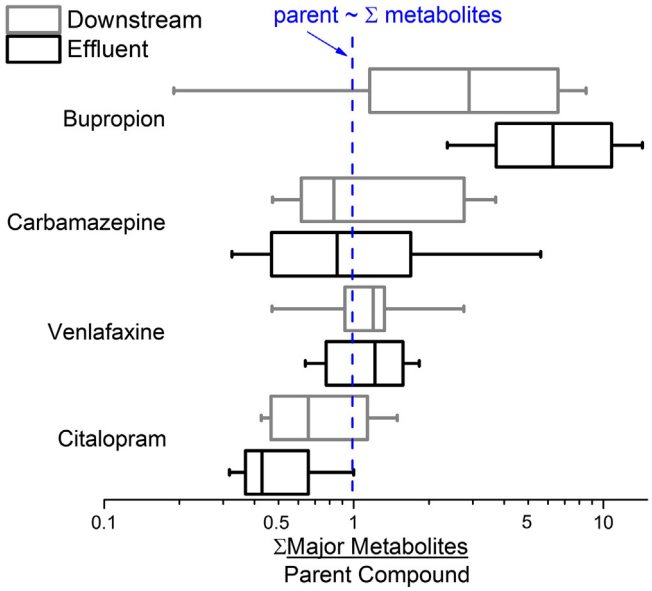

Fig. 4. Ratio between the sum of major metabolites and parent neuro-active compounds in wastewater treatment plant effluent and downstream; box diagram corresponds to quartile ranges, line represents the median, whiskers represent 10–90% values, blue reference line indicates when the metabolite concentrations are equivalent to the parent compound concentration. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

from effluent outfalls. These Phase I metabolite compounds (e.g. threo/erythro hydrobupropion, DMV), also can be neuro-active (Howell et al., 1993; Haas et al., 2004) and generally are found at much higher concentrations in human urine (based on pharmacological studies) than the parent compounds, as indicated by the following examples; threo-hydrobupropion:bupropion (19:1, Haas et al., 2004), cbz-DiOH: carbamazepine (70:1, Reith et al., 2000), DMCit (1:1, Dalgaard and Larsen, 1999), and DMV:venlafaxine (6:1, Howell et al., 1993). Because the ratios between metabolites and parent compounds in wastewater effluent (Table 2) were considerably smaller than reported in urine: 1) the metabolites are either more labile relative to the parent compounds and being degraded by wastewater treatment processes, 2) the parent compounds are being reformed from the metabolites, and/or 3) parent compounds are present at higher concentrations due to the disposal of unused medications. Additionally, while pharmacokinetic work has shown that the 10-OH-CBZ is not a significant human metabolite of carbamazepine (Reith et al., 2000), our results indicate that 10-OH-CBZ is routinely found at concentrations higher than carbamazepine, and detected more frequently than other carbamazepine metabolites (Fig. 3). Other studies identified the presence of 10-OH-CBZ in WWTP effluents, although conflicting information exists on the relative importance of this metabolite and these studies were based on a limited number (one to three) of wastewater treatment plants (Miao et al., 2005; Leclercq et al., 2009). One possible explanation is that 10-OH-CBZ is being transformed from oxcarbazepine (increasingly prescribed as an alternative to carbamazepine, Schmidt and Sachdeo, 2000); however, there was no correlation between oxacarbazepine and 10-OH-CBZ detection and concentrations. Widespread identification of 10-OH-CBZ in this study was based on the most comprehensive sampling of neuro-active compounds in wastewater effluents to date, but it is unclear whether this is due to changes in patient treatment (switching from carbamazepine to oxcarbazepine) or microbial formation of 10-OH-CBZ from carbamazepine and/or other metabolites in the wastewater treatment processes. Because influent concentrations of neuro-active compounds were not measured as part of this study, observations on treatment efficacy and potential mechanisms of removal should be regarded as preliminary yet valuable findings that warrant further investigation.

3.3. Transport of psychoative drugs to rivers and streams

Neuro-active pharmaceuticals and associated metabolites were frequently detected at locations downstream from the wastewater effluent outfalls (Fig. 2). In contrast, the targeted neuro-active pharmaceuticals and associated metabolites were only sporadically detected in receiving waters upstream from the wastewater effluent outfall (Fig. 2). Lamotrigine, carbamazepine, and venlafaxine were detected with the most frequency in upstream sample locations (25%, 21%, 8%, respectively). Metabolites were detected at a lower frequency in upstream samples than the parent compounds and only the following target metabolites were identified; threo-hydrobupropion (detected in 8% of upstream samples, n = 24), 10-OH-CBZ (8%), DMV (17%). Median hydraulic transfer times between the effluent outfall and the corresponding downstream sampling ranged from 6 min to 300 min (Table 2). Variability in wastewater discharge and associated contaminant loading to receiving waters is substantial (Nelson et al., 2011). Therefore, unless the same parcel of water is sampled from the point where the effluent and upstream waters completely mix to the downstream sampling point, quantitative evaluation of environmental fate is not possible. Nonetheless, preliminary observations can be made about the environmental persistence of these neuro-active pharmaceuticals. Limited attenuation of these compounds was observed in the downstream sample based on an evaluation of the ratio between the load (measured concentration × measured discharge) at the downstream sampling location and the total loading from upstream and effluent sources. Additionally, there was no significant relation between hydraulic transfer time and the ratio between downstream mass load and the combined mass load from effluent and upstream sources. In general, the greater the fraction of wastewater effluent at the downstream sampling location (f_{WW}) the higher the observed concentration of neuro-active compounds. Considerable variation was observed in metabolite:parent ratios between different effluents and between paired effluent and downstream samples (Table 2), indicating the influence of source variability and multiple attenuation mechanisms. The majority of sites in which more than 3 neuro-active compounds were detected (shown as underlined site numbers in Fig. 1) were in rivers having WWTPs 5 to 30 km upstream (Lee et al., 2011) and illustrate the environmental persistence of these compounds. Metabolites appear to be less persistent due to their lower frequency of detection. Further research evaluating environmental attenuation processes of these compounds is warranted.

4. Conclusion

Wastewater and environmental processes transform neuro-active pharmaceuticals into metabolites that are potentially bio-active (Haas et al., 2004; Howell et al., 1993; Lajeunesse et al., 2011), but are infrequently studied due to a limited knowledge of their prevalence. In this study we used LC–TOF-MS to identify metabolites of neuro-active pharmaceuticals in 24 municipal WWTPs from around the state of Minnesota. The widespread existence of neuro-active metabolites in municipal wastewater effluent and associated receiving waters from diverse sources and demographics clearly illustrates that these compounds are widespread in surface waters and need to be considered when evaluating the environmental relevance of neuro-active pharmaceuticals.

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Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2013.04.099.

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