UNIVERSITAT JAUME·I

Anàlisi de pesticides en aigües i sols mitjançant cromatografia líquida micel·lar

Tesi presentada per Pasqual Roca Genovés,

per a obtindre el grau de Doctor

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CERTIFIQUEN

Que la present Memòria, ***Anàlisi de pesticides en aigües i sols mitjançant cromatografia líquida micel·lar***, constitueix la tesi doctoral de:

PASQUAL ROCA GENOVÉS

Així mateix, certifiquen haver dirigit i supervisat les parts teòriques, metodològiques, instrumentals i aplicacions dels diferents treballs, així com també la seva redacció.

I perquè conste als efectes oportuns, signem la present.

Josep Esteve i Romero

Juan Peris Vicente

Castelló de la Plana, 9 de Juny de 2017

AGRAÏMENTS

Mai oblidaré com va sorgir la idea de realitzar la tesi doctoral. Recorde que em quedaven unes poques assignatures per finalitzar Llicenciatura en Química. Havia sortit amb la bicicleta muntanya cap a Eslida juntament amb el meu director de tesi Josep, que aleshores ja era un amic i també un dels meus professors a la Universitat. Recorde que en arribar a Eslida, ens vam prendre un breu descans i després d'una estona de xerrada li vaig dir "quan acabe la carrera i faça el màster, m'agradaria realitzar la tesi i que sigues el meu director". Uns anys més tard, em trobe en el laboratori escrivint aquestes paraules per finalitzar el que anys enrere vam comentar enmig de la muntanya.

En primer lloc, vull agrair als meus directors de tesi, Josep Esteve i Juan Peris la seua gran ajuda i suport durant aquests anys, ja que sense ells no hauria pogut aconseguir aquest propòsit.
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Índex

Pròleg

El treball presentat en aquesta Memòria s'emmarca en una línia d'investigació desenvolupada pel grup de "Química Bioanalítica" del Departament de Química Física i Analítica de la Universitat Jaume I, amb codi 029 de l'Oficina per a la Cooperació en Investigació i Desenvolupament Tecnològic. Aquesta línia té com a objectiu el desenvolupament de nous mètodes analítics en l'àrea de protecció del medi ambient, fent ús de la cromatografia líquida micel·lar (MLC) i de quimiometria. L'estudi que es presenta es va elaborar en col·laboració amb l'empresa dedicada a la gestió del cicle integral de l'aigua Fomento Agrícola Castellonense, S.A. (FACSA) a través dels contractes de col·laboració nº 11I036.01/1 "*Activitats d'assessorament i assistència tècnica en l'àrea de la determinació de melamina i altres contaminants industrials en mostres d'aigües residuals*" i nº 11I358.01 "*Determinació toxicològica de contaminants (melamina, pesticides, etc.) en aigües residuals d'estacions depuradores d'aigües residuals (EDAR)*", i el subministrament de mostres d'aigües residuals i fangs. També ha rebut el finançament de la Universitat Jaume I, per mitjà del projecte P1-1B2012-36: "*Modificació dels mecanismes de retenció a través de la introducció d'equilibris secundaris per a la separació de compostos bàsics en cromatografia líquida d'alta resolució*".

El medi ambient és l'entorn en el qual es desenvolupa l'activitat dels éssers vius, i que permet la interacció entre ells. Engloba la natura, la societat i la cultura existents en un moment i localització determinats. Està format per components biòtics (la població humana, la flora, la fauna i els microorganismes), abiòtics (clima, la geologia, la geografia, la litosfera, la hidrosfera i l'atmosfera), artificials (context socioeconòmic, activitat laboral, urbanització, conflictes socials, moral, estètica, tradicions) i les relacions entre ells (relacions alimentàries, competència i col·laboració entre éssers vius, transformació de l'energia i l'intercanvi de matèria entre els components biòtics i abiòtics). Per tant, el medi ambient és el producte de la interacció dinàmica de tots els elements, objectes i éssers vius presents en un lloc. La relació que s'estableix entre aquests elements és el que, des d'una visió integral, conceptualitza el medi ambient com a un sistema. Els éssers vius i el medi ambient estan en íntima relació. Les

característiques d'una espècie biòtica (morfologia, comportament i estructura social) depenen del seu entorn, i es modifiquen enfront de qualsevol canvi d'aquest, per a adaptar-se i sobreviure. Així mateix, l'activitat dels éssers vius produeix un canvi en el medi ambient, els més rellevants són els relacionats amb l'obtenció de recursos i d'aliments, la producció de residus, la transformació intencionada i l'acumulació dels individus en una àrea reduïda.

Els éssers humans han exercit una influència notable en nombrosos aspectes sobre el medi ambient des de l'Antiguitat, a causa de la urbanització, la producció d'aliments i la manufactura tecnològica. De tots aquests, la contaminació d'origen humà ha estat l'amenaça més gran per a l'equilibri ecològic i per a la mateixa població. La contaminació ambiental es defineix com a la presència d'agents químics, físics o biològics a la biosfera, que poden tindre efectes nocius per a la seguretat i la salut dels éssers vius. Aquest problema esdevingué rellevant a partir de la Revolució Industrial. La població humana va començar a augmentar exponencialment, així com la producció de materials i el vessament de residus al medi ambient cada volta més variats, no-biodegradables, i tòxics. Aquests compostos es dispersaren ràpidament per la biosfera, causant un enverinament de moltes espècies animals i vegetal, i el malbaratament de sols i aqüífers. L'impacte general sobre el medi ambient va ser intens, global, nociu i d'efectes acumulatius, de manera que un segle després ja es podia apreciar clarament. En pràcticament totes les àrees habitades (i en menor mesura en la resta), l'ecosistema es va modificar fortament, reduint la qualitat de vida de la flora i la fauna autòctones. En alguns casos extrems, l'equilibri ecològic s'hi ha destruït o es troba en una mala situació. Aquesta pol·lució també afecta la població, directament per contacte o a partir de la cadena tròfica, estimulant l'aparició de malalties, o de forma indirecta per la desaparició d'espècies considerades útils. Hui en dia, la societat és plenament conscient dels problemes causats per la contaminació i la necessitat de protegir el medi ambient. Per tant, els poders públics han aprovat nombroses lleis i regulacions per disminuir i avaluar el nivell de contaminació a la biosfera. Una de les majors fonts de contaminació són els pesticides, a causa del seu extens ús i la seua persistència.

Els pesticides o plaguicides són substàncies o mescles de substàncies utilitzades per a prevenir (preemergència), controlar, combatre o eliminar (postemergència), les plagues mitjançant la seua repulsió, incapacitació, inhibició del creixement o de la reproducció, o la mort. Aquest concepte és molt ambigu, i, des d'una òptica antropocèntrica, s'aplica a éssers vius, el comportament natural dels quals resulta perjudicial, des d'un punt de vista econòmic,

de benestar, estètic o de salut, per als éssers humans: parasitar, danyar, o competir pels aliments contra els humans o altres espècies animals o vegetals criades i/o mantinguts amb finalitats alimentàries, decoratives o afectives, deteriorar la propietat, transmetre, induir o provocar malalties, o causar molèsties de qualsevol classe. S'utilitzen principalment en agricultura precollita, silvicultura i horticultura contra les espècies indesitjades de plantes, animals, bacteris o fongs, que poden interferir en les etapes de creixement, maduració, i perjudicar la salut o l'aspecte de la planta o l'arbre corresponent, així com disminuir la producció i la salubritat dels fruits i de les flors. El terme inclou també substàncies destinades a utilitzar-se com a reguladors del creixement de les plantes, defoliant, antiparàsites, dessecants, agents per a reduir la densitat de fruita o inhibidors de la germinació. En agricultura postcollita, s'utilitzen a les etapes d'emmagatzemament, elaboració, transport i distribució del producte agrícoles, aliments processats i pinsos per a mantenir l'aspecte i les propietats organolèptiques i nutricèutiques òptimes. En veterinària, s'administren als animals per a combatre ectoparàsits. En l'àmbit domèstic, públic i industrial també s'utilitzen en el manteniment de vies de transport, basses i corrents d'aigua, jardins, plantes ornamentals d'interior, i per a evitar problemes d'higiene o salut pública. Els desinfectants són pesticides que es gasten per a eliminar patògens en estat vegetatiu en objectes i ambients en contacte en l'home. Per tant, el seu nivell de toxicitat es redueix per a evitar que afecte la salut i la qualitat del material tractat. Es gasten en l'interior d'edificis, intal·lacions diverses, equipament i material d'ús quotidià i laboral, superfícies de ciment, fusta, metall o teles, en productes sanitaris d'aplicació dèrmica i inclús en aliments com a conservants.

Els pesticides més habituals són compostos orgànics i xenobiòtics que se sintetitzen a escala industrial. S'administren en forma sòlida (pols sec, pols humectable, pols soluble, gràmuls, microgràmuls, gels, ceres, tauletes, esquers), líquids (concentrats solubles, suspensions concentrades, emulsions concentrades, concentrats emulsionables, líquids miscibles, microencapsulats) i de gasos (fumigant, aerosol, gasos liquats). En aquestes formulacions, van acompanyats per diversos adjuvants que els estabilitzen i milloren la seua eficàcia: tensioactius, adhesius, emulsionants, estabilitzants, fotoprotectors, antitranspirants, colorants, repel·lents, vomitius, i en alguns casos els antídots. Alguns d'aquests compostos o els seus derivats tenen una toxicitat i persistència apreciables, i també representen un perill pel medi ambient.

Segons el seu objectiu, es poden classificar com a: algicida (algues), aracnicida (aràcnids), avicida (ocells), bactericida (bacteris), feromona (alteren el comportament sexual), fitoregulador (alteren el creixement natural de les plantes), fungicida (fongs), germicida (microorganismes), herbicides (males herbes), insecticida (insectes), micocida (xampinyons), miticida i acaricida (àcars), mol·lusquicida (mol·luscs), nematicida (cucs), parasiticida (paràsits), pediculicida (polls), icticida (peixos), predacida (depredadors), ovicides (ous d'insectes i àcars), rodenticida (rodadors) i termiticida (termites). Alguns poden servir contra unes quantes classes de plagues, o només contra espècies específiques dins de cada categoria. També es poden classificar a partir de la seua estructura química: alcaloide, arsenical, azol, carbamat, cumarina, dinitrocompost, fenòlic, neonicotinoide, piretroide, pirimidina, organoclorat, organofosfat, organometàl·lic, rotenoide, urea i triazina.

Els pesticides són molt beneficiosos per a la nostra societat, ja que ajuden a prevenir malalties, augmentar la producció d'aliments, a regular l'estètica del nostre entorn i a millorar la higiene pública i privada. Als últims anys, alguns inconvenients de la forta dependència dels plaguicides s'han tornat cada vegada més evidents. Els riscos mediambientals de l'ús de formulacions de pesticides són causades principalment pel seu extens ús (agrícola, industrial, domèstic i públic), la seua persistència al medi ambient, la seua toxicitat intrínseca (que va més enllà de l'objectiu inicial del pesticida), la seua mobilitat per la biosfera i a la bioacumulació en alguns éssers vius. Aquestes circumstàncies causen la contaminació de la biosfera, afecten el cicle de vida dels animals i les plantes autòctons, pertorben la cadena alimentària, trenquen l'equilibri ecològic, i estimulen l'emergència de plagues resistents als pesticides. La població també pot entrar en contacte amb els plaguicides per inhalació, dèrmica i ingestió, a través d'aliments, aigua, partícules en suspensió contaminades, materials tractats o durant el seu ús. L'exposició als pesticides està relacionada amb diverses malalties i disfuncions, com alteracions endocrines, problemes respiratoris, resposta al·lèrgica, malalties neurodegeneratives, la diabetis, la neurotoxicitat, la interrupció cardiovascular, i fins i tot en alguns casos de càncer, i per tant ha esdevingut un problema de salut pública global de la màxima importància. La Unió Europea, a través de la xarxa NORMAN, ha elaborat una gran llista de plaguicides prohibits o catalogat com a "contaminants emergents", els quals han de ser especialment controlats i regulats a causa dels seus potencials riscos ambientals i sanitaris. Entre ells, es pot destacar el tiabendazol, imazalil, diuron, terbutilazina i terbutrina. A causa del seu intens ús, també resulta interessant el control del o-fenilfenol, pirimetanil, clorpirifos i

dels octilfenols. Aquests compostos són derivats dels alquilfenols polietoxilats, que s'usen com a tensioactiu en nombroses formulacions. Per aquestes raons, la UE, els governs nacionals i alguns municipis han desenvolupat una gran quantitat de polítiques, lleis i regulacions per conciliar les preocupacions ambientals i de salut, amb les realitats econòmiques i estil de vida. En general, s'obliga a disminuir el vessament de pesticides i adjuvants al medi ambient, mitjançant la reducció del seu ús de les formulacions de plaguicides i el reciclatge dels abocaments. També és necessari el monitoratge de la quantitat de plaguicides en aigües residuals, en aqüífers, sols i sediments, per a comprovar el grau de pol·lució, la salubritat de la zona i el compliment amb les normes mediambientals.

Existeixen una gran diversitat de plaguicides amb estructures i propietats fisicoquímiques molt diverses. Per tant, s'ha desenvolupat un nombre molt elevat de mètodes analítics per a determinar aquests compostos en aigües residuals i sols, per mitjà de diverses tècniques, especialment per la cromatografia líquida. També proposen un pretractament de la mostra, que en aigües és una extracció i purificació, mentre que en el cas del sol és necessari lixiviar la mostra per a aïllar el pesticida al sobrenedant, el qual es tracta com l'aigua residual.

El grup de Química Bioanalítica s'inicià en la recerca sobre cromatografia liquida als anys 90, primer a la Universitat de València i a l'Institut de Recerca en Aliments de Norwich (Regne Unit) i després es va especialitzar en cromatografia liquida micel·lar (MLC). A l'inici, la recerca estava orientada al coneixement del fonament teòric d'aquesta tècnica innovadora, la qual va atraure el seu interés per la seua versatilitat, la seua accessibilitat, i el seu baix impacte sobre el medi ambient; i després, un cop establits els fonaments, els estudis es van dirigir cap al desenvolupament de noves aplicacions analítiques, en els camps de la química clínica, mediambiental, de seguretat alimentària, toxicologia i forense, basades en l'MLC.

La cromatografia líquida micel·lar (MLC) és una variant de la cromatografia líquida d'alta resolució de fase reversa (RP-HPLC), que es basa en l'ús de dissolucions de tensioactius neutres o carregats per damunt de la concentració micel·lar crítica (dissolucions micel·lars) com a fases mòbils. Per tant, les molècules de tensioactiu es troben formant micel·les. Aquestes estructures mantenen una gran diversitat de punts de solubilització: orgànic a l'interior, polar i, eventualment, electrostàtic a l'exterior, i miscel·lanis al punt intermedi. Aquestes dissolucions són heterogènies a escala microscòpica, ja que coexisteixen

dos medis marcadament diferents: el pseudomicel·lar i altre aquós a l'exterior. Per això, poden dissoldre tant compostos polars com hidròfobs. En l'MLC es poden gastar fases mòbils micel·lars pures (només amb el tensioactiu), però és més habitual afegir un xicotet volum de dissolvent orgànic per a millorar la qualitat de la resolució cromatogràfica, resultant en fases mòbils híbrides. L'ús de dissolucions micel·lars modifica la natura de la fase mòbil aquosa, a causa de la presència de les micel·les, i de la fase estacionaria, ja que els monòmers de tensioactiu es fixen sobre la seua superfície. El mecanisme de retenció és més complex que en les fases mòbils hidroorgàniques, per què els anàlits es reparteixen entre tres medis en lloc de dos: la fase estacionària, la fase mòbil i la micel·la. A causa d'aquest fenomen, l'MLC constitueix una fabulosa opció per a la separació de mescles de soluts catiònics, aniònics, i neutres dins d'un elevat rang d'hidrofobicitat, mitjançant elució isocràtica. També cal dir a aquest respecte que el mecanisme de retenció és altament estable i reproduïble, el que permet la seua modelització amb gran exactitud, emprant les tècniques quimiomètriques adequades.

D'altra banda, atés que les micel·les solubilitzen els compostes hidròfobs que formen fases separades de l'aigua, l'MLC permet la injecció directa de les mostres aquoses amb contingut oliós directament, o preferentment diluïdes amb dissolucions micel·lars, sense cap risc per al sistema cromatogràfic. Això representa un gran avantatge enfront d'altres tècniques de separació que requereixen llargs processos d'extracció, i simplifica la feina al laboratori. Així mateix l'elevat poder de solubilització fa de les dissolucions micel·lar excel·lents solvents per a extraure els anàlits de matrius sòlides mitjançant lixiviació. A més a més, el sobrenedant es pot injectar directament a la columna.

L'MLC presenta diversos aspectes pràctics interessants. La quantitat de compostos tòxics i inflamables que es fa servir en mètodes basats en l'MLC és molt menor que la utilitzada als basats en RP-HPLC clàssica, considerant tant la preparació de la mostra com l'etapa de resolució cromatogràfica. Per tant, resulten més respectuosos per al medi ambient i més segurs per al personal de laboratori. A causa de les seues característiques, els mètodes basats en MLC resulten més econòmics, senzills, i automatitzables que els de RP-HPLC. Des del seu inici en 1980, la MLC ha evolucionat fins a convertir-se en una alternativa pràctica a la RP-HPLC hidroorgànica. Gràcies a la gran quantitat d'estudis previs realitzats, i a nombrosos articles publicats dirigits al coneixement d'aquesta tècnica, els fonaments de l'MLC estan avui dia fermament establits.

La disponibilitat de noves ferramentes analítiques que possibiliten la millora dels procediments analítics, l'experiència prèvia del grup en el desenvolupament i validació de mètodes analítics, l'interés per involucrar-se en temes de defensa del medi ambient, i el repte analític, va portar la proposta d'una nova Tesi Doctoral sobre la determinació de pesticides mitjançant cromatografia líquida micel·lar, la Memòria de la qual es presenta a continuació. En aquesta tesi es mostrarà el desenvolupament de mètodes analítics basats en MLC per a la determinació de diversos plaguicides i un derivat de tensioactiu en aigües residuals i sols. Es descriurà la fase d'optimització de la preparació de la mostra i de les condicions cromatogràfiques. Posteriorment, els mètodes es validaran per a avaluar la seua capacitat per a identificar i quantificar els anàlits, i determinar el rang de concentracions analitzable. Finalment, els procediments desenvolupats s'aplicaran a mostres mediambientals, per a demostrar la seua aplicabilitat en anàlisi rutinària. Així doncs, els treballs desenvolupats tindran un fort impacte social en el camp de la protecció mediambiental.

Aquesta memòria consta de set capítols. A la introducció es descriu de forma detallada les característiques de la cromatografia líquida micel·lar i del procés de validació de mètodes analítics, i al segon es detallen els objectius generals i específics. Als capítols de tres a sis, es descriuen de forma específica els anàlits i la necessitat de la seua determinació, es detalla el procediment experimental i es discuteixen els resultats obtinguts a cada estudi. Finalment, al capítol set es presenten les conclusions generals estretes de la totalitat del treball realitzat. Per ser una Tesi escrita parcialment en anglés (Art. 24 de la NORMATIVA DELS ESTUDIS DE DOCTORAT, REGULATS PEL R.D. 99/2011, EN LA UNIVERSITAT JAUME I (Aprovada pel Consell de Govern núm. 19 de 26 de gener de 2012)), aquesta tesi ha de contenir un apartat prou ampli en valencià o castellà, que ha de formar part de l'enquadernació de la tesi on s'incloga necessàriament:

- Objecte i objectius de la investigació.
- Aportacions originals.
- Conclusions obtingudes i futures línies d'investigació.

Chapter 1

Introduction

1. Micellar liquid chromatography

Micellar liquid chromatography (MLC) is well-established branch of reversed-phase liquid chromatography. Almost three decades of experience have resulted in an increasing production of analytical applications. Current concern about the environment also reveals MLC as an interesting technique for "green" chemistry because it uses mobile phases containing >85% water, and innocuous and biodegradable chemicals. These micellar mobile phases have a low toxicity and, then the risk for the health of the laboratory staff by handling them and the amount of hazardous waste is minimized. The stationary phase is modified with an approximately constant amount of surfactant monomers, and the solubilizing capability of the mobile phase is altered by the presence of micelles, giving rise to a great variety of interactions (hydrophobic, hydrophilic, ionic and steric) with major implications in retention and selectivity. From its beginnings in 1980, the technique has evolved up to becoming a real alternative in some instances (and a complement in others) to classical RP-HPLC with aqueous-organic mixtures, owing to its peculiar features and unique advantages. The addition of an organic solvent to the mobile phase was, however, soon suggested in order to enhance the low efficiencies and weak elution strength associated with the mobile phases that contained only micelles. An important feature of MLC is the direct injection of food, medicinal and biological samples, without risk for the chromatographic system, by the solubilizing power of micellar solutions. This simplifies and expedites treatment, which confers analytical procedures greater accuracy and a lower cost.

An interesting advantage of MLC is the high stability and reproducibility of retention, which permits its modelling by empirical and mechanistic models with great accuracy to predict the retention changes when the mobile phase composition varies (surfactant and organic solvent concentrations), thus facilitating the optimization of separation conditions. In addition, the different equilibria inside the column among the solute, the mobile phase, and the modified stationary phase by monomers of surfactant have been exhaustively studied. In a sequential strategy, the retention of the solutes is not known *a priori*, and each set of mobile phases is designed by taking into account the retention observed with previous eluents. By contrast, in an interpretative strategy, the experiments are designed before the optimization

process and used to fit a model that will allow the prediction of the retention of each solute. This strategy is more efficient and reliable. The sequential strategy will be inadequate when several local and/or secondary maxima exist, as frequently occurs in chromatography, and may not give the best maximum, that is to say, the optimum. More often than not, the complexity of the mixtures of compounds studied and the relevant modification of their chromatographic behaviour when changing the mobile phase composition requires the use of computer-assisted simulations in MLC to follow the modifications in the chromatograms in detail. These simulations can be done with sound reliability thanks to the use of chemometric tools.

1.1 Description

Surfactants are amphiphilic compounds, containing a nonpolar hydrocarbon-like chain (tail) bonded to a polar group (head). According to the charge of the polar group, surfactants are classified as anionic, cationic, neutral or zwitterionic. Solved in water, they have a propensity to locate in the air- or oily liquid-water interface, thus altering the interfacial tension and free energy [1]. At concentrations over the critical micellar concentration (CMC), the surfactant monomers tend to aggregate to form a spherical-shaped structure: the normal-phase micelle. The hydrophobic tails stay in the core, whereas the hydrophilic and, for ionic surfactants, charged heads form an outer layer, oriented to the water. The palisade layer is the intermediate region (miscellaneous), and it consists of the inner atoms of the hydrophilic groups and the first few carbon atoms. Therefore, they have nonpolar, polar (and electrostatic for ionic surfactants) and intermediately polar solubilization sites, respectively. The number of monomers per micelle (number of aggregation) remains nearly constant for each surfactant. Therefore, a variation of the quantity of surfactant results in the subsequent modification of the number of micelles, whereas the concentration of the free monomer remains nearly constant and equal to the CMC. The structure of the micelle is supported by the hydrophobic interactions between the carbon-chained tails of the monomers. Micelles are dispersed in water, forming a colloid solution named micellar solution. These solutions are heterogeneous at a microscopical glance, as they contain two differentiated environments: the micellar pseudophase and the aqueous phase. Therefore, these solutions are able to solubilize

compounds in a high range of hydrophobicity and charge, even those slightly or non water-soluble [2,3].

Micellar liquid chromatography (MLC) is a form of reverse phase (RP)-HPLC, based on the use of micellar solutions as mobile phases. The addition of a surfactant to the mobile phase in RP-HPLC changes the chromatographic behaviour, if compared to that occurs using aqueous-organic mixtures. The presence of surfactant associated with the stationary phase and as micelles in the mobile phase in RP-HPLC implies a change in the retention mechanisms, which affects the retention time and selectivity. Solutes associated to micelles experience a microenvironment that is different from that of bulk solvent. Neutral and charged surfactants are used, which has major implications in both stationary and mobile phases. The versatility of MLC is due to the wide variety of interactions that are established among the eluted solutes, the modified stationary phase, the aqueous phase and micelles. Their eluent characteristics allow the analysis of compounds with a wide range of polarities and charge. The presence of a surfactant not only modifies the interactions established inside the column, but also reduces the necessary amount of organic solvent in the mobile phase and its evaporation rate. These characteristics are genuinely interesting given current concerns about reducing organic contaminant residues in laboratories. As the pH has an important role in the retention mechanism, a buffer must be added to the micellar mobile phase [4,5].

MLC shares the basic components of RP-HPLC systems, that is, a non-polar stationary phase and a polar aqueous mobile phase. However, hydro-organic mobile phases in conventional RP-HPLC are homogeneous, whereas micellar solutions are microscopically heterogeneous, being composed of two distinct media: the amphiphilic, and eventually charged, micellar aggregates (micellar pseudophase) and the surrounding bulk water or aqueous-organic solvent that contains surfactant monomers in a concentration approximately equal to the CMC. On the other hand, the stationary phase is modified by the adsorption of surfactant monomers, and reducing silanophilic interactions. With nonionic surfactants, only the polarity of the stationary phase changes, whereas with ionic surfactants, a net charge (positive or negative) appears on its surface with major implications [2,6,7].

The variety of interactions found in MLC do not exist in any homogeneous aqueous-organic mobile phase. Owing to the amphiphilic nature of surfactants, solutes can associate with both micelles and the surfactant-coated stationary phase through a combination of

electrostatic, hydrophobic, and steric interactions. For this reason, micellar mobile phases are compatible with a wide range of solutes (soluble to insoluble in water, and with different ionization states). The main strength of MLC lies precisely in the capability of performing and controlling the separation of mixtures of cationic, anionic, and uncharged polar and nonpolar solutes, with isocratic elution [2].

The major drawback of applying MLC to practical separations is still the low chromatographic efficiency, which is caused by resistance to mass transfer in the processes involving micelles and a surfactant-modified stationary phase. This is especially important since the increase of the micelle concentration causes a decrease in plate number. To overcome this problem, a common strategy is the addition of an organic solvent, giving rise to hybrid micellar mobile phases. The most commonly used are acetonitrile and short-chained monoalcohols. The organic solvent decreases the polarity of the aqueous solution, alters the micelle structure, and diminishes the hydrophobicity (and the charge for ionic surfactants) of the modified stationary phase. This favours and accelerates the transfer of the analyte from the stationary phase to the micelles, resulting in a reduction of the retention time and the broadness of the peak. Although the separation mode is still predominantly micellar in nature, the micelle is perturbed by the organic solvent. This can change the micellar parameters, such as the CMC and surfactant aggregation number. A high percentage of organic solvent can disrupt the micelle structure. The maximal allowable concentration depends on the type of organic solvent and surfactant [5,8]. Therefore, almost all applications in MLC use with hybrid micellar mobile phases in a buffered medium, that contains micelles, surfactant monomers, molecules of organic solvent, and water [2,6,8-10].

1.2 Characteristics of the micellar mobile phase

1.2.1 Surfactant selection

The selection is often limited to the following surfactants: the anionic sodium dodecyl sulphate (SDS), the cationic cetyltrimethylammonium bromide (CTAB), and the nonionic Brij-35 and Triton X-100, whose main characteristics are summarized in Table 1.1 [6].

A suitable surfactant for MLC should have a low CMC. A high CMC would imply operating at high surfactant concentration, which would result in viscous solutions, giving undesirable high system pressure and background noise in UV detectors. The CMC values of these surfactants in pure water are low enough for MLC. It should also be taken into account that the CMC is strongly affected by the presence of an organic solvent. The changes are related to the modification of the structure of the micelle, which also induces, at least partially, the reduced retention in MLC [5].

Table 1.1. Main parameters (in aqueous solution) of the surfactants most used in MLC [6].

	SDS	CTAB	Brij-35	Triton X-100
Formula	$C_{12}H_{25}SO_4Na$	$C_{19}H_{42}NHBr$	$C_{12}H_{25}(C_2H_4O)_{23}OH$	$C_{14}H_{22}O(C_2H_4O)_{9.5}$
Hydrophobic tail	Linear hydrocarbon	Linear hydrocarbon	Linear hydrocarbon	Branched hydrocarbon-phenyl
Polar head	Sulphate	Quaternary amine	Polyethylene oxide chain	Polyethylene oxide chain
Molecular mass (g/mol)	288.4	364.5	1225	625
CMC (mM)	8.1	0.83	0.06	0.3
Aggregation number at 25ºC	62	90	41	140
Partial specific volume (L/mol)	0.246	0.364	1.12	0.743
Krafft point (ºC)	16	26	-----	-----
Cloudy point at 1-6 % solution (ºC)	-----	-----	100	64

The Krafft point is defined for ionic surfactants as the temperature at which the solubility of a surfactant monomer becomes equal to the CMC. Below the Krafft point temperature, the solubility is quite low and micelles cannot be formed. Chromatographic work in MLC should be conducted above this temperature to avoid surfactant precipitation. This means that the Krafft point should be well below room temperature. Nonionic surfactants also have a specific temperature (Cloud point) that exceeded, phase separation

occurs, leading of a cloudy suspension of surfactant. The chromatographic analyses using these surfactants should be conducted below this temperature [11].

SDS is the most selected surfactant for bioanalytical applications, because it holds a strong ability to denature proteins and a Krafft point below the room temperature. In addition, it would interact with most of the drugs, which have an amino-group and would be neutral or positively charged. Besides, sodium dodecyl sulfate (SDS) is an easily manageable powdered solid, commercially available at elevated purity, harmless, biodegradable, highly soluble in water, relatively inexpensive, its aqueous solutions hold low viscosity and give a relatively low increase of the system pressure [8,12].

1.2.2 pH of the Mobile Phase

MLC employs the same packing materials as classical RP-HPLC, which for conventional columns have a limited working pH range of 2.5–7.5. Appropriate pH values depend on the nature of the analytes and the surfactant selected. The pH of the micellar mobile phase is commonly fixed with phosphoric or citric acid buffers [4,7]. For mobile phases containing SDS, potassium salts are not recommended as potassium dodecyl sulphate presents a high Krafft point and precipitates from aqueous solutions at room temperature [4].

The pH has a strong effect on the retention, especially for analytes with acidic/basic activity, as it can influence the charge and the dipole distribution of the solutes. Therefore, the pH must be optimized considering the desired charge of the solute. The pH of the mobile phase must be far from the pKa of the analytes in order to avoid the coexistence of two forms of the same compound. Besides, the free silanols (pKa between 4 - 6) of the silica particle surface remain protonated at low pH, thus reducing the silanofilic interactions. This contributes to the amelioration of the peak shape, especially for the analysis of basic drugs [13].

Finally, the pH must be optimized on the basis of the charge of the acidic and basic forms, their chemical properties and the behavior of the other compounds. The pH must be fixed so as to avoid between- and within run variation of the analyte, micelles and stationary phase properties, to increase the robustness of the method and compare the results obtained at different chromatographic analysis [2].

1.2.3 Organic Solvents: Types and Concentration

The addition of an organic solvent increase the elution strength of the mobile phase and the efficiency of the peaks. This effect augments at higher hydrophobicity and proportion in the mobile phase. The selection of the appropriate organic solvent modifier in MLC should consider the polarity of the solutes. For polar compounds, sufficiently short retention times (below 20 min) are obtained with acetonitrile and methanol. Moderately hydrophobic analytes require larger alcohols, like ethanol and 1-propanol, whereas for nonpolar compounds or compounds with high affinity for the surfactant adsorbed on the stationary phase, stronger solvents as 1-butanol or 1-pentanol are needed [14]. However, it should be noted that the two latter alcohols give rise to microemulsion formation at sufficiently high concentration. It should be noted that at high proportions of organic solvent, the micelles disaggregate and the mobile phase contains only free surfactant molecules. The maximal proportion of organic solvent contents that preserve the integrity of micelles are: 30% for acetonitrile, methanol and ethanol, 22% for 1-propanol, 10% for 1-butanol, and 6% for 1-pentanol [5]. Therefore, a mobile phase must contain proportions of organic solvents below these values. These contents are low in comparison with those needed in classical RP-HPLC. Besides, the other chemicals (surfactant, buffer, *etc.*) are innocuous and biodegradable. Therefore, MLC-analyses are eco-friendly and relatively safe for the operator, which may become prominent for “green chemistry”. Besides, the cost for waste segregation and treatment is reduced Also, the stabilization of the organic solvent in the micellar media decreases the risk of evaporation, by its interaction with SDS. This means that micellar mobile phases can be preserved in the laboratory for a long time without significant changes in their composition [15].

1.3 Modified Stationary Phase

1.3.1 Surfactant Adsorption

The alkyl-bonded reverse-phase columns, such as C18, C8 and ciano, are the stationary phase most widely used in MLC. Alkyl-bonded phase columns are strongly modified when SDS is incorporated into the mobile phase. Solid-state nuclear magnetic resonance studies for SDS have proven that the hydrocarbon tail is associated with the alkylbonded phase, whereas the sulphate group is oriented to the mobile phase, creating a structure similar to an open micelle. This creates a negatively charged hydrophilic layer affecting the penetration depth of solutes into the bonded phase, and increases the hydrophobicity of the inner layer. Surfactant coating masks the bonded-stationary phase (Figure 1.1). This means that a full similar coating would render the stationary phases all similar [8].

Surfactant adsorption on the porous RP-HPLC packing affects drastically the chromatographic retention, owing to the change of diverse surface properties of the stationary phase (*e.g.*, polarity, structure, pore volume, and surface area). Surfactant molecules coat the stationary phase pores, reducing appreciably their volume. This has a deep impact on solute interactions [16]. Besides, it also masks the free silanols, preventing their interaction with cationic solutes, and then reducing the peak tailing [5]. In order to keep constant the modifications of the stationary phase, the number of SDS-monomers must remain inter- and intra-run invariant, or showing small changes, with mobile phase composition. In this way, a stable stationary phase is obtained (in a reversible process) with features remarkably different from those of the underlying bonded phase. Several studies have demonstrated that C18; C8 and ciano columns become saturated when mobile phases containing above 10; 300 and 400 mM of SDS, respectively. As we prefer to work with less concentrated mobile phases (as indicated in section 1.2.1), C18-columns are often selected [2].

Ionic compounds are frequently added to micellar mobile phases for pH buffering and, eventually, ionic strength adjustment. Salt addition may change the amount of adsorbed ionic surfactant due to the reduction of both electrostatic repulsion and surfactant CMC, and the enhancement of hydrophobic interactions [4].

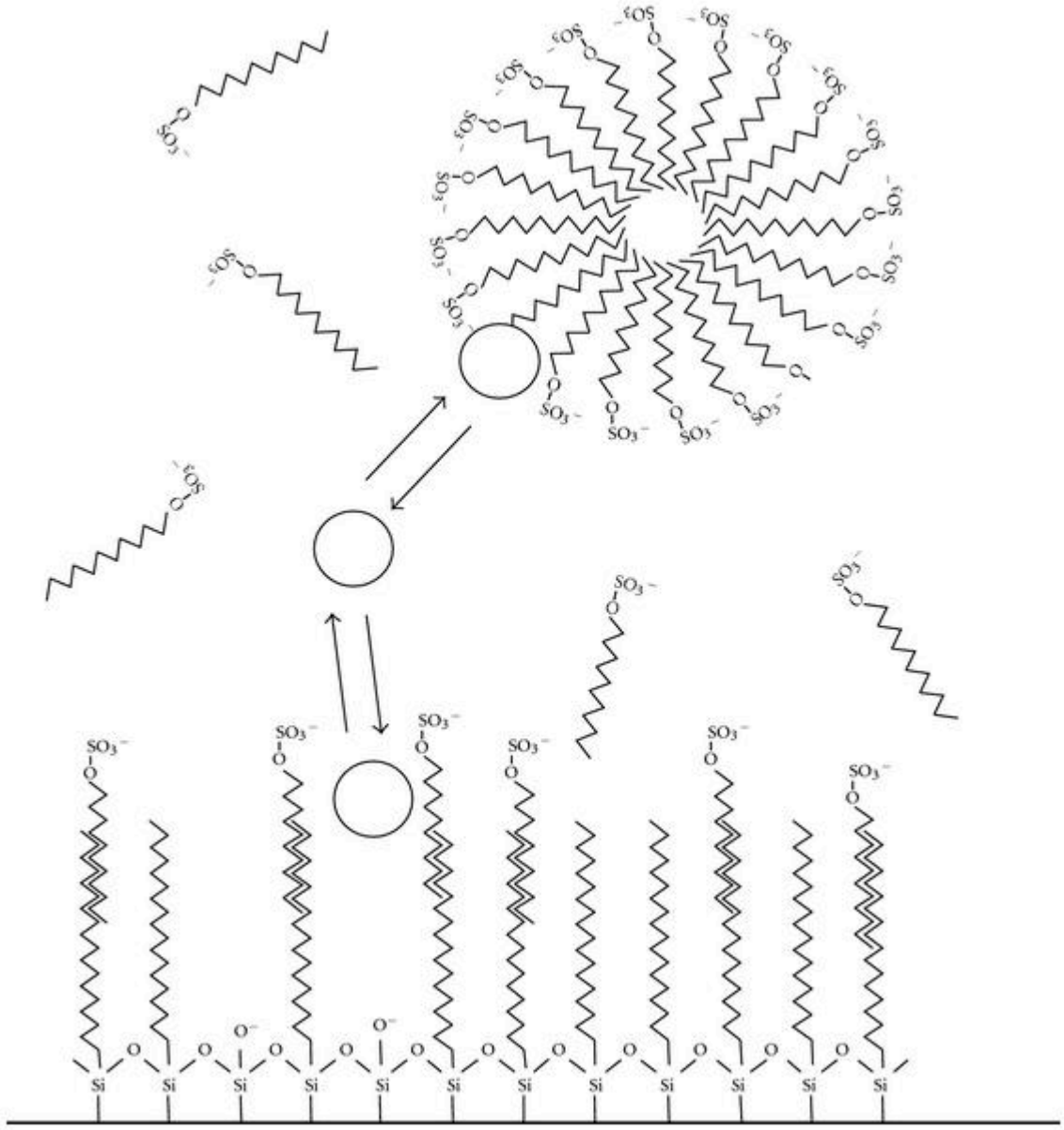

Figure 1.1: Solute environment in a chromatographic system using octadecyl-bonded phase, and mobile phase containing the anionic SDS. Equilibria between bulk solvent, micelle, and surfactant-modified stationary phase are depicted.

1.3.2 Presence of an Organic Solvent in the Mobile Phase

Competition between alcohols and surfactant molecules for adsorption sites on the stationary phase explains the linear reduction in the amount of adsorbed surfactant with increasing concentration of alcohol in the mobile phase. In fact, mobile phases rich in organic solvent can sweep completely the adsorbed surfactant molecules from the bonded phase, another reason to limit the concentration of organic solvent in the micellar mobile phase [5].

The organic solvents are incorporated to the stationary phase, with the carbon chain associated to the C18-tail, and the hydrophilic group (hydroxy or ciano), face to the water. This increases the polarity and reduced the negative charge of the stationary phase [11].

1.4 Solute-Micelle and Solute-Stationary Phase Interactions

The unique capabilities of micellar mobile phases are attributed to the ability of micelles to selectively compartmentalize and organize solutes at the molecular level. However, the association of the surfactant monomers to the bonded phase has deep implications with regard to retention and selectivity. The chromatographic behaviour in an RP-HPLC system of a solute eluted with a mobile phase containing a surfactant above the CMC can be explained by considering three phases: stationary phase, bulk solvent, and micellar pseudophase. Figure 1.2 illustrates the three-phase model. Solutes are separated on the basis of their differential partitioning between bulk solvent and micelles in the mobile phase or surfactant-coated stationary phase. For water-insoluble species, partitioning can also occur via direct transfer of solutes between the micellar pseudophase and the modified stationary phase [4,17].

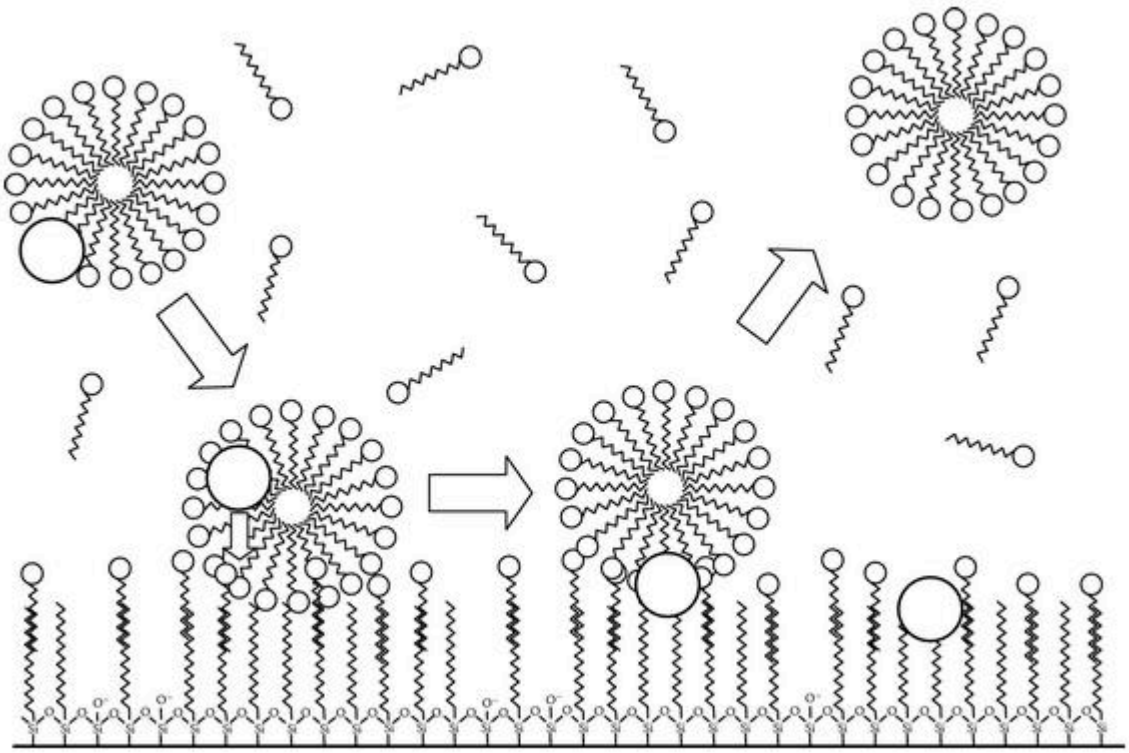

Figure 1.2. Direct transfer of highly hydrophobic solutes between micelle and surfactant-modified stationary phase.

The partitioning equilibria in MLC can be described by three coefficients: P_{WS} (partition between aqueous solvent and stationary phase), K_{AM} (between aqueous solvent and micelles), and P_{MS} (between micelles and stationary phase). The coefficients and account for the solute affinity to the stationary phase and micelles, respectively, and have opposite effects on solute retention: as P_{WS} increases, the retention increases, K_{AM} whereas as increases, the retention is reduced due to the stronger association to the micelles [17,18].

The retention behaviour depends on the interactions established by the solute with the surfactant-modified stationary phase and micelles. Neutral solutes eluted with SDS will only be affected by nonpolar, dipole-dipole, and proton donor-acceptor interactions. Besides these interactions, charged solutes will interact electrostatically (*e.g.*, with the charged surfactant

layer on the stationary phase and the charged outer layer of micelles). In any case, the steric factor can also be important [5,17].

Many situations are possible according to the charges of solute. Positively and polar compounds interact with the anionic hydrophilic external coat of the modified stationary phase and the outer layer of the micelles. The neutral form of a substance would be more hydrophobic, and then would interact with the hydrophobic tails of the stationary phase and the core of the micelle. In these cases, a strong retention would occur, which may be reduced by augmenting the number of micelles in the mobile phase ("binding behavior"). Combined electrostatic attraction and hydrophobic interactions with the modified stationary phase may give rise to strong retention in MLC. On the other hand, a negatively charged substance should be repelled by SDS either coated on the stationary phase or micellized, and then would remain in the bulk mobile phase and be eluted earlier ("unbinding behavior"). As has been observed, the behaviour of the same compound can strongly vary at different protonation states, which must be considered for resolution. Therefore, mixtures of compounds within a large range of hydrophobicity and charge can be resolved, using a mobile phase containing the appropriate concentration of SDS, kind and proportion of organic solvent, and pH, running under isocratic mode [4,5,8].

1.5 Modelling of the retention behavior

The chromatographer is concerned with the achievement of the optimum mobile phase that permits the maximal separation between the solutes at the minimum time (to reduce reagent consumption). This task may be really difficult when two or more variables are involved in this process. The optimization strategy may be sequential or interpretative. In a sequential strategy, the retention of the solutes is not known a priori, and each set of mobile phases is designed by taking into account the retention observed with previous eluents. In contrast, in an interpretative strategy, the experiments are designed before the optimization process and used to fit a model that will permit the prediction of the retention of each analyte. This strategy may be much more efficient and reliable. A sequential strategy will be inadequate when several local (or secondary) maxima exist (as occurs in chromatography) and may not give the best maximum, that is, the optimum [4,9].

The necessity for an adequate experimental design becomes especially important when dealing with forms of liquid chromatography suitable for the simultaneous analysis of ionic and nonionic compounds, such as MLC, where several variables should be controlled, like type and concentration of surfactant and organic modifier, pH, temperature, and ionic strength, flow-rate and temperature. The method development strategy must provide the chromatographer with an answer to which variables should be used, and how to set up initial experiments to search the appropriate variable space in an effective way, thus minimizing the number of experiments. The separation process in a micellar chromatographic system requires a structured approach in the development of practical applications [4,9].

The retention mechanism in a hybrid micellar mobile system can be modelled using a procedure that utilizes the retention data of only five mobile phases: four measurements at the corners of the selected two-dimensional variable space, defined by the concentrations of surfactant and modifier, and the fifth in the center [4]. The chromatographic data obtained (retention factor, efficiency, and asymmetry factor) are used to fit some equations, which can be used to model the retention time, peak shape (for each solute), and elementary (for each peak or paired peaks) and global resolution (for the whole chromatogram). Therefore, it is possible the prediction of these parameters, at intermediate concentrations of SDS and organic solvent by interpolation, as well as the quality of the separation. This is extremely helpful for the selection of the mobile phase. The accuracy of the equations of the model here-described has been confirmed by the high goodness for fit and by comparison between the theoretical and the experimental values for a wide range of compounds [2,6,8-10,19].

1.5.1 Empirical models for the retention factor

The models used for the prediction of retention factor are [9,10,19]:

$$1/k = c_0 + c_1\,[\mathrm{M}] \tag{1.1}$$

$$1/k = c_0 + c_1\,[\mathrm{M}] + c_2\,\varphi + c_{12}\,[\mathrm{M}]\varphi \tag{1.2}$$

$$1/k = c_0 + c_1\,[\mathrm{M}] + c_2\,\varphi + c_{12}\,[\mathrm{M}]\varphi + c_{22}\,\varphi^2 \tag{1.3}$$

k is the retention factor for a given mobile phase composition; [M] is the concentration of surfactant in the mobile phase; φ is the volume fraction of the organic

solvent; c_0, c_1, c_2, c_{12} and c_{22} are empirical fitting coefficients, corresponding to the retention in absence micelles and organic solvent, the effect of the surfactant, the effect of the organic solvent, the interaction and the quadratic effect of the organic solvent.

Retention in micellar mobile phases with a fixed amount or without organic solvent has been extensively proven to be described by the hyperbolic relationship shown in (1.1). Several models were considered where $1/k$ values were related to the surfactant concentration and the proportion of organic modifier. Equation (1.2) is the simplest equation giving acceptable predictions for both polar and moderately polar compounds, in a relatively large range of concentrations of surfactant and modifier. However, for highly hydrophobic compounds, the plots are nonlinear. An additional term is required to achieve more accurate descriptions, as shown in (1.3). The parameters in (1.2) and (1.3) should be obtained by fitting the data in experimental designs with at least four and five mobile phases, respectively. However, at least one additional measurement should be taken to check the accuracy of the fittings [9,10].

1.5.2 Mechanistic models for the retention factor

The parameters of the empirical models of MLC are related to physicochemical constants that describe the interactions of the solutes with the three environments involved in micellar mobile phases: bulk water, micelles, and the stationary phase. A better understanding of the retention mechanism in micellar systems is provided by these models [10, 19]:

$$k = K_{AS}/(1+K_{AM}[\mathrm{M}]) \quad (1.4)$$

$$k = [K_{AS}/(1+K_{AD}\,\varphi)]/[1+K_{AM}\,[\mathrm{M}]\,(1+K_{MD}\,\varphi)/(1+K_{AD}\,\varphi)] \quad (1.5)$$

$$k = [K_{AS}/(1+K_{AD1}\,\varphi+K_{AD2}\,\varphi^2)]/[1+K_{AM}\,[\mathrm{M}]\,(1+K_{MD}\,\varphi)/(1+K_{AD1}\,\varphi+K_{AD2}\,\varphi^2)] \quad (1.6)$$

$$k = [K_{AS}\,(1+K_{SD}\,\varphi)/(1+K_{AD}\,\varphi)]/[1+K_{AM}\,[\mathrm{M}]\,(1+K_{MD}\,\varphi)/(1+K_{AD}\,\varphi)] \quad (1.7)$$

$$k = K_{AS}/[1+K_{AM}\,[\mathrm{M}]\,(1+K_{MD1}\,\varphi+K_{MD2}\,\varphi^2)/(1+K_{AD1}\,\varphi+K_{AD2}\,\varphi^2)] \quad (1.8)$$

where K_{AS} is the product of the solute- (A) stationary (S) partition coefficient by the phase ratio, K_{AM} and the solute-micelle (M) association constant; K_{AD} and K_{MD} measure the relative variation produced in the concentration of solute in bulk water and micelle,

respectively, in the presence of modifier, taking the pure micellar solution as a reference; K_{AD2} corresponds to a quadratic hyperbolic variation in K_{AS} and K_{AM} with φ.

The mechanistic models are based on (1.1), which is the classical equation proposed for micellar mobile phases at a fixed volume fraction of organic modifier. Equation (1.1) can be written as (1.4), which relates the retention of a solute to the concentration of monomers of surfactant in the form of micelles. For hybrid micellar mobile phases, (1.2) can be expressed as (1.5), while (1.3) gives rise to (1.6), which may suggest an excessive dependence of the retention on the organic solvent concentration and produce high errors when an extrapolation is made in a region of high proportion of organic solvent [10,19].

As a result, (1.7) was proposed as an alternative model for highly hydrophobic solutes, and it takes into account the additional change in the concentration of solute associated with the stationary phase produced by the presence of modifier. In (1.7), K_{SD} and K_{AD} describe the modification of the water-stationary phase equilibrium. These changes are caused by the decrease in the polarity of water and the modification of the interactions of the solute with micelles and stationary phase, when an organic solvent is added. Equation (1.7) provides an accurate description of the retention of solutes of a wide range of polarities, when they are eluted with hybrid mobile phases of SDS and alcohol (1-propanol, 1-butanol or 1-pentanol) [10]. For acetonitrile and tetrahydrofuran [19], (1.8) fits better.

1.5.3 Peak shape modelling

The major drawback of applying MLC to practical separations is still the low chromatographic efficiency, which is caused by resistance to mass transfer in the processes involving micelles and a surfactant-modified stationary phase. This is especially important since the increase of the micelle concentration causes a decrease in plate number. Thus, it is very important to include the expected peak shape in the expression of the chromatographic quality. The complexity of the chromatographic process does not allow the use of simple equations to describe peak profiles. The best peak-profile predictions are achieved using a Gaussian equation where the standard deviation depends polynomially on the distance to the peak time (polynomial modified Gaussian model) [8,10]:

$$h(t) = H_0 e^{-0.5((t-t_R)/(s_0+s_1(t-t_R)+s_2(t-t_R)^2+\cdots))^2} \quad (1.9)$$

where *h(t)* is the predicted signal at time *t*, H_0 the maximal peak height, t_R the retention time, and s_0 is related to the width (standard deviation of the curve), and s_i (i>0) quantifies the asymmetry of the chromatographic peak. For a given solute and mobile phase, t_R and s_i are ideally invariable, whereas H_0 depends on the concentration and the sensitivity. Better descriptions of peak profiles are achieved by increasing the degree of the polynomial. The use of a larger number of coefficients improves the fittings, but decreases the practical application of the model. A linear standard deviation in (1.9) approximates real peak profiles satisfactorily. The linear equation is also useful for simulating chromatograms.

1.5.4 Strategies to Measure the Peak Resolution

The appropriate elementary resolution criterion should be decided. Some criteria have been based on conventional elementary measurements, such as the modified selectivity in (1.10), peak-to-valley measurements in (11), and overlapping fraction measurements in (1.12) [10,20], which are described below:

$$r_{i;i+1} = 1 - k_i/k_{i+1} = 1\text{-}1/\alpha_{i;i+1} \quad (1.10)$$

$$r_{i;i+1} = 1 - h_1/h_2 \quad (1.11)$$

$$r_{i;i+1} = 1 - o'_i/o_i \quad (1.12)$$

k_i and k_{i+1} and are the retention factors of two neighboring peaks, $\alpha_{i;i+1}$ and is the selectivity; h_1 represents the height of the signal at a specific time depicting the valley location, and h_2 is an interpolated height, measured at that time, from the baseline to the line obtained by joining the maximums of the two neighboring peaks; o'_i is the area under a given peak overlapped by the chromatogram yielded by the remaining peaks, and o_i the total area of the peak.

Criterion (1.10) is not very useful, as it does not consider the shape of the peak. However, the calculations are relatively easy to carry out. The resolution ranges from 0 (elution at the same time) to 1 (asymptote, infinite separation between the peaks).

In (1.11), the valley between two consecutive peaks can be measured at the time giving the largest possible distance, measured orthogonally. If the valley is observed orthogonally, this point is obvious even when there is substantial overlap. The resolution depends on the difference of elution times and the peak shape, and is especially affected by the asymmetry. The resolution ranges from 0 (peaks fused at their maximum) to 1 (the two peaks are separated by a null baseline).

The criterion of overlapping fractions (1.12) takes into account not only positions, but also peak profiles; it isolates the contribution of each component in a mixture, associating a value to each individual peak, which is not affected by the identity of its neighboring peaks, and the intrinsic normalization facilitates understanding of the information obtained in the optimization process. However, it requires the drawing of the shape of the overlapped peaks, which may be an important source of error. Besides, it does not measure the separation of the peaks. An adequate resolution is reached at r_i=1; inferior values (even slightly) are not accepted.

After the selection of the elementary resolution function, the appropriate global resolution criterion must be selected [10]. Different measurements of diverse complexity have been proposed to depict chromatographic performance. Optimization criteria based on the calculation of an individual or elementary resolution measurement, r_i, for the least resolved peak or peak pair is a very widely used procedure in chromatographic practice, because of its simplicity:

$$Z = \text{MIN}\,(r_i) \qquad 1 < i < p \tag{13}$$

where p is the number of peaks or peak pairs, and Z is the global resolution. This criterion is reasonable, but it considers the resolution of only one peak or peak pair, and is insensitive to the other peaks. In many cases, a practically identical resolution of the worst peak can be obtained, while the resolution of the other peaks can be improved. The product of

peak resolutions solves this drawback, since it optimizes the resolution of all peaks in the chromatogram.

The normalized-by-mean product (1.14) is conventionally applied. This treatment normalizes the resolution approximately, using the mean r_i of all the peaks in the chromatogram instead of the extreme values.

$$Z = (\Pi^{p}_{i=1}\, r_i)/\ [\Sigma^{p}_{i=1}\ r_i/p] \qquad (1.14)$$

The unnormalized product of (1.15) seems to be a better alternative, although it can be used only with intrinsically normalized resolution measurements. This product varies from 0 (complete overlapping between at least two peaks) to 1 (full resolution of every peak in the chromatogram).

$$Z = \Pi^{p}_{i=1}\ \ r_i \qquad (1.15)$$

1.5.5 Computer optimization

Although these equations are very useful, they required complex calculations to be fitted and interpreted. Computer optimization attempts to mimic the methodology followed by an experienced chromatographer so as to reduce the time and the effort required [10,19]. A software application was developed to assist the chromatographer in the selection of the optimal composition of the mobile phase in MLC [21]. The software combines the equations obtained for the retention time and peak shape of each analyte to draw simulated chromatograms and represent the global resolution in a surface plot. Therefore, the chromatographer can quickly and easily visualize the variations of the chromatograph shape and the global resolution when the concentration of surfactant and organic solvent changes, without performing the analysis. This information can be employed in a straightforward manner to select the optimal composition of the mobile phase, from the results obtained by a few experiments. This requires equations predicting peak retention and profile as accurate as possible, considering the selected model and the adjustment of the constants.

1.6 Direct injection

The remarkable properties of SDS-micellar solutions are considerably useful for the simple pretreatment of a large variety of samples, including waste water and solid samples [5].

Hydrophobic liquids are solved because of their interaction with the micelles, rather than forming drops dispersed in the colloidal solutions. Non-polar small molecules (matrix or analytes) are also solubilized, and can be distributed evenly in the micellar solution, instead of remaining concentrate in the drops of the organic solvent or precipitating on the walls of the glassware. Therefore, samples of water also containing oily compounds can be directly injected, after a filtration to remove the soil particles, in the chromatographic system without risk for the column. Indeed, the released analytes are free to interact with the stationary phase and can be determined, whereas micelle-bound oily liquids are harmless eluted near the dead time, rather than being strongly linked to the stationary phase [6,22-24].

Pure SDS-micellar solutions are excellent solvents to isolate the analyte from the solid sample, as the analytes (either hydrophobic or polar) are attracted to the micellar solution because of their wide solubilization power. The powdered sample can be leached in a micellar solution, by shaking and/or ultrasonication, to extract the analyte and other compounds from the solid matrix to the liquid phase with a high recovery. The supernatant can be filtered and directly injected to the chromatographic system. The extraction time, the composition of the extraction solvent and the ratio sample/supernatant must be optimized to maximize the sample throughput, the sensitivity, homogeneity and fluidity of the supernatant (for and easier and reproducible filtration) and the absence of interfering compounds [6,9,10,11,15,18].

The treatment of the supernatant and the waste water only involves a dilution, filtration and direct injection, and instead of tedious, and time- and reagent-consuming extraction or cleanup steps. Thus, the sample is quantitatively introduced in the chromatographic system, and then and internal standard is not required. The leaching is semi-automated, uses only biodegradable and innocuous chemicals and usual laboratory material and apparatus. Therefore, the time, effort, participation of the operator, economic and laboratory resources, and impact on the environment is minimized. This reduces significantly

the potential sources of variance, the probability of loss of the analyte (either by incomplete recovery or chemical change), thus obtaining low bias and variability. This simplification permits the laboratory staff to process a high number of samples per day, which is useful for routine analysis [6,9,10,11,15,18,22-24].

2. Method Validation

Reliable analytical methods are required for compliance with national and international regulations in all areas of the analysis. Accordingly, it is internationally recognized that a laboratory must take appropriate measures to ensure that it is capable of providing data of the required quality. For this reason, the performances and the limitations of the method, as well as the external influences which may modify these features, must be determined prior its use. Validation plays a major role in achieving this goal [25]. The most accurate definition of validation is that provided by ISO 9000:2000 as the confirmation, by means of a thorough examination and the obtaining of realistic and unequivocal evidences, that the procedure is effectively applicable for its indented purpose [26].

Validation is the act of proving that any approach, strategy, experimental procedure, process, laboratory staff, instrumentation, reagents, and room conditions selected for the method will function in a proper way under a fixed set of conditions. Besides, it can be used to individually evaluate the appropriateness of these factors [27]. The validation evaluates the concentration range and conditions of applicability, and checks if every future measurement in routine analysis will provide a concentration of the analyte close enough to the true value [28]. In addition, it can also quantify the degree of coincidence of a measured concentration and the true value, by the calculation of the bias and the uncertainty associated with the result [29]. Therefore, the validation verifies if the method is suitable to be used as a quality control tool and for research support. Consequently, validation is an essential step in analytical chemistry practice [30]. Validation must be conducted after development or first time implemented. Once a methodology is validated, it remains 'validated' while applied in the same laboratory and using the same experimental conditions [20]. According to ISO/IEC 17025:2005, a laboratory must validate all the used methods. The methods will be separately validated for each matrix and working range, even dealing with the same analyte. A full

validation is required when implementing a new method: in-house developed, taken from a bibliographic source, transferred from other laboratories, and reference one [25,30,31].

The validation consists in the determination of well-defined quality statistical parameters: selectivity, specificity, calibration curve, linearity, calibration range, quantitation limits and range, detection limit, accuracy, trueness, precision, ruggedness, stability, system suitability, comparison with other methods and other ones. These parameters are preferably measured in a matrix close to the real samples, like or spiked samples. The meaning and the way to calculate them have been defined by different working groups of national and international committees and are widely described in the literature and in the validation guidelines. Unfortunately, some of the definitions vary between the different organizations [20,25]. The results from method validation evince the quality and consistency of the analytical results obtained in future determinations in real samples.

The fitness-for-purpose is the extent in which the performances of the method match the characteristics that have been agreed between the analyst and the end-user of the results and/or the requirements of the government institutions and selected validation guide [32]. The analytical requirements are not always the same, and must be individually established on the basis of the scope of the method, the analyte, the matrix, possible interfering, kind of the sample, the expected interval concentration and the local regulations. The validation parameters which have to be determined and the acceptance criteria should be completely specified before starting the development of the method [33]. If the method characteristics do not match the minimal analytical requirements, then it must be modified, and the validation process must be repeated. This iterative process of development and evaluation should follow until the validation parameters meet the fixed requirements; then the method is fit-for purpose and can proceed. The final results of validation must be documented to be always available for consulting by laboratory staff, clients and accreditation agencies, and ready to be transferred to other laboratories [34].

Many industry committees and regulatory agencies and individual researchers have published reviews and technical reports about validation strategies, quality assurance and regulatory purposes. Aware of its importance, many international renowned organizations have offered along the years guidance about method validation. Consequently, many validation guidelines with different scopes are available for researchers, describing the

statistical parameters to-be-studied, the way to determine each one and their acceptance criteria. The different published documents agree about what type of studies should be done, but they show a great diversity in how the validation should be conducted [20,30]. The selection of the guide is made on the basis of the purpose and the scope of the analysis, the geographic zone and the kind of sample [20,30]. Between them we can highlight the *Validation and Peer Review of U.S. Environmental Protection Agency Chemical Methods of Analysis*, laucnched by The Environmental Protection Agency (EPA),which is has launched a guideline specifically for the analysis of environmental samples (water and soil) [35] and the *ICH Harmonised Tripartite Guideline Validation of Analytical Procedures: Text and Methodology Q2(R1)*, developed by the International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use, which aims to develop consensus criteria for about the analytical performances required by the analytical methods used in quality control of pharmaceutical formulations for its registration in the US, Japan, and the European Community, and then thoroughly describes a wide range of valdiation parametes [36].

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Chapter 2

Objectives

L'objectiu principal de la recerca realitzada ha estat el desenvolupament d'una sèrie de nous mètodes senzills, fiables i selectius d'anàlisi per a la identificació i quantificació de diversos pesticides, d'intens ús i que representen un perill mediambiental i de salut a l'àrea de Castelló (Espanya), en mostres mediambientals mitjançant cromatografia líquida micel·lar, amb l'ús d'eines quimiomètriques, i variant la detecció segons les característiques de cada anàlit.

- Els plaguicides usats en la producció o tractament de cítrics: tiabendazol, pirimetanil, o-fenilfenol i imazalil en aigües residuals agrícoles, d'indústries de processament de cítrics, i de corrents d'entrada i eixida d'EDAR emprant la detecció d'absorbància UV-Visible.
- Els pesticides tiabendazol i o-fenilfenol, que a més tenen un elevat ús domèstic, públic i industrial com a desinfectants en les mateixes mostres d'aigües per detecció de fluorescència. Comprovar si els abocaments i les diverses aigües residuals (urbans, agrícoles, de la indústria agroalimentària i d'EDAR) compleixen la normativa imposada pel municipi de Vila-real (província de Castelló).
- Els pesticides tiabendazol i clorpirifos (emprant sobretot en l'àmbit agrícola) i el 4-*tert*-octilfenol, un derivat dels tensioactius alquilfenols polietoxilats, que s'afegeixen com a adjuvants en formulacions de pesticides, i per tant donen una idea del nivell de la quantitat global de plaguicides que s'usen en la zona, en aigües residuals (urbanes, agrícoles, de la indústria agroalimentària i d'EDAR) per detecció d'absorbància UV-Visible.
- Els herbicides diuron, terbutilazina i terbutrina, usats a nivell, públic, domèstic i industrial, en aigües residuals agrícoles i urbanes, d'EDAR, i emprades amb funció decorativa, i en una gran varietat de sols potencialment contaminats mitjançant detecció per absorbància UV-Visible.

Els mètodes han de ser capaços d'analitzar una gran quantitat de mostres en poc de temps amb un cost econòmic i mediambiental mínim. Per altra banda, la sensibilitat ha de ser suficient per a quantificar l'anàlit a les concentracions que es puguen trobar a les mostres.

El fet que les dissolucions micel·lars tinga un elevat poder extractant d'anàlits de baix pes molecular, amb una elevada diversitat de propietats físico-químiques, i que l'MLC facilite la injecció directa del sobrenedant i de mostres aquoses en contingut oliós i amb una simple filtració, en mode isocràtic, tal vegada siguen les característiques que més avantatges aporta enfront d'altres tècniques analítiques revisades en la bibliografia, que, per les seues

característiques intrínseques necessiten instrumentació més costosa, llargs i complexos procediments previs d'extracció, ús de gradients, i conseqüentment incrementant la quantitat de reactius contaminants usats i la durada de l'anàlisi. Un altre dels objectius ha sigut la validació dels mètodes desenvolupats seguint els criteris definits per una guia oficial. Els laboratoris han de demostrar que els mètodes recentment desenvolupats proporcionen resultats quantitatius pròxims al valor real, i determinar l'interval de concentracions aplicable, atés que la informació que donen serveix per a prendre decisions. Els mètodes desenvolupats s'aplicaran en el camp de la protecció mediambiental, es podran aplicar a l'avaluació del grau de pol·lució dels abocaments, l'eficàcia del tractament de purificació a l'EDAR, estimar l'ús urbà i agrícola de formulacions de pesticides, monitorar el grau de contaminació de les aigües residuals urbanes, agrícoles i industrials. Així doncs, el cost econòmic i la repercussió social d'aquestes decisions pot ser molt elevat, ja que poden afectar a la salut del consumidor i a l'equilibri ecològic de la regió.

Per a assolir aquests objectius generals es proposen els següents objectius específics, que són comuns a tots els treballs:

- Estudi dels paràmetres físico-químics dels anàlits (pKa, hidrofobicitat, solubilitat, i propietats espectroscòpiques).
- Establir les condicions cromatogràfiques generals (fase estacionària, volum d'injecció, cabdal...)
- Avaluar l'efecte de la concentració de tensioactiu i dissolvent orgànic en el comportament cromatogràfic de cada anàlit. Estudi i modelització de la retenció i la resolució, mitjançant eines quimiomètriques.
- Determinació de la composició de la fase mòbil (concentració de tensioactiu, modificador orgànic i pH), per a eluir els anàlits sense interferències en el mínim temps d'anàlisi.
- Optimització de les condicions instrumentals del detector.
- Optimització dels paràmetres de lixiviació dels sols amb una dissolució micel·lar.
- Selecció de la guia de validació adequada segons els anàlits i la matriu.
- Determinació dels paràmetres de validació exigits per la guia de validació seleccionada: selectivitat, sensibilitat (límit de detecció i de quantificació), linealitat, interval lineal, exactitud, precisió, robustesa i estabilitat.

- Comprovar que els valors obtinguts a la validació cobreixen les concentracions d'anàlits possibles a les mostres estudiades. En el segon treball s'han d'assolir els límits màxims per a abocaments i corrents d'aigües residuals indicades a la normativa municipal de Vila-real.
- Determinar l'estabilitat dels pesticides en les condicions habituals d'emmagatzemament, per a estimar el temps prudencial al qual es poden guardar les mostres abans de l'anàlisi, i al medi ambient, per a esbrinar la cinètica de la degradació.
- Quantificar els pesticides a les diverses aigües residuals i sols mostrejats en diversos punts de la província de Castelló. Estimar si representen un risc per a la salut de la població o per al medi ambient i compleixen la normativa legal, si escau.

Chapter 3

Use of micellar liquid chromatography for rapid monitoring of fungicides post harvest applied to citrus in wastewater

Abstract

A method based on micellar liquid chromatography has been developed to simultaneously monitor four pesticides largely post-harvest applied to citrus: thiabendazole, pyrimethanil, o-phenylphenol and imazalil. Water samples were filtered and directly injected without other treatment, thus avoiding extraction steps. The composition of the mobile phase was optimized using a chemometrical approach to achieve and excellent resolution to 0.07 mol/L SDS/5%, v/v 1-pentanol buffered at pH 3. Mobile phase run through a C18 column at 1 mL/min at room temperature. The detection was performing by UV-Visible absorbance using a wavelength program: 0-10 min, 305 nm (for thiabendazole); 10-12, 265 nm (for pyrimethanil) and 12–18, 220 nm (o-phenylphenol and imazalil). The developed method was validated following the guidelines of the US Environmental Protection Agency in terms of: quantitation range, (0.5–4 to 15 mg/L), linearity (r^2>0.9995), sensitivity (LOD, 0.18-1.4 mg/L), precision (<9.2%), trueness (93.9%–103.7%), and ruggedness (<9.9%). It was found that the fungicides remain up to eight days in surface water at outdoor conditions. The method was used to screen the presence of the analytes in several waste water samples, and was proved to be useful in routine analysis.

1. Introduction

The production of citrus and the related fruit processing industry have as strong importance in the Castellón area (Spain). In fact, the exportation of fruits has an important weight in its economy [1]. One of the problems of fruit trading is the spoilage of citrus during storage and transportation, caused by microorganism, fungi and insects. This reduces the economic yielding of the agricultural activity. To prevent this fruit decay, pesticides are post-harvest added to fruits [2]. Thiabendazole (TBZ), pyrimethanyl (PYR), o-phenylphenol (OPP), and imazalil (IMZ) are pesticides widely post-harvest applied by citrus traders and fruit-processing industry to protect crops during storage and transportation, because of their broad spectrum and strong fungicide activity [3,4]. TBZ and PYR are also pre-harvest used to protect the tree and citrus during growing against mold and fungi. They are applied to the soil or sprayed over crop fields [4,5].

Because of their intensive use and persistence, pesticides represent an important source of contamination of environmental water, especially those near areas with strong fruit-related activity. These pesticides are highly toxic and potentially carcinogenic, and then represent a serious threat to the local flora and fauna [3]. The population is also exposed to these hazardous compounds by dermal contact, accidental ingestion, or inhalation of polluted water. Moreover, the main danger is the ingestion of animals of vegetables, which have been in contact with polluted water, due to because their high bioaccumulation in edible tissues of living organisms [6]. For these reasons, the European Water Framework Directive recommends the implementation of actions to avoid these compounds arrive to underground and surface water [7].

These pesticides are present in wastewater from agricultural fields, drained by rain water [6], and in sewage water from fruit-processing plants. To avoid the pollution of environmental water by pesticides, sewerage and waste water are purified in wastewater treatment plants (WWTP) before discharging to the river streams. In order to evaluate the quality of waste and sewerage water, several local governments have implemented programs to periodically perform pesticide screening in water samples from their area. Moreover, the

fruit processing industry has been requested to monitor these hazardous compounds in their own wastewater before throwing it, in order to reduce the environmental impact of their activity. The effectiveness of the WWTP treatment must also be evaluated by analyzing the influent and effluent flow [7]. Indeed, they must dispose of a reliable, easy-to-use and sensitive analytical method to simultaneously quantify thiabendazole, o-phenyl-phenol, pyrimethanyl and imazalil in water.

The preferred analytical methods to perform multiresidue pesticide analyses in wastewater are gas chromatography and liquid chromatography, coupled to mass spectrometry [8]. However, this instrumentation is costly and requires expensive maintenance; thus, the analyses are sold at higher prices. In the current context of economic crisis, industries and government institutions are forced to reduce their budgets, and increasingly demand less expensive methods. Liquid chromatography can be coupled to affordable and reasonably selective detector, as UV-Visible, to detect TBZ [9,10], PYR [11,12], OPP [13,14] and IMZ [11,15] in water and aqueous samples. However, the resolution of a pesticide mixture is normally performed using gradient-programmed mobile phases, complicating the screening of a large amount of samples [9,10,13]. Nevertheless, wastewater samples may contain sludge particles and oily compounds dispersed in water, which must be removed before analysis [16]. Thus, tedious and time-consuming clean-up steps must be introduced, as a liquid-liquid [10,11,13] or solid phase [9,12,14,15] extraction. These steps require specific chemicals and materials, and increase the possibility of loss of analyte by low yielding or operator error.

Liquid chromatography using hybrid micellar mobile phases, containing sodium dodecyl sulfate (SDS) as surfactant and short-chain alcohol (to improve the elution power and the efficiency) as additive, is an interesting alternative to analyze contaminants in wastewater. The lipophylic environment inside the micelle allows the solubilization of hydrophobic compounds. Therefore, after a simple filtration, the water sample can be directly injected in the chromatographic system, thus expediting the experimental procedure [17]. The retention mechanism is different from hydroorganic reverse phase-high performance liquid chromatography (RP-HPLC), because the monomer surfactant modifies the nature of the stationary phase, and the analyte also can interact with the core of the micelles. Hence,

compounds with dissimilar hydrophobicity can be resolved in the same run under isocratic conditions. The behavior of the analytes in micellar liquid chromatography (MLC) is highly steady and reproducible. The retention parameters can be accurately predicted at different SDS/alcohol concentration by means of a statistical treatment from the results obtained by testing only few mobile phases. Moreover, SDS solutions are more stable, less toxic, non-flammable, biodegradable, and uses less amount of organic solvent (up to 12.5%, v/v), in comparison to hydroorganic mobile phases used in HPLC [18]. MLC has been already used to detect carbamate pesticides in water [19,20,21].

The aim of the work is to develop an analytical procedure based on micellar liquid chromatography for the screening of TBZ, PYR, OPP and IMZ in water. The method must be simple, rapid, inexpensive, reliable and environmental friendly. The sample preparation must be simplified to facilitate the study of a large amount of samples, in order to apply it to routine analysis. The analytical procedure would be in-house validated by the Validation and Peer Review of U.S. Environmental Protection Agency (EPA) Chemical Methods of Analysis guideline in terms of selectivity, quantitation range, linearity, sensitivity, precision, trueness and ruggedness [22]. The analytical method would be applied to evaluate the stability of the fungicides in outdoor conditions, and to detect the concentration of pesticides in sewage and WWTP treated water streams.

2. Materials and methods

2.1 Reagents and solutions

The pesticides thiabendazole, pyrimethanil, o-phenyl-phenol and imazalil (purity >99.9%) were purchased from Dr. Ehrerstorfer (Augsburg, Germany). The characteristics are described in Table 3.1 [23]. SDS (purity >99%) was supplied by Merck (Germany). Methanol, 1-butanol and 1-pentanol (HPLC grade) were from Scharlab (Spain). Sodium dihydrogen phosphate monohydrate, sodium hydroxide (analytical grade), hydrochloric acid (37.0%), and 1-propanol (HPLC grade) were ordered from Panreac (Barcelona, Spain). Ultrapure water was in-situ generated using a Simplicity UV ultrapure water generator device

(Millipore S.A.S., France). This ultrapure water was used to prepare all the aqueous solution throughout the whole work.

2.2 Solutions and mobile phase preparation

Stock solutions containing 100 mg/L of each pesticide were prepared by weighting the appropriate amount of solid standard and solving in methanol, and stored at -4°C. Standard solutions were prepared by successive dilutions of stock solutions in water or in wastewater from sample 12, free of the studied fungicides. These solutions were not stored. The mobile phases were prepared by weighting the adequate amount of SDS and sodium dihydrogen phosphate. These reagents were solved in water and the pH was adjusted by adding 0.1 mol/L HCl or 0.1 mol/L NaOH. The adequate volume of alcohol was added to achieve the desired concentration. The solution was filled up with water to reach the final volume, ultrasonicated and filtered through 0.45 μm nylon membranes (Micron Separations, USA).

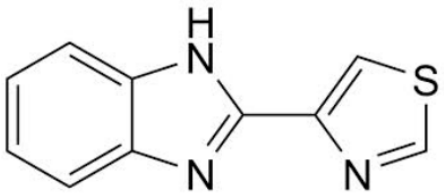
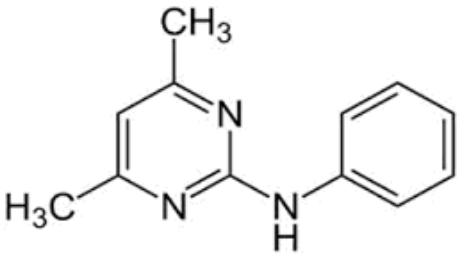
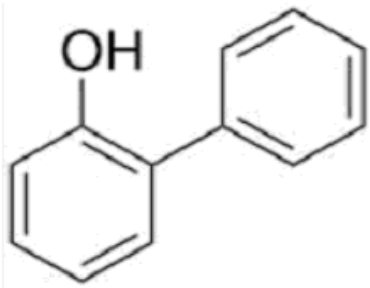
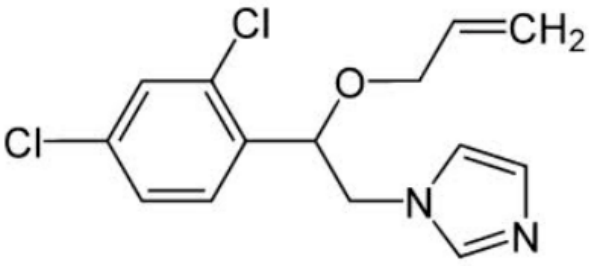
2.3 Apparatus and instrumentation

Solids were weighted using a Mettler–Toledo analytical balance (Switerland). The pH measurements were performed using a GLP 22 potentiometer (Crison, Spain) equipped with a combined Ag/AgCl/glass electrode. An Ultrasons-H ultrasonic bath (Selecta, Spain) was used to achieve the complete dissolution of the mobile phases.

The chromatographic system was an Agilent Technologies Series 1100 (USA). It was equipped with an isocratic pump, a degasser, an autosampler and UV-Visible diode array detector (DAD). The signal was acquired by a personal computer connected to the chromatograph by means of an Agilent Chemstation version B.01.01.

Chromatograms were treated using Michrom software [24] to extract the chromatographic parameters: retention time (t_R), retention factor (k), dead time (t_0), efficiency (N), asymmetry (B/A) and peak area (A). The meaning of the chromatographic parameters has been described in [25].

Table 3.1. Structure, pKa and log P*o/w* for the studied pesticides [23].

Compound (group)	Structure	pKa	log P*o/w*
Thiabendazole (benzimidazole)		4.73/12.00	2.39
Pyrimethanil (anilinopyrimidine)		3.52	2.84
o-phenylphenol (phenol)		9.4	3.18
Imazalil (imidazole)		6.49	2.65

2.4 Chromatographic conditions

The stationary phase chosen for the analysis was coated in a Kromasil C18 column, with the following characteristics: length 150 mm; internal diameter, 4.6 mm; particle size, 5 μm; pore size 100 Å. The selected mobile phase was an aqueous solution of 0.07 mol/L SDS - 5%, v/v 1-pentanol - 0.01 mol/L phosphate buffer at pH 3, flowing at 1 mL/min in isocatic mode at room temperature. The injection volume was 20 μL. The detection was performed by switching the absorbance wavelength as follows: 0–9.20, 305 nm; 9.20–12.0, 265 nm; 12.0–20.0, 220 nm. The special care required for liquid chromatographic instrumentation when dealing with micellarmobile phases has been detailed in [26].

2.5 Sample preparation

Wastewater samples were collected and supplied to the laboratory by Fomento Agricola Castellonense, S.A. (FACSA, Spain), a company managing the integral water cycle and the evaluation of the water quality in the Spanish province of Castellón. The samples were taken from fruit-processing wastewater, in the influent and effluent stream water in wastewater treatment plants, as well as in the agricultural sewage water (Table 3.2). The samples were kept in laboratory at 4°C and analyzed in less than three days. Before the analysis, the samples were put out the fridge and maintained in the laboratory for 30 min to warm up to room temperature.

The standard solutions and water samples were analyzed by filtering an aliquot using a 0.45 μm nylon membrane and directly injected in the chromatographic system.

Table 3.2. Concentrations (mg/L) of TBZ, PYR, OPP and IMZ detected in sewage water samples.

Origin of water sample	Sample	Location	TBZ	PYR	OPP	IMZ
Sewage agricultural water	1	Villareal	n.d.	0.25 - 0.5	n.d.	n.d.
	2	La Vilavella	0.18- 0.5	0.6	n.d.	n.d.
	3	Betxí	n.d.	0.25 - 0.5	n.d.	n.d.
	4	Alcora	0.18- 0.5	n.d.	n.d.	n.d.
	5	Onda	0.18-0.5	0.7	n.d.	n.d.
	6	Nules	n.d.	1.0	n.d.	n.d.
Collector basin of wastewater from a citrus-processing plant	7	Real Export (Vila-real)	2.0	1.0	n.d.	1.4 - 4
	8	Invicto (Vila-real)	1.4	n.d.	1- 3	n.d.
	9	Serifruit (Vila-real)	0.9	1.5	1- 3	n.d.
	10	Eurococi (Betxí)	1.7	1.3	n.d.	1.4 - 4
Wastewater from WWPT	11	Influent (Nules-La Vilavella)	1.3	0.8	n.d.	n.d.
	12	Effluent (Nules-La Vilavella)	n.d.	n.d.	n.d.	n.d.
	13	Influent (Vora Riu)	1.4	1.1	1- 3	1.4 - 4
	14	Effluent (Vora Riu)	n.d.	n.d.	n.d.	n.d.
	15	Influent (Mancomunada OBVA)	0.6	n.d.	3.1	n.d.
	16	Effluent (Mancomunada OBVA)	n.d.	n.d.	n.d.	n.d.

3. Results and discussion

3.1. Optimization of chromatographic conditions

The stationary phase, flow-rate and injection values were taken as the standard values used in MLC, a C18 column, 1 mL/min and 20 μL, respectively. The composition of the mobile phase (SDS, alcohol and pH) and the detection wavelength were optimized. The standard solution used for the optimization analysis was a mixture of 2 mg/L of TBZ and PYR, 5 mg/L of OPP and 7 mg/L of IMZ solved in ultrapure water.

3.1.1 pH selection

The pH values can modify the retention mechanism of the pesticides with acidic/basic activity. The mobile phase was buffered to avoid the oscillations of pH can affect the retention conditions. As the chosen column has a working pH range of 1.5-7.5, only neutral and acidic pHs were tested.

The influence of the pH was evaluated by analyzing the standard solution of the pesticides using the optimal mobile phase buffered at pH 3; 5 and 7. For the four pesticides, the retention times were similar at the three studied pH. However, a strong tailing was observed for TBZ at pH 5 and 7; but at pH 3 the obtained peak was quite Gaussian. It was observed that the shape of PYR, OPP and IMZ do not change by varying the acidity of the mobile phase. Therefore, the pH 3 was selected as optimal.

3.1.2 Selection of surfactant and alcohol

The selected surfactant was SDS, the most widely used in MLC, according to its moderate CMC (8.3 mmol/L), low Krafft point (15°C), high solubility in water, biodegradability and low viscosity of the solutions [18].

According to the medium-high log Po/w, the pesticides are too hydrophobic to be eluted at a reasonable retention time using a C18 column and a pure aqueous SDS solution them in a useful retention time using a C18 column. The addition of a short chain alcohol would be necessary to increase the elution power of the mobile phase to obtain more adequate retention times, and additionally improve the efficiency [27]. Therefore, the selection of the alcohol was performed by studying the retention times obtained by analyzing the standard solution at several hybrid mobile phases containing 1-propanol, 1-butanol and 1-pentanol.

The concentrations of SDS and organic modifier in the tested mobile phases were chosen following a full factorial design plus the center: four points, combining the minimum and maximum concentration of SDS and alcohol typically recommended for MLC, and a

central point taking the intermediate concentrations [18]. In this case, the studied mobile phases were aqueous solutions buffered at pH 3 of:

- SDS/1-propanol (mol/L/%, v/v): 0.05/2.5; 0.05/12.5; 0.10/7.5; 0.15/2.5 and 0.15/12.5.
- SDS/1-butanol (mol/L/%, v/v): 0.05/1; 0.05/7; 0.10/4; 0.15/1 and 0.15/7.
- SDS/1-pentanol (mol/L/%, v/v): 0.05/2; 0.05/6; 0.10/4; 0.15/2 and 0.15/6.

The four pesticides show a binding behavior with SDS-micelles: their retention time decreases at higher concentrations of SDS. Moreover, the retention time also diminishes by increasing the concentration and the length of the carbon chain, as it is usual in MLC. In nearly all the tested mobile phases, the elution order was maintained: t_R(IMZ) > t_R(OPP) > t_R(PYR) > t_R(TBZ). Besides, even using the mobile phase with the higher elution power (0.15 mol/L/6% 1-pentanol), the less retained pesticide (TBZ) was eluted enough far (≈4 min) from the dead time (1.04 min).

The use of 1-propanol was discarded, because even at the more eluent conditions (0.15 mol/L/12.5%, v/v 1-propanol), the retention time of IMZ was too high. Comparing 1-butanol and 1-pentanol, this last one provide less retention times in all the SDS/alcohol amount combinations. Therefore, 1-pentanol was selected as the more adequate organic modifier.

3.1.3 Optimization of SDS/1-pentanol concentration

The optimization criterion was to obtain a mobile phase that allows the complete separation of the pesticides in an appropriate analysis time. The concentration of SDS and 1-pentanol were simultaneously optimized using an interpretative strategy based on a statistical model described in [28]. It allows the prediction of the chromatographic behavior of each analyte depending on the composition of the mobile phase.

The retention factor of a compound is related to the concentration of SDS and 1-pentanol in the mobile phase using the following equation:

$$k = \frac{K_{AS}\dfrac{1}{1+K_{AD}\varphi}}{1+K_{AM}\dfrac{1+K_{MD}\varphi}{1+K_{AD}\varphi}[M]} \quad (3.1)$$

where, [M] (mol/L)) and φ (%, v/v) are the concentration of SDS and 1-pentanol. The constants mean partition coefficients characteristics of each analyte, and has been described in [28]. Besides, the peak shape, and then the efficiency and asymmetry, were modeled using the Eq. (3.2), which calculates the signal h(t) at each time of the chromatographic run (t):

$$h(t) = H_0 e^{-0.5\left(\frac{t-t_R}{s_0+s_1(t-t_R)}\right)^2} \quad (3.2)$$

where, s_i are constants depending on t_R (min), N (number of theoretical plates) and B/A (dimensionless) [28]. They are ideally the same for each studied compound and mobile phase. H_0 (absorbance unit) depends on the concentration of the pesticide.

Michrom software [24] requires the experimental values of retention factor, N and B/A obtained at five mobile phases to adjust the Eqs. (3.1) and (3.2). Thus, the values for each pesticide obtained using the SDS/1-pentanol mobile phases described in section 3.1.2 were processed. Once known, the statistical model was able to estimate the theoretical values of the chromatographic parameters (retention factor, efficiency and asymmetry) and peak shape for TBZ, PYR, OPP and IMZ at intermediate concentrations of SDS/1-pentanol (0.05-0.15 mol/L/2-6%, v/v, respectively) by interpolation. It can utilize this information to draw the corresponding simulated chromatograms without performing the analysis and to calculate the theoretical resolution (r_{ij}) for each pair peak (following the valley-peak criterion) and the predicted global resolution of the chromatogram (Z) calculated as the minor r_{ij} [28].

Applying the maximum resolution-minimum analysis time, the optimal mobile phase proposed by the statistical model was 0.07 mol/L SDS - 5%, v/v 1-pentanol at pH 3 (Z = 0.9997). The pesticide standard solution was analyzed (n = 3) under these conditions (Fig.

3.1). The obtained chromatogram shows completely resolved peaks with adequate shape in <18 min. This indicates the specificity of the method, because each pesticide can be unambiguously identified. The experimental chromatographic parameters (t_R; N; B/A) were: TBZ (8.29; 2272; 1.098), PYR (10.95; 3398; 0.973), OPP (13.19; 2176; 1.159), IMZ (15.70; 6637; 12.78). The error in the prediction of the retention factor was <5%.

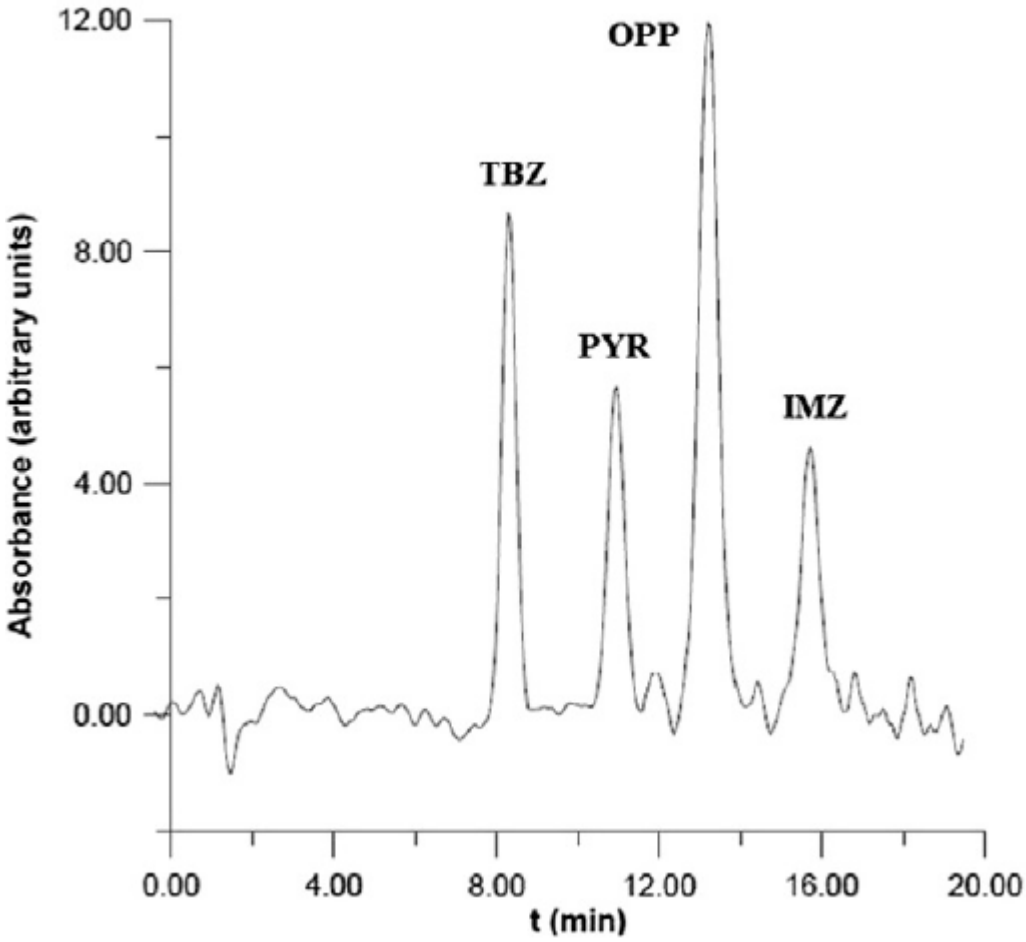


Figure 3.1. Chromatogram obtained by the analysis of a standard solution of 2 mg/L of thiabendazole (TBZ) and pyrimethanil (PYR), 5 mg/L of o-phenylphenol (OPP) and 7 mg/L of imazalil (IMZ) in water.

The selected mobile phase permits the resolution of the pesticide mixture in a short time. Besides, as the isocratic run is used, the stabilization time between two injections is not needed. For these reasons, the achievement of many successive chromatographic runs is

facilitated. Moreover, the mobile phase contains lower amount of toxic solvent (5%) than hydroorganic HPLC (up to 100%), and only requires inexpensive reagents and basic chromatographic instrumentation.

3.1.4 Detection conditions

The pesticide standard solution was analyzed using the optimal micellar mobile, and UV-Visible spectra of each pesticide were registered between 200 and 400 nm using a diode array detector. Therefore, the spectra of each pesticide were obtained for each pesticide in the same environment furthermore used for the analysis.

The maximum absorbance wavelength of each pesticide was taken as the optimal value for the analysis: 305 nm for TBZ, 265 nm for OPP and 220 nm for PYR and IMZ. The baseline noise is higher for PYR and IMZ, because 220 nm is a less selective wavelength, and the absorption of the background increases, especially for aqueous solutions. The wavelength detection was then modified during the chromatographic run to quantify each pesticide at its optimal value.

3.2 Method validation

The method was in-house validated following the US EPA review for chemical methods of analysis, applicable for the analysis of environmental water samples, to check the concentration range of applicability and the reliability of the obtained data. The studied validation parameters were: linearity, quantitation range, inter- and intraday trueness and precision, and ruggedness [22]. The whole validation was performed using standard solutions of TBZ, OPP, PYR and IMZ in effluent wastewater from a WWTP (sample 12), initially without the analytes. The four pesticides were simultaneously studied.

3.2.1 Quantitation range and linearity

For calibration studies, several solutions containing increasing concentrations of pesticides were analyzed (n = 3) at increasing concentrations up to 15 mg/L. The lowest concentration was different for each analyte (see Table 3.3). The slopes, y-intercepts and determination coefficients were obtained by plotting the peak area versus the corresponding concentration by the least-square linear regression. This procedure was carried out five days during a 3-months period, and the calibration curve parameters were taken as the average values of the five measurements. The quantization range covers from the limit of quantification (see below) to 15 mg/L.

The limit of quantification (LOQ) is the minimal concentration at which the analyte can be reliably quantified. It was taken as ten times (10 s-criterion) the standard deviation of the blank, taken as the standard deviation of the residual, divided by the sensitivity (slope of the calibration curve) [28]. Levels under LOQ were removed, and the calibration parameters changed accordingly. The final results are shown in Table 3.3. The excellent values for determination coefficient (r^2>0.9990) indicate a good linearity in the considered range.

The limit of detection (LOD) is the lowest concentration that provides a signal significantly over the baseline noise. Between LOD and LOQ, the analyte is detected, but it cannot be quantified with enough trueness and precision. The LOD was determined as 3 times the standard deviation of the blank divided by the sensitivity (3 s-criterion) [29].

Table 3.3. Calibration parameters and quantitation range (concentrations in mg/L).

Fungicide	Slope	y-intercept	r^2	LOD	LOQ
TBZ	110.3 ± 1.0	0 ± 14	0.99990	0.18	0.5
PYR	81.2 ± 1.5	5 ± 6	0.99990	0.25	0.5
OPP	89.3 ± 1.3	- 50 ± 30	0.9992	1.0	3.0
IMA	38.5 ± 1.1	- 48 ± 6	0.9990	1.4	4.0

n = 5; Quantitation range: LOQ to 15 mg/L

3.2.2 Trueness and precision

For the intraday measurements, the standard solution was analyzed 6-times in the same day. Intraday precision (repeatability) was determined as the relative standard deviation (RSD) of the peak areas. The intraday trueness was calculated as the quotient of the average value of the concentration provided by the method and true value. For the interday values, the same procedure was performed five different days over a 3-months period, by renewing the solution at each occasion. The interday precision (reproducibility) was the RSD of all the taken peak area, whereas the interday trueness was calculated as the average value of five intraday values.

The results are shown in Table 3.4. The measures show good recovery (93.9%-103.7%) and low variability (<9.2%) for the quantitative data provided by the method. The high quality of the results is due to the quantitative transferring of the aliquot by the direct injection, which reduces the probability of loss of analyte.

3.2.3 Ruggedness

The modification of the elution power and the sensitivity at slight, but deliberate changes in the main chromatographic parameters (SDS concentration, 1-pentanol, pH and flow-rate) are studied. The influence of each parameter was separately studied by analyzing (n = 3) the standard solution using three mobile phases at: the optimal value, slightly under and slightly over, and remaining the others constant. The RSD of the three measurements was calculated for retention time and peak area of the three measurements. The results are shown in Table 3.5.

The method was considered quite robust, as low variations were observed for retention times (<9.9%) and peak area (<8.8%).

Table 3.4. Intra- and inter-day trueness and precision for the studied pesticides.

		Intra-day[a]		Inter-day[b]	
Pesticide	Concentration (mg/L)	Trueness (%)	Repeatability (RSD, %)	Trueness (%)	Reproducibility (RSD, %)
TBZ	0.5	102.9	4.1	93.9	8.1
	2	101.1	3.2	97.2	1.3
	15	98.7	6.3	98.3	2.9
PYR	0.5	101.8	6.9	103.7	2.8
	2	98.6	4.4	96.5	1.9
	15	97.8	2.9	98.5	5.4
OPP	3	103.2	3.6	95.5	8.3
	5	103.0	5.1	101.2	3.5
	15	98.9	4.5	103.2	4.7
IMZ	4	94.5	9.2	98.7	7.2
	7	104.3	2.1	103.3	0.6
	15	97.8	4.4	97.8	3.8

[a]n=6; [b]n = 5

Table 3.5. Evaluation of the ruggedness of the MLC-method.

Pesticide	Chromatographic parameters	Level	Retention time (R.S.D.; %)	Peak area (R.S.D; %)
Thiabendazole	SDS concentration (mmol/L)	65 - 75	4.3	2.6
	1-pentanol amount (%, v/v)	4.9 - 5.1	4.0	0.8
	pH	2.9 - 3.1	3.5	2.5
	Flow rate (mL/min)	0.95 - 1.05	5.4	3.1
Pyrimethanil	SDS Concentration (mmol/L)	65 - 75	5.0	4.8
	1-pentanol amount (%, v/v)	4.9 - 5.1	3.1	4.5
	pH	2.9 - 3.1	5.5	1.1
	Flow rate (mL/min)	0.95 - 1.05	5.6	3.3
o-phenylphenol	SDS Concentration (mmol/L)	65 - 75	4.8	8.6
	1-pentanol amount (%, v/v)	4.9 - 5.1	4.5	2.3
	pH	2.9 - 3.1	3.1	3.6
	Flow rate (mL/min)	0.95 - 1.05	5.4	8.8
Imazalil	SDS Concentration (mol/L)	65 - 75	2.9	1.6
	1-pentanol amount (%, v/v)	4.9 - 5.1	9.9	5.5
	pH	2.9 - 3.1	6.5	4.5
	Flow rate (mL/min)	0.95 - 1.05	5.9	8.2

n = 3

3.3 Stability in surface water

The stability of the pesticides in water under environmental conditions was evaluated. Thus, the effect of the oscillations of the temperature and the irradiation was considered. A solution of 10 µg/mL of TBZ and PYR, and 15 mg/L of OPP and IMZ spiked in water was kept for eight days reproducing the outdoor weather conditions: without controlling the temperature, under the sunlight and warm during the day and in darkness and cold during the night. The flask was thoroughly sealed to avoid water evaporation. An aliquot was analyzed each day (nearly at noontime), and the peak area was measured for each pesticide. The results can be seen in Fig. 3.2.

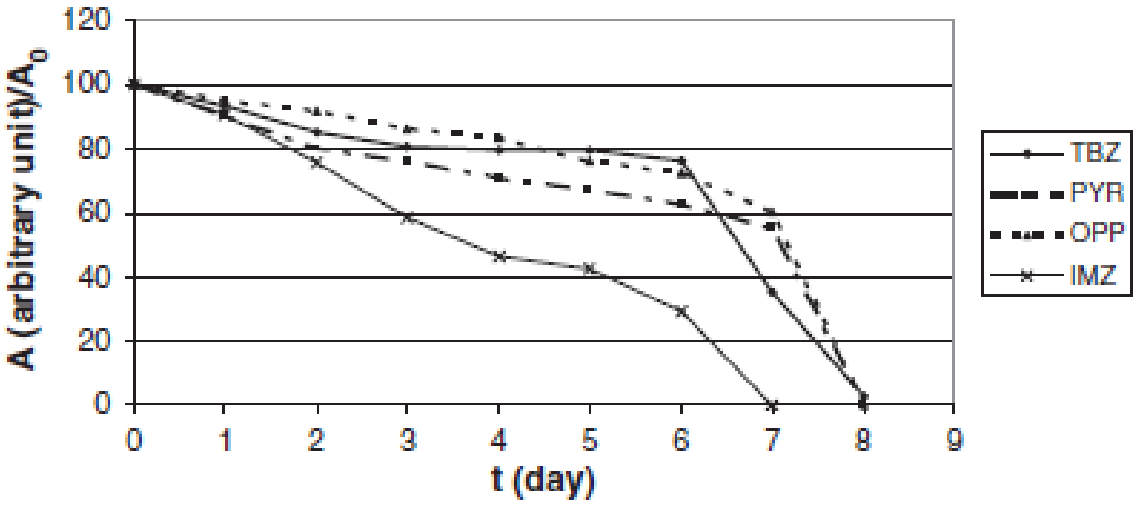


Figure 3.2. Plot of the ratio peak area/initial peak area for the studied pesticides v.s. storage time under outdoor conditions.

The four pesticides show a detectable and continuous degradation process. On the first day, the peak area has diminished (5%-10%). This decrease continues during the following days, and the analytes become undetectable at the eighth day. TBZ undergo a slow decomposition rate during the first six days, since the peak area diminishes nearly 20% during this period. Furthermore, the degradation accelerated, and in only two days the concentration of TBZ falls up to below the detection limit. PYR and OPP show a similar behavior, the peak area lessens at a nearly constant rate of 7% per day during the seven first days, and fully decompose in one more day. On the other hand, IMZ undergoes a rather linear degradation during seven days, when the concentration attains undetectable values.

It was deduced that the four pesticides are significantly affected by the sun radiation and the high temperatures in aqueous media, and remain a short period. Even using high concentration as initial conditions, they fall to undetectable levels in only eight days.

3.4 Analysis of wastewater samples

The concentration of TBZ, PYR, OPP and IMZ was determined in several samples from agricultural sewage, fruit-processing industry waste, and WWTP influent and effluent

water from the Castelló area. The origin and the concentrations can be seen in Table 3.2. The analytes were resolved without interferences. Fig. 3.3 shows the chromatogram obtained by analysis of sample 15.

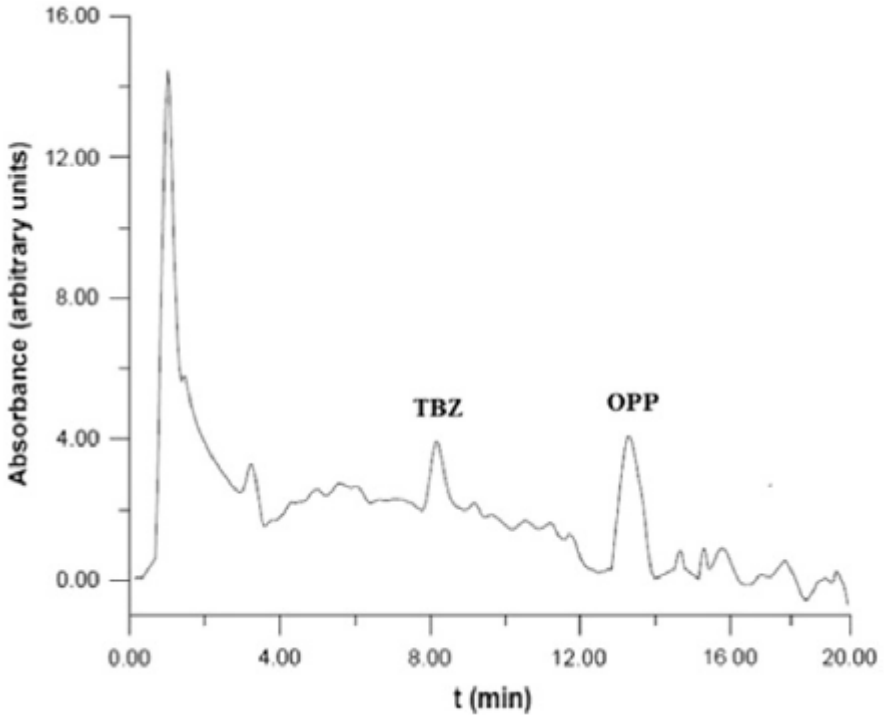


Figure 3.3. Chromatogram of sample 15, collected from the influent water stream of a wastewater treatment plant.

3.4.1 Optimization of the experimental procedure

Several samples had sludge or oily drops suspended in the aqueous matrix. These samples were filtered, and directly injected, as the aliquot. The pressure does not change, and then neither precipitation nor obstruction in the needle, column or chromatographic tubes was noticed. Thus, the sample was not diluted, allowing to keep a reasonable sensitivity level.

This possibility of direct injection, previous filtration, without dilution, clean-up or extraction step can be considered as the main advantage of the method. The simplification of the experimental procedure allows to reach good quality of the validation results, while

minimizing the participation of trained staff, and strongly shortening the analysis time, then favoring the analysis of a large amount of samples. Besides, neither chemical nor extraction instrumentation are needed, reducing the cost, the environmental impact and the safety of the operator.

3.4.2 Results in real samples

Wastewater samples from agricultural origin show a significant concentration of TBZ and PYR. This indicates that these pesticides are pre-harvest applied to crops, and remain in sludge, prior to be dragged to rain water to the wastewater. The collector basins of the fruit-processing plants contain a significant amount of TBZ, OPP and IMZ, those used for postharvest protection. However, considering the large quantity of pesticides needed to assess a correct storage of the fruits, the results indicate that the plants have implemented a purification treatment to partially purify the wastewater prior discharge.

The influent stream of WWTPs contains a moderate concentration of the TBZ, PYR, OPP and in one case, IMZ, because they come from areas with a strong agriculture-related activity. The pesticides in effluents were lower than in influent, ensuring the validity of the water purification process.

4. Conclusions

The here-described MLC-method can be used to monitor TBZ, PYR, OPP and IMZ in routine analysis of sewage water. The sample was directly injected into the chromatographic system after a simple filtration, thus avoiding complex and time consuming intermediate steps. The analytes were identified and eluted without interferences from the waste water matrix in less than 18 min. As main features, we highlight the low global analysis time, and the easy-to-handle sample preparation, which permits the analysis of a large amount of samples per day. The method was validated in terms of quantitation range, linearity, precision, trueness and ruggedness, following the Validation and Peer Review of US EPA for chemical methods of analysis. The method meets the requirements of “green chemistry”, as

low amount of toxic reagents are used, then reducing the waste of pollutants and minimizing the danger for the operator health. Besides, the method is quite inexpensive, and then making it accessible even to laboratories with low economic power. The stability of the pesticides in water at outdoor conditions was evaluated. Finally, the method was applied to determine the concentration of TBZ, PYR, OPP and IMZ in sewage water from an area with a strong fruit-related activity, and suspected to be contaminated.

5. Acknowledgements

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Chapter 4

Development and validation of a method to determine thiabendazole and o-phenylphenol in wastewater using micellar liquid chromatography-fluorescence detection

Abstract

A micellar liquid chromatographic method to determine thiabendazole (TBZ) and o-phenylphenol in wastewater is described here. The sample was directly injected without any additional treatment other filtration. The pesticides were resolved in <11 min, using a mobile phase of 0.10 M SDS-6% 1-pentanol buffered at pH 3 running through a C18 column at 1 mL/min. The detection was performed by fluorescence at 305/360 and 245/345 nm excitation/emission wavelengths for TBZ and o-phenylphenol, respectively. The method was validated following the directives of the Validation and Peer Review of U.S. Environmental Protection Agency Chemical Methods of Analysis guidelines in terms of selectivity, quantitation range (0.01-0.02 to 2 mg/L), detection limit (0.005-0.008 mg/L), trueness (92.1-104.2%), precision (<13.9%), robustness (<6.6%), and stability under storage conditions. The procedure was applied to the screening of TBZ and o-phenylphenol in wastewater samples from citrus packing plants, agricultural gutters, urban sewage, as well as in influent and effluent wastewater treatment plants.

1. Introduction

Thiabenzadole (p*K*a = 4.7/12.0; log Po/*w* = 2.39; solubility in water = 28 mg/L) is a broad spectrum fungicide and antiparasitic [1, 2]. O-phenylphenol (OPP) (p*K*a = 9.4; log Po/*w* = 3.18; solubility in water = 700 mg/L) is a general biocide, with germicide, fungicide, bactericide, and nematicide activity, usually supplied as its sodium salt [2,3]. These pesticides are largely utilized in the agro-food industry as preservative agents. They are included in the post-harvest treatment of many fruits and vegetables commercialized for fresh consumption, to control fungal and mold diseases during storage and transportation, in order to maintain their nutraceutical properties and an attractive appearance. They are deposited on the surface to form a protective film by dipping, drenching or surface waxing [1,3]. They are also used in seed treatments to avoid spoilage [4].

Thiabendazole (TBZ) is used in pre-harvest agriculture and in urban gardening to protect bulbs and trees, by spraying on leaves, fruits and soil [1]. It has also been prescribed as an anthelmintic drug to treat infections caused by parasitic worms in human, livestock and pets [5]. O-phenylphenol (OPP) is the main active ingredient of many disinfectant formulations used to sterilize hard surfaces in agricultural, medical, commercial, institutional, residential and public access premises and equipment. It is also added as preservative to a broad range of materials, such as paints, stains, cement and wood products. It can be also found in cosmetics, as well as in household aerosols and cleaning products [3,6].

Because of their large variety of applications and massive use [1,3-6], TBZ and OPP can be found in wastewaters from diverse sources, such as urban sewage systems, agro-food industry discharges, and agricultural gutters [7, 8]. From there, they can diffuse into the atmosphere and may arrive to the environmental water [9]. They can attain remarkable levels in water [2] and in river sludge [1, 3]. Due to their high toxicity, they can damage the aquatic wildlife and the population, either by dermal contact or by inhalation [1, 3, 10, 11]. Wastewaters are purified in wastewater treatment plants (WWTP), but this process may be incomplete [12]. Besides, a large concentration of TBZ and OPP in influent waters reduces the capacity of the WWTP, and then the quality of the effluent stream [13].

Many municipal governments have launched actions to minimize the pollution level of the wastewaters in their area [14]. Therefore, maximum permitted levels have been fixed

for spills and municipal sewage waters, and the effectiveness of the WWTP must be evaluated. Besides, the agrofood industry has been requested to depurate the waste [7]. Therefore, TBZ and OPP must be continuously monitored in the different wastewaters.

We described in a previous paper a functional, reliable, easy-to-handle, rapid, ecofriendly, and economic procedure to determine thiabendazole (TBZ) and OPP, together with other fungicides, in wastewater by micellar liquid chromatography (MLC) DAD, avoiding the major drawbacks of hydroorganic RP-HPLC [15]. In the present work, we explore the possibility to detect TBZ and OPP in wastewater by fluorescence detection (FLD), in order to increase the sensitivity and the selectivity. Indeed, TBZ [16] and OPP [17] have been determined in water by HPLC-FLD. Besides, fluorescence is enhanced in organized media [18]. The new MLC-FLD method should be validated following the guidelines of the Validation and Peer Review of Environmental Protection Agency (EPA) Chemical Methods of Analysis [19]. Finally, it has to be applied to wastewater samples from several sources in the Spanish town of Villarreal (Castelló province). Therefore, the sensitivity should be sufficient to detect the maximal tolerated concentrations in wastewaters set by the Municipal Regulation of Villarreal (0.5 mg/L for a single spill and 0.1 mg/L as the diary average concentration) [20].

2. Materials and methods

The reagents, apparatus, and the preparation of the mobile phases were the same as in [15].

2.1 Chromatographic conditions

The chromatographic analyses were carried out using an Agilent Technologies Series 1100 (Palo Alto, CA, USA), equipped with an isocratic pump, a degasser, an autosampler, and a fluorescence detector, connected to a computer. The software Chemstation version B.01.01 was used to control the instrument, as well as to acquire and process the signals.

The analytes were eluted using a hybrid micellar mobile phase of 0.10 M SDS/6% 1-pentanol at pH 3 (fixed with 0.01 M phosphate buffer), running at 1 mL/min under isocratic

mode through a Kromasil (Sigma-Aldrich, Saint-Louis, MO, USA) C18 column (150 × 4.6 mm; particle size, 5 μm; pore size, 10 nm). The injection volume was 20 μL. The fluorescence signal was registered using the following excitation/emission (nm) wavelengths program: 0-8.0 min, 305/360; and 8.0-11.0 min, 245/345.

2.2 Sample collection and processing

Wastewater samples (Table 4.1) were collected at several points of the Castelló province, in Spain, between February and May, 2015. The samples were stored in amber bottles in a fridge (+4ºC), and analyzed before 2 weeks.

The samples were thawed 30 min at room temperature before processing. Thereafter, an aliquot was filtered and directly injected.

3. Results and discussion

3.1 Optimization of the chromatographic conditions

The main chromatographic conditions (see Section 2.1) were taken from [15]. TBZ is positively monocharged and OPP is neutral, and both are moderately hydrophobic. Therefore, they would be adequately retained in a C18 column.

The SDS and 1-pentanol concentrations in the mobile phase and the detection conditions were optimized to determine TBZ and OPP.

Table 4.1. Origin, sampling way and date, and TBZ/OPP concentration (mg/L) of the studied samples (supplied by FACSA, Castelló, Spain).

Origin	Nº	Date	Sampling	Place	TBZ	OPP
Wastewater collector basin from a fruit packing plant	1	07/02	Spot	Real Export (Villarreal)	1.09	0.78
	2	14/02	Spot	Serifruit (Villarreal)	0.78	1.1
	3	28/02	Spot	Invicto (Villarreal)	0.89	0.95
	4	28/02	Spot	Eurococi (Betxí)	0.09	0.51
Agricultural gutter	5	11/02	Continuous 08:00-18:00 h	Villarreal	0.31	n.d.
	6	24/02	Continuous 24 h	La Vilavella	0.44	n.d.
	7	26/02	Continuous 24 h	Alcora	0.51	n.d.
	8	27/03	Continuous 24 h	Betxí	0.24	n.d.
	9	13/05	Continuous 08:00-18:00 h	Onda	0.18	n.d.
Urban sewage water	10	05/03	Continuous 24 h	Nules I	n.d.	0.02
	11	15/04	Continuous 08:00-18:00 h	Nules II	0.01	0.05
	12	22/04	Continuous 08:00-18:00 h	Villarreal I	0.06	0.03
	13	13/05	Continuous 08:00-18:00 h	Villareal II	n.d.	0.06
	14	14/05	Continuous 08:00-18:00 h	Alcora	n.d.	n.d.
	15	27/05	Continuous 08:00-18:00 h	Onda I	n.d.	n.d.
	16	25/06	Spot	Onda II	0.05	0.07
Wastewater from the WWTP Vora Riu (Villarreal)	17	05/03	Spot	Influent	1.57	1.34
	18	05/03	Spot	Effluent	0.02	n.d.
Wastewater from the Joint WWTP OBVA (Onda/ Betxí/Vilareal/ Alquerías)	19	06/04	Spot	Influent	0.84	1.75
	20	12/06	Spot	Decanted influent	0.91	1.83
	21	06/04	Spot	Effluent	n.d.	n.d.
Wastewater from the WWTP Nules, La Vilavella	22	19/02	Spot	Influent	0.71	1.1
	23	19/02	Spot	Effluent	n.d.	n.d.

The places refer to several towns from the Castelló Province (Spain) - n.d. = not detected

3.1.1 Optimization of the SDS/1-pentanol concentration

The SDS/1-pentanol concentrations were optimized following the criterion of maximal resolution–minimum analysis time using an interpretative strategy [15, 21-23].

The influence of SDS and 1-pentanol on the elution strength of the mobile phase was examined using a full factorial experiment. The experimental retention factors of TBZ and OPP were measured by five mobile phases containing a combination of the minimum and maximum concentration of SDS (M) and 1-pentanol (%) recommended for MLC (0.05/2; 0.05/6; 0.15;2 and 0.15/6), and another one at the intermediate values (0.1/4) [15]. In the five cases, TBZ was eluted before OPP, according to its higher polarity, and their peak shapes were quite Gaussian for the five mobile phases. Both pesticides show a binding behavior with SDS-micelles, as their retention factor diminishes when the number of micelles increases. Besides, the analytes were less retained by increasing the proportion of 1-pentanol, as usual in MLC.

The retention factors and the modified selectivity were modelled from the concentration of SDS and 1-pentanol in the mobile phase. Thereafter, the most adequate values were selected by response surface methodology.

3.1.1.1 Modeling of the retention behaviour

The retention factor (k) was modeled using the following empirical equation, applicable to moderately hydrophobic compounds [21]:

$$1/k = c_0 + c_1 [SDS] + c_2 \varphi + c_{12} [SDS] \varphi \quad (4.1)$$

where φ is the proportion of 1-pentanol and the c_i are experimental constants.

For each pesticide, the data obtained from the factorial experiment were processed to fit this equation by nonlinear least-squares regression (curve fitting) [22]. The concentrations of SDS and 1-pentanol were normalized from their ranges (0.05 - 0.15 M) and (2 - 6 %), respectively, to (-1; +1), in order to compare the obtained constants. According to the

obtained multiple regression coefficients (R^2), this hyperbolic model provides accurate predictions. The adjusted equations were:

TBZ $1/k=(0.164\pm0.003)+(0.072\pm0.004)[SDS]+(0.037\pm0.004)\varphi+(0.017\pm0.004)[SDS]\varphi$ $R^2=0.998$ (Eq 4.2)

OPP $1/k=(0.105\pm0.003)+(0.044\pm0.003)[SDS]+(0.017\pm0.003)\varphi+(0.010\pm0.003)[SDS]\varphi$ $R^2=0.997$ (Eq 4.3)

The values of the constants and their corresponding standard deviation allow us to infer interesting statements about the retention behaviour of both pesticides. The constants c_1; c_2 and c_{12} have a low relative standard deviation and a positive value, that means the rising of SDS or 1-pentanol amount effectively provokes a significant diminishing of the retention. In addition, the augmentation of the elution strength caused by the increase of the SDS concentration is more intense at higher proportions of 1-pentanol, and *vice versa*. The most influent parameter was the concentration of SDS, followed by the proportion of 1-pentanol and, finally, their interaction. Otherwise, the theoretical retention time was higher for OPP than for TBZ in the overall studied ranges. These assertions coincide with that previously observed in the qualitative interpretation of the untreated experimental results obtained from the factorial experiment.

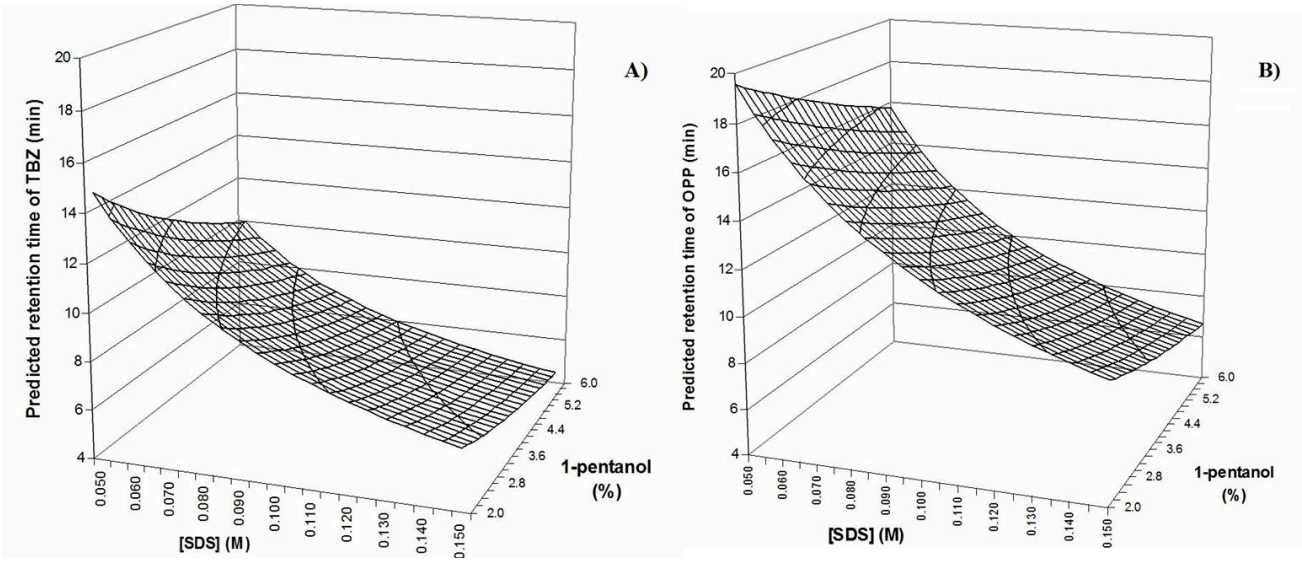

Figure 4.1. Predicted A) thiabendazole and B) o-phenylphenol retention time, *versus* the concentration of SDS and 1-pentanol.

3.1.1.2 Modeling of the chromatographic resolution

The selectivity (α) and the modified selectivity (Z) were modeled by the following equation [23]:

$$Z = 1\text{-}1/\alpha = 1\text{-}\ k(TBZ)/k(OPP) \quad (4.4)$$

$$Z = 1 - (0.164+0.072[SDS]+0.037\varphi+0.017[SDS]\varphi)/(0.105+0.044[SDS]+0.017\varphi+0.010[SDS]\varphi) \quad (4.5)$$

The theoretical modified selectivity can be seen in Figure 4.2.

3.1.1.3 Selection of the SDS/1-pentanol concentrations

SDS and 1-pentanol concentrations were selected considering their effect on the duration of the chromatographic run and the separation of both analytes. We can see that the maximal modified selectivity (0.3911) was achieved at 0.05 M SDS/6 % 1-pentanol, but with a too high analysis time, as OPP was eluted at nearly 15 min. At 6 % 1-pentanol, the modified selectivity was barely affected by the SDS concentration, while the retention times were significantly reduced. Finally, the selected mobile phase was an aqueous solution of 0.10 M SDS/6 % 1-pentanol phosphate-buffered at pH 3, which provides a theoretical modified selectivity of 0.389 and an expected retention time of 9.0 min for OPP. The error in the prediction of the retention factors was < 3.0 %.

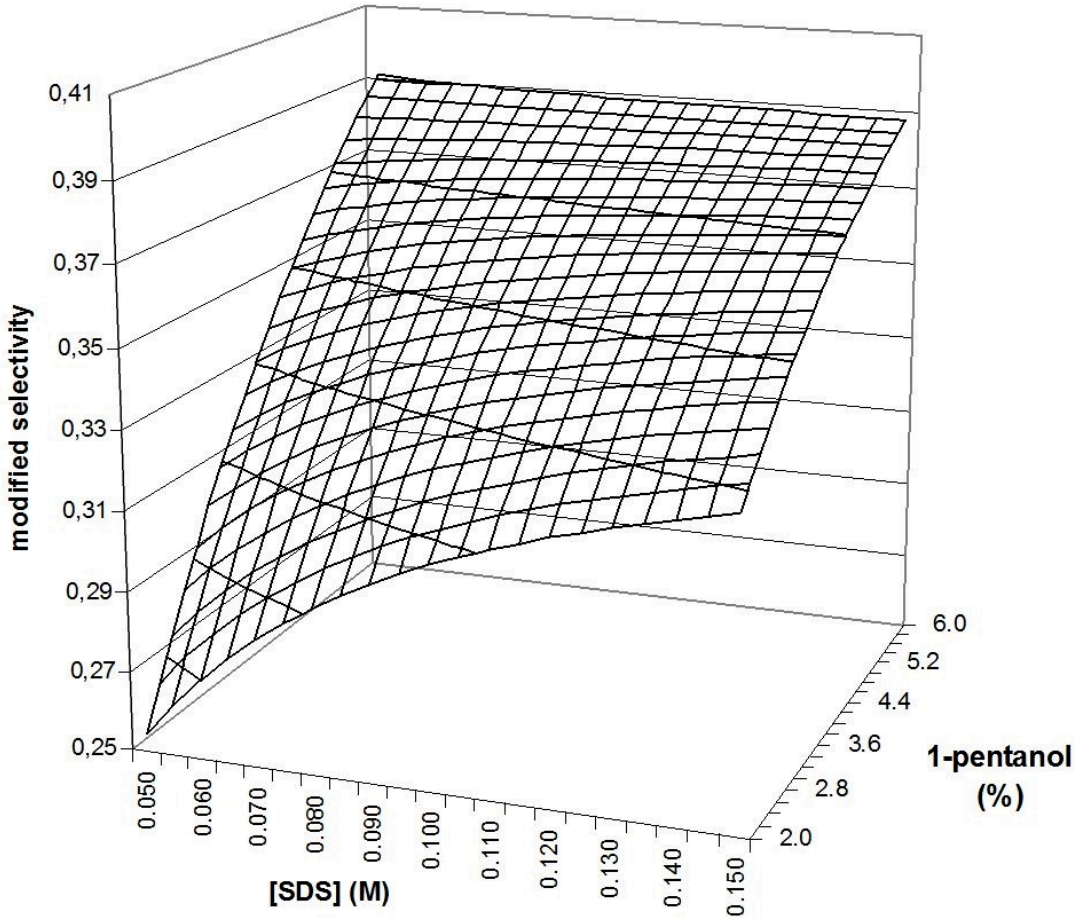

Figure 4.2. Predicted modified selectivity *versus* the concentration of SDS and 1-pentanol.

The selected mobile phase was an aqueous solution of 0.10 M SDS/6% 1-pentanol phosphate buffered at pH 3. Under these conditions, TBZ and OPP were eluted at 6.0 and 9.2 min, respectively (total analysis time, <11 min), with a quite Gaussian peak shape (Fig. 4.3A). The limited efficiency, as usual in MLC [21], was not a disadvantage, because the analytes do not overlap and no other peaks were detected.

3.1.2 Detection conditions

The detection conditions were optimized by a chromatographic analysis of both pesticides maintaining the other instrumental parameters at their previously selected values. The excitation and emission spectra were on-line measured using the fluorescence detector of the chromatograph, and the optimal excitation/emission wavelengths were selected by

iteration to maximize the S/N of the emitted luminescence. These values were 305/360 and 245/345 for TBZ and OPP, respectively.

The excitation/emission wavelengths were adjusted in time to quantify each pesticide under its optimal conditions. Thus, the signal was monitored by the following program: 0-8.0; 305/360 (for TBZ) and 8.0-11.0; 245/345 nm (for OPP). No sudden variation of the baseline signal was noticed at the change time, and the baseline noise was relatively narrow.

3.2 Method validation

The analytical procedure was within-laboratory validated following the directives of the Validation and Peer Review of the EPA Chemical Methods of Analysis. The studied parameters were instrument calibration, quantitation limits and range, detection limit, inter- and intraday trueness and precision, robustness [19], and stability under storage conditions.

Seven solutions containing increasing concentrations of the pesticides (0.01–2 mg/L) were analyzed (n = 3). For each pesticide, the peak area was correlated to the corresponding concentration (mg/L) by least-square linear regression [23]. The resulting equations were:

TBZ A = (1524 ± 14)[TBZ] + (9 ± 4); r^2 = 0.9995; calibration range = 0.01-2.0 mg/L
OPP A = (525 ± 23)[OPP] + (1.0 ± 2.6); r^2 = 0.9998; calibration range = 0.02-2.0 mg/L

These calibration data refer to the concentration (mg/L) in the injected sample. As neither dilution nor preconcentration steps were included in the analytical procedure, the concentration in the sample equals that in the injected solution. Therefore, the quantitation range is the same as the calibration range.

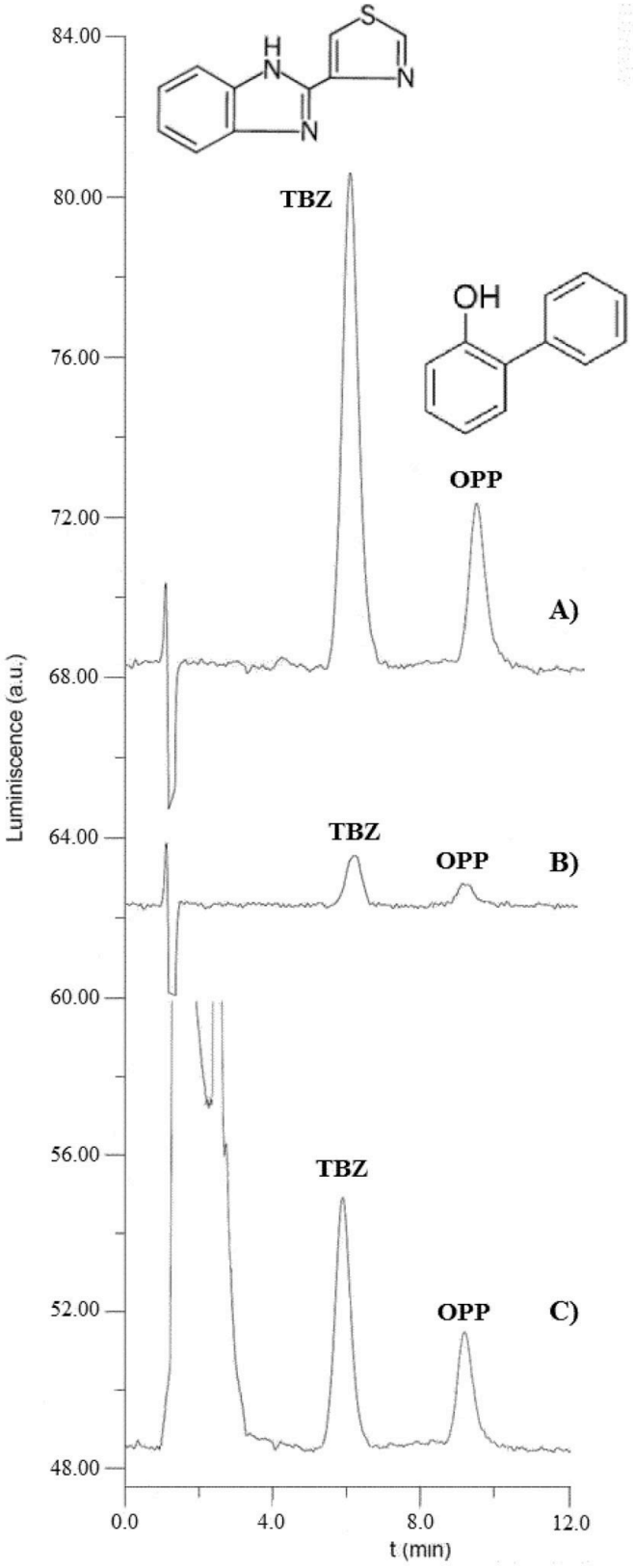

Figure 4.3. Chromatograms obtained by the analysis of a standard solution of TBZ and OPP (A) at 0.1 mg/L and (B) at their LLOQ, and (C) sample 16.

The lower (LLOQ) and upper (ULOQ) LOQ were taken as the smallest and the highest concentration of the calibration curve, respectively [23]. The LOD was calculated by the 3.3 criterion: TBZ, 0.005 mg/L and OPP, 0.008 mg/L. The chromatogram obtained by the analysis of a water sample spiked at their corresponding LLOQ can be seen in the Fig. 4.3B. The intra- and interday trueness and precision were studied as described in [15], at four concentrations: LLOQ, both regulatory levels (0.1 and 0.5 mg/L), and ULOQ. The method shows good recovery (92.1-104.2%) and low variability of the signals (<13.9%). The robustness was examined as indicated in [15], inside the following ranges: SDS concentration, 100 ± 5 mM; 1-pentanol proportion, 6.0 ± 0.2%; and pH, 3.0 ± 0.1. Insignificant variations of retention time (<6.5%) and peak area (<6.6%) were noticed.

A blank sample was spiked at 0.5 mg/L of TBZ and OPP and kept in a fridge as indicated in Section 2.2. During 2 weeks, an aliquot was daily taken and analyzed (n = 3), and no significant reduction of the peak area was observed.

The quantitation range includes the maximum permitted limits in discharges to the sewage system set by the municipal bylaw of Villarreal (Castelló, Spain) [20]. Besides, the sensitivity is able to detect moderate concentrations of TBZ and OPP in polluted water. Otherwise, the method provides reliable quantitative results, because of the high recovery, low variability, and the stability of the experimental response. In addition, the samples can be kept until 2 wk before analysis. Therefore, the developed procedure can be used to evaluate the compliance of the spills and the urban sewage with the regulation.

3.3 Analysis of wastewater samples

The concentrations of TBZ and OPP were measured in the wastewater samples described in the Table 4.1. In all cases, the matrix was barely retained, and the analytes were resolved without interferences. The chromatogram obtained from the analysis of the sample 16 is shown in Fig. 4.3C. Concentrations over the maximum permitted limit (0.1 mg/L) stated by the Municipal Regulation of Villarreal, were considered "high."

The collector basins from citrus packing plants showed an elevated quantity of both pesticides (except sample 4), as they are largely used for postharvest conservation purposes.

Agricultural gutters contained a significant concentration of TBZ, while OPP was not detected. Indeed, TBZ is preharvest applied in trees and fruits, and it is further dragged by irrigation and rain water to the gutters. On the contrary, OPP is not directly used on crops. In urban wastewater, low concentrations of OPP and TBZ were detected. The higher occurrence of OPP was probably due to its higher domestic and public applications.

TBZ and OPP were found at high concentrations in influent WWTP streams. The occurrence of both pesticides is due to the presence of densely populated urban centers, strong industrial (for OPP) and fruit-agricultural activity in the area, both production (for TBZ) and processing (for TBZ and OPP).

Effluent streams contained no detectable quantities of both contaminants. Therefore, the purified water can be released to the environment.

4. Concluding remarks

The here-described procedure provides interesting advantages over that in [15]. First, the duration of the chromatographic run was significantly shortened from 14 to 11 min, because the present study was focused on two compounds. This permits the study of a larger number of samples per day, which is interesting for routine analysis. The use of FLD, instead of UV absorbance, also improved the quality of the method. The sensitivity was significantly augmented, as the detection limits were nearly 100 times lower (0.005-0.18 and 1.0-0.008 mg/L, for TBZ and OPP, respectively) and the baseline was narrower. This is a significant improvement, insomuch as the present method allows the detection of pollution at lower levels. Besides, the signals from the front of the chromatograms were lower, and less matrix compounds were detected, which enhances the specificity of the method. The other features were similar to [15]. Therefore, this MLC-FLD procedure is an interesting alternative for laboratories of public agencies, treatment plants, and the agro-food industry to monitor TBZ and OPP in wastewater samples, because of its interesting analytical performances.

5. Acknowledgements and conflict of interest disclosure

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The authors have declared no conflict of interest.

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Chapter 5

Analysis of thiabendazole, 4-tert-octylphenol and chlorpyrifos in waste and sewage water by direct - injection micellar liquid chromatography

Abstract

A micellar liquid chromatographic method has been developed for the simultaneous quantification of the pesticides thiabendazole and chlorpyrifos, as well as an alkylphenol, which is a derivative of the surfactants alkylphenol polyethoxylates included in pesticide formulations, *i.e.*, 4-tert-octylphenol, in water. A sample was filtered and directly injected, avoiding large extraction steps using toxic solvents, thus expediting the experimental procedure. The contaminants were eluted without interferences in <17 min, using a mobile phase of 0.15 M sodium dodecyl sulfate - 6% 1-pentanol buffered at pH 3, running through a C18 column at 1 mL/min under the isocratic mode. This optimal mobile phase was selected using a statistical approach, which considers the retention factor, efficiency and peak shape of the analytes measured in only a few mobile phases. The detection was carried out by measuring absorbance at 220 nm. The method was successfully validated in terms of specificity, calibration range (0.5 to10 mg/L), linearity (r^2>0.994), limit of detection and quantification (0.2-0.3; and 0.5-0.8 mg/L, respectively), intra- and interday accuracy (95.2–102.9%), precision (<8.3%), and ruggedness (<9.3%). The stability in storage conditions (at least 14 days) was studied. The method was safe, inexpensive, produced little pollutant and has a short analysis time, thus it is useful for the routine analysis of samples. Finally, the method was applied to analyse wastewater from the fruit-processing industry, wastewater treatment plants, and in sewage water belonging to the Castelló area (Spain). The results were similar to those obtained by an already reliable method.

1. Introduction

Pesticide formulations are used in agriculture and food-processing plants to protect crops during growing, storage, and in gardening to maintain house plants, from annoying pests. They are made of a pesticide, as the active principle component, mixed with other materials such as stabilizers, solvents, adjuvants, foaming agents, dispersants, suspensors or emulsifiers [1,2]. Non-ionic alkylphenol polyethoxylates (APEs) are among the surfactants that are most commonly included in pesticide formulations. They are also added in household detergents [3], cosmetics and office products [4]. Because of their proven toxicity, persistency in the environment and bioaccumulation, pesticides [5,6] and APEs [3,4] represent an important source of contamination of natural water.

These hazardous compounds are incorporated into agricultural and food-processing plant waste and municipal sewage water, which are further processed by wastewater treatment plants (WWPT) to remove the contaminants. Depending on the pollutant, its concentration in the influent water and the purification technique applied in the WWPT, the elimination may be incomplete. Hence, some amount of pesticides and APEs can remain in the effluent water, which is discharged to the river [7,8]. The occurrence of these contaminants in natural water causes serious damage to local flora and fauna [2,3]. The population is also directly exposed to this contamination by accidental inhalation, dermal and oral contact with polluted water [3,9], and through the food chain, by the consumption of edible tissue of animals and plants grown with contaminated water [10,11]. Actually, these chemicals are cataloged as “Emerging Pollutants”, hazardous compounds that have to be controlled and regulated due their potential environmental and health hazards. The European Union, through the “EU Water Framework Directive” [12] and the US Environmental Protection Agency [13] have implemented programs and policies to monitor these compounds in surface water.

Thiabendazole (TBZ) is a fungicide and antiparasitic, which is largely used as a post-harvest preservative for various fruits and vegetables. Thiabendazole health effects include damage to red blood cells, liver and thyroid. It is even carcinogenic at high concentrations [14]. Chlorpyrifos (CPF) is an insecticide, which is extensively used pre-harvest in

agriculture to protect crops such as cotton, corn, almonds, orange and apples, and in households to protect ornamental plants, lawn, pets and wooden objects [5] CPF is quite toxic and it causes diseases and disrupting effects on the nervous system with short term contact [15]. The short APE 4-tert-octylphenol (4-tOP) is a product of degradation by aerobic hydrolysis of long APEs spiked in formulations [16]. APEs show endocrine disruption effects, thus altering the hormonal system. In addition, 4-tOP shows higher toxicity and bioaccumulation than its long APE precursors [3,4]. These compounds are largely used in the Castelló area, due to its strong fruit agriculture and fruit-processing industry, which introduces a high risk of water contamination. Thus, the monitoring of TBZ, CPF and 4-tOP in waste and sewage water is required to protect population health and the environment.

A high amount of analytical methodologies has been developed to detect pesticides [17] and alkylphenols [18,19] in several types of water. Among them, those based on both gas chromatography (GC) and liquid chromatography (LC) are predominant. HPLC coupled to mass spectrometry (MS) has been proposed for routine analysis of pesticides [20] and APEs [21-24] in water samples, although GC-MS is still being used [25,26]. However, a mass spectrometer is an expensive instrument, hence, the analyses of water samples are high-priced. HPLC coupled with UV-Visible absorbance (DAD) is an economic alternative and has been shown to be successful in several reports [27-30]. Waste and sewage water usually contain suspended sludge and oily compounds, and requires sample preparation to avoid the introduction of harmful substances in the chromatographic system. The experimental procedure involves tedious and time consuming clean-up steps, such as solid/liquid [20-22,24,28,30] and liquid/liquid [27,29] extraction, which increases toxic waste and the risks related to the handling of hazardous reagents. The introduction of additional steps can also cause sample loss or experimental error. Recently, new efforts have been taken to develop analytical methodologies to avoid these problems [31].

Micellar liquid chromatography, using hybrid mobile phases containing sodium dodecyl sulfate (SDS) as the surfactant and a short-chain alcohol, has been demonstrated as an interesting alternative to hydroorganic-RP-HPLC [32]. Micellar solutions are able to solubilize compounds within a wide range of polarities. Therefore, samples with hydrophobic compounds can be directly injected, without the risk of column damage. Moreover, the surfactant monomer coat on the external layer of the stationary phase, changes its

characteristics. The analyte is partitioned between three environments (stationary phase, mobile phase and micelles), thus improving the versatility of MLC [33]. The strong reproducibility and stability of the chromatographic behavior of the analytes allows the prediction of the solute retention using a statistical model, from the experimental data, which can be obtained in several mobile phases, thus expediting the optimization of the mobile phase composition. Moreover, micellar mobile phases are non-flammable, less toxic, more environmentally friendly, and relatively inexpensive than those used in hydroorganic-HPLC.34 Micellar liquid chromatography has been previously proposed to detect chemical pollutants in wastewater [35], and the pesticide carbaryl [36] in environmental water.

The aim of this work is to develop a rapid, easy-to-handle, inexpensive, environmentally friendly and reliable method to detect the pesticides TBZ, CPF and the short APE 4-tOP in water samples, in order to apply it to routine analyses. The features of MLC are exploited to allow the direct injection of the sample and resolve the mixture of analytes in a short chromatographic run. The method is validated in terms of calibration, linearity, sensitivity, intra- and interday accuracy and precision, ruggedness and stability to prove its reliability [37]. Finally, the developed analytical method was used to quantify the analytes in WWPT influent and effluent, industrial waste from the fruit-processing industry, and sewage water samples, which were collected at several points in the Castelló area. The results are compared with those obtained by a reference method based on LC-MS.

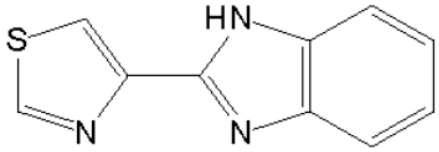
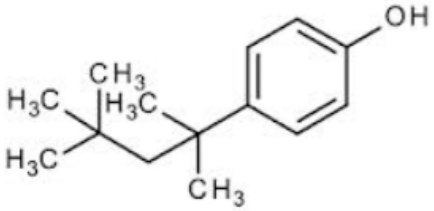
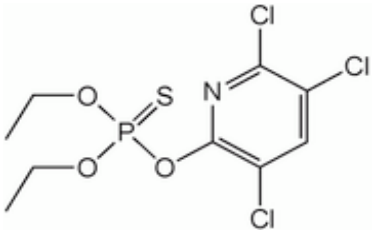
2. Material and methods

2.1. Chemicals and equipment

Standards of TBZ, CPF and 4-tOP (purity > 99.0%), were purchased from Dr Ehrenstorfer-Schäfers (Augsburg, Germany). The structures and main physicochemical characteristics of these compounds are shown in Table 5.1. SDS (purity > 99.0%), methanol, 1-butanol, 1-pentanol (HPLC grade) were obtained from Scharlab (Barcelona, Spain). Hydrochloric acid (37.0%), sodium hydroxide, 1-propanol and sodium dihydrogenphosphate monohydrate (analytical grade) were supplied by Panreac (Barcelona, Spain). The additives triethylamine (TEA) and 1-ethyl-3-methylimidazolium tetrafluoroborate ($EMIMBF_4$), both of

HPLC grade, were obtained from J.T. Baker (Deventer, The Netherlands) and Sigma-Aldrich (St. Louis MO, USA), respectively. Ultrapure water was in-laboratory produced from deionized water using an ultrapure water generator device, Millipore S.A.S. (Molsheim, France). This ultrapure water was used in all aqueous solutions.

Table 5.1. Structure and physicochemical parameters of the analytes.

Compound	Structure	pKa	Charge at pH = 3	$\log P_{o/w}$
Thiabendazole [15]		4.73/12.00	+ 1	1.62
4-tert-octylphenol [3]		10.7	0	4.12
Chlorpyrifos [17]		Not applicable	0	4.70

2.2 Preparation of solutions and mobile phases

The stock solutions of the pesticides were prepared by weighing a portion of the pesticide and dissolving it in methanol, in order to obtain concentrations of 100 mg/L. Working solutions were prepared by diluting these stock solutions in methanol to reach the desired concentration. All the solutions were protected from light and stored at 4 °C.

The micellar mobile phases were prepared by dissolving the appropriate amount of SDS and sodium dihydrogenphosphate. in ultrapure water. An adequate volume of TEA or $EMIMBF_4$ was added, and then the pH was adjusted by adding drops of HCl or NaOH solution to reach the desired value. Furthermore, an adequate volume of short-chain alcohol was added, the solution was filled up to the final volume with ultrapure water, ultrasonicated and filtered.

All the solutions and mobile phases were filtered through 0.45-μm-Nylon membranes (Micron Separations, Westboro, MA, USA).

2.3 Apparatus and instrumentation

The solid standard and reagents were weighted on a Mettler-Toledo analytical balance (Greifensee, Switzerland). A GLP 22 potentiometer (Crison, Barcelona) equipped with a combined Ag/AgCl/glass electrode was used to measure pH values. The ultrasonication of the mobile phases was performed in an ultrasonic bath; model Ultrasons-H (Selecta, Abrera, Spain).

The separation and quantification was performed using an Agilent Technologies HP 1100 Series (Palo Alto, CA, USA) chromatographic system equipped with an isocratic pump, a degasser, an auto sampler and a UV-visible variable wavelength detector (VWD). The signal was obtained by a personal computer connected to the chromatographic system using an Agilent Chemstation version B.01.01. The chromatographic parameters such as retention time (tR, min), peak area (A, arbitrary units), dead time (t_0, min), retention factor (k), efficiency (N, theoretical plates) and asymmetry (B/A) were obtained from the registered chromatograms using the Michrom software [38]. The meaning of these chromatographic parameters can be found in [39].

2.4 Chromatographic conditions

The stationary phase was coated on a Kromasil C18 column (125 × 4.6 mm, 5 μm, 100 Å) from Scharlab. The mobile phase was an aqueous solution of 0.15 M SDS - 6% 1-pentanol buffered with 0.01 $NaH_2PO_4.H_2O$ at pH 3, running under the isocratic mode at 1 mL/min at room temperature. The injection volume was 20 μL and the absorbance detection wavelength was set at 220 nm. The special care required for the chromatographic system when dealing with micellar mobile phases can be seen in [40]. Under these conditions, the column has a lifespan of nearly 1000 injections [40].

2.5 Sample treatment

Water samples were provided by FACSA (Castelló, Spain), the company which manages the water monitoring and treatment in the Castelló province in Spain. The samples were collected during the February-May period from several places where the presence of TBZ, CPF or 4-tOP is suspected: influent and effluent of WWPT, fruit-processing plant wastewater and sewage water (see section 3.3). The samples were placed in a fridge protected from light (amber glass) until analysis. Prior to analysis, sample water or standard solutions were taken out of the fridge and maintained for 30 min to reach room temperature. Then, they were filtered and directly injected into the chromatographic system.

3. Results and Discussion

3.1 Optimization of the chromatographic conditions

The column, injection volume and flow rate were taken as the usual conditions in MLC, whereas the composition of the mobile phase and the detection condition were optimized. A standard solution containing 2 mg/L of TBZ, CPF and 4-tOP was used for optimization.

3.1.1 Optimization of the pH

The pH was selected in the working range of the column (1.5-7.5). The mobile phase was buffered to avoid variation of pH when the sample was injected in the mobile phase flow.

Three mobile phases with the composition indicated in section 2.4, but buffered at pH 3; 5 and 7 were tested. At the three pH, the retention times were similar for the three studied compounds. However, a strong tailing was observed for TBZ at pH 5 and 7, whereas the peak shape was quite Gaussian at pH 3. For CPF and 4-tOP, the peak shape was comparable at the three pH. As a consequence, pH 3 was selected for the analyses.

3.1.2 Selection of the organic modifier

According to the strong hydrophobicity of 4-tOP and CPF, a pure SDS solution would be unable to elute them from a C18 column in a reasonable retention time [41]. Therefore, SDS/1-propanol SDS/ 1-butanol and SDS/1-pentanol hybrid mobile phases were tested.

The mobile phases containing the maximal concentration recommended for SDS and each short-chain alcohol were tested: 0.15 M SDS/12.5% 1-propanol, 0.15 M SDS/7% 1-butanol and 0.15 M SDS/6% 1-pentanol.32 In the three mobile phases, the elution order was: tR(TBZ) < tR(4-tOP) < tR(CPF), and these retention times increases when the lenght of the carbon chain of the alcohol decreases. Finally, the mobile phases containing 1-butanol and 1-propanol were discarded because the analysis time was too high. Thus, 1-pentanol was selected.

3.1.3 Optimization of SDS/1-pentanol concentration

The concentrations of SDS and 1-pentanol were simultaneously optimized using an interpretative strategy. The experimental design consists of four mobile phases containing a combination of the minimum and maximum amount recommended for SDS and 1-pentanol in MLC, and the average value. Therefore, the mobile phases tested were SDS (M)/1-pentanol% (v/v): 0.05/2; 0.05/6; 0.1/4; 0.15/2 and 0.15/6 [32]. The experimental chromatographic

parameters: (retention factor; efficiency and asymmetry) for each mobile phase were taken for the three analytes. From these preliminary studies, it was deduced that TBZ, 4-tOP and CPF show a bending behavior face to SDS, and the retention factor and the efficiency decrease at higher SDS concentrations. As expected, the elution power and the peak shape increased with larger amount of 1-pentanol.

The more adequate mobile phase composition was obtained using a statistical model. The relationship between the retention factor of a specific compound and the SDS ([M]) and 1-pentanol (φ) concentrations of the mobile phase are related by the following equation [32]:

$$k = \frac{K_{AS}\dfrac{1}{1+K_{AD}\varphi}}{1+K_{AM}\dfrac{1+K_{MD}\varphi}{1+K_{AD}\varphi}[M]} \qquad (5.1)$$

The constants signify partition coefficients between phases. Where: K_{AS}, is the partition constant between the stationary phase and aqueous environment times the phase ration; K_{AM}, is the partition coefficient between the micelle and the aqueous environment, and K_{AD} and K_{MD}, are the relative variation in the solute concentration in pure water and micelles due to the presence of 1-pentanol, as compared to a pure micellar solution [42]. Another equation allows for the modeling of the peak shape (N and B/A) at several SDS/1-pentanol concentrations [32].

For each analyte, the experimental values of *k*, N and B/A, which were obtained from the five tested mobile phases were processed by the Michrom software as "calibration levels" in order to calculate the constants of the equations. Therefore, the mathematical model was able to predict the chromatographic behavior (the values of *k*, N and B/A) of TBZ, 4-tOP and CPF in mobile phases containing intermediate SDS and 1-pentanol concentrations, 0.05–0.15 M, and 2–6%, respectively. The software also predicted the resolution of each pair (r_{ij}), which was calculated using the valley peach criterion, and the global resolution (R) was taken as the least r_{ij}. This information was used to draw simulated chromatograms, in order to allow the operator to visualize the variations of *k*, N and B/A of the analytes when the SDS and 1-pentanol concentrations in the mobile phase change [32,38].

According to the statistical model, using a mobile phase of 0.15 M SDS - 6% 1-pentanol at pH 3, the three analytes would be completely resolved (R = 1) in the minimum analysis time (<20 min). A solution containing 2 mg/L of each studied pollutant was analyzed. The experimental chromatographic parameters (t_R; N and B/A) were as follows: TBZ (3.82 min; 1490; 0.97), 4-tOP (7.43 min; 1340; 1.08) and CPF (14.16 min; 1110 and 1.06). The obtained chromatogram can be seen in Fig. 5.1A. As predicted, the mixture was completely resolved in an adequate time (<17 min), thus proving the high specificity of the method. The errors in the expected retention factors were below 6%.

3.1.4 Optimization of additive concentration

The additives triethylamine (a tertiary amine) and 1-ethyl-3-methylimidazolium tetrafluoroborate (an ionic liquid) has been used in liquid chromatography to block the protonated silanol groups [43]. This avoids their interaction with the column, thus preventing the formation of tailing and improving peak shape. Therefore, two mobile phases containing 0.5% of TEA and EMITBF4 were tested. In both cases, the retention factors of the analytes increased without improvement in the peak shape when compared with the mobile phase selected in section 3.1.3. For this reason, the use of these additives was discarded.

3.1.5 Optimization of the detection conditions

The mixture of TBZ, 4-tOP and CPF was analyzed through the previously selected optimized conditions, at wavelengths ranging from 200 and 300 nm by measuring at 10 nm intervals. Thus, we obtained the absorbance of each compound in the chemical environment formed by the already selected micellar mobile phase. A strong absorbance was observed by the three analytes at 220 nm, with low baseline noise. Therefore, this value was taken for the analysis, and the whole chromatogram was registered at the same wavelength.

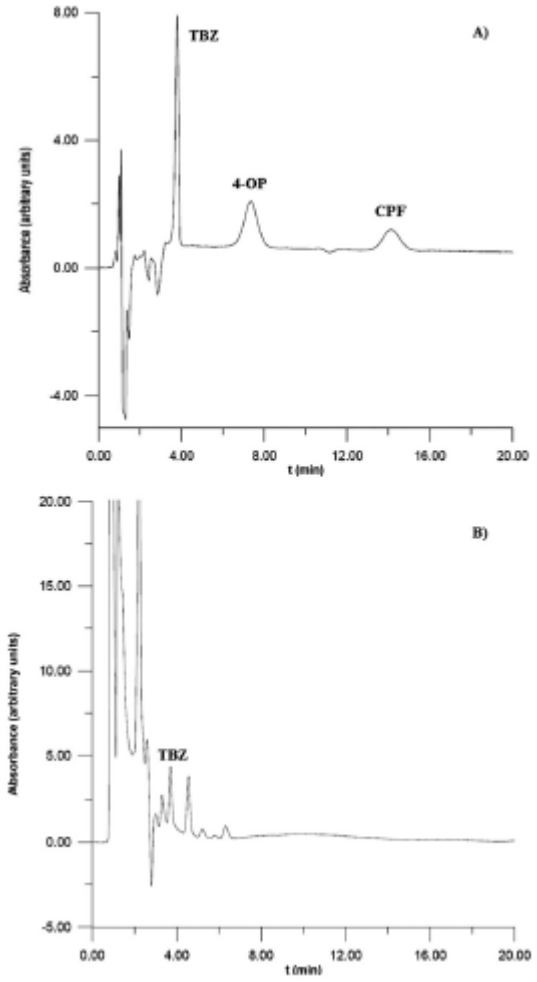


Figure 5.1. Chromatogram obtained by the analysis of: (A) a mixture of 2 mg/L of TBZ, 4-tOP and CPF and (B) water sample 13 collected from the wastewater collector basin in the fruit-processing plant Invicto, Villarreal, Spain. Chromatographic conditions: C18 column, mobile phase 0.15 M SDS - 6% 1-pentanol - pH 3; detection at 220 nm.

3.1.6 General discussion

One of the main features of the optimized procedure is the reduction of time for analysis and the simplification of the experimental procedure. This is possible because of the possibility to directly inject the sample, which allows the elimination of intermediate extraction steps. In addition, the elution of the analytes was performed in <17 min using the isocratic mode, due to the use of micellar mobile phases. Thus, the stabilization time between

two successive injections, which is required in the gradient mode, is not needed. This characteristic facilitates the successive analysis of a large amount of samples.

Another interesting advantage is the minor environmental impact of the analysis and the reduction of the risks related to handling hazardous reagents. The experimental procedure does not require any chemicals, and the optimized mobile phase uses a lesser amount of organic solvents (6% 1-pentanol), than typically used in hydroorganic HPLC (up to 100%).

The analysis can be performed at a low cost because the method only requires basic chromatographic instrumentation and a small amount of inexpensive reagents are used. In addition, the analysis of a large amount of samples per day is possible.

All of these features make the method feasible even for laboratories with low economic power, thus allowing them to sell these analyses at low prices, and also extremely useful for the routine analysis of water samples for pollution monitoring.

3.2 Method validation

The method was validated to check the quality of the quantitative data and evaluate its performance. The validation parameters were: calibration range, linearity, intra- and interday accuracy and precision, ruggedness and stability [37]. The entire calibration was performed in ultrapure water.

3.2.1 Calibration and sensitivity

For calibration purposes, eight solutions containing increasing concentrations of TBZ, 4-tOP and CPF in the range 0.5 to 10 mg/L were analyzed in triplicate. The slope, y-intercept, regression coefficients and determination coefficients were obtained by plotting the peak area (average of three measurements) vs. concentration using the least-square linear regression method. The study was repeated five days over a 3-month period, by preparing standard solutions each time. The calibration curves were taken as the average values of these five regression curves and the results are shown in Table 5.2. Excellent linearity (r>0.997 and r^2>0.994) was observed for the three contaminants in the range LOQ to 10 mg/L (see below).

Table 5.2. Calibration and sensitivity parameters of the studied pollutants.

Compound	Slope	Intercept	*r*	r^2	LOD	LOQ
Thiabendazole	0.8 ± 0.1	-0.05 ± 0.06	0.997	0.9946	0.20	0.5
4-tert-octylphenol	0.60 ± 0.03	-0.04 ± 0.03	0.998	0.9966	0.25	0.6
Chlorpyrifos	0.42 ± 0.02	-0.03 ± 0.06	0.9993	0.9993	0.30	0.8

Slope and y-intercept: average value ± standard deviation, Concentrations in mg/L; n = 5:

The limit of detection (LOD), is the lowest pesticide concentration in a sample, which produces a response that is detectable above the noise level of the system. The LOD was obtained by visual appreciation following the 3 signal-to-noise ratio criterion, which is the concentration value providing a signal 3 times the baseline noise. The baseline noise was measured for each analyte, by analyzing a blank and measuring the width of the baseline at the corresponding retention time [37]. The LOQ was taken as the lowest point of the calibration curve with a precision <20% and accuracy between 80% to 120% (see section 3.2.2) [37] The results can be seen in Table 5.2. The values indicate that the method is able to detect the presence of these compounds in contaminated waste and sewage water.

3.2.2 Accuracy and precision

The intra- and inter-day accuracy and precision were determined at three concentration levels (1; 2 and 5 mg/L). The intra-day accuracy was calculated as the ratio concentration provided by the method (average value of 6 analyses taken the same day)/true value. The intraday precision was the RSD of the peak area obtained by six analyses on the same day. Interday accuracy was calculated as the average of the intraday values obtained in five different days over a 3-month period, using renewed solutions. Interday precision was measured as the RSD of the peak area of days over a 3-month period. The results are shown in Table 5.3. The method shows high recovery (95.2%–102.9%) and low variability (<8.3%) in the determination of TBZ, 4-tOP and CPF in water, thus confirming the reliability of the quantitative data.

Table 5.3. Intra- and inter-day accuracy and precision for TBZ, 4-OP and CPF.

Compound	Concentration (mg/L)	Intra-day[a]		Inter-day[b]	
		Accuracy (%)	Precision (RSD, %)	Accuracy (%)	Precision (RSD, %)
Thiabendazole	1	103.3	0.7	101.8	1.4
	2	95.5	0.8	99.4	1.4
	5	101.2	0.4	96.9	3.4
4-tert-octylphenol	1	110.1	4.4	102.1	7.5
	2	93.8	1.5	96.7	1.1
	5	98.2	0.3	97.5	1.5
Chlorpyrifos	1	102.9	3	100.5	8.3
	2	88.2	3.6	95.2	3.3
	5	102.8	0.5	101.3	1.5

[a]n = 6; [b]n = 5

3.2.3 Ruggedness

The ruggedness was examined by considering the variation in the elution power and the sensitivity area face to minor, but deliberate variations in the surfactant concentration, 1-pentanol amount, pH and flow rate. To study the influence of a determinate condition, a standard solution containing 2 mg/L of each analyte was analyzed in three mobile phases: at its optimal value, slightly under and slightly over, while maintaining the other conditions constant. Thus, the influence of each parameter was separately studied. The considered ranges were as follows: SDS concentration (0.145-0.155 M), 1-pentanol (5.9%-6.1%), pH (2.9-3.1) and flow rate (0.95-1.05 mL/min) in triplicate and the RSD of the measured retention times and peak areas were then calculated.

The small experimental oscillations in the main chromatographic conditions that may happen during routine analysis had no significant influence in the retention time (RSD < 5.1%) and the peak area (RSD < 9.3%) of TBZ, 4-tOP and CPF.

3.2.4 Stability

The stability of the analytes in water was studied at +60 °C and in fridge storage conditions (+4 °C in darkness). Although 60 °C is rarely reached in real situations, the results would provide interesting information about the thermostability of the analytes. In both cases, a solution containing 1 mg/L of TBZ, 4-tOP and CPF was used.

The pollutant standard solution was heated to 60 °C in a water bath. An aliquot was analyzed at 20 min intervals during a 3 h period. The peak area corresponding to the contaminants remained almost constant. Therefore, TBZ, 4-tOP and CPF are quite thermostable and cannot be removed by heating.

The standard solution was kept in a fridge, at +4 °C and in darkness. Daily, an aliquot was analyzed, and no significant diminution in the peak area was observed up to 14 days. Therefore, a water sample can be collected and stored in a fridge until 14 days prior to analysis, without analyte degradation.

3.3 Analysis of real samples from sewerage and wastewater

The developed method was applied to the analysis of samples provided to us by FACSA. The samples were collected from sewerage, industrial waste, and influent and effluent WWPT water from several towns located in the Castelló area (Spain), where the occurrence of TBZ, 4-tOP and CPF is suspected. We analyzed the water samples at a maximum of three days after we received them. Previously, FACSA analyzed the samples using its own standardized LC-MS method. For confidentiality reasons, FACSA has not provided us the characteristics of this method. The origin of each sample and the content of TBZ, 4-tOP and CPF can be seen in Table 5.4. Despite the presence of suspended sludge in several samples, neither obstruction nor damage was noticed in the column, needle or tubes. Fig. 5.1B shows the chromatogram obtained by analyzing sample 13, which indicates the other water contaminants were eluted far from the retention time of the analytes.

Table 5.4. Concentrations (μg mL^{-1}) of TBZ, 4-tOP and CPF detected in real water samples.

			TBZ		4-tOP		CPF	
Origin of water sample	Sample	Location	MLC	LC-MS	MLC	LC-MS	MLC	LC-MS
Sewerage receiving agricultural wastewater	1	Vila-real	< 0.5	0.29	0.65	0.75	n.d.	0.24
	2	La Vilavella	< 0.5	0.41	n.d.	n.d.	n.d.	n.d.
	3	Betxí I	< 0.5	0.12	n.d.	0.1	n.d.	0.14
	4	Betxí II	< 0.5	0.23	n.d.	0.21	n.d.	n.d.
	5	Onda	< 0.5	0.30	n.d.	0.15	n.d.	n.d.
Sewerage not receiving agricultural wastewater	6	Alcora	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	7	Nules	< 0.5	0.04	n.d.	n.d.	n.d.	n.d.
	8	Vila-real I	< 0.5	n.d.	n.d.	0.21	n.d.	n.d.
	9	Alcora	< 0.5	0.08	n.d.	0.14	n.d.	n.d.
	10	Vila-real II	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	11	Onda	n.d.	n.d.	n.d.	0.14	n.d.	n.d.
Collector basin of wastewater from a fruit processing plant	12	Real Export (Vila-real)	1.1	0.93	n.d.	n.d.	< 0.8	0.18
	13	Invicto (Vila-real)	0.9	0.85	n.d.	0.15	n.d.	0.12
	14	Serifruit (Vila-real)	0.5	0.42	0.6	0.63	n.d	n.d.
	15	Eurococi (Betxí)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Wastewater from WWPT	16	Influent (Nules-La Vilavella)	< 0.5	0.52	2.0	1.8	n.d.	0.21
	17	Effluent (Nules-La Vilavella)	< 0.5	0.12	n.d.	n.d.	n.d.	n.d.
	18	Influent (Vora Riu)	1.9	1.71	0,8	0.71	n.d.	0.12
	19	Effluent (Vora Riu)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	20	Influent (Mancomunada OBVA)	0.8	0.84	0.8	0.88	n.d.	n.d.
	21	Decanted influent (Mancomunada OBVA)	< 0.50	0.41	1.1	1.2	n.d.	n.d.
	22	Effluent (Mancomunada OBVA)	n.d.	0.12	n.d.	n.d.	n.d.	n.d.

n.d. = not detected (< LOD)

The concordance of the results obtained by the two methods was evaluated by plotting the data obtained by MLC vs. those obtained by LC-MS, using least-square linear regression [44]. Only the samples providing reliable concentration values (over LOQ) were taken. The obtained curve was:

$$[\text{MLC}] = (1.13\pm0.08)\,[\text{LC-MS}] + (-0.09\pm0.08)\quad r^2=0.96;\ \text{freedom degrees} = 9 \qquad (5.2)$$

The two values show an adequate correlation. A statistical hypothesis test was performed to assess the equivalence of the two values of each pair: null hypothesis H_0 slope = 1 and y-intercept = 0. Considering a significance level of $\alpha = 0.05$ and a two-tailed test, the tabulated students-t test value was 2.26 ($t_{0.05;9;\,2tails}$). Thus, the confidence intervals were [0.96 to 1.34] and [-0.28 to 0.13] for the slope and y-intercept, respectively, thus the null hypothesis was accepted. Consequently, the results obtained by our MLC method were close to those obtained by FACSA from LC-MS. Although the sensitivity is lower, the analysis can be performed at a lower price. Moreover, the MLC methods can be applied to samples with a high contamination degree.

CPF was only detected in one sample, indicating that it remains in crops and sludge, rather than reaching water. We can see that TBZ occurs in almost all samples, due to its extended use. In fact, even the sewerage which does not receive agricultural waters contained TBZ. The contamination of the sewerage water which received wastewater from fruit production was slightly higher, indicating that pesticides are moderately applied to crops and they arrive diluted to the sewerage.

The wastewater from the fruit-processing plants showed a moderate/low concentration of TBZ and 4-tOP, indicating that these industries partially purge the wastewater before discharge. The influent samples from WWPT show higher concentrations than the effluent, thus confirming that the analyte are removed from wastewater and ensuring the validity of the water purification treatment.

4. Conclusions

The obtained data indicate that micellar liquid chromatography can be used to analyze TBZ, 4-tOP and CPF in highly contaminated waste and sewerage waters. The use of an interpretative strategy base on chemometrics has allowed the optimization of the two main parameters (SDS and 1-pentanol), by testing only five mobile phases. The main features of the developed method are the direct injection of the sample, after filtration, and the quick elution of the studied pollutants without overlapping in less than 17 min. The method was validated in terms of specificity, calibration range, linearity, accuracy, precision and ruggedness, and was successfully compared with an LC-MS established method, thus confirming its reliability. Besides, the method is safer for the operator and is environmental friendly, thus making it more attractive. Due to its interesting performance facilities, this method is suitable for routine analyses of water samples with a high concentration of contaminants, such as illegal spills from production plants or consumers, to ensure environmental safety at a low price. The method was also used to evaluate the stability of TBZ, 4-tOP and CPF in several situations (heated and stored in a fridge). The contamination of several waste and sewerage waters because of agriculture-related activity was determined.

5. Acknowledgements and Conflict of interest disclosure

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Chapter 6

Determination of diuron, terbuthylazine and terbutryn in wastewater and soil by micellar liquid chromatography

Abstract

An analytical method for the quantification of the herbicides and algaecides diuron, terbuthylazine, and terbutryn in wastewater and soil by micellar liquid chromatography was developed. The sample preparation was expedited to reduce the number of intermediate steps and the use of chemicals. The analytes in soils were recovered by ultrasonication in the mobile phase. The obtained supernatant and the water samples were directly injected, thus avoiding intermediate steps. The chromatographic behavior of the analytes, depending on the surfactant and alcohol was studied, in order to optimize the chromatographic run, by a chemometrical approach. The herbicides were resolved in <16 min using a C18 column and a mobile phase of 0.07 M sodium dodecyl sulfate/6% 1-pentanol phosphate buffered at pH 3, running under isocratic mode at 1 mL/min. The detection absorbance wavelength was set to 240 nm. The method was successfully validated in terms of selectivity, detection limit (0.06 mg/L in water and 0.3 mg/kg in soil), quantitation range (0.2 to 2 mg/L in water and 1 to 10 mg/kg in soil), trueness (-6.1 to +5.0%), precision (<9.4%), and ruggedness (<8.3%). The procedure was reliable, practical, easy-to-handle, available, short-time and ecofriendly and useful for routine analysis. Its applicability to real samples was evaluated by analyzing several wastewater, decorative reservoir, and soil samples from agricultural and urban sources.

1. Introduction

The growth of wild weeds and algae causes economic, esthetic, and practical disturbances in many human activities. Indeed, they compete with useful plants for water and nutrients, grow out of control, poison humans and livestock, provide unpleasant odors, or hinder the passage of pedestrians and vehicles. Therefore, the pesticide industry manufactures a wide range of synthetic chemicals to fight against these pests [1,2]. The phenylurea diuron (DIU; without acid/basic activity, log Po/w = 2.9, solubility in water 35.6 mg/L) and the triazines terbuthylazine (TBA; pKaTBA-H+ = 1.9, log Po/w = 3.4, solubility in water = 6.6 mg/L) and terbutryn (TBT; pKaTBT-H+ = 4.3, log Po/w = 3.7, solubility in water = 25 mg/L) are broad-spectrum herbicides, microbiocides, and algaecides with strong and rapid effects, which acts by the inhibition of the photosynthesis [3]. The structures can be seen in Fig. 6.1. They are used to prevent and control the growth of weeds, grasses, and mosses in agricultural crops, forestry, plant nursery, gardens, urban woodland and landscaped areas, lawns, and rights of ways. They are applied by spraying on the leaves and soil, and by aerial drift, and absorbed by roots and foliage. They are also used as aquatic herbicides for control of submerged and free-floating weeds and slime-forming algae in industrial and ornamental watercourses, reservoirs, fountains, water cooling systems, aquaria, and ponds as well as in fisheries. In these cases, they are directly added to water [3-7].

Despite their beneficial effects, the prolonged use of DIU, TBA, and TBT is a great concern since they are an important source of contamination of the environment and humanactivity zones. Indeed, these herbicides are moderately to highly persistent in water and soils, and toxic for plants (other than weeds), animals, and humans. Diuron, terbuthylazine, and terbutryn are able to remain in agricultural, residential, and public soils for a long time after use [3-6, 8, 9]. They can arrive to urban sewerage waters, due to the discharging of wastewater from green, domestic, and industrial areas [10], and in agricultural gutters, where they arrive by drainage [11]. These wastewaters are usually purified in wastewater treatment plants (WWTP) in order to remove the contaminants and then released to the aquatic ecosystem. However, some areas even lack of effective wastewater sanitation services [12]. In the surface water, they tend to adsorb on sediments and then pollute river

and estuary sludge [4,8,13]. Besides, diuron has the potential to leach into the soil and may also pollute groundwater, water springs, and water wells [3,4,9,14].

The presence of these herbicides in natural water and soils represent a major threat for the autochthonous flora and fauna. In addition, they can induce serious diseases in the population, which is exposed to these compounds either directly from a polluted source by dermal, eye, and oral way or by food chain, as they bioaccumulate in edible tissues of some consumed living beings [3-9]. These negative effects are enhanced in densely populated areas [15]. Therefore, DIU, TBA, and TBT have been classified as emerging pollutants, hazardous compounds which occurrence must be monitored and regulated in all the potentially contaminated locations [16]. Because of its higher danger, terbutryn was banned in 2003 for use in the European Union (EU), but its occurrence is suspected because of its persistence or current illegal utilization [7]. Local authorities, supported by the scientific community, have implemented actions and policies to reduce the pollution and monitor the quality of wastewaters, treated waters, and soils in their area [17]. Therefore, diuron, terbuthylazine, and terbutryn must be simultaneously screened in water and soils using a reliable a practical analytical method.

Nowadays, hydoorganic gas- and reverse-phase liquid chromatography (RP-HPLC) coupled to mass spectrometry have become the technique of choice for the multiresidue analysis of organic pesticides in water and soil samples [17]. Indeed, liquid chromatography-mass spectrometry (LC-MS) has been used to quantify diuron, terbuthylazine, and terbutryn in urban wastewater [18], diuron and terbuthylazine in agricultural wastewater [11], diuron [11] and terbuthylazine [11, 19] in soils, and terbutryn in sediments [13]. In spite of its performances, this instrumentation is quite expensive, considering both acquisition and maintenance, and delicate. Therefore, it requires an extremely careful sample purification, to avoid the introduction of solid particles and solved salts, and highly specialized staff [20]. Several authors have proposed the use of liquid chromatography-absorbance detection (LC-UV) to determine these three herbicides in urban and agricultural wastewater [21], diuron in urban [22] wastewater, as well as diuron [14,22-28], terbuthylazine [23,29] and terbutryn [29-31] in soils and sludge, to use an affordable detector with a reasonable level of sensitivity and selectivity.

Wastewater samples must be treated to remove interfering and harmful compounds before the HPLC analysis. The most used procedures are based on cartridge solid-phase extraction [11,13,22], solid-phase microextraction [30], and liquid/liquid [26] extraction. Soils must be processed to extract the herbicides from the particles to a liquid environment as clean as possible. The most common methods involve ultrasonication [11,13,22,30], mechanical shaking [14,23,28], stand-overnight [26], microwave-assisted [27,31], Soxhlet [24,29], simple flowing-through-sample [25], simple pressurized solvent [28] extraction using a solution containing a high proportion of organic solvent (60-100%), and QuEChERS [19]. Some of them also include a further purification by cartridge solid-phase extraction [13,14,18,25,26,28], molecular imprinting solid-phase extraction [29], and column switching [27]. However, these procedures are large, tedious, and complex and require multiple operations and specific equipment. These characteristics increase the sources of variance and enlarge the analysis time and price, as well as the use of reagents and toxic solvents. These drawbacks have been partially solved in water analysis by the introduction of automated purification procedures, such as on-line solid-phase extraction [18], in-tube solid-phase microextraction and direct column switching [21]. Therefore, the development of reliable, easy-to-operate, cost-effective, and green methods based on more available instrumentation is quite interesting for routine analysis [32].

Micellar liquid chromatography (MLC), using a C18 column and a hybrid mobile phase, containing sodium dodecyl sulfate (SDS) as surfactant, running under isocratic mode has been used as an interesting alternative to hydroorganic RP-HPLC to determine toxic organic pollutants both polar and hydrophobic in water, such as melamine [33], phenols [34,35], imidazoles [34, 35], organophosphates [34], anilinopyrimidines [35], and carbamates [36], as well as carbaryl in soil [36]. Under these conditions, the retention behavior of compounds with average hydrophobicity is highly reproducible in SDS mobile phases. Hence, it can be modeled using appropriate and well-established equations, which can be adjusted by testing a few mobile phases [37]. The micellar environment also enhances the spectrophotometric activity. Besides, hydrophobic compounds dispersed in water can be easily solubilized in the micellar medium. Therefore, filtered aqueous solutions and suspensions can be directly injected without risk of column degradation or clogging, expediting the sample preparation. The pesticides can also be recovered in an aqueous

micellar liquid phase by a simple stirring, due to their affinity for the micellar pseudophase. As micellar mobile phases use a low proportion of hazardous and volatile organic solvent (maximum of 30%), they are relatively stable, harmless, non-pollutant, and non-flammable [20].

The aim of the work was the development of a reliable method for the determination of the herbicides diuron, terbuthylazine, and terbutryn in wastewater and soils by micellar liquid chromatography. The method should hold practical performances (easy-to-conduct, rapid, ecofriendly, and inexpensive) in order to be applicable for routine analysis. Its analytical quality must to be proven by validation following the recommendations of the Validation and Peer Review of US Environmental Protection Agency (EPA) Chemical Methods of Analysis guideline, in terms of selectivity, calibration and quantitation range, sensitivity, trueness, precision, and ruggedness [38]. Finally, it should to be applied to determine the concentration of DIU, TBA, and TBT in several wastewater and soil samples from diverse sources (agricultural, urban, input WWTP streams, and decorative water reservoirs and right-of-way) in order to verify its suitability for pollution monitoring.

2. Materials and methods

2.1 Standard and reagents

The powdered standards of terbuthylazine, terbutryn, and diuron (purity >99.0%) were supplied by Dr. Ehrenstorfer-Schäfers (Augsburg, Germany). SDS (>99.0%), 1-butanol, and 1-pentanol (HPLC grade) were purchased from Scharlab (Barcelona, Spain). Hydrochloric acid (37.0%), sodium dihydrogen phosphate monohydrate (analytical grade) and 1-propanol (HPLC grade) were bought from Panreac (Barcelona, Spain). Ultrapure water was produced in the laboratory from distilled water, provided by the University Jaume I (Castelló, Spain) using an ultrapure water generating device Simplicity UV (Millipore S.A.S., Molsheim, France). This ultrapure water was used to prepare all the aqueous solutions.

2.2 Apparatus and instrumentation

The solid standards were weighted using an analytical balance Mettler-Toledo (Greifensee, Switerland). A GLP potentiometer (Crison, Barcelona, Spain) equipped with a combined Ag/AgCl/glass electrode was used to measure the pH of the aqueous solutions. The vortex shaker and the Ultrasons-H ultrasonic bath were from Selecta (Abrera, Spain).

The chromatograph was an Agilent Technologies Series 1100 (Palo, Alto, CA, USA) equipped with an isocratic pump, a degasser, an autosampler, and a UV-Visible absorbance diode array detection (DAD), connected to a personal computer. The software Chemstation version B.01.01 (Agilent Technologies) was used to control the instrument and the acquisition and processing of the signal. The experimental dead time was ≈1.0 min.

2.3 Solutions and mobile phases

Individual stock solutions containing 100 mg/L of each pesticide were prepared by weighting the appropriate quantity and solving it in methanol. The working solutions were prepared by successive dilutions with ultrapure water. These solutions were kept at +4 °C for a maximum of 2 weeks.

Mobile phases were prepared by solving the adequate amount of SDS and $NaH_2PO_4 \cdot H_2O$ in ultrapure water, and then drops of HCl solutions were added to reach the desired pH. Furthermore, the appropriate volume of alcohol was added, and then the flask was filled up with ultrapure water. Afterwards, the mobile phases were ultrasonicated for 5 min and filtered through a 0.45-µm-Nylon membranes (Micron Separations, Westboro, MA, USA) with the aid of a vacuum pump. All the mobile phases were stored in amber bottles.

2.4 Chromatographic conditions

The stationary phase was in a Kromasil C18 column (Sigma-Aldrich, Saint-Louis, MO, USA) with the following characteristics: length, 150 mm; internal diameter, 4.6 mm; particle size, 5 µm; pore size, 10 nm). The mobile phase was an aqueous solution of 0.07 M SDS/6% 1- pentanol buffered to pH 3 with 0.01 M phosphate salt. It runs at 1 mL/min under

isocratic mode. The injection volume was 20 μL, and the absorbance detection wavelength was set to 240 nm. Standard solutions and samples were filtered through 0.45-μm Nylon membranes before introduction into the vials. The analyses were carried out without controlling the temperature. The special care required when dealing with micellar mobile phases can be seen in [39].

2.5 Sample treatment

The samples were taken in several towns in from densely populated areas, belonging to the Castellón Province (Spain). Wastewater samples were collected and supplied to the laboratory by FACSA (Castellón). This company is in charge of the control of the quality of waste, sewerage, and surface water, as well as the management of the integrated water cycle in the studied area. The soil samples were collected at the surface by the laboratory staff. Both kinds of samples were taken and stored in amber bottles. The source and characteristics of the samples can be seen in Tables 6.1 and 6.2, respectively. The samples were kept in a fridge and analyzed at a maximum of 2 weeks after collection.

Samples were thawed 30 min at room temperature before processing. Water samples were filtered and directly injected. Soil samples were air-dried for 30 min and powdered in an agate mortar, and then 1 g was weighed and mixed with 5 mL of mobile phase (sample/supernatant ratio 1:5 w/v). The mixture was stirred for 1 min and ultrasonicated for 5 min to extract the studied herbicides. After decantation, the supernatant was filtered and injected. In spiking samples, the appropriate volume of standard solution was introduced in the soil before adding the mobile phase. The resulting sample was stored 1 day to assure the evaporation of the solvent and the deposition of the herbicides on the solid particles. These artificially herbicides-added samples imitated those "naturally" contaminated [40].

Table 6.1. Characteristics and herbicide concentrations (mg/L) in the several water samples.

Source	Nº	Location	DIU	TBA
Public fountains	1	Villarreal	1.4	2.1
	2	Castelló	0.98	1.3
Private aquaria	3	Villarreal	0.84	0.76
	4	Almassora	0.52	0.68
Agricultural gutters	5	Villarreal	0.43	0.62
	6	La Vilavella	0.24	n.d.
	7	Betxí I	0.32	0.48
	8	Betxí II	0.28	<0.2
	9	Onda	<0.2	0.43
Urban sewage water streams	10	Alcora	<0.2	n.d.
	11	Nules	0.44	0.36
	12	Vilarreal I	n.d.	0.45
	13	Alcora	<0.2	n.d.
	14	Vilarreal II	n.d.	0.22
	15	Onda	<0.2	0.34
Influent wastewater	16	WWTP Vora Riu (Villarreal)	<0.2	0.31
Influent wastewater	17	Joint WWTP OBVA (Onda/Betxí/Vilareal/Alquerías)	0.51	<0.2
Influent wastewater	18	WWTP Nules, La Vilavella	0.31	0.25

The places refer to several towns from the Castelló Province (Spain) - n.d. = not detected

Table 6.2. Characteristics and herbicide concentrations (mg/kg) in the several soil samples

Source	Nº	Location	DIU	TBA
Agricultural fields	19	Villarreal	5.7	2.1
	20	La Vilavella	8.5	5.4
	21	Alcora	4.1	1.9
Private gardens	22	Villarreal	2.2	3.2
	23	Burriana	2.1	5.1
Public gardens	24	Castelló	2.1	4.5
	25	Villarreal	4.3	2.8
Soil near to a road	26	Onda	5.8	n.d.
Soil near a railroad	27	Villarreal	3.7	n.d.

The places refer to several towns from the Castelló Province (Spain) - n.d. = not detected

3. Results and discussion

3.1 Optimization of the chromatographic conditions

The principal chromatographic conditions (injection volume, 0.020 mL; stationary phase, C18; flow-rate, 1 mL/ min running under isocratic mode; surfactant, SDS, buffer, a phosphate salt; and pH 3) were taken from previously published papers related to the determination of pesticides in water [34,35]. The concentration of SDS, the nature and proportion of the organic solvent, and the absorbance wavelength detection were optimized. These studies were performed using a standard solution of 1 mg/L DIU, TBT, and TBA in ultrapure water.

3.1.1 Selection of the organic solvent

The studied pesticides are moderately hydrophobic (log Po/w 2.9-3.7). Using a C18 column, the use of hybrid mobile phases was envisaged to obtain useful retention times, and additionally, improve the shape of the peak. The shortchained monoalcohols 1-propanol, 1-butanol, and 1-pentanol were tested as organic solvents [20].

The analytes were analyzed using three mobile phases containing 0.1 M SDS and 7.5% 1-propanol, 5% 1-butanol, and 4% 1-pentanol. The herbicides were less retained and the efficiency was enhanced by increasing the length of the carbon chain, as expected in MLC [20]. The retention time of TBT was too high using 1-propanol (44.9 min) and 1-butanol (65.4 min). Therefore, 1-pentanol was selected for the analysis.

3.1.2 Modeling of the retention behavior

The effect of the concentrations of SDS and 1-pentanol in the retention factor was studied by an interpretative strategy, using a complete factorial design plus the central point. Therefore, the retention factor of DIU, TBA, and TBT were measured by five mobile phases containing a combination of the minimum and the maximum concentration of SDS (M) and 1-pentanol (%, v/v) recommended for MLC and the following average values: 0.05/2, 0.05/2, 0.10/4, 0.15/2, and 0.15/6 [20].

In all the mobile phases, the elution order was the same: t_R(TBT) > t_R(TBA) > t_R(DIU). The three herbicides show a binding behavior face to the SDS-micelles, as the retention factor decrease at increasing values of SDS in the mobile phase. Otherwise, the addition of 1-pentanol enhances the elution strength of the mobile phase, as expected in RP-HPLC.

An empirical hyperbolic model was used to describe the retention behavior of each herbicide. Equation 6.1 allows to predict the retention factor (dependent variable) at several concentrations of SDS ([SDS] in M) and 1-pentanol (φ in %, v/v) in the mobile phase (factor), for moderately hydrophobic compounds [37]:

$$1/k = c_0 + c_1\,[SDS] + c_2\varphi + c_{12}[SDS]\varphi \qquad (6.1)$$

The constants c_1, c_2, and c_{12} represent the influence of each factor and their interaction, respectively. The equation was adjusted taking the experimental values of the retention factors of the analytes obtained at the five mobile phases above indicated, by curve-fitting non-linear least-square regression [41]. The concentration of SDS and the proportion of 1-pentanol were normalized from 0.05 to 0.15 M and 2 to 6% to (-1)-(+1), respectively, in order to better compare the calculated constants. The finally fitted equations were as follows:

DIU $1/k = 0.174 + 0.090\ [SDS] + 0.042\ \varphi + 0.037\ [SDS]\varphi$ $R^2 = 0.995$ (6.2)

TBA $1/k = 0{:}1329 + 0.0677\ [SDS] + 0.0293\ \varphi + 0.0304\ [SDS]\ \varphi$ $R^2 = 0.9997$ (6.3)

TBT $1/k = 0.097 + 0.073\ [SDS] + 0.034\ \varphi + 0.021\ [SDS]\ \varphi$ $R^2 = 0.98$ (6.4)

These equations are able to provide an accurate theoretical retention time of the three herbicides at values of SDS and 1-pentanol concentrations in the 0.05-0.15 M and 2–6% range, respectively, by interpolation (Fig. 6.1), according their adequate multiple determination coefficient (R^2). The order of the predicted retention times (TBT > TBA > DIU) was maintained in the whole interval of SDS/1-pentanol amounts.

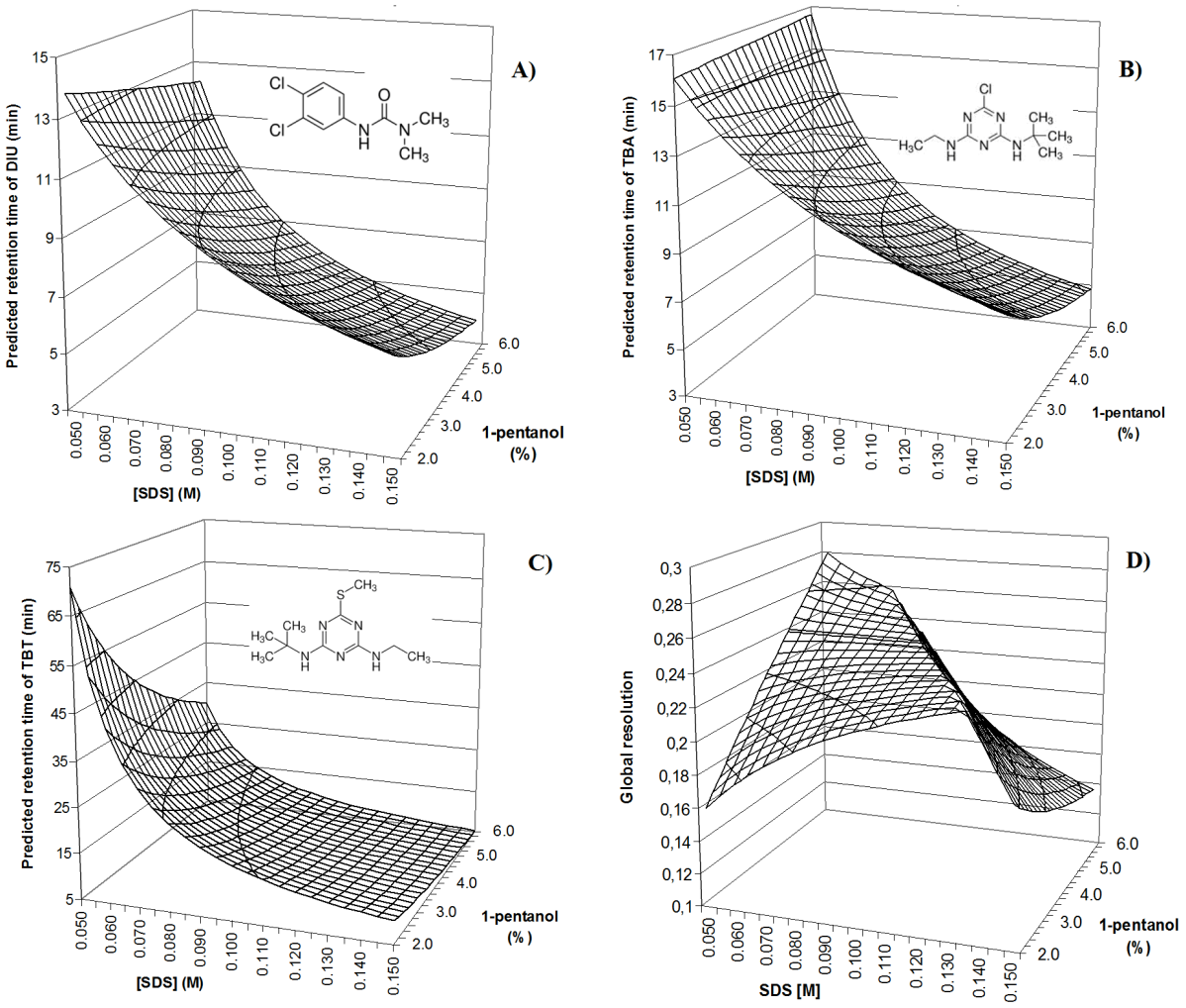

Figure 6.1. 3-D plot of the predicted retention time of A) diuron, B) terbuthylazine, and C) terbutryn, and D) global resolution, v.s. the concentration of sodium dodecyl sulfate and 1-pentanol in the mobile phase.

The influence of each factor and their interaction of the retention factor can be inferred from the empirical constants. According to the positive values of all the constants, the augmentation of the concentration of the surfactant and the alcohol effectively improves the elution strength of the mobile phase. These assessments match to those extracted from the qualitative interpretation of the unprocessed values obtained by the experimental design. Besides, the diminishing of the retention caused by the increase of the SDS concentration is enhanced at higher values of 1-pentanol, and vice versa. Indeed, the presence of the alcohol causes a diminishing of the critical micellar concentration and then the increase of the number of micelles [42]. The relative influence of each factor is as follows (in decreasing order): SDS concentration, 1-pentanol proportion, and their interaction. These findings were applicable for the three studied herbicides.

3.1.3 Optimization of the SDS/1-pentanol concentration

The concentrations of SDS/1-pentanol in the mobile phase were selected by surface response methodology using the mathematical model developed in the section 3.1.2, to maximize the global resolution and minimizing the analysis time.

The elementary resolution between each pair of consecutive peaks (DIU and TBA, and TBA and TBT), described as the modified selectivity (α′) [37], was calculated as follows (Eqs. 6.6 and 6.3, respectively):

$$\alpha'(\text{DIU-TBA}) = 1-k(\text{DIU})/k(\text{TBA}) \quad (6.5)$$
$$\alpha'(\text{DIU-TBA}) = 1-(0.1329+0.0677[\text{SDS}]+0.0293\varphi+0.304[\text{SDS}]\varphi)/(0.174+0.090[\text{SDS}]+0.042\varphi+0.037[\text{SDS}]\varphi$$

$$\alpha'(\text{TBA-TBT}) = 1-k(\text{TBA})/k(\text{TBT}) \quad (6.6)$$
$$\alpha'(\text{TBA-TBT}) = 1-(0.097+0.073[\text{SDS}]+0.034\varphi+0.021[\text{SDS}]\varphi)/(0.1329+0.0677[\text{SDS}]+0.0293\varphi+0.0304[\text{SDS}]\varphi$$

The global resolution (*Z*) was modeled as the minimum elementary resolution (Fig. 6.1D) [37]. The maximal theoretical Z (0.280) was reached at 0.05MSDS/6%1-pentanol, but with a too long retention time of TBT (30.0 min). We can see in Fig. 6.1, that the augmentation of [SDS] from this point provokes a reduction of the retention time, without excessively diminishing the global resolution. Finally, the mobile phase SDS/1-pentanol 0.07 M SDS/6% was selected, with a theoretical Z of 0.261 and a retention time of 14.6 min. In

addition, the retention time of the first eluted (DIU) is 8.2 min, which is relatively far from the front of the chromatogram, and then would not be disturbed by the less retained compounds of the matrix.

A mixture of the three herbicides (1 mg/L each one) was analyzed using the optimized mobile phase. The experimental retention times (min) were as follows: DIU, 8.0; TBA, 10.8, and TBT, 14.6 min, and then each chromatographic run would take ≈16 min. The error in the prediction of the retention time was <3%.

3.1.4 Optimization of the detection conditions

The absorption spectrum of each herbicide was on-line taken using the DAD of the chromatograph, by adjusting the other instrumental parameters at their previously fixed values. The optimized mobile phase was selected, in order to consider the influence of the solvent in the absorptivity of the chromogenes. Under these conditions, the wavelengths of maximum absorbance were as follows: diuron, 235 nm; terbuthylazine, 265 nm; and terbutryn, 230 nm. The wavelength detection was set to an average value of 240 nm, at which the three herbicides show a high absorptivity, in order to avoid changes in the detection conditions during the chromatographic run.

3.2 Sample preparation

3.2.1 Wastewater

Wastewater can contain variable amounts of solid particles and oily liquids suspended in the aqueous phase, depending on its source and cleanliness degree. These aggregates are the responsible of the turbidity, usually noticeable at a glance, of the aqueous sample. Besides, the oily phase is potentially harmful for the column, as it can be adsorbed on the stationary phase (because of its high hydrophobicity) and may slowly modify its properties [43]. The optimization of the experimental protocol was performed on the basis of eliminating the potential danger of these compounds without including an extraction step. The studies were performed using a waste water sample directly taken from the urban

sewerage system, which was especially dirty (blank sample). This sample was collected for a previous study and does not contain the studied pesticides.

A filtration was proposed to remove the sludge and solid particles suspended in the waste water. In order to verify the behavior of wastewater after injection, the filtered sample was diluted in optimum mobile phase, and then the oily drops were solubilized and only one phase was observed. We suppose that the hydrophobic phase was introduced inside the micelles.

This blank sample was filtered and directly injected to investigate the chromatographic behavior of these oily compounds. Only the front of the chromatogram (dead time to 3.0 min) was observed, with a relatively low signal. We suppose that the oily compounds bounded to the micelles scarcely interact with the stationary phase. Anyway, they did not interfere with the studied pesticides, thus assessing the selectivity of the method.

The effect of the direct injection of filtered dirty waste water on the column was examined by 50 successive analyses of the blank sample, spiked at 1 mg/L DIU, TBA, and TBT. The chromatogram obtained by the first analysis can be seen in Fig. 6.2A. No variation of the system pressure and retention time of the pesticides was observed, indicating that the injection of the oily drops is harmless for the chromatographic system. Therefore, no dilution was required to maintain a reasonable sensitivity level. Finally, the sample processing was limited to filtration and direct injection in the chromatographic system.

3.2.2 Soil samples

The optimization of the main extraction conditions (extraction solution, ultrasonication time, and sample/supernatant) was carried out using a soil sample taken from a private garden, where the studied herbicides have been never used.

The extraction solvent and the time of ultrasonication and the sample/supernatant ratio were optimized to maximize the recovery, which was evaluated by comparing the area of the chromatographic peaks corresponding to each herbicide. The ability of the mobile phase to recover the herbicides was compared to that of methanol, which is usually used to extract pesticides from soil samples [23, 31]. In both vases, clear chromatograms were obtained, and the recovery was similar. Therefore, the mobile phase was selected, due to its lower content

of toxic organic solvent to improve the normality of the peak shape [44]. The ultrasonication time was tested from 1 to 10 min, at 1-min steps. For the three herbicides, the recovery increased up to 5 min and remained nearly constant at higher values. Therefore, 5 min was considered as the most adequate ultrasonication time.

The soil/supernatant (So/Su) ratio (w/v) considered were 1:1, 1:2, 1:5, 1:10, and 1:20. In all cases, the number of solid particles was not significant, and no obstruction of the filter was noticed during the filtration of the supernatant. In this case, the product peak area times the So/Su was used to compare the extraction capacity. This value increased from 1:1 to 1:5 and remained nearly constant from 1:5 to 1:20. Indeed, at values under 1:5; the mobility of the soil particles in the supernatant was hindered, reducing the contact between the two phases. This slows down the transfer of the herbicides from the solid particles to the supernatant. No advantage was found to use higher So/Su ratios higher than 1:5, and then this value was selected, in order to avoid an excessive diminishing of the sensitivity.

Finally, the blank sample was analyzed, in order to check the presence of contaminant. No peak was observed at and near to (±2.00 min) the window time of the analytes, thus demonstrating the selectivity of the procedure. This was confirmed by the analysis of the blank soil sample spiked at 5 mg/kg of each analyte (Fig. 6.3A).

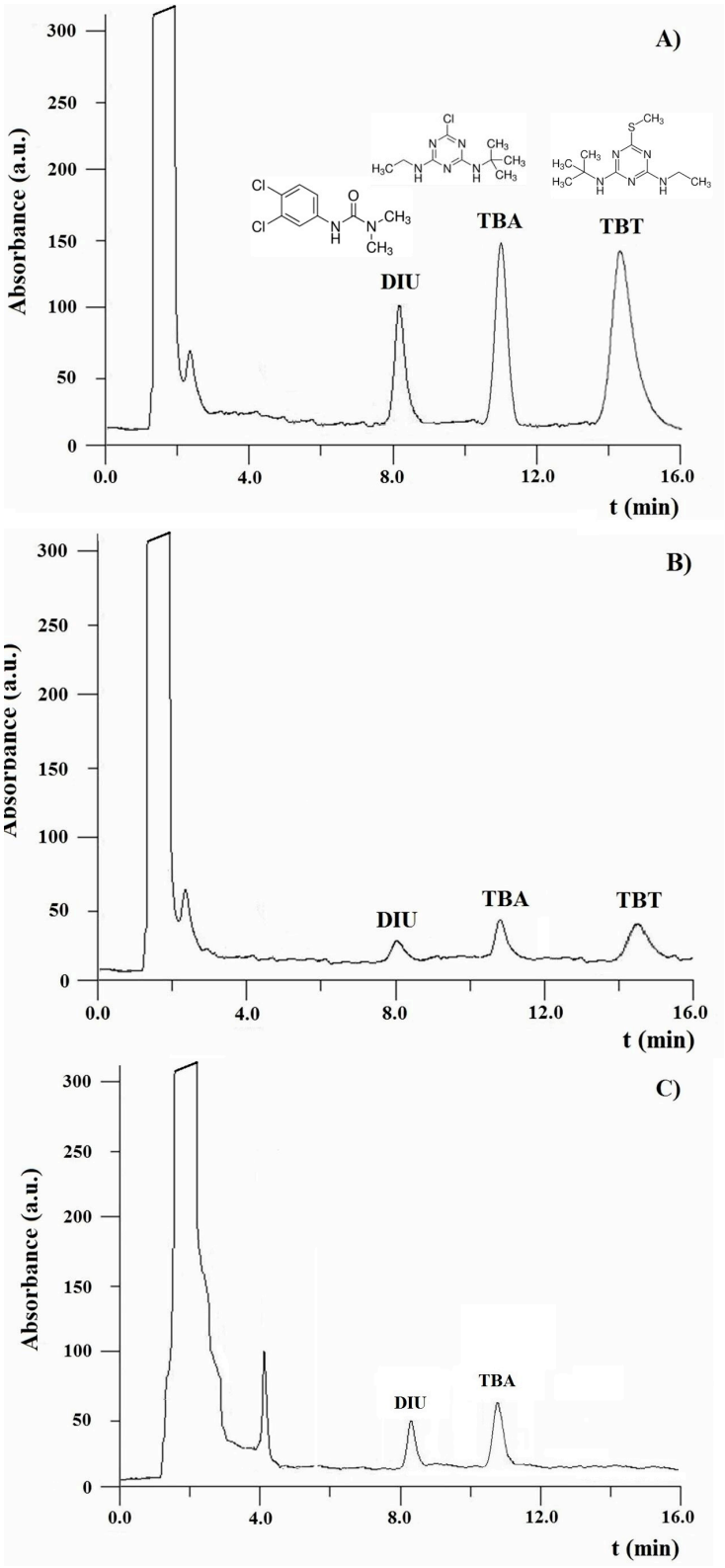

Fig. 6.2. Chromatograms obtained by the analysis of a blank wastewater sample spiked at A) 1 mg/L and B) LLOQ, of the three studied herbicides, and C) sample 11, taken from an urban sewerage water stream, and containing DIU 0.44 and TBA 0.36 mg/L

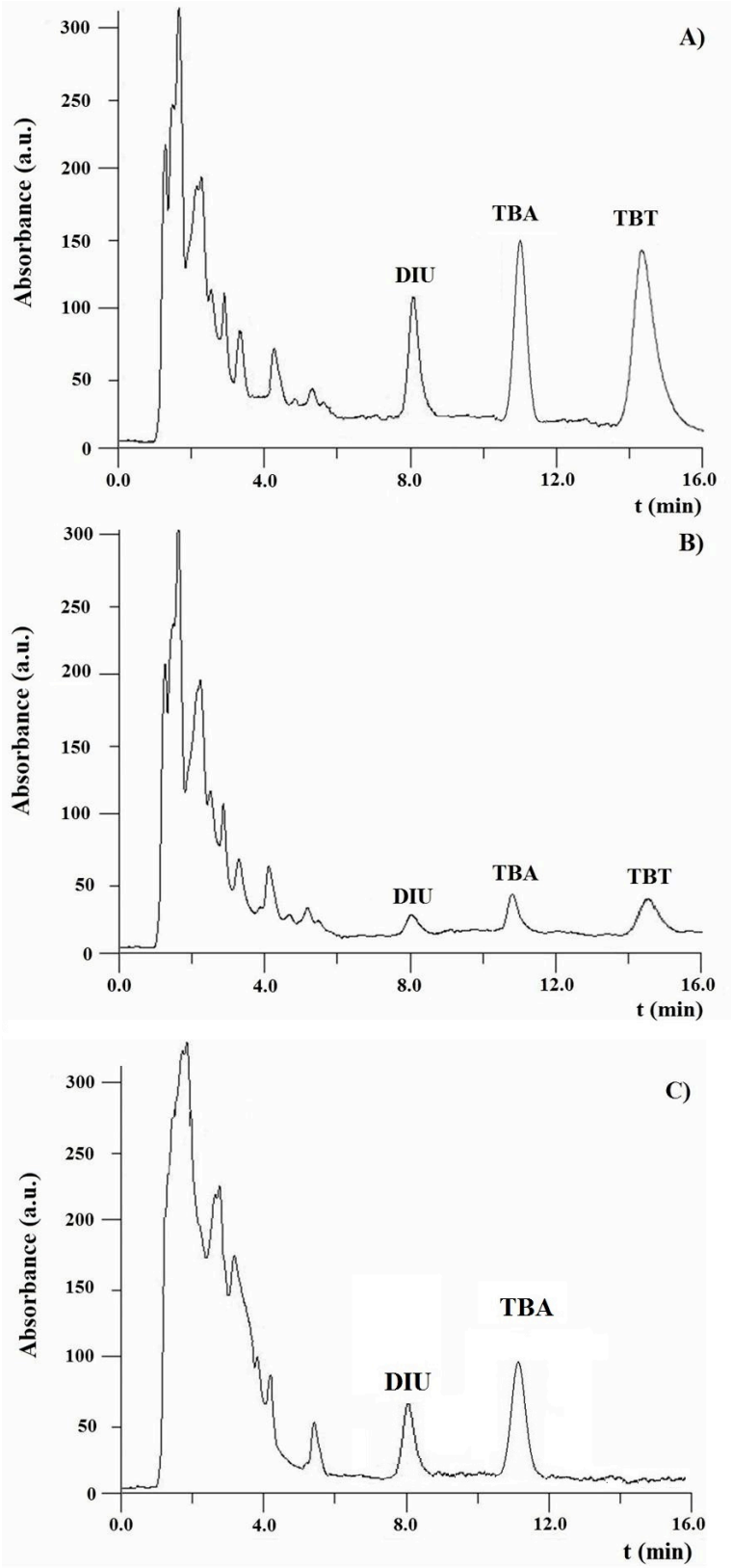

Figure 6.3. Chromatograms obtained by the analysis of a blank soil sample spiked at A) 1 mg/kg and b) LLOQ of each herbicide, and C) sample 22, taken from a private garden and containing 2.2 and 3.2 mg/kg of DIU and TBA, respectively

3.3 General comments about the procedure

The developed method holds several practical advantages over those based on hydroorganic RP-HPLC, because of the use of micellar mobile phases. These characteristics make the method meets the requirements of "green analytical chemistry", which fits the current trend in analytical chemistry [32].

The main feature of the method is, undoubtedly, the strong simplification of the sample preparation, which was limited to a one-step treatment. The transference of the herbicides from the solid particles to the liquid phase was a single ultrasonication. The wastewater and the soil extract were directly injected (after filtration), involving a minimal participation of the operator, thus avoiding long and tedious clean up steps with variable recovery. Otherwise, it reduces the risk of exposure to other chemicals and the contamination of the processed sample.

The soil extract solution and the micellar mobile phase contain innocuous and biodegradable reagents (SDS and phosphate salts) and require less proportion of toxic, volatile, and flammable solvent (<6%) than typically used in hydroorganic RP-HPLC (up to 100%). Besides, the interaction of 1-pentanol with SDS-micelles even decreases its evaporation rate. No chemicals are involved in the treatment of wastewater. Therefore, the operator is barely exposed to hazardous chemicals and the waste contains a minimum amount of pollutants. This makes the developed procedure ecofriendly and relatively safe for the laboratory staff.

Another interesting advantage is the shortening of the analysis time. Samples can be processed in nearly 1-2 and 6-7 min, for water and soil, respectively. Moreover, as the mobile phase flows in isocratic mode, the column does not require a stabilization time between two successive injections, as in gradient programs. These characteristics facilitate the study of a high number of samples per day.

The developed procedure only required basic laboratory material and chromatographic instrumentation, a small amount of common chemicals, and the participation of a unique operator, which can even coordinate perform this activity with other tasks. In addition, a high number of samples per day can be analyzed and the expenditure related to segregation and waste treatment is inferior. Therefore, the analysis of wastewater

and soils potentially contaminated with these herbicides can be carried out at an affordable price, which is reasonably advantageous in the current context of economic crisis.

3.4 Method validation

The developed analytical method was *in-lab* validated following the directives of the Validation and Peer Review of the US EPA Methods of Analysis. The evaluated parameters were instrument calibration, trueness, precision, ruggedness (in ultrapure water), quantitation range and limits, detection limits [38], and recovery in blank wastewater and soil.

3.4.1 Instrument calibration

Five solutions containing increasing concentrations of DIU, TBA, and TBT (calibration range from 0.2 to 2 mg/L) were analyzed (n = 3). For each herbicide, the corresponding peak area was plotted versus the concentration by least-squares regression [45]. The slope, y-intercept, determination coefficient, and the Cook's squared distance (CD^2), calculated for each level to detect possible outliers, were (concentrations in mg/L):

DIU	A = (175.1±2) [DIU]+(8.6±3)	$r^2 = 0.9996$	$CD^2 = 0.69$	(6.7)
TBA	A = (315.6±5) [TBA]+(10±5)	$r^2 = 0.9996$	$CD^2 = 0.74$	(6.8)
TBT	A = (684±10) [TBT]+(25±11)	$r^2 = 0.9996$	$CD^2 = 0.31$	(6.9)

The values of the goodness of fit criteria ($r^2 > 0.99$ and $CD^2 < 1$) demonstrate the high reliability of the statistical model.

3.4.2 Trueness and precision

These parameters were evaluated at the extremes of the calibration range (0.2 and 2 mg/L) and 0.5 mg/L.

For the intraday measurements, a standard solution of DIU, TBA, and TBT at the appropriate concentration was analyzed six times by consecutive injections. The trueness was calculated as the bias (difference of the average of the found concentrations and the true value, divided by the true value), while the precision (repeatability) was the closeness of agreement (relative standard deviation, RSD) of the peak areas. To determine the interday values, the same procedure was carried out five different days with renewed standard solutions over a 2-month period. The interday trueness was the average of the five intraday bias values, whereas the interday precision (reproducibility) was the RSD of the five daily averages of the peak areas. The results are shown in Table 6.3. The low bias (-6.1 to +5.0%) and low variability of the measures (<9.4%) indicate the high reliability of the chromatographic determination.

Table 6.3. Intra- and inter-day trueness and precision for the studied herbicides.

		Intra-day[a]		Inter-day[b]	
Herbicide	Concentration (mg/L)	Trueness (error, %)	Precision (RSD, %)	Trueness (error, %)	Precision (RSD, %)
DIU	0.2	- 5.2	8.9	- 3.0	7.4
	0.5	+4.2	4.2	+2.1	5.4
	2	+0.1	1.0	+0.5	2.8
TBA	0.2	-5.3	9.4	- 6.1	7.4
	0.5	+3.7	3.3	+2.9	4.2
	2	+0.8	1.1	+1.9	3.0
TBT	0.2	+5.0	8.3	+4.1	5.9
	0.5	+0.8	3.0	+3.1	1.4
	2	+0.5	0.4	-0.2	3.1

[a]n=6; [b]n = 5

3.4.3 Ruggedness

The real composition of the mobile phase may fluctuate in a short range around the indented value, because of the random errors arising during its preparation. In order to evaluate the effect of this annoyance on the instrumental response, the variation of the retention time and the peak area for each analyte was examined at minor deviations of the main components of the mobile phase from the optimal value: SDS concentration, 70 ± 5 mM; 1-pentanol proportion, 6.0 ± 0.2%; and pH, 3.0 ± 0.2%. The effect of each one was separately investigated.

A working standard solution of 1 mg/L of each herbicide was analyzed by triplicate, using three mobile phases, containing the selected value, the selected value minus the studied deviation, and the selected value plus the considered deviation, respectively, for the examined constituent, whereas the other ones were maintained at their optimal value. The results are shown in Table 6.4. Insignificant variations of the retention times (RSD <8.3%) and peak area (RSD <5.8%) were noticed, and then the instrument responses are robust enough to be unaffected by possible oscillations of the mobile phase in the studied range.

Table 6.4. Evaluation of the robustness of the MLC-method (n=3)

Herbicide	Chromatographic parameter	Level	Retention time (R.S.D.; %)	Peak area (R.S.D; %)
DIU	SDS concentration (mM)	65 – 75	8.3	4.6
	1-pentanol amount (%)	5.8 – 6.2	7.4	5.8
	pH	2.8 – 3.2	3.4	2.9
TBZ	SDS Concentration (mM)	65 – 75	7.5	3.8
	1-pentanol amount (%)	5.8 – 6.2	6.6	4.2
	pH	2.8 – 3.2	2.0	2.1
TBT	SDS Concentration (mM)	65 – 75	6.4	2.6
	1-pentanol amount (%)	5.8 – 6.2	5.9	4.5
	pH	2.8 – 3.2	1.5	1.5

3.4.4 Quantitation range

The lower limit of quantitation (LLOQ) was taken as the smallest concentration that can be quantified with a reasonable level of trueness and precision. The higher limit of quantitation (HLOQ) was the highest expected concentration of contaminants in water. For wastewater, the quantitation range was the same as for the calibration range, as neither dilution nor preconcentration steps are included in the sample preparation. For soils, the amount in soils (mg/kg) was five times the concentration in the injection solution, because of the solid/liquid extraction. Therefore, the lower/upper quantitation ranges were 0.2/2 mg/L and 1/10 mg/kg for wastewater and soils. The chromatograms obtained from blank samples of wastewater and soil spiked with DIU, TBA, and TBT at their corresponding LLOQ can be seen in Figs. 2B and 3B, respectively.

The detection limit (LOD) is the lowest concentration that provides a signal clearly above the baseline noise, but not accurately quantifiable [45]. The detection limit was measured by the 3.3 s criterion (3.3 times the standard deviation of the blank divided by the sensitivity). The standard deviation of the blank was calculated as the standard deviation of the residuals, and the sensitivity was the slope of the calibration curve, divided by the dilution ratio. For the three herbicides, the LOD were 0.06 mg/L and 0.3 mg/kg for wastewater and soils, respectively. Therefore, the sensitivity is sufficient to detect moderate quantities in wastewaters and soils.

3.4.5 Recovery

The effect of the matrix on the bias of the measurements was evaluated by analyzing blank samples of wastewater and soils (same as used in section 2.5), spiked at several amounts of diuron, terbuthylazine, and terbutryn. The analysis was performed six times within the same day, repeating at each case the whole experimental procedure. The average and the RSD of the quotient found concentration/true value were measured for each level. The results can be seen in Table 6.5.

The developed procedure shows good recovery (89.4 to 103.2%) through the whole quantitation range, indicating that the matrix barely affects the results. This is mainly due to

simplification and the short time of the sample preparation, which minimizes the risk of loss or chemical change of the analyte and the arising of random errors.

Table 6.5. Recovery (%) for the studied herbicides in wastewater and soil (n=6)

	Wastewater		Soil	
Herbicide	Concentration (mg/L)	Recovery	Amount (mg/kg)	Recovery
DIU	0.2	93.6±7.3	1	90.1±7.7
	0.5	101.4±4.1	5	95.4±5.5
	2	99.1±1.1	10	97.1±3.0
TBA	0.2	94.8±8.4	1	89.4±8.8
	0.5	96.8±4.8	5	94.2±3.9
	2	98.4±2.1	10	93.8±4.1
TBT	0.2	103.2±7.5	1	97.4±8.0
	0.5	102.0±2.9	5	94.5±4.1
	2	99.1±0.9	10	93.9±3.5

3.5 Analysis of wastewater and soil samples

The method was applied to samples from several kinds of wastewater (agricultural gutters, urban sewerage), influent WWTP streams, decorative water pools, and soils (garden and right-of-way) from the Castelló area (Spain). If necessary, the quantitation range was extended by augmenting the dilution ratio. The results are shown in Tables 6.1 and 6.2 for water and soil samples, respectively.

Terbutryn was not found in any sample, thus indicating that the users comply with the regulation, and there is no residue from the time before the ban. DIU and TBA were detected in the studied samples, indicating the suitability of the method to the quantification of the studied herbicides in these samples. The chromatograms obtained by the analysis of samples 11 (urban sewerage wastewater) and 22 (soil from a private garden) are shown in Figs. 6.2C and 6.3C, respectively.

A high amount was detected in the primary sources of pollution, soils, and decorative water reservoirs, mainly because their intense use. Indeed, the local climate (Mediterranean)

is characterized by warm temperatures and sunny days in the major part of the year and stimulates the growing of weeds in soils, especially those irrigated, and algae in outdoor reservoirs, which must be frequently fumigated to avoid spoilage. In addition, their persistence motivates their accumulation in soils. The concentration of DIU and TBA in the decorative water pools is limited, because the treated water is frequently renewed, and then the addition of low amount of herbicides each time is preferable. Besides, there is a major risk of involuntary contact with the population. The concentration found in aquaria was lower than in fountains. Indeed, a less amount of herbicides must be added, to reduce the danger to the fishes.

Agricultural, sewerage, and WWTP-influent wastewaters show lower concentrations of DIU and TBA. Indeed, when a treated water stream discharges to the wastewater system, it results in its dilution with other herbicide-free spills. Otherwise, in the fields, the herbicides have a slow tendency to move from soils to water. Besides, the herbicides tend to move from the wastewater stream to the walls and sediments of the channels. According to the results, these water streams must be treated before releasing into the environment.

4. Conclusions

MLC-DAD was demonstrated a suitable technique for the routine monitoring of diuron, terbuthylazine, and terbutryn in water and soil. The samples were injected directly (for water) or after an uncomplicated one-step extraction (for soils), despite of the complexity of the matrices, due to the particular properties of the micellar media. Therefore, the sample preparation was as simple as possible, thus minimizing the sources of variance. A mathematical relationship between the retention factor of the herbicides and the composition of the mobile phase (SDS and 1-pentanol) was found, expediting its optimization by surface response methodology. The analytes were resolved without interferences in <16 min, using the isocratic mode. The method was validated by following the guidelines of the Environmental Protection Agency in terms of selectivity, calibration and quantitation range, limit of detection, trueness, precision, ruggedness, recovery, and uncertainty. The method is short-time, easy-to-handle, eco-friendly, relatively safe for the operator, inexpensive, and useful for routine analysis, if compared to other procedures based on hydroorganic RP-

HPLC. Finally, the method was successfully applied to quantify DIU, TBA, and TBT in different kinds of water (agricultural and urban wastewater, input WWTP streams, and decorative water pools) and soil (agricultural, garden, and right-of-way) samples, suspected to be polluted with these herbicides.

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Chapter 7

General Conclusions

En la present memòria es desenvoluparen i validaren una sèrie de mètodes analítics per a la determinació de diversos pesticides (tiabendazol, pirimetanil, o-fenilfenol, imazalil, clorpirifos, diuron, terbutilazina i terbutrina) i un derivat dels tensioactius emprats en formulacions de plaguicides (4-*tert*-octilfenol) en mostres mediambientals (aigües residuals urbanes, agrícoles, de la indústria agroalimentària, d'EDAR, aigües decoratives i sols). Aquestes formulacions de pesticides s'utilitzen quotidianament en diferents àmbits: agricultura (pre- i postcollita), a les llars, per al manteniment d'edificis públics i privats (plantes decoratives, aquaris, neteja d'instal·lacions, material divers i superfícies), en espais verds (jardins i fonts), vies de transport, etc. Malgrat els seus avantatges, els pesticides són una de les principals amenaces pel medi ambient, a causa del seu excessiu ús, la seua persistència, mobilitat, bioacumulació i toxicitat. Poden danyar la flora i fauna autòctones i alterar l'equilibri ecològic, i deteriorar la salut de la població. Per eixos motius, és necessari evitar (reduint el seu ús i aplicant tractaments de purificació adequats) i avaluar la seua presència a la biosfera. Es va fer ús d'una estratègia analítica basada en la injecció directa (per a les aigües) o lixiviació (per als sols) - cromatografia líquida micel·lar - detecció per absorbància UV-Visible i fluorescència per a identificar i quantificar els anàlits. Per a cada procediment, es descriu i es discuteix l'optimització dels paràmetres experimentals, realitzada mitjançant tècniques quimiomètriques, i l'avaluació de les qualitats analítiques i pràctiques: senzillesa, baixa manipulació, semiautomatització, millora de la seguretat laboral, baix impacte mediambiental, reducció del cost de les anàlisis, fiabilitat dels resultats dins un ample interval de concentracions, sensibilitat adequada i possibilitat d'analitzar una elevada quantitat de mostres en poc de temps. Els resultats positius en tots aquests paràmetres demostraren que aquests mètodes són una eina aplicable a l'anàlisi de rutina de mostres d'abocaments i d'aigües residuals de diferent origen, i en sols contaminats. Per tant, representen un avanç interessant en la protecció del medi ambient i l'entorn natural, una àrea de gran importància social.

Els compostos estudiats es classificaren en diversos grups, a partir de les seues aplicacions i característiques, que fan que es puguen trobar simultàniament en les corresponents mostres estudiades. Així mateix, es va proposar un pretractament diferent per a cada matriu (aigües residuals i sols). Primerament, es va desenvolupar un mètode per a la determinació dels pesticides majoritàriament usats en el processament de cítrics (tiabendazol,

pirimetanil, o-fenilfenol i imazalil) en aigües residuals agrícoles, abocaments de la indústria agroalimentària, i corrents d'entrada i eixida d'EDAR, usant la detecció per absorbància UV-Visible (P1). Posteriorment, es va quantificar el tiabendazol i el o-fenilfenol, que també tenen un ample ús domèstic i públic, emprant la detecció per fluorescència en les mateixes mostres que per a P1, i a més a les aigües residuals urbanes (P2). En tercer lloc, es va determinar el tiabendazol i clorpirifos, utilitzats en agricultura precollita i en l'àmbit domèstic, i el 4-tert-octilfenol, que proporciona informació de la quantitat de pesticides usats a la zona, per detecció per absorbància a les mateixes mostres que P2 (P3). Finalment, es va mesurar la quantitat dels herbicides diuron, terbutilazina i terbutrina, amb detecció per absorbància, en aigües residuals agrícoles, urbanes, punts d'entrada i eixida d'EDAR, aigües amb funció decorativa i una ampla varietat de sols (P4).

El principal avantatge dels mètodes desenvolupats fou la simplificació i la rapidesa de la preparació de la mostra (la més crítica per als procediments basats en tècniques separatives), a causa de la gran capacitat de les dissolucions micel·lars de solubilitzar compostos diversos, com dissolvents hidròfobs i xicotetes molècules poc solubles en aigua. De fet, les mostres d'aigües residuals es filtraren i s''injectaren directament, a pesar de la presència de gotes de líquids oliosos dispersats. El tractament dels sols fou més complex, per la seua natura sòlida. Després d'assecar i triturar, la mostra fou lixiviada, mitjançant agitació (1 min) i ultrasonicació (5 min) en la fase mòbil, en proporció 1/5 w/v. Posteriorment, el sobrenedant es va tractar com les mostres d'aigües residuals. Es va optimitzar els paràmetres de l'extracció, per a maximitzar el rendiment, i evitar un temps d'anàlisi i dil·lució excessius. Als dos casos, es va comprovar que la dissolució injectada fos homogènia, no danyés la columna i l'absència de compostos interferents. Els filtres no es varen obstruir, pel que els filtrats foren representatius de la mostra inicial. A més a més, foren introduïts quantitativament a la columna, no s'usà un patró intern. No fou necessari aplicar llargs i tediosos procediments d'extracció d'anàlits i/o de purificació de la mostra, els quals són habituals en HPLC hidroorgànica). Es reduïren el nombre d'etapes intermèdies, la manipulació per part de l'operador, i la quantitat de reactius emprats. Per tant, es va disminuir la probabilitat de pèrdua de l'anàlit, per recuperació ineficient o canvis físico-químics, durant la preparació de la mostra, el que facilità l'estudi simultani d'una gran quantitat de mostres en poc de temps.

La composició de la fase mòbil fou seleccionada per a maximitzar la resolució entre els anàlits, evitar el solapament amb els compostos de la matriu, i minimitzar la durada de la cursa cromatogràfica. A tots els casos, es gastaren columnes apolars C18 i fases mòbils micel·lars híbrides de SDS tamponades circulant en mode isocràtic a 1 mL/min. Es va demostrar que es requeria fixar el pH 3 i l'addició de pentanol (a causa de l'elevat temps d'elució dels pesticides més retinguts de cada grup) com a dissolvent orgànic, per a obtenir uns cromatogrames adequats. Les concentracions de tensioactiu i de pentanol s'optimitzaren (independentment per a cada un dels quatre grups de pesticides estudiats) mitjançant una estratègia interpretativa pel mètode de resposta de superfície. Es basa en l'ús d'equacions que modelitzen amb gran exactitud el comportament cromatogràfic dels anàlits (factor de retenció i resolucions individual i global) a partir de la composició de la fase mòbil, gràcies a l'estabilitat i reproductibilitat del mecanisme de retenció en MLC. Una volta ajustades, aquestes equacions es poden representar en un gràfic de superfície, el que facilita la seua interpretació i la selecció dels valors òptims. Es va fer un estudi complet del comportament cromatogràfic dels pesticides en fases mòbils de SDS (0.05-0.15 M) i 1-pentanol (2-6%). Les concentracions provades es triaren mitjançant un disseny factorial amb cinc punts: quatre amb les combinacions dels valors mínim i màxim de l'interval considerat, i un amb els valors centrals. Els valors experimentals s'usaren per a ajustar el model matemàtic, el qual ja es podia emprar per a valors intermedis de SDS i 1-pentanol. Aquesta informació es completà per una avaluació del perfil de pic. Aquesta estratègia va reduir notablement el temps i esforç necessaris per a l'estudi simultani d'aquests dos factors dins d'un gran interval de concentracions, ja que només fou necessari assajar cinc combinacions de tensioactiu i alcohol, en lloc de totes les possibles. Ulteriorment, es va demostrar que l'ús d'additius com les amines quaternàries o els líquids iònics no aportava cap avantatge. Per a cada grup de pesticides, la fase mòbil òptima i el temps global de l'anàlisi cromatogràfic foren:

- P1: 0.07 M SDS - 5%, v/v 1-pentanol - pH 3; < 17 min
- P2: 0.10 M SDS - 6%, v/v 1-pentanol - pH 3; < 11 min
- P3: 0.15 M SDS - 6%, v/v 1-pentanol - pH 3; <17 min
- P4: 0.07 M SDS - 6%, v/v 1-pentanol - pH 3; <16 min

Una volta optimitzats els mètodes, es varen analitzar les matrius no contaminades. Es va observar una banda a l'inici del cromatograma, però abans del pesticida menys retés, i posteriorment ja no s'apreciaren pics d'interés a la finestra d'elució de cada compost. Els

compostos oliosos de les aigües residuals, es troben units preferentment a les micel·les de la fase mòbil, i gairebé no interaccionen amb la fase estacionaria o els anàlits. Així mateix, l'ús de l'MLC contribueix a evitar la interferència de la matriu (*via* solapament cromatogràfic i efecte matriu).

Per al grup P2 es va triar la fluorescència per a la seua major selectivitat i sensibilitat, ja que el tiabendazol i o-fenilfenol presenten fluorescència natural. Als altres casos, es seleccionà l'absorbància UV-Visible. Es va prioritzar l'ús de tècniques de detecció barates i accessibles, sense introduir etapes de derivatització. Les longituds d'ona aplicades per a registrar el senyal s'optimitzaren a partir de la mesura dels corresponents espectres, durant la carrera cromatogràfica i amb les altres condicions ja optimitzades, per a considerar l'efecte del medi organitzat en el resultat. Per a la quantificació de cada pesticida, es triaren els valors de longituds d'ona (d'absorbància o excitació/emissió, segons el cas) que maximitzaven la relació senyal/soroll:

P1: 0-10 min, 305 nm (TBZ); 10-12, 265 nm (PYR); i 12-18, 220 nm (OPP i IMZ)

P2: 08.0 min, 305/360 nm (TBZ) i 8.0-11.0 min, 245/345 nm (OPP).

P3: 220 nm.

P4: 240 nm.

Els canvis de condicions de detecció dins d'un mateix cromatograma s'aplicaren lluny dels pics, i no afectaren l'estabilitat de la línia base.

Aquests resultats demostren que l'MLC és efectivament aplicable a la resolució i identificació dels pesticides potencialment presents als abocaments, aigües residuals i sols estudiats. L'elevada versatilitat de l'MLC, a causa de la diversitat d'entorns químics i equilibris de repartiment (fase estacionària modificada, fase mòbil i micel·la) i de punts d'interacció a la fase estacionària i a la micel·la (polar, hidrofòbica, intermèdia i aniònica), facilita la resolució de cada grup de plaguicides en una única cursa cromatogràfica. L'addició del dissolvent orgànic augmenta el poder d'elució i l'eficàcia, el que contribueix significament a l'obtenció de cromatogrames acceptables. El mode isocràtic elimina el temps d'estabilització necessari entre dues injeccions, incrementa l'estabilitat de la línia base i resulta menys agressiu per a la columna.

L'última i més important etapa del desenvolupament dels mètodes fou la validació, per a verificar la qualitat analítica i proporcionar rigorositat a l'estudi. Es va fer en matrius fortificades (P1, P4) i aigua pura (P2, P3), ja que la variabilitat entre les diverses aigües

residuals podia ser important, i segons les directrius de la "*Validation and Peer Review of US Environmental Protection Agency (EPA) Chemical Methods of Analysis*", la qual va ser específicament redactada per a la determinació de contaminants orgànics en mostres mediambientals. Es va intentar assolir la màxima sensibilitat, sense complicar excessivament la manipulació experimental. Els paràmetres estadístics avaluats i els resultats es descriuen a continuació: selectivitat (es va demostrar que els pesticides es podien resoldre sense interferències en l'etapa d'optimització de la separació cromatogràfica), interval de quantificació (per damunt de 0.2-4 mg/L, en aigües per absorbància, 0.01-0.02 a 2 mg/L en aigües per fluorescència i 1 mg/kg en sols; fins a valors alts), linealitat (r^2> 0.994), límit de detecció (0.18-0.8 mg/L, en aigües per absorbància, 0.005-0.008 mg/L en aigües per fluorescència i 0.3 mg/kg en sols), exactitud (92.1%-104.2%), precisió (<13.6%) i robustesa (les respostes analítiques són aproximadament constants front a canvis xicotets en les condicions experimentals). Per als plaguicides usats en agricultura postcollita, es va trobar que en condicions ambientals es degradaven al voltant de huit dies en aigua. S'arribà a una major selectivitat i sensibilitat (de prop de dos ordres de magnitud) emprant la detecció mitjançant fluorescència, a causa de les propietats d'aquest fenomen físic. Es varen obtenir valors de recuperació, reproductibilitat i sensibilitat vàlids a causa de la simplificació del tractament de la mostra i a la introducció quantitativa sense dilució de les aigües residuals i el sobrenedant obtingut a partir de la lixiviació dels sols, a la baixa proporció sol/dissolució extractora, i a la minimització de fonts de variança en aquesta etapa. Finalment, els procediments s'aplicaren amb èxit a l'anàlisi de mostres d'aigües i sols procedent de diversos punts de la província de Castelló. Es va provar que els mètodes proporcionen valors qualitatius i quantitatius fiables als nivells de contaminació de cada classe de mostra, i dins d'un ample interval de concentracions. A més a més, per al grup P2 es detectaren els pesticides per sota dels nivells fixats per la regulació local per als abocaments i les aigües residuals.

Les anàlisis es poden realitzar emprant instrumentació, material i reactius analítics barats, robustos, d'ús general i fàcilment accessibles. A més a més, els procediments són senzills i relativament ràpids, sobretot el d'anàlisi d'aigües que no va necessitar cap pretractament. Els reactius principals (SDS i tampó fosfat) són biodegradables i innocus, i només es va gastar una proporció mínima de dissolvent orgànic tòxic, volàtil i inflamable (<6% pentanol) per a les fases mòbils i per a la dissolució extractora dels sols. Per tant, es va

minimitzar la manipulació i vessament de dissolucions perilloses per a la salut dels treballadors i per al medi ambient. A més a més, els mètodes faciliten l'anàlisi successiva d'una elevada quantitat de mostres, el que és útil si el laboratori rep moltes mostres en una època determinada i/o el client exigeix el resultat en poc de temps. Per tant, es poden utilitzar per al monitoratge, en anàlisi de rutina, de les aigües residual urbanes i agrícoles, abocaments industrials i sols (per a avaluar el seu nivell de contaminació) i punts d'entrada i d'eixida d'EDAR (per a comprovar el rendiment de la purificació de l'aigua), que en conjunt pot servir per a reduir la quantitat de pesticides al medi ambient.

Annex 1. Aportacions originals

Els estudis descrits han sigut realitzats gràcies al finançament rebut del Plà per a la Promoció de la Investigació de la Universitat Jaume I (projectes: P1-1B2012-36) i de FACSA (contractes 11I036.01/1 i 11I358.01), organismes que consideraren que la seva importància i la rellevància social de la recerca mereixia el seu suport. El treball realitzat ha permès la publicació de les següents aportacions originals en revistes i reunions científiques de primer nivell:

Articles en revistes de recerca científica

1. Peris-Vicente J, Marzo-Mas A, Roca-Genovés P, Carda-Broch S, Esteve-Romero J (2016) of micellar liquid chromatography for rapid monitoring of fungicides post harvest applied to citrus wastewater. *J. Environ. Sci. (China)* 42, 284-292, doi: 10.1016/j.jes.2015.12.012

2. Juan Peris-Vicente J, Pasqual Roca-Genovés P, Tayeb-Cherif K, Esteve-Romero J (2016) Development and validation of a method to determine thiabendazole and o-phenylphenol in wastewater using micellar liquid chromatography-fluorescence detection. *Electrophoresis* 37, 2517-2521, doi: 10.1002/elps.201500580.

3. Romero-Cano R, Kassuha D, Peris-Vicente J, Roca-Genovés P, Carda-Broch S, Josep Esteve-Romero J (2015) Analysis of thiabendazole, 4-tert-octylphenol and chlorpyrifos in waste and sewage water by direct injection - micellar liquid chromatography. *Analyst* 140,1739-1746, doi: 10.1039/c4an01782j

4. Pitarch-Andrés S, Roca-Genovés P, Peris-Vicente J, Esteve-Romero J (2017) Determination of diuron, terbuthylazine, and terbutryn in wastewater and soil by micellar liquid chromatography. *Anal. Bioanal. Chem.* 409, :2037-2049. doi: 10.1007/s00216-016-0151-3

Ponències en congressos internacionals de recerca científica

20th International Symposium on Separation Science (20th ISSS). 30 Agost - 2 de Setembre 2014; Praga, República Checa

Publicació: "Book of proceedings of the 20th International Symposium on Separation Science", (Editores = A. Horna, P. Jandera) Ed. Radanal, Pardubice, Czech Repulik, 2014. ISBN: 978-80-7395-777-3

1) "Application of factorial design to optimization strategies in micellar liquid chromatography" (P9 - p. 64) Esteve-Romero J, Carda-Broch S, Peris-Vicente J, Kassuha D, Roca-Genovés P, Fabregat-Safont D, Álvarez-Rodríguez L

2) "Micellar liquid chromatography: an interesting tool for determination of biological samples in bioanalytical chemistry" (P66 - p. 121) Esteve-Romero J, Carda-Broch S, Peris-Vicente J, Roca-Genovés P, Tayeb-Cherif K, Romero-Cano R, Monferrer-Pons L

3) "Development of a new method for the determination of antifalciparium malaria" (P119 - p. 174) Roca-Genovés P, Esteve-Romero J, Raviolo MA, Rambla-Alegre M, Carda-Broch S, Peris-Vicente J

4) "Determination of some banned aromatic amines in waste water using micellar liquid chromatography" (P130 - p. 185) Roca-Genovés P, Esteve-Romero J, Carda-Broch S, Fabregat-Safont D, Romero-Cano R, Peris-Vicente J

14th International Nutrition and Diagnostics Conference (14th INDC). 02 - 05 de Setembre 2014; Praga, República Checa

Publicación: "Book of proceedings of the 14th International Nutrition and Diagnostics Conference" (Editor: A. Horna) Ed. Radanal, Pardubice, Czech Repulik, 2014. ISBN: 978-80-7395-776-6

5) "Analysis of biogenic amines in anchovy sauce using micellar liquid chromatography" (P130 - p. 187) Roca-Genovés P, Esteve-Romero J, Peris-Vicente J, Rambla-Alegre M, Carda-Broch S, Romero-Cano R, Monferrer-Pons L

6) "Simple chromatographic method for determination of thiram, cabendazim and ziram in agricultural samples" (P131 - p. 188) Roca-Genovés P, Esteve-Romero J, Peris-Vicente J, Bose D, Durgbanshi A, Carda-Broch S, Garrido-Cano I, Álvarez-Rodríguez L

7) "Green method to determine melamine in dietetic supplements" (P132 - p. 169) Roca-Genovés P, Esteve-Romero J, Peris-Vicente J., Beltrán-Martinavarro B, Carda-Broch S, Marco-Peiró S

European Symposium on the Practical Applications of Analytical Technologies in the Biopharmaceutical Industry (ATEurope 2016). 15-18 Març 2016; Viena, Austria

8) "Comparing the Effect of the Improvement of Tamoxifen Treatment Efficiency in Breast Cancer Patients With a Poor Metabolizer Genotype in Men and Women" (LB-03a) Albiol-Chiva J, Ochoa-Aranda E, Peris-Vicente J, García-García A, Roca-Genovés P, Esteve-Romero J, Carda-Broch S

9) "Comparison of the Concentration of Antiretroviral in Serum Samples of Men And Women Using Micellar Liquid Chromatography" (LB-03b) Albiol-Chiva J, Roca-Genovés P, Carda-Broch S, Esteve-Romero J, García-García A, Peris-Vicente J, Ochoa-Aranda E

10) "Relationship Between Tamoxifen/Endoxifen Concentration Ratio and CYP2D6 Genotipe in Men and Women" (LB-03c) Albiol-Chiva J, Roca-Genovés P, Ochoa-Aranda E, Esteve-Romero J, Peris-Vicente J, Tayeb-Cherif K, García García A

11) " Development and Validation of a Method to Detect Eight Fluoroquinolones in Honey Using Micellar Liquid Chromatography - Fluorescence Detection" (LB-03d) Albiol-Chiva J, Tayeb-Cherif K, Peris-Vicente J, Roca-Genovés P, Esteve-Romero J, Carda-Broch S

12) "Use of Micellar Liquid Chromatography to Analyse Thiabendazole, Tert-octylphenol and Chlorpyrifos in Wastewater" (LB-03e) Albiol-Chiva J, Peris-Vicente J, Esteve-Romero, J Carda-Broch S, Roca-Genovés P

13) "Determination of Melamine in Several Matrices by Micellar Liquid Chromatography" (LB-03f) Albiol-Chiva J, Carda-Broch S, Roca-Genovés P, Peris-Vicente J, Esteve-Romero J

14) "Use of Micellar Liquid Chromatography to Quantify Several Quinolones in Porcine and Bovine Flesh" (LB-03g) Albiol-Chiva J, Tayeb-Cherif K, Carda-Broch S, Roca-Genovés P, Esteve-Romero J, Peris-Vicente J

15) "Quantification of the Antidepressants Citalopram, Paroxetine and Fluoxetine in Plasma, Urine and Tablets by Micellar Liquid Chromatography" (LB-03h) Albiol-Chiva J, Esteve-Romero J, Peris-Vicente J, García-García A, Carda-Broch S, Roca-Genovés P

16) "Analytical Determination of Paracetamol in Serum and Urine by Micellar Liquid Chromatography With Electrochemical Detection" (LB-03i) Albiol-Chiva J, Esteve-Romero J, Carda-Broch S, García-García A, Peris-Vicente J, Roca-Genovés P

17) "Determination of Antibiotics in Pharmaceuticals and Physiological Samples by Micellar Liquid Chromatography" (LB-03j) Albiol-Chiva J, Carda-Broch S, Tayeb-Cherif K, García-García A, Peris-Vicente J, Roca-Genovés P, Esteve-Romero J

Annex 2. Futures línies de recerca

Durant l'elaboració de la Tesi Doctoral, he treballat en una de les línies d'investigació del grup de Química Bioanalítica, que té com a finalitat el desenvolupament de nous mètodes ràpids, simples, econòmics, segurs, fiables i ecològics per cromatografia líquida micel·lar, mitjançant tècniques quimiomètriques, aplicables en el camp de la protecció mediambiental i continuaré participant dins d'aquesta línia.

En un futur, tinc previst analitzar els pesticides que s'han determinat només en aigües, en mostres de sols, per a completar l'estudi començat a la Tesi Doctoral. També es pretén l'optimització i validació de procediments per a la quantificació d'altres pesticides en aigües residuals i sols. Més avant, també considere interessant treballar en la detecció de pesticides en aliments, en totes les fases de la cadena de producció i distribució (producció al camp, collita, emmagatzematge, transport, venda al públic i conserva i emmagatzematge a les llars particulars) per a comprovar si es tracten adequadament i si la seua ingesta implica risc per al consumidor. Per últim, es pretén avaluar la possibilitat d'utilitzar fases mòbils i dissolucions extractants micel·lars mixtes, on el dissolvent orgànic és substituït per un altre tensioactiu no-contaminant. S'haurà de mesurar el comportament cromatogràfic dels pesticides en aquest medi, realitzar estudis teòrics sobre el mecanisme de retenció, i el desenvolupament d'equacions i dissenys factorials per a la modelització. Així mateix, caldria optimitzar les condicions de lixiviació, en el cas de les mostres sòlides. Es disposaria d'una col·lecció de mètodes analítics completament segurs i ecològics.

Annex 3. Acceptació dels coautors de les publicacions que integren la tesi, de què el doctorand presenta el treball com a tesi i renúncia expressa d'aquests a presentar-ho com a part d'una altra tesi doctoral (segons Art. 23 de la NORMATIVA DELS ESTUDIS DE DOCTORAT, REGULATS PEL RD 99/2011, EN LA UNIVERSITAT JAUME I (Aprovada pel Consell de Govern núm. 19 de 26 de gener de 2012)

Josep Esteve Romero, director de la present tesi, declara que els coautors de les publicacions que es presenten en aquesta tesi, i que passe a enumerar: S. Carda Broch, J. Peris Vicente, A. Marzo Mas, K. Tayeb-Cherif, R. Romero Cano, D. Kassuha i S. Pitarch Andrés no utilitzarem el material que ací es presenta per a formar part d'altres tesis. I perquè conste on convinga, signe la present.

Josep Esteve Romero	Samuel Carda Broch	Juan Peris Vicente	Ana Marzo Mas
			
K. Tayeb-Cherif	R. Romero Cano	D. Kassuha	S. Pitarch Andrés

Universitat Jaume I, 9 de Juny de 2017

Annex 4. Abreviatures i acrònims

Pesticides i compostos relacionats

4-tOP: 4-tert-octilfenol/*4-tect-octylphenol*

APE: alquilfenols polietoxilat/*Alkylphenol polyethoxylate*

CPF: Clorpirifos/*Chlorpyrifos*

DIU: diuron

IMZ: imazalil

OPP: o-fenilfenol/*o-phenylphenol*

PYR: pirimetanil/*Pyrimethanil*

TBZ: tiabendazol/*Thiabendazole*

TBA: terbutilazina/*terbuthylazine*

TBT: terbutrina/*terbutryn*

Reactius de laboratori

CTAB: Bromur d'hexadeciltrimetilamoni/*cetyltrimethylammonium bromide*

$EMIMBF_4$: tetrafluoroborat d'1-etil-3-metilimidazoli/*1-ethyl-3-methylimidazolium tetrafluoroborate*

HCl: Hydrochloric acid

$NaH_2PO_4.H_2O$: Dihidrogenfosfat de sodi monohidratat/*sodium dihidrogenophosphate monohydrate*

NaOH: Hidròxid de sodi

SDS o $C_{12}H_{25}SO_4Na$: dodecil sulfat sòdic/*sodium dodecyl sulphate*

TEA: trietilamina/*triethylamine*

Tècniques o instrumentació analítiques

C8: octasilà/*octasilane*

C18: octadecilsilà/*octadecylsilane*

DAD: detecció per matriu de diodes/*diode array detection*

FLD: detecció per fluorescència/*fluorescence detection*

GC: cromatografia de gasos/*Gas Chromatography*

LC, HPLC: cromatografia líquida d'alta resolució/*High Performance Liquid Chromatography*

MLC: cromatografia líquida micel·lar/*Micellar Liquid Chromatography*

MS: espectrometria de masses/*mass spectrometry*

RP: fase reversa/*reverse phase*

UV: ultraviolat/*ultraviolet*

Associacions i Organismes

AOAC: Associació Oficial de Químics Analítics/*Association of Official Analytical Chemists*

EDAR/WWTP: Estació Depuradora d'Aigües Residuals/*Wastewater Treatment Plants*

EFSA: Autoritat Europea de Salut Alimentària/*European Food Safety Authority*

EPA: Agència de protecció del medi ambient/*Environmental Protection Agency*

EURACHEM: Grup Europeu de Química Analítica/*European Analytical Chemistry Group*

FACSA: Fomento Agrícola Castellonense, Sociedad Anónima

FAO: Organització per als Aliments i l'Agricultura/*Food and Agricultural Organization*

ICH: Conferència Internacional d'Armonització/*Internacional Conference on Harmonization*

IEC: Comissió Electrotècnica Internacional/*International Electrotechnical Commission*

ISO: Organització Internacional de Normalització/*International Organization for Standardization*

IUPAC: Unió Internacional de Química Pura i Aplicada/*International Union of Pure and Applied Chemistry*

UE/EU: Unió Europea/*European Union*

Paràmetres cromatogràfics

α_{ij}: selectivitat cromatogràfica

α'_{ij}: selectivitat modificada

φ: proporció de dissolvent orgànic (v/v)

A, o_i: àrea total d'un pic

B/A: Factor d'asimetria

$c_{..}$: constants empíriques en la modelització del factor de retenció

H_0: alçada del pic al temps de retenció

h_1: senyal cromatogràfica al punt vall entre dos pics

h_2: alçada interpolada fins a la línia que uneix dos pics als seus corresponents temps de retenció, al punt vall

h(t): senyal del cromatograma

k: factor de retenció o factor de capacitat

K_{AS}: Constant de repartiment de l'anàlit entre l'aigua pura i la fase estacionària pel volum de la fase estacionària, dividit pel volumn mort.

K_{AM}: Constant de repartiment de l'anàlit entre l'aigua pura i la micel·la

K_{AD}, K_{AD1}, K_{A2}: mesura de la variació de la concentració de l'anàlit en la fase aquosa a causa de l'addició del dissolvent orgànic.

K_{MD}: mesura de la variació de la concentració de l'anàlit en la micel·la a causa de l'addició del dissolvent orgànic.

N: nombre de plats teòric (eficàcia)

o'_i: àrea de la part d'un pic solapat amb altres

P_{MS}: Constant de repartiment de l'anàlit entre la micel·la i la fase estacionària

P_{WS}: Constant de repartiment de l'anàlit entre l'aigua pura i la fase estacionària

r_{ij}: resolució per parells de pic

$s_{..}$: constants relacionades amb el perfil de pic

S/N: relació senyal-soroll/*signal-to-noise ratio*

t_0: temps mort

t_R: temps de retenció

Z: resolució global d'un cromatograma

Paràmetres químics

CMC: concentració micel·lar crítica/*critical micellar concentration*

Ka: constant de desprotonació d'un àcid

Po/w : coeficient de repartiment octanol-aigua/*octanol-water partition coefficient*

Paràmetres de validació

CD^2: distància quadràtica de Cook/*Cook's squared distance*

HLOQ, ULOQ: límit de quantificació màxim/*Higher or Upper Limit of Quantification*

LLOQ: límit de quantificació mínim/*Lower Limit of Quantification*

LOD: límit de detecció/*Limit of Detection*

LOQ: límit de quantificació/*Limit of Quantitation*

r^2: coeficient de determinació/*determination coefficient*

R^2: coeficient de regressió múltiple/*multiple regression coefficient*

RSD: desviació estàndard relativa/*Relative Standard Deviation*