NICOLE ENNIS

DEVELOPMENT OF NEW ANALYTICAL METHODS FOR THE ANALYSIS OF AN EMERGING PHARMACEUTICAL POLLUTANT (IBUPROFEN)

ANALYSIS BY CAPILLARY ELECTROPHORESIS AND HPLC COUPLED WITH OFF-LINE SPE

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Faculdade de Ciências e Tecnologia

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Erasmus Mundus Master in Quality in Analytical Laboratories

Trabalho efetuado sob a orientação de:
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Professor Marina Franco Maggi Tavares

UNIVERSIDADE DO ALGARVE

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I declare to be the author of this work, which is original and unpublished. Authors and works consulted are duly cited in the text and are included in the list of references.

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Nicole Ennis

Abstract

Keywords: high performance liquid chromatography, micellar electrokinetic chromatography, capillary electrophoresis, ibuprofen, metabolites, validation, wastewater, aerobic granule

Pharmaceuticals have become priority emerging environmental pollutants. Environmental monitoring and toxicological studies are essential to establish maximum permissible limits in the environment. Furthermore, innovative wastewater treatment is required for their degradation. Águas do Algarve is constructing an aerobic granule plant to modernize wastewater treatment for the cities of Faro and Olhão. The company is interested in research regarding granule ability to degrade pharmaceuticals detected in wastewater influent.

Two aerobic granule sequencing batch reactors were constructed and operated at laboratory scale using different cycles (anaerobic/aerobic and aerobic). The efficiency of the reactors and type of operating conditions was investigated for the degradation and removal of ibuprofen and its metabolites 2-hydroxyibuprofen, 1-hydroxyibuprofen and carboxyibuprofen.

Two methods, high performance liquid chromatography (HPLC) and micellar electrokinetic chromatography (MEKC), a form of capillary electrophoresis, coupled with UV/Vis, were developed to detect and quantify ibuprofen and its metabolites. Solid phase extraction was used to preconcentrate the compounds to method detection limits, however recoveries were problematic. Eluents were dried and reconstituted in solvent (milli-q water) for compatibility to both methods. Separation conditions for the compounds were optimized. Both methods were validated in solvent (milli-q water) for linearity, recovery, LOD, LOQ, precision (repeatability and intermediate precision), range and selectivity. Selectivity could be obtained for both methods. However, acceptable linearity, LOD, LOQ, repeatability and recovery could not be established in the desired working range. Additionally, robustness problems particularly for the MEKC method may have affected validation results. For some compounds, both methods failed to meet statistical tests applied for acceptance of linearity and repeatability. Solvent and synthetic wastewater calibration were compared using student's t-test. There was no statistically significant difference between the curves for the HPLC method in contrast to those of the MEKC method.

The methods were applied to synthetic wastewater samples taken from the SBRs. Each method detected different analytes with 1-hydroxyibuprofen observed in HPLC and carboxyibuprofen observed in MEKC. The calculated concentrations exceeded method range. Revalidation of both methods and reanalysis of the samples is required to verify accuracy of results and confirm suitability for intended application.

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List of Abbreviations and Symbols

α	Selectivity
1OH	1-hydroxyibuprofen
$\Delta\varphi$	change in organic solvent (%B) during gradient
σ_1, σ_2, σ_3	User selected adjustable weights
2OH	2-hydroxyibuprofen
a	y-intercept
AOAC	Association of Official Analytical Chemists
b	Slope
%B	Change in organic solvent
BA	Benzoic acid
BGE	Background electrolyte
CBX	Carboxyibuprofen
CE	Capillary electrophoresis
COD	Chemical Oxygen Demand
COX	Cyclooxygenase
CEF	Chromatographic exponential function
CRF	Chromatography response function
CRS	Chromatographic resolution statistic
CV	Coefficient of variance
d^2_c	column internal diameter
DAD	Diode array detector
EOF	Electroosmotic flow
EU	European Union
f	Number of degrees of freedom
F	Flow rate
F_{max}	Calculated Fmax value for Hartley's F_{max} test
F_{maxo}	Critical Fmax value for Hartley's F_{max} test
FASS	Field-amplified sample stacking
FASI	Field-amplified sample injection
HCl	Hydrochloric acid
HPLC	High Performance Liquid Chromatography
ICH	International Conference on Harmonization
i.d	Internal diameter
k	Number of factors for experimental design
k	Isocratic retention factor
k*	Gradient peak retention factor
L	Column length
L_{det}	Length to detector
L_{tot}	Total length
LC-MS	Liquid chromatography-mass spectrophotometry
LOD	Limit of detection
LOQ	Limit of quantification
MEKC	Micellar electrokinetic chromatography
n	Number of result: for response function – number of solutes in sample
np	Number of peak pairs
NSAID	Nonsteroidal anti-inflammatory drug
ppb	Parts per billion
ppm	Prt per million

Q_{crit}	Calculated value of Dixon's Q test
Q_1	Smallest calculated value of Dixon's Q test using smallest result
Q_n	Calculated value of Dixon's Q test using number of results
r	Correlation coefficient
RP	Number of resolved peaks
R_{cp}	Resolution of critical peak pair
$R_{i, i+1}$	Resolution between adjacent solute pairs
R_m	Average resolution of all solute pairs
R_{min}	Minimum acceptable resolution
R_{opt}	Optimum resolution
R_s	Chromatography resolution
R_{sum}	Resolution sum
SBR	Sequencing batch reactor
SD	Standard deviation
SD^2_{min}	Smallest standard deviation
SD^2_{max}	Largest standard deviation
SDS	Sodium dodecyl sulphate
SPE	Solid phase extraction
t	Calculated value for student's t test
t_{ap}	Analyte migration time
t_{eo}	Time of EOF marker
t_0	Column dead time
t_i	Migration time of first solute
t_{crit}	Critical value for student's t test
t_f	Migration time of final solute
t_G	Gradient time
t_m	Migration time
t_{max}	Maximum desired runtime
t_{min}	Minimum desired runtime
t_n	Migration time of final solute
t_r	Retention time
THF	Tetrahydrofuran
μ_{ef}	Effective mobility
μ_{eof}	Electroosmotic flow mobility
UAlg	Universidade do Algarve
USP	Universidade do São Paulo
UV/Vis	Ultraviolet visible
V	Applied voltage
V_{eof}	Electroosmotic velocity
V_{ef}	Effective velocity
V_{ap}	Apparent velocity
V_m	Column dead volume
x	Analyte concentration corresponding to the measured signal
x_1	Smallest value in result series
x_n	Largest value in result series
W_b	Peak width at baseline
WWTP	Wastewater treatment plant

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Objective of Study

The first objective of the study was to develop, optimize and validate new analytical methods for the detection and quantification of ibuprofen and the three metabolites, 1-hydroxyibuprofen, 2-hydroxyibuprofen and carboxyibuprofen in synthetic wastewater. The compounds were chosen due to frequent detection in wastewater treatment plants (WWTP) influent and effluent, surface (rivers/lakes/streams), ground and drinking waters. The methods developed were high performance liquid chromatography (HPLC) initiated at the Universidade do Algarve (UAlg) and completed at the University of São Paulo and capillary electrophoresis (CE) at the University of São Paulo (USP). Both methods were used to detect and quantify the compounds in synthetic wastewater samples taken from two aerobic granular sequencing batch reactors (SBRs). Separation conditions for both methods were optimized for resolution of compound peaks and analytical time, then validated for the application. Solid phase extraction was performed to pre-concentrate the analytes, facilitating analysis at the trace concentration range commonly detected in the environment and for the dosage applied to the reactors. The performance of both methods would be compared statistically using student's t test for the results of sample analysis. Solvent and synthetic wastewater calibration curves would also be compared using student's t-test to assess matrix effect on validation parameters.

The second objective of the study was the construction and operation of two aerobic granule SBRs at laboratory scale. Both SBRs were operated using different cycles, anaerobic/aerobic and aerobic conditions respectively to compare the efficiency of this wastewater treatment and the type of operating cycle on the degradation and removal of ibuprofen and the metabolites. Samples for analysis were taken at selected timepoints during one cycle for analysis by the HPLC and CE methods.

1. Introduction and Literature Review

1.1 Pharmaceuticals: Emerging Pollutants in Water

Pharmaceuticals are indispensable to the preservation of global public health[1]. Consumption has increased concurrent to prolonged human life expectancy, shifting population demographics, rising global population, rapid urbanization, research advancement and enhanced market availability and affordability[1–3]. Consequently, pharmaceuticals, their metabolic and transformation products are increasingly detected in trace concentrations (ng to μg/L) in the aquatic (surface, ground and drinking waters) and terrestrial (soil and manure) environment and wastewater treatment plants, garnering great concern as emerging environmental pollutants[2–7].

Extensive environment studies have been published, with emphasis on the predominantly used therapeutic classes such as nonsteroidal anti-inflammatory drugs (NSAIDs). Current knowledge is limited on the definitive effects of all detected pharmaceuticals. Nevertheless, research has given evidence to the potential damage resulting from the bioaccumulative effects of chronic exposure and continuous introduction into the environment[2,3,7–9]. Their potentially dangerous environmental impact arises from their inherent ability for unhindered biological activity at sub-therapeutic concentrations[2,8,10,11].

Apprehension is greatest for aquatic pollution due to the inescapable life-cycle exposure of these organisms and possible disruption of the food chain at the primary producer level[6,8]. Metabolic or transformation products may exhibit similar or higher toxicity than parent compounds[12,13]. A database compiled by the United Nation’s Strategic Approach to International Chemicals Management revealed the detection and quantification of 671 pharmaceuticals inclusive of their metabolic and transformation products in 71 countries. The data, while collected mainly from Western Europe, United States and Canada, indicate that this is a global problem[2].

Pharmaceuticals are introduced to the aquatic and terrestrial environment through anthropological activities including industrial production, animal husbandry, household and clinical use and effluent from wastewater treatment plants (WWTPs) (Figure 1) [2,3,7,14]. WWTPs are the dominant emission pathway, particularly for the aquatic environment. Human and animal excreta (faeces and urine), remnants of industrial production and discarded clinical and domestic pharmaceutical waste are

transported via the sewage system to WWTPs and released via wastewater effluent into the environment[2,7,10,15,16]. The pharmaceuticals detected and their concentration are contingent upon location of the WWTP, season and geographical region[2,3]. This problem arises from the operational intent of WWTPs, which are designed for the treatment of biological waste to reduce carbon, nitrogen and phosphorus loads. Additionally, WWTPs employ a high degree of dilution during treatment and release, hindering the contact time[9]. Many pharmaceuticals, their metabolites and transformation products are resistant to the treatment, hence their persistence in WWTP effluent[2,17].

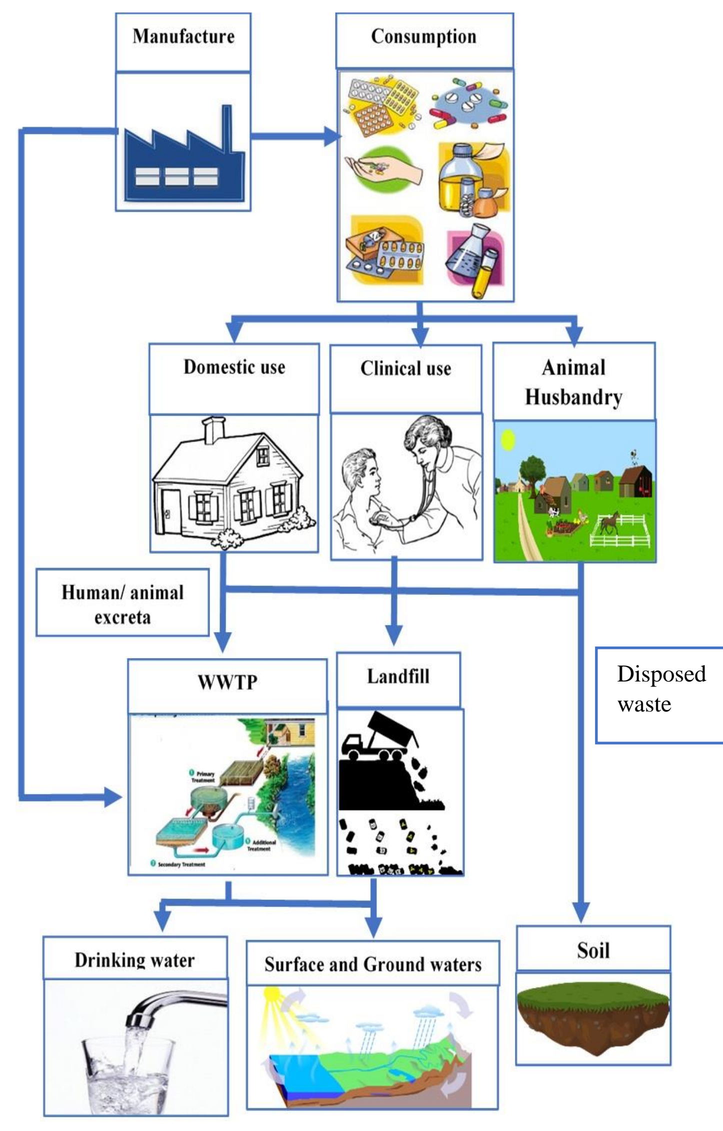


Figure 1. Pharmaceutical emission pathways into the aquatic and terrestrial environment

1.2 Compounds of Study: Ibuprofen and Metabolites

This thesis focuses on ibuprofen (IBU), one of the most popular NSAIDs available on the market, and its metabolites - 1hydroxyibuprofen (1OH), 2-hydroxyibuprofen (2OH) and carboxyibuprofen (CBX). Ibuprofen, discovered in 1950[18], is designated as an essential medicine by the World Health Organization. The pharmaceutical is easily available via prescription and over the counter and used in pain, inflammation and other chronic arthritic treatment[19,20]. Brazil is a substantial consumer of pharmaceuticals concomitant to its large population but accurate, reliable data on volume and therapeutic classes used, is difficult to obtain[21]. For Portugal, the latest INFRAMED data shows that 2011 sales of over two million packages [22].

Pain and/or fever due to infection and/or injury stimulate the cyclooxygenase (COX) pathway where COX-1, COX-2 are involved in prostaglandin synthesis. This activates the vertebrate and invertebrate inflammatory response to initiate curative measures. Ibuprofen acts as a non-selective inhibitor through reversible binding to both COX isozymes, reducing their activity and consequently prostaglandin synthesis and inflammation (Figure 2). These enzymes also play critical roles in prostaglandin synthesis in the reproductive, gastro-intestinal, blood and nervous systems [23–25].

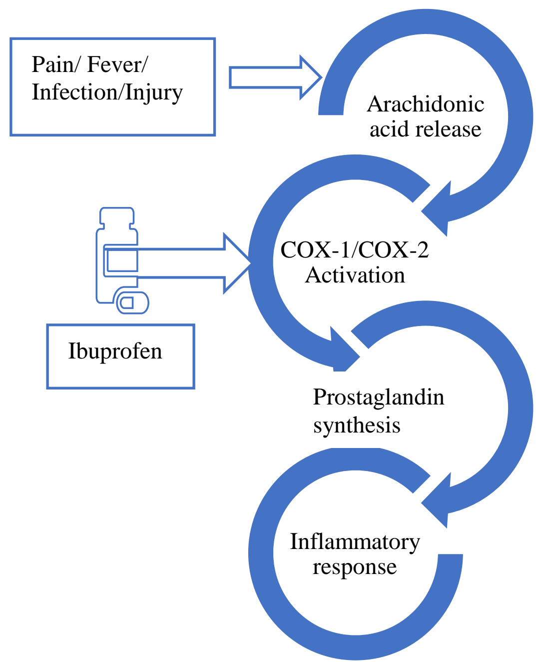


Figure 2. Ibuprofen mode of action on the COX pathway

Human metabolism produces 1-hydroxyibuprofen, 2-hydroxyibuprofen and carboxyibuprofen [20,26]. Their structure and conversion from the parent compound are shown in Figure 3[27]. These products are observed in environmental studies of ibuprofen biodegradation in wastewater, except that CBX is produced first and generally has the higher concentration [27–32]. The pharmaceutical is almost completely metabolized, with 15% of ingested IBU excreted in urine, 25-26% as 1OH and 2OH and 37-46% as CBX[20,33]. The compounds are weak hydrophobic acids, possessing carboxyl (COOH) and aromatic benzene ring functional groups. Their chemical names and physicochemical properties are given in Table 1[27].

Table 1. Structure and physico-chemical properties of compounds of interest

Compound	Chemical Name	Molecular Weight g/mol	pKa	Log K_{ow}
Ibuprofen	2-(4-Isobutylphenyl)propanoic acid	206.281	4.91	3.97
1-hydroxyibuprofen	2-[4-(1-Hydroxy-2-methylpropyl)phenyl]propionic acid	222.284	4.55	2.69
2-hydroxyibuprofen	2-[4-(2-Hydroxy-2-methylpropyl)phenyl]propionic acid	222.284	4.63	2.37
Carboxyibuprofen	3-[4-(1-carboxyethyl)phenyl]-2-methylpropanoic acid	236.267	3.97	2.78

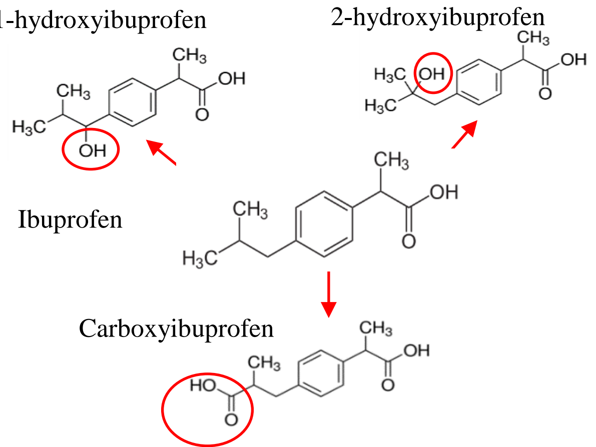


Figure 3. Chemical structure of IBU, 1OH, 2OH and CBX

1.3 Water Quality Legislation and Monitoring

The United Nations highlights the fundamental importance of ecological conservation and sustainability of good, safe and adequate water and treatment of urban wastewater for public health and socioeconomic development[34]. Countries have enacted regional and/or national legislation to meet this global priority. Brazil legislates water quality and conservation by the National Water Resource Policy and National Water Resource Management System, reinforced by additional state regulations (Figure 4)[35]. The European Union (EU) uses the Water Framework Directive 2000/60/EC to establish policies and measures for water quality, safety, resource conservation and pollution reduction. Supplementary directives[36–38] are enacted for prioritization and environmental monitoring of selected compounds [39–42], environmental quality standards[42], wastewater collection, treatment and discharge regulation [43,44] and sewage sludge use in agriculture[45] (Figure 5). Recognizing pharmaceuticals as emerging environmental pollutants, Directive 2013/39/EU, updates the Water Framework Directive to initiate their inclusion to the watch list of environment priority compounds for monitoring and specific action. The aquatic environment is the predominant matrix of concern. The list is subject to revision arising from research identifying other pharmaceuticals as environment risks.

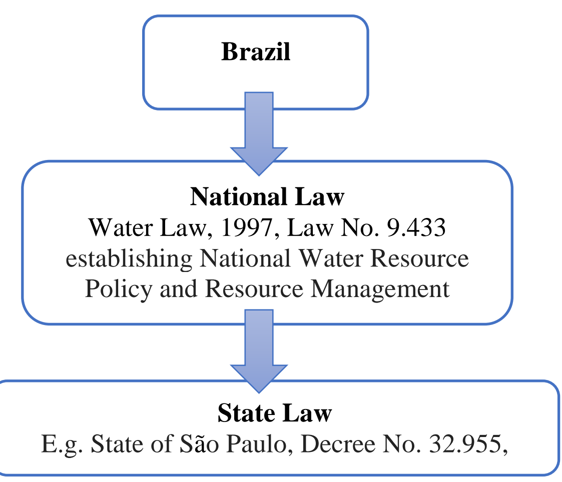


Figure 4. Water quality and safety legislation in Brazil

Individual member states such as Portugal, enact EU directives through national-specific legislation (Figure 5)[35,46].

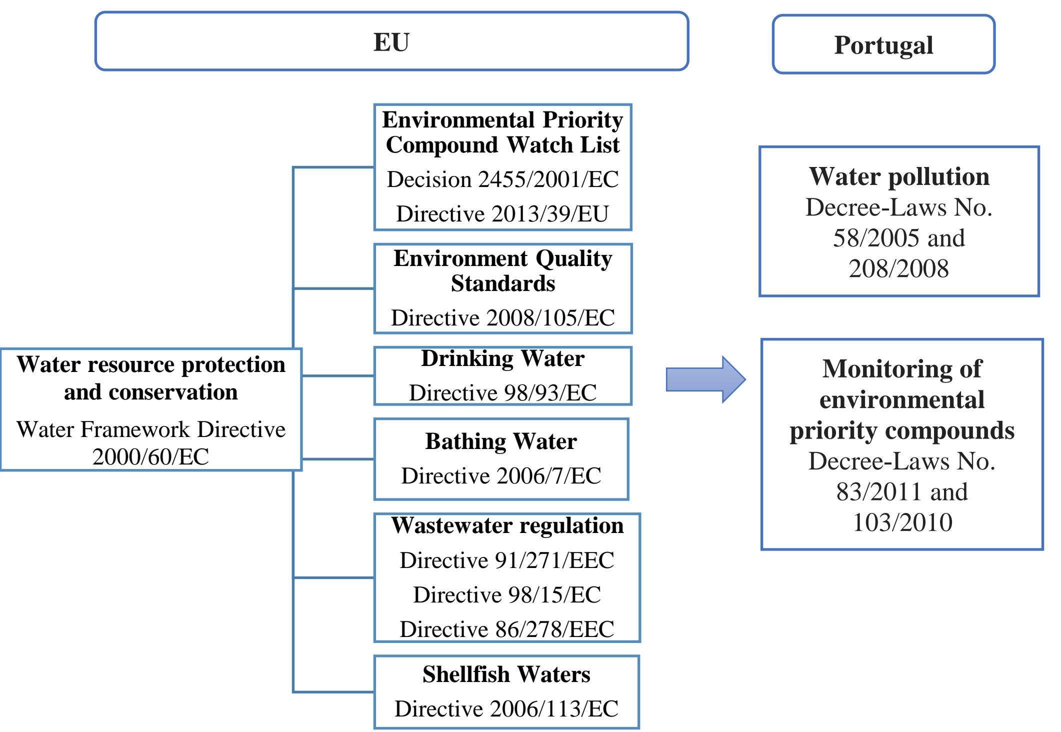


Figure 5. Water quality and safety legislation in the EU and Portugal

It is recommended that environmental regulatory agencies focus monitoring programs by selecting priority pharmaceuticals based on country specific consumption and environment risk assessments. This allows the calculation and evaluation of pharmaceutical environmental liability by predicted or measured environmental concentration and toxicity data to three different trophic levels of the aquatic ecosystem (algae, daphnids and fish). However, the certainty of minimal environmental impact from pharmaceuticals deemed to have no or low risk (≤1), has been questioned for several reasons. Risk assessments are conducted on individual pharmaceuticals, excluding their realistic occurrence in the environment as mixtures with the same or different therapeutic classes and other compounds. Some studies proved significant additive effect and higher toxicities of pharmaceutical mixtures from same or different therapeutic class on algae[47–53]. Additionally, the ecotoxicological impact of chronic exposure over the lifespan of aquatic organisms and the toxicity of pharmaceutical metabolites are rarely studied [54–56]. These considerations along with the inherent pharmaceutical ability for biological activity at sub-therapeutic concentrations, have prevented the establishment of maximum permissible limits for ibuprofen and other

pharmaceuticals in surface and ground waters and treated wastewater effluent. Monitoring data and assessments of chronic ecotoxicological effects are required to define these limits and select pharmaceuticals for monitoring programmes[22,54,57,58].

1.4 Environmental Detection and Risk Assessment of Ibuprofen and Metabolites

The EU is considering the addition of ibuprofen to the watch list of priority pharmaceuticals of environmental concern, due to the frequent detection in the aquatic environment and potential impact [59–61].

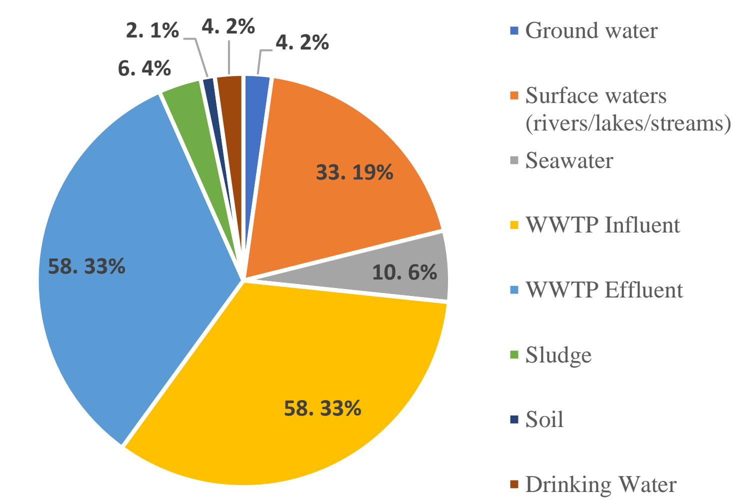


Figure 6. Distribution detection of Ibuprofen and environmental matrix of study

Its medical popularity means it has been extensively studied to evaluate its presence and potential risk in the environment. In literature review for this thesis, wastewater influent and effluent was the most studied environmental matrix and had the highest rate of ibuprofen occurrence as shown in Figure 6 [17,22,32,54,57,62–101]. In the United Nation Strategic Approach to International Chemicals Management database, ibuprofen is ranked as the third most detected pharmaceutical in surface, drinking and ground waters and is detected in 47 countries[2]. The concentrations measured and aquatic matrix studied are given in Table 2. Few studies include the metabolites, which were typically found in greater concentration than the parent compound, particularly in WWTP effluent. This indicates degradation and metabolite formation before entry into WWTPs and during the treatment process[32,87,102]. In the last few years, researchers have recommended extending environmental studies to include metabolites[12,55,103,104]. Higher concentrations are measured in colder countries, colder seasons and WWTPs close to densely populated areas[57,101]. In Brazil, studies of several Rio de Janiero WWTPs measured average ibuprofen concentration ranges in influent of 1-27 µg/L and effluent of 4.7- 40 µg/L, consistent to a large population[80]. In Portugal, measured mean influent concentrations were 3, 3.9, 5.3, 9.80 µg/L in spring, summer, autumn and winter respectively with lower effluent concentrations

of 0.16, 0.3, 1.5, 1.8 µg/L in spring, summer, autumn and winter respectively [22,57]. The highest concentrations are observed in the Algarve, Alentejo and Center regions in the winter influenced by population size and colder temperatures, where individual WWTPs concentrations could be as high as 30 µg/L.

Table 2. Measured ibuprofen and metabolite concentration ranges and aquatic matrix of study

Aquatic Matrix	Ibuprofen Concentration Range µg/L	Metabolite Concentration Range µg/L	
		Hydroxyibuprofen	Carboxyibuprofen
WWTP influent	4-228	1.9-2.0	41-120
WWTP effluent	0.01-4	2.8-3.6	n.d
Seawater	n.d.-40	0.2	1.2
Surface water (rivers/lakes/streams)	0.008-2		
Drinking water	0.04-0.05		
Groundwater	0.02-0.05		

n.d-below limit of detection

Numerous studies have been conducted to assess the environment toxicity and risk of ibuprofen with differing results. Some have classified ibuprofen as low risk in surface and wastewaters[22,57,105]. In contrast, several authors mark ibuprofen as a significant environmental risk[21,55,77,86,106–108]. No risk assessments have been performed for the metabolites. It is believed they have no pharmacological activity[109,110], however one study has shown the toxicity of 1OH and 2OH to luminescent bacteria[111].

Toxicity studies have proven that ibuprofen is an aquatic environmental hazard, as a single compound or magnified with other pharmaceuticals[49,107]. Through its therapeutic action as a COX inhibitor, it causes toxic inhibition of growth, reproductive and inflammatory response of organisms at the trace concentrations commonly measured in the aquatic environment. These include primary producers - algae[50,112–117], phytoplankton[50,112], rotifers[118] and zooplankton[50] and common food sources such as fish[119–123], mussels[124–130], clams[131] and oysters[132]. It also promotes the proliferation of cyanobacteria in competition with algae[112]. The targeting of primary producers and food sources has potential disruption to the food chain and entire ecosystem.

1.5 Wastewater Treatment of Ibuprofen and Metabolites

Conventional biological and/or chemical methods are used to treat WWTP influent for the production of effluent that meets quality and safety standards[43,44,133,134]. Pharmaceutical removal from WWTPs is reliant upon its physicochemical properties, the treatment method, operational conditions and season[135–137]. Research has demonstrated the success of several methods in the removal of some pharmaceuticals

including aerobic and anaerobic biodegradation, sorption to WWTP sludge and oxidation [9,134,138]. Biological treatment is greatly favoured for its high efficiency, low cost and utilization of the metabolism of naturally occurring microbial communities to remove or transform compounds to nontoxic products[139].

Ibuprofen has a high removal percentage in WWTPs ranging from 80-100% from influent to treated effluent, dependent on season and treatment employed[22,57,65,69,78,83,84,93,135,140,141]. The metabolites exhibit similar removal, but it is uncertain whether this is mineralization or transformation to other compounds [27,142]. Aerobic biodegradation has been confirmed as the key removal method of these compounds in WWTPs[9,30,143–146]. Studies have isolated the responsible bacterial strains[147–152]. Their chemical structure allows easy degradation by hydrolysis of the carboxylic acid group and carbon atoms on the aromatic ring (cleaving the ring). Removal by sludge sorption is minor, due to their hydrophobicity and negative electrostatic charge derived from their Log K_{ow} and pKas (Table 1) [9,143,144,153,154].

Despite its high removal in WWTP, ibuprofen is considered an emerging pharmaceutical pollutant. Widespread usage results in the presence of ibuprofen and the metabolites in WWTP effluent, surface, ground and drinking waters[9,22,54,93]. Additionally, ibuprofen appears to be merely transformed into compounds of similar complexity and properties instead of being completely degraded. Most studies of its removal, identify and quantify the parent compound only, excluding the metabolites. Furthermore, ibuprofen exists in two isomeric forms and the bacteria responsible for its biodegradation preferentially utilize the pharmacologically inactive isomer, resulting in higher concentration and slower degradation of the pharmacologically active isomer[28,32,88]. These dual factors increase the likelihood of environment impact at trace concentrations of ibuprofen and its metabolites and highlight the need for innovation on wastewater treatment methods despite the high removal in conventional WWTPs.

1.6 Aerobic Granular Sludge

The aerobic granular sludge system is an innovative advancement in biological wastewater treatment[155,156]. It has several advantageous characteristics in comparison to conventional WWTPs, including high biomass retention, faster settling properties, capability of simultaneous COD, nitrogen and phosphate removal, good biosorption properties and high resilience to chemical toxicity and toxic shock load. Its simplicity and flexibility facilitates adaptability of operational cycles and conditions to

characteristics of wastewater influent, weather conditions, actual sludge conversion rates and desired effluent conditions with added benefits of lower operational costs and required operational land space[157–161].

Extensive research has been conducted in lab scale to optimize the operational parameters including understanding the mechanisms and bacterial processes[162–167]; influential factors for granule formation and maintenance such as optimal diameter, influent composition and start-up inocolum[162,164,168–173]; reactor design, dimensions, operational conditions[156,166,174–182] and the compounds that can be degraded by the granules[158,159,167,183–185]. The granules form by self-aggregation of microbial communities and have a stratified structure of aerobic and anaerobic layers (Figure 7). During aeration, polyphosphate and glycogen accumulating organisms employ growth metabolic processes using oxygen in the outer layer. Simultaneously, nitrite oxidation by nitrite oxidizing bacteria in the aerobic zone and nitrite reduction by denitrifiers in the anaerobic zone occur respectively. Hence the granule capability for concurrent removal of COD, phosphorus and nitrogen [162–168,179,186]. Their stability and efficiency is directly dependent on their size (optimum diameter of 2-4 mm), to which dissolved oxygen saturation levels are critical. Studies have shown the granules are highly resilient to toxic compounds. Their significant potential for application to pharmaceuticals and other toxic compounds lies in the adaptability of their biodegradation properties through tailoring of carbon source, influent composition and operational cycle[158,159,167,183–185,187,188].The system is operated in sequencing batch reactors (SBRs) with a four-phase operational cycle as illustrated in Figure 8. Early studies of aerobic granule systems employed mainly aerobic cycles[156]. Subsequent optimization studies for granule stability, efficiency and effluent production conforming to EU standards, have determined that the anaerobic/aerobic cycle is best suited. Operational differences lie in the longer length of influent feeding and shorter aeration period in the anaerobic/aerobic cycle versus the aerobic cycle. SBRs can be started using activated sludge or already formed granules as inoculum. Several parameters can be monitored and/or measured to assess SBR efficiency and granules applicable with the desired research objective [156,166,174–182]. Some key factors

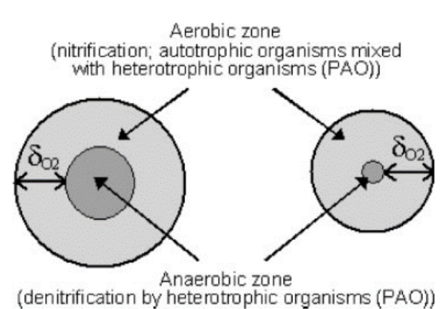


Figure 7. Schematic representation of aerobic granule stratified structure[164]

consistently managed in operation of aerobic granule SBRs at lab-scale and considered in SBR construction for this study are described in Table 3.

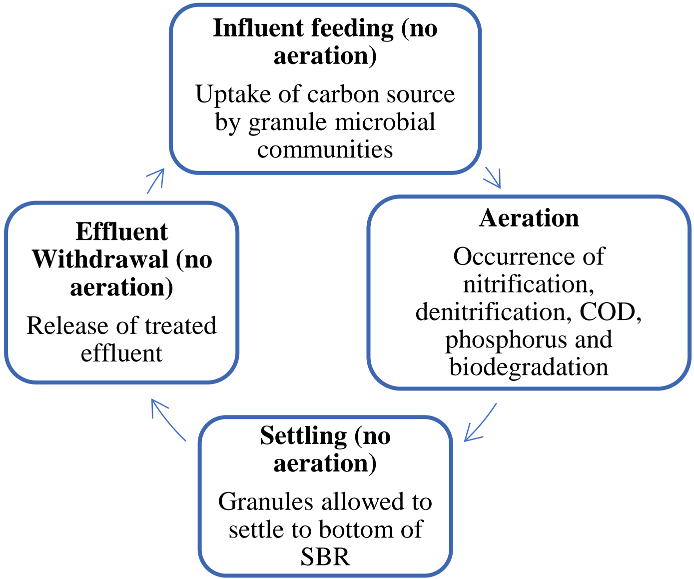


Figure 8. Operating cycle of aerobic SBRs

Pilot plants were installed across Europe under the Nereda® company, initiating aerobic granules as a full scale urban wastewater treatment[166,189–191]. In Portugal, Águas do Portugal has operated the Lisbon Frielas Nereda plant since 2011 producing effluent conforming to EU urban wastewater regulations[192]. With its success, Águas do Algarve commenced the construction of a Nereda® WWTP plant in 2017, to service the cities of Faro and Olhão. The project was undertaken to address the current limitations of the existing Faro Noroeste and Olhão Nascente WWTP lagoon treatment systems to produce effluent that meet EU regulations due to increased capacity. Águas do Algarve aims to enact protection, rehabilitation, and sustainability measures to reduce pollution of the Ria Formosa Lagoon. The current systems are dual stage treatments, with influent first processed in activated sludge reactors, followed by UV treatment before release into the Ria Formosa[193–197].

Studies have shown the great potential of aerobic granules in the treatment of wastewater containing pharmaceuticals (fluoroquinones, chirals, antibiotics and NSAIDs), with degradation occurring after adaptation to the compounds[198–203]. Zhao et al. conducted the sole study on aerobic granule efficiency on ibuprofen degradation. An aerobic SBR operated with synthetic wastewater, was dosed with a mixture of ibuprofen, naproxen, prednisolone, norfloxacin, and sulfamethoxazole at a concentration of 50 µg/L respectively. The results showed a removal of only 34-45% compared with the other compounds which had low removal rates in the early stages but increased during the

operational period. The author noted that the result may be due slower rate of aerobic biodegradation for ibuprofen. No studies thus far have examined the removal of the metabolites formed during wastewater treatment[204]. Further research is required to confirm the capability of aerobic granules to reproduce similar and/or better degradation patterns as found in conventional WWTPs.

Table 3. Factors critical to aerobic granule formation and SBR operation

Condition	Recommendation	Impact on Granule Formation and Reactor Operation
Ratio of SBR height to SBR internal diameter	Large height to internal diameter ratio	Promote high shear force, microorganism proliferation and granule formation through selection of fast settling particles and washout of floccular sludge during settling time and effluent withdrawal
Temperature	Room temperature (20-25^{0}C)	Promotes granule stability as an optimum condition for COD, nitrogen and phosphorus removal processes
Aeration	Controlled air flow that produces small air bubbles introduced at bottom of SBR	Promotes granule mixing with influent and aeration for COD, nitrogen and phosphorus removal processes
Dissolved oxygen saturation	≥ 20%	20-50% DO ensure granule stability and efficiency, promoting a larger anaerobic layer with decreased penetration depth of oxygen and promote nitrification/denitrification processes.
pH	Monitored and controlled as necessary 7.0±0.2	Optimum condition for COD, nitrogen and phosphorus removal processes
Hydraulic retention time	< 8 h	Promote microorganism proliferation and granule stability by decreasing suspended biomass growth by washout in effluent withdrawal
Influent	• Controlled flow introduced at bottom of SBR • High COD	Good interaction with granules Promote microorganism proliferation, granule formation and stability.
Effluent withdrawal	• At least 40% volume exchange ratio (percentage difference between reactor volume and volume refilled after withdrawal) • Effluent withdrawal at approximately SBR mid height	Volume exchange ratio > 40% selects for granule formation. Withdrawal height selects for fast settling particles

2. Analytical Methodology

2.1 Analytical Methods Employed to Analyse Ibuprofen in the Aquatic Environment

The method of choice for pharmaceutical environment monitoring is high performance liquid chromatography (HPLC) coupled to tandem mass spectrometry (LC-MS/MS), because of high selectivity via unique mass to charge ratio of ionized analytes[5,55,60]. Literature review reveals numerous analytical methods for ibuprofen with other pharmaceuticals. Ultraperformance liquid chromatography dominates the field [17,27,30,62,74,87,93,94,97,136,148,150,205–210], followed by LC-MS/MS[85,101,204,209,211–216]. Gas chromatography requires derivatization of the acidic compound, increasing analytical time[32,55,62,95,96,98–100,217–221]. Other detectors used are ultraviolet visible (UV/Vis) diode array detectors (DAD)[147,222,223], DAD coupled to fluorescent[86,153,224–227] and mass spectrometer[221]. Sample pretreatment by an extraction method was always performed for all studies to enrich pharmaceutical concentration to method detection and quantification limits. Very few methods include the metabolites[27,30,87,142].

Several capillary electrophoresis (CE) methods have been developed for the quantification of ibuprofen in pharmaceutical analysis [228–239] and environmental analysis in surface water [240,241], tap water [242], wastewater[92,243] and bottled water[244]. The quantity pales in comparison to liquid and gas chromatography. Ahrer et al. assessed ibuprofen analysis in surface water using HPLC-MS and CE-MS and determined that the methods were comparable. However, two extraction procedures were used to boost CE method detection limit comparable to HPLC, one was used for HPLC[240]. CE has gained recognition as a viable alternative on par with HPLC, for pharmaceutical and clinical analysis with potential application to environmental analysis[232,245–249].

Research potential remains for the development of rapid, simple, accurate and precise methods for environmental monitoring of specific pharmaceuticals, that include their metabolites[5,55]. Budget and experience limitations may restrict implementation of the complex liquid chromatography methods coupled with mass spectrometry. HPLC coupled to UV/Vis detectors can still be a feasible and cost effective analytical method with desired sensitivity, accuracy and precision. Environment method development can be difficult due to the complex matrices, potential interferences and the low limits of detection and quantification required (ng to μg/L).

2.2 High Performance Liquid Chromatography (HPLC)

The foundation for HPLC was laid in the 1900s by Tswett[250,251]. More than a century later, research advancements have transformed HPLC into a universal separation technique for numerous compounds of wide-ranging physico-chemical properties. Its favoured advantages lie in fast, reliable separation, reproducibility, precision and accuracy of results and re-usability of chromatographic columns[245,252]. Figure 9 presents the typical HPLC modular system configuration[245]. High pressure pumps drive mobile phase through the column at a selected flow rate. Samples are injected manually or by autosampler, passing through the column where separation occurs and analytes generate a response in the detector. The column is installed inside an oven whose temperature can be regulated. The entire system is monitored and controlled by a computerized software. Responses are displayed in chromatograms with peak area as a function of retention time. Analyte concentration in samples is calculated by comparison of response to that of reference standards[245,253]. HPLC separates analytes by their differing affinity and interactions that creates different distributions to a solid column stationary phase and the liquid mobile phase[251,254].

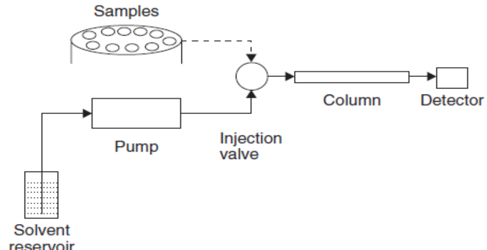


Figure 9. Schematic representation of HPLC system[245]

Solvent flow path →

Liquid chromatographic techniques can be sub-divided according to the stationary phase of chromatographic column as shown in Figure 10. Various detectors exist for sample specific, bulk property measurement and hyphenated techniques including UV/Vis, fluorescent, mass spectrometer, tandem mass spectrometer[251,254].

2.2.1 Reverse Phase Chromatography

Reverse phase chromatography works by differing analyte adsorption to the stationary phase and is recommended for separating compounds with the same functional group[251,254]. The stationary phases are non-polar and hydrophobic, while mobile phases are polar and hydrophilic. The principal chromatographic conditions controlling analyte separation are shown in Figure 10 [251,254,255].

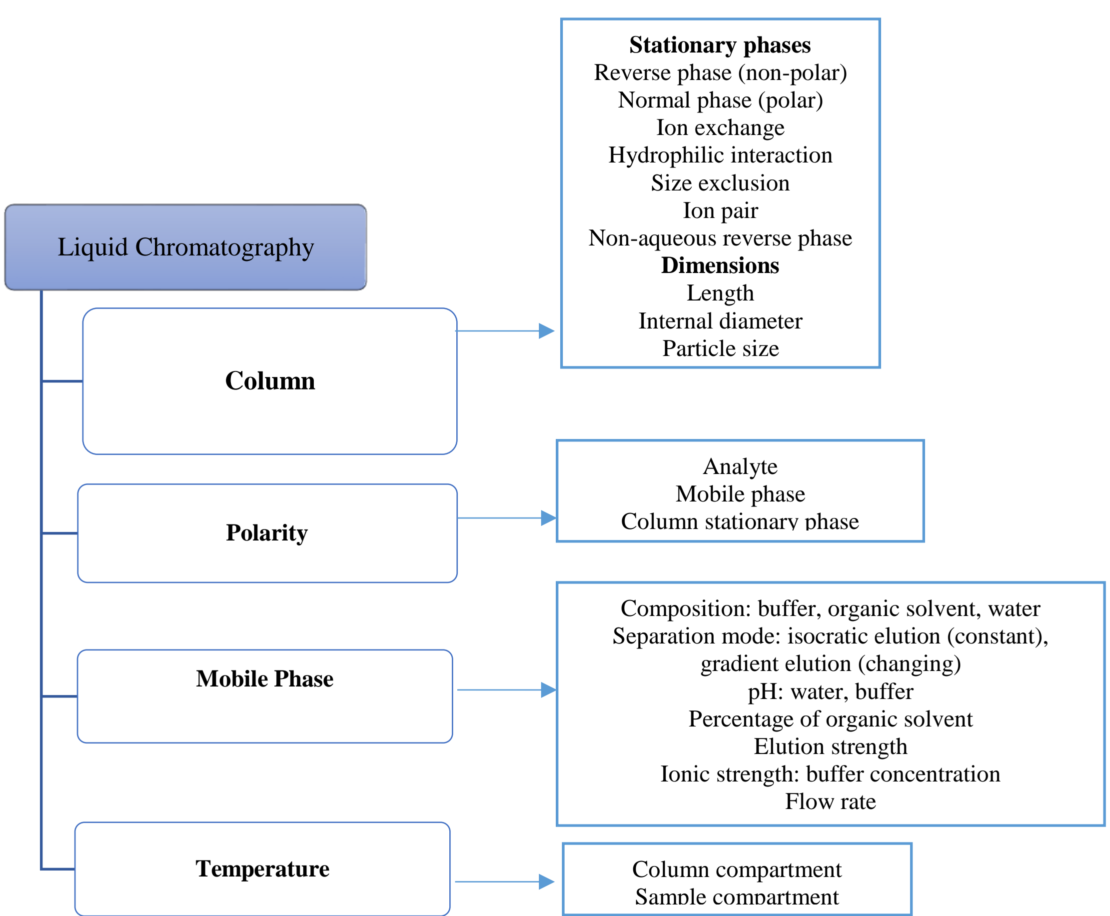


Figure 10. Important factors for liquid chromatography separation

Isocratic or gradient elution modes are employed. Isocratic elution separates analytes using constant mobile phase composition, while gradient elution separates by increasing the percentage of organic solvent (%B) to continuously change mobile phase composition. A linear gradient is the simplest, maintaining constant change in %B during the run. The most appropriate gradient shape for analyte separation is identified in method development. Researchers recommend an exploratory linear gradient run to assess whether isocratic or gradient separation is most suited [255]:

$\Delta tr/t_G$ Equation 1

where Δt_r is the difference in retention times of the final and first peaks. t_G is the gradient time. Isocratic elution is possible for values ≤ 0.25, while gradient elution is recommended for values ≥ 0.40. For intermediate values, the best elution mode is determined by experimentation. %B to be used to test isocratic elution is calculated by:

isocratic $\%B \approx 6.3\ (t_{ravg} - t_D) - 2$ Equation 2

where t_{ravg} is the average of the retention times of the first and last peaks and t_d is the hold-up time and is determined by dividing the dwell volume by flow rate.

1. **Columns**

Column selectivity is determined by type of stationary phase and analyte molecular structure that cause differing interactions such as hydrophobic and dipole-dipole. The strength of these interactions differs with analyte polarity. Analytes elute from least to most hydrophobic (highest to least polarity)[245,251,254]. Alkyl silica particles bonded to ligands such as C18 is the preferred reverse phase stationary phase, with pH range 2.5 - 7.5. The silica particle surfaces can also be chemically modified. Column length (typically 10-25 cm) and internal diameter (i.d.) (typically 2.0-4.6 μm) have a direct proportional effect on retention time. This results in reduced analysis time with shorter columns and smaller volumes of mobile phase used as internal diameter decreases. Particle size (typically 1.5–5 μm) is indirectly proportional to column efficiency, which decreases as particle size increases[245,251,252].

2. **Mobile Phase**

Reverse phase mobile phases are composed of water or buffer (solvent A) with volatile organic solvents usually methanol, acetonitrile or tetrahydrofuran (solvent B). Mobile phase polarity and elution strength are dependent on its composition (water, buffer type and concentration, %B, organic solvent and pH). This dictates analyte retention time, changing their affinities due to the different polarities of the stationary phase and mobile phase. Water is the weakest solvent, followed by methanol and then acetonitrile. Acetonitrile commonly applied for short analysis time. pH controls selectivity according to analyte pKa. pH can be adjusted to convert analytes to neutral or ionic form, changing retention time and elution order[245,254,256]. Elution strength is also controlled by separation mode, remaining constant during isocratic elution, but increasing incrementally during gradient elution[245,256].

3. **Temperature**

Analyte retention is indirectly proportional to temperature, with retention time decreasing with increasing temperature which lowers mobile phase density[245,256].

2.2.2 Chromatographic Quality Parameters

The following parameters are investigated during method development and optimization and then evaluated to assess chromatographic quality and separation:

1. **Retention**

The retention factor of an analyte is the ratio of the quantity of analyte in the stationary phase to the quantity in mobile phase and calculated using[245,257]:

Isocratic retention factor, $k = (t_r - t_0)/t_0$ Equation 3

where t_r is the analyte retention time; t_0, column dead time, is the retention time of the solvent peak. t_0 is obtained from visual inspection of the chromatogram for the first baseline disturbance, estimated using flow rate and column dimensions or determined by injection of an unretained compound such sodium nitrate.

Gradient retention factor $(k^*) = (0.87\ t_G F)/\ V_m \Delta\varphi S$ Equation 4

Where t_G is the gradient time; $\Delta\varphi$ is the change in %B, S is assumed to be 4 for analytes with molecular weights of 100 to 500 Da; F is flow rate and V_m is the column dead volume, which can be directly measured or calculated from t_0 by:

$V_m = t_0 F \approx 5x10^{-4} L d^2_c$ Equation 5

where L is column length (mm) and d_c is column internal diameter (mm). Chromatographic separation is controlled by adjusting experimental conditions to achieve $1 \leq k/k^* \leq 10$. If this is not possible, $0.5 \leq k/k^* \leq 20$ is acceptable. Retention time is used for identification of a compound in HPLC[245,257].

2. **Selectivity**

Selectivity is the separation factor (α) between two peaks and calculated as[245,257]: $\alpha = k_2/k_1$ Equation 6

Where k_2 is the retention factor of the second peak and k_1 the retention factor of the first peak.

3. **Resolution**

Resolution (R_s) measures the distance of separation between two peaks and is the chief objective of method development, particularly for the peak pair (critical peak pair) with the lowest separation (critical resolution). Baseline separation is necessary for accuracy of quantitative analysis and therefore resolution ≥ 2 is desired. If this is unachievable, resolution ≥ 1.5 is accepted[245,257]. Chromatographic selectivity and efficiency control resolution by increasing distance between the peaks and reducing peak width respectively. R_s is calculated:

$$Rs = \frac{2\,(t_{r(2)} - t_{r(1)})}{W_{b(1)} + W_{b(2)}}$$ Equation 7

Where $t_{r(2)}$ is the retention time of the second peak; $t_{r(1)}$ is the retention time of the first peak; $W_{b(1)}$ and $W_{b(2)}$ are the peak widths at baseline for both peaks respectively.

4. Efficiency

Chromatographic efficiency is defined by the plate number (N) which measures peak width compared to its retention time by the equations[245]:

$$N = \frac{16\,(t_r^2)}{W_b}$$ Equation 8 or $$H = \frac{L}{N}$$ Equation 9

Where efficiency is measured per meter column with H as plate height and L as the column length.

Band broadening is controlled by retention times and peak widths, influenced by three effects summarized in the Van Deemter equation and illustrated in Figure 11:

$$H = A + B/u + Cu$$ Equation 10

Where A is the effect of column packing, B is the effect of longitudinal diffusion as analyte molecules pass along the column and C is the effect of resistance to mass transfer due to analyte affinity between the mobile phase and stationary phase that dictates its retention in either phase.

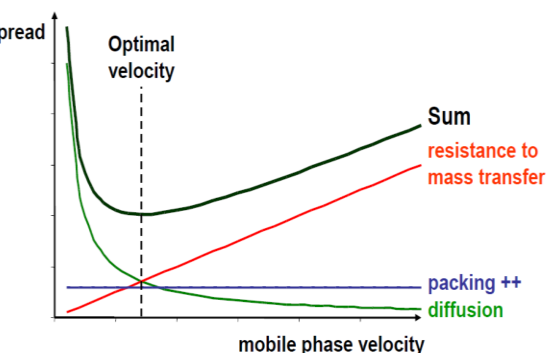


Figure 11. Graphical representation of Van Deemter's equation[320]

Mobile phase velocity (flow rate) affects plate efficiency due to the differing relationships with each effect, A is independent, B is inversely proportional while C is directly proportional.

5. Tailing Factor

Peak asymmetry affects the separation between two peaks and is evaluated by tailing factor. A tailing factor of 1.0 demonstrates good peak separation, > 2 indicates potential problems with separation and creates difficulties for peak integration in quantitative analysis[245,255].

2.2.3 Optimization of Chromatographic Separation

Method development focuses on the relationship between plate efficiency, selectivity and retention factor and their contribution towards chromatographic separation (resolution), described by the Purnell equation[245,255,257]:

$$R_s = (\sqrt{N}/4)\ (\alpha\text{-}1/\alpha)\ (k_2/1+k_2) \qquad \text{Equation 11}$$

Systematic adjustment is conducted to optimize each condition until the critical resolution is $R_s \geq 2$. Table 4 summarizes the typical chromatographic condition adjustments and their effect in retention factor, selectivity and efficiency, which helps to focus experimental investigations during method development and optimization. The different effect on retention factor and selectivity between isocratic and gradient elution due column length and flow rate results from the change in %B unless gradient time is changed proportionally to maintain same conditions[245].

Table 4. Effect of selected chromatographic separation conditions on retention (k/k*), selectivity (α) and plate number (N)[245]

Chromatographic Condition	Effect		
	k	α	N
Column			
Stationary phase	Minor,	major	small
Column length	Isocratic: no effect Gradient: major	Isocratic: no effect Gradient: major	major
Particle size	no effect	no effect	major
Mobile Phase			
%B	major	minor	small
Organic solvent	minor	major	small
pH for ionizable analytes	major	major	minor
Buffer concentration for ionizable analytes	minor	minor	small
Flow rate	Isocratic: no effect Gradient: major	Isocratic: no effect Gradient: major	minor
Temperature	minor	minor	minor

2.3 Capillary Electrophoresis (CE)

CE is a separation technique based on differing analyte electrophoretic mobilities (resulting from charge to mass ratio) as charged ions in a capillary under the influence of high electric field and background electrolyte (BGE). Compound migration is also controlled by the electroosmotic flow (EOF) induced in the electrolyte by the electric field, that carries analytes towards the detector[258]. The technique was developed by substantive innovation of Tiselius's work in the 1980s and 90s [259].

CE is comparable to HPLC in versatility, capacity to separate analytes of varying physico-chemical properties and flexibility in instrument parameters and experimental conditions. Furthermore, the same detectors used in chromatography can be coupled to CE instrumentation. Several specific techniques exist based on separation

mechanism [246,260]. It has significant advantages over the chromatographic techniques including potentially faster separations, lower reagent and consumable consumption, smaller sample demand (typically nL injected), less waste, use of a single capillary to separate different samples, better separation of enantiomers and simpler equipment. Additionally, detection at low wavelength (190-200 nm) is possible with the short capillary path length, allowing direct detection of acidic analytes without the derivatisation required in gas chromatography. Therefore, it has good potential in small organic and inorganic compound analysis[246,258,261,262]. The significant shortcomings in comparison to chromatographic techniques are poorer precision as result of migration time variance and method sensitivity (detection and quantification limits) resulting from small injection volumes and short capillary path length (defined by µm internal diameter). Precision is generally corrected using internal standards, while off-line (such as SPE) and online-preconcentration is applied to improve method sensitivity [246,247,263–265].

Figure 12 shows a representation of a typical system. The sample is injected into the capillary, followed by the electrolyte (from its reservoir). Electrodes apply voltage to create the electric field, charging the analytes which migrate by their own mobility and are also carried by the EOF. In normal voltage polarity, EOF flow moves from injection at the anode (positive) to detection at the cathode (negative), in inverted flow, the charges of the electrodes are reversed. Analyte response is generated and displayed as an electropherogram. Hydrodynamic (with pressure) or electrokinetic (with voltage) injection can be performed. The former is preferred to reduce the bias problem that plagues electrokinetic injection, which works based on analyte electrophoretic mobility and EOF[266]. Figure 13 shows the important factors that affect CE separation.

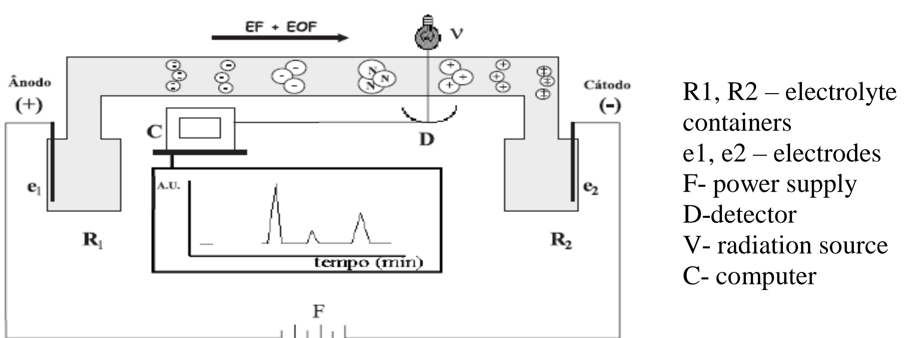


Figure 12. Schematic representation of a CE system showing ion separation by mass and charge and generated electropherogram[321]

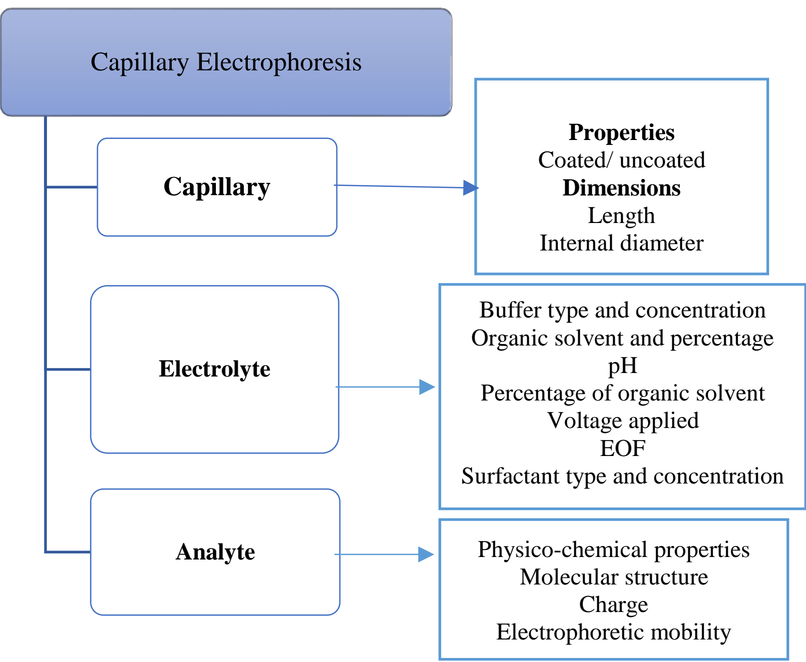


Figure 13. Important factors for CE separation

Capillary zone electrophoresis, also called free solution, is the simplest CE method. Analyte separation is determined by BGE composition (buffer concentration/ionic strength, solvent, additives), pH and experiment conditions (temperature, voltage) that control EOF and analyte electrophoretic mobility. Voltage is applied with caution as high values can induce Joule effects (heating) through the capillary. This alters electrolyte viscosity and EOF, causing deterioration in separation and the sample, non-reproducibility, solvent boiling and cessation of analysis[260,267]. Mobility calculations from theoretical equations is difficult, therefore the values are derived from experiments to measure analyte migration time and EOF marker with[267]:

$$\text{Electrolyte electroosmotic flow } (v_{eo}),\ \text{cm/s} = \frac{\text{Length to detector } (L_{det})}{\text{Time of EOF marker } (t_{eo})} \qquad \text{Equation 12}$$

Equation 13

$$\text{Electrolyte electroosmotic mobility } (\mu_{eo}),\ 10^{-5}\text{cm}^2\text{V}^{-1}\ \text{s}^{-1} = \frac{v_{eo} \text{ x Total length } (L_{tot})}{\text{Applied voltage (V)}}$$

$$\text{Analyte apparent velocity } (v_{ap}),\ \text{cm/s} = \frac{L_{det}}{\text{Analyte migration time } (t_{ap})} \qquad \text{Equation 14}$$

$$\text{Analyte effective velocity } (v_{ef}),\ \text{cm/s} = v_{ap} - v_{ap} \qquad \text{Equation 15}$$

$$\text{Analyte effective mobility } (\mu_{ef}),\ 10^{-5}\text{cm}^2\text{V}^{-1}\ \text{s}^{-1} = \frac{v_{ef} \text{ x Total length } (L_{tot})}{\text{Applied voltage (V)}} \qquad \text{Equation 16}$$

A response function can be calculated from the effective mobilities to identify the best pH for separation. The effective mobilities of each compound are organized in increasing value at a specific pH (0 to 12). The product of the difference between adjacent effective mobilities and the difference between maximum and minimum effective mobilities were calculated. The response function was determined at each pH by dividing the product by the difference. The maximum response function corresponds to the best pH for separation. The effective mobility curves for each compound and response function was plotted as a function of pH as shown in Figure 30. The maximum value for response function occurs at pH 5.

CE migration time is equivalent to HPLC retention time and also used for compound identification. EOF time (t_{eo}) is the migration time of a neutral solute or the solvent marker (first significant baseline disturbance). Resolution, tailing factor and efficiency is also used to evaluate quality of CE separation[268,269].

1. Capillary

Narrow bore fused silica (silicon dioxide) capillaries are commonly used, with internal diameters of 25 –200 µm and length of 50 –100 cm for the benefits of precision, low electrical conductivity, high thermal conductivity, mechanical resistance and optical transmission over a wide UV range. Capillaries are conditioned when first prepared and before analysis with sodium hydroxide, followed by milli-q water and finally the electrolyte, to ensure the surface is uniformly and fully charged and to remove any residue from previous experiments respectively. Electroosmosis occurs due to analyte interactions with the weakly acidic silanol groups on the capillary wall surface, with some groups dissociating in the aqueous electrolyte to give negative charge to the capillary wall. The inner capillary wall can be chemically modified to change analyte interactions. Joule effects are controlled by the large ratio between the internal surface area and volume and high electrical resistance of the silica material[260,267].

2. Background Electrolyte

The BGE is necessary to transport and separate samples in the electric field[270]. Buffer concentration controls EOF flow by changing analyte adsorption to the capillary wall. Buffer type affects band tailing and symmetry by closeness of electrolyte mobility to anion mobility. pH controls analyte ionization and resulting electrophoretic mobility as a product of the distribution and relative concentrations of

ionic and neutral form and degree of ionization of silanol groups on the capillary surface. These conditions are therefore selected considering analyte molecular structure and physico-chemical properties that influence behaviour in buffer of choice and give desired separation, while avoiding heat close to Joule effects, band distortion and broadening and erratic EOF which reduces migration time reproducibility[258,271]. Low buffer absorption at analytical wavelength should be observed. High pH buffers such as borate are recommended for acidic compound separation such as ibuprofen and the metabolites, which are converted to anions at these pHs with effective mobilities that allow fast separation[246]. The application of high electric field gives high separation efficiency, resolution and short analysis time through control of EOF flow[258,260,267,270].

Additives such as organic solvents (methanol, acetonitrile and tetrahydrofuran) and surfactants are added to BGEs to alter EOF flow (reduce, increase, invert or suppress), analyte electrophoretic mobility, electrolyte viscosity, solubilize analytes and analyte adsorption to the capillary wall[258,260,272]. However, it is recommended to limit maintain organic solvent to < 40%, otherwise contradictory results, erratic migration times and electric breakdown can occur[228]. The effect of EOF control and voltage polarity on the migration of anions is depicted in Figure 14 and are considered when selecting the appropriate CE experimental conditions[258,273]. Figure 14A shows possible separation of slow anions (eg. aromatic compounds) whose smaller mobility (v_{ef}) is overwhelmed by that of the EOF (v_{osm}) and carried towards the cathode. In contrast, fast anions (such as inorganic and short chain carboxylic acids) migrate towards the anode, away from the detector, leaving the capillary. Figure 14B is possible for rapid anions although analysis time is lengthened, due to their migration in counterflow to the EOF. Figure 14C presents the use of a surfactant and further discussed in Section 2.3.1. The separation in Figure 14D is a result of special inner lined capillaries however, the analysis time is long.

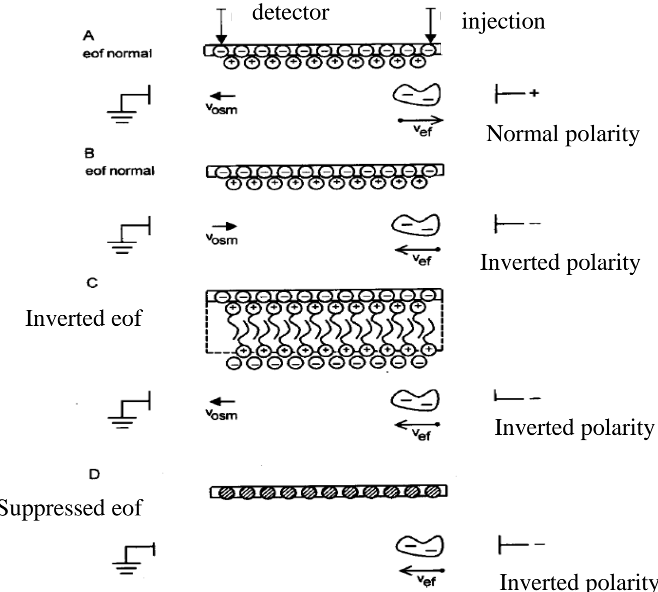


Figure 14. Schematic representation of anion migration in
(A) normal EOF with normal polarity (B) normal EOF with inverted polarity (C) EOF inverted by cationic surfactant and inverse polarity (D) suppressed EOF and inverted polarity[258]

2.3.1 Micellar Electrokinetic Capillary Chromatography

Micellar electrokinetic capillary chromatography (MEKC) employ surfactants in BGEs to alter the EOF by formation of a micelle dispersed (secondary) phase moving at a different velocity to the electrolyte (primary phase). When an electric field is applied, charged (acidic and basic) and neutral compounds are separated by differences in electrophoretic movement, solubility-based partitioning and ion pair interaction with the charged micelle[246,260,274].

The surfactants (micelles) are amphiphilic molecular aggregates possessing long hydrophobic tails and polar heads, ranging from anionic (e.g. sodium lauryl sulphate, SDS), cationic (e.g. cetrimonium bromide) to neutral. Their properties determine individual effect on the EOF (reduce, reverse, suppress) and analyte mobility due to hydrophobic and electrostatic interactions. Concentration in the electrolyte determines the magnitude of their effect, hence its likeness to reverse phase chromatography and should be used above critical micellar concentration to promote rapid, stable formation and dynamic equilibrium. Analyte selectivity in MEKC is determined by the surfactant selected, buffer type (analyte, its electrophoretic mobility and electrolyte interactions), pH (migration faster as pH increases) and temperature (solubility, equilibrium and kinetics)[246,260,274].

Figure 14C depicts the MEKC mechanism with a cationic surfactant, which adsorps to the capillary wall, leading to dispersion and the establishment of a two dimensional semimicelle structure on the capillary wall surface. The positive heads of the surfactant face the electrolyte, creating a diffuse electric double layer from the anions which position at the surface-solution interface. When the electric field is applied, the EOF flow is inverted with migration extending through all layers, causing anion movement towards the anode by electrophoresis and electroosmosis[258]. MEKC using SDS, has been used in ibuprofen in analysis of pharmaceutical products[237,238,275].

2.3.2 On-line Preconcentration: Stacking

Off-line or on-line preconcentration techniques can be used to resolve method detection limit problems in CE[276–280]. Extraction methods form the basis of off-line procedures such as solid phase extraction (SPE)[240,281]. On-line preconcentration techniques include stacking by ionic strength, pH, large volume and field amplified. Large volume and field amplified modes have been applied to ibuprofen analysis[230,242,282] or off-line and on-line techniques have been combined[279] to improve method sensitivity such as field amplified and SPE techniques[243,283]. Field-amplified sample stacking (FASS), the simplest approach, is a result of conductivity differences between the sample solution and BGE. Hydrodynamic injection of the low conductivity sample (prepared in dilute buffer or water) into the capillary with high conductivity BGE results in the higher application of the electric field on the sample zone in comparison to the BGE. This results from the inverse proportion of electric field strength to electrical conductivity. The electrophoretic mobility of ionized

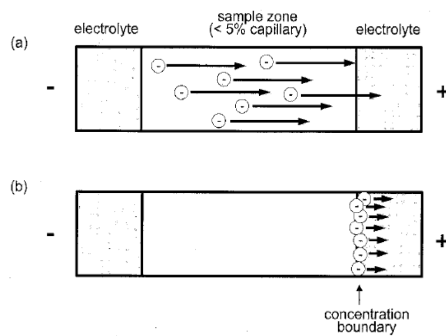
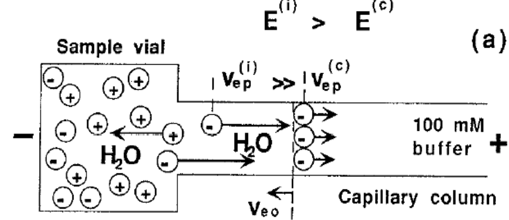


Figure 15. Schematic representation of FASS (a) immediately after voltage application (b) after stacking is completed[318]



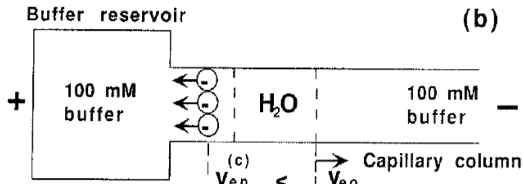


Figure 16. Schematic representation of FASI

(a) introduction of short plug of water into the capillary, followed by sample injection under negative voltage.
(b) Separation with high conductivity buffer and normal polarity[286]

analytes is therefore faster in the sample zone and they move with high velocity towards the boundary of sample and BGE. At the boundary, the low field strength decreases their velocity decreases, creating the build-up of the analytes (stacking) at the boundary of sample and BGE (Figure 15). By increasing the injection time of the sample, increase in sensitivity up to 100 fold can be achieved[277]. Field-amplified sample injection (FASI) builds on FASS by injecting a water plug hydrodynamically, followed by the electrokinetic injection of the sample to provide a high electric field strength as shown in Figure 16. For anions, the injection is performed with reverse polarity, then switched to normal polarity for separation[284–286].

2.4 HPLC and CE UV/Vis Detectors

The UV/Vis detectors used in HPLC and CE instrumentation (general operating range 190 - 600 nm) directly measure analyte responses, following Beer's Law that absorbance is directly proportional to analyte concentration[245]. The detectors are sample specific, measuring the response of compounds that absorb at a specific wavelength. The customary light sources are deuterium, mercury and tungsten lamps as fixed wavelength, diode array (spectrum) or variable wavelength. These detectors are applicable to a vast range of compounds except for those with no UV activity (e.g. sugars). Mobile phase and BGE solvents must be selected so that they do not absorb at the detection wavelength and are mistaken for the target analyte. Determining the analyte absorption maximum is useful to pinpoint detection range, however experiments during method development can decide the selection of final method detection wavelength, based on observed strength of absorbance, sensitivity increase or decrease, visibility of other compounds in sample and signal to noise ratio[245].

Detector optic configuration differs between instruments and manufacturer in the type of UV/Vis light source (such as deuterium, mercury, tungsten), use of multiple lamps, addition of filters (holmium oxide) or UV selecting filters.

2.5 Experimental Design

Experimental design is used for the simultaneous evaluation of multiple factors at a selected number of levels in a defined number of experiments[287]. It is extremely valuable to best experimental conditions by evaluation of the whole experimental domain, detect the significant factors and their influence on the response of critical method variables[288]. It avoids inefficient trial and error investigations or monitoring

one variable at a time that consumes time and resources and prevent consideration of the interactive effects. Experimental design is gaining popularity as useful tool in pharmaceutical and environmental method development[287–290], particularly for sample preparation, instrument and method conditions for analyte separation[289].

Experimental designs can be classified as screening designs such as full factorial (Table 5) [291,292], response surface and mixture designs. Screening designs are used to identify the most significant factors on response variables and may be used to select their optimal levels. Response surface designs help to determine optimal level, while mixture designs investigate factors as a fraction in a mixture[287,290]. The appropriate design is selected depending on desired information, number of factors to be studied, operational and resource availability and ability to implement the chosen design[291]. Preliminary experiments are conducted to identify the significant response variables for evaluation of method performance, the factors and the levels to be investigated[291]. The responses are evaluated by response functions[293] or other statistical tests such as analysis of variance[290,291]. Common HPLC variables investigated are mobile phase composition, flow rate, temperature and pH while investigated CE variables include BGE composition, pH, separation voltage and additives [287].

Table 5. Full factorial screening designs and their parameters

Design	Factors		Number of experiments
	Type	Number of factors (k)	
Two-level full factorial	Numerical categorical	$2 \leq k \leq 5$	2^k

2.6 Chromatographic Response Functions

The quality of chromatographic separations can be evaluated and classified using response functions based on separation goal of the developed method. The commonly used response functions are chromatographic resolution statistic (CRS), chromatographic response function (CRF), chromatographic exponential function (CEF), resolution sum (R_{sum}), resolution of critical peak pair (R_{cp}) and number of resolved peaks (RP)[293–296]. Originally developed for HPLC, the functions have been applied to CE[295] and are defined as:

$$R_{sum} = \sum_{i=1}^{np} R_{i,\,i+1} \quad \text{Equation 17}$$

$$CRS = \left\{ \sum_{i=1}^{np} \left\{ \frac{R_{i,\,i+1} - R_{opt}}{R_{i,\,i+1} - R_{min}} \right\} \frac{R_{opt}}{R_{i,\,i+1}} + \sum_{i=1}^{np} \frac{R^2_{i,\,i+1}}{np R^2_m} \right\} \frac{t_f}{n} \quad \text{Equation 18}$$

$$CRF = \sum_{i=1}^{np} R_{i,\,i+1} + \delta_1 RP + \delta_2(t_{\max} - t_f) - \delta_3(t_{\min} - t_1) \quad \text{Equation 19}$$

$$CEF = \left(\sum_{i=1}^{np} \left(1 - e^{\delta_1 (R_{opt} - R_{i,i+1})}\right)^2 + 1 \right)\left(1 + \frac{t_N}{t_{max}}\right) \quad \text{Equation 20}$$

Where np is the number of peak pairs, $R_{i,\,i+1}$ is resolution between adjacent solute pairs, R_{opt} is the optimum resolution, R_{min} is the minimum acceptable resolution, R_m is the average resolution of all solute pairs, t_f is the migration time of final solute, n is the number of solutes in the sample. t_{max} is the maximum desired runtime, t_{min} is the minimum desired runtime, t_f is the migration time of final solute, t_i is the migration time of first solute, σ_1, σ_2, σ_3 are user selected adjustable weights ($\sigma_1 = 3$, $\sigma_2 = 2$, $\sigma_3 = 1$).

These multi-variate functions allow the ranking of electropherogram results by evaluation of separation resolution, retention time, separation time and uniformity of peak distribution to select the optimal conditions for separation. Large R_{sum} values are obtained from electropherograms with well resolved solute pairs. The smallest CRS and CEF value indicates those with the best resolved peaks while the largest CRF indicates the electropherograms with the best separation conditions. The CEF improves on the CRS by reducing sensitivity to peak pairs with resolution larger than the desired maximum and emphasis on separation time unless the desired maximum is exceeded.

The response functions offer an simpler alternative to chemometric software in the event of unavailability in the laboratory or due to small number of analytes in a sample and simple separation conditions.

2.7 Solid Phase Extraction (SPE)

Sample preparation is the fundamental basis of accurate and precise analysis, from point of collection to instrument analysis[245]. SPE (manual or automated) is a prevalent sample preparation method in environmental pharmaceutical analysis for several advantages. It is a facile, highly efficient, robust, economic, versatile technique with low organic solvent consumption. It removes the target analyte from the matrix of study, minimizing the possible effects of interferences and enhancing selectivity in analytical methods. Finally, trace analyte concentrations usually found in the environment are enriched to levels detectable by the method and measuring instrumentation[5,55,245,252,257].

SPE works by same principles as HPLC. The differing affinities between the functional groups of the target analytes and those of the SPE sorbent are enhanced or decreased through critical factors that must be considered in developing and selecting an SPE procedure. These factors include sample pre-treatment, sorbent type (silica, polymeric) and the solvents for conditioning, washing and elution. SPE conditions are chosen upon evaluation of the physico-chemical properties of the target analytes (pKa, polarity, structure, hydrophobicity), expected analyte concentration, matrix composition and objective of the SPE procedure[257,297]. The typical SPE procedure performed under vacuum, consists of the following typical steps (Figure 17)[257,297]:

1. Conditioning: the passage of an appropriate solvent, typically methanol or acetonitrile, is used to solvate the sorbent and remove any impurities. Water is then passed through the cartridge to remove the conditioning solvent.
2. Retention: the analyte solution is loaded and passed through cartridge where the target analytes are retained.
3. Washing: Interferents are removed by rinsing with an appropriate solvent of intermediate strength. The solvent can be water or organic solvent/ water. The latter is used if water only is insufficient and the amount of organic solvent is controlled to remove the interferences but not lose the target analyte.

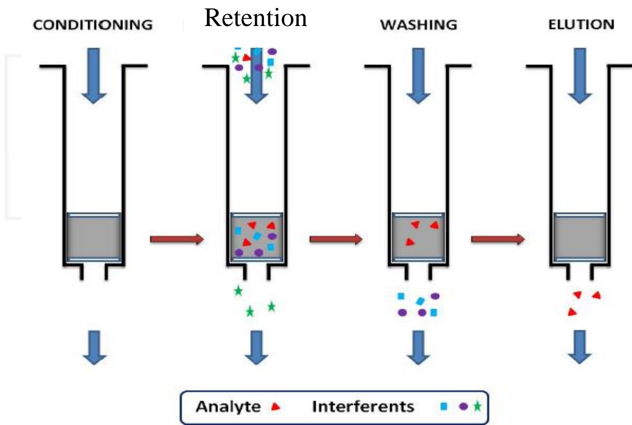


Figure 17. Schematic representation of typical SPE procedure[297]

4. Elution: appropriate solvent of appropriate strength (e.g. methanol, acetonitrile, water or buffer) strong enough to recover the target analytes from the SPE sorbent. Additional steps that can be employed are:

1. Sample pre-treatment: sample solution properties can be changed via a pH adjustment or other additives to suppress analyte ionization and maximize the selectivity of the target analytes to the SPE sorbent in the retention step.
2. Drying: This can be performed after the washing step using vacuum or heat to ensure the complete removal of the wash solvent and any interferences.

3. Drying of elution and reconstitution: the elution can be dried by vacuum concentration or evaporation and the extracts reconstituted in a weaker solvent that is more compatible with the analytical methods.

Off-line or online SPE using silica based or polymeric sorbents is the most prevalently method for pharmaceuticals [60,297]. Literature review reveals several SPE methods developed and validated for ibuprofen and the metabolites, which can be used in this study. The best recoveries were obtained with the following SPE conditions:
Sample pretreatment: pH of all aquatic matrices (surface, ground, wastewater) is adjusted to the acidic range (pH 2 -3)[22,27,29,72,147,212,240,298,299] to ensure the acidic compounds remain in neutral form.
Sorbent: Reversed phase silica C18 and polymeric sorbents such as Oasis HLB, Oasis MCX and Strata-X are most commonly applied. These non-polar sorbents display selectivity with the hydrophobic compounds via nonpolar-nonpolar and van der Waals interactions[257,297]. The narrower pH stability range of the C18 sorbent (pH range 2-8) slightly reduces its versatility in comparison to polymeric sorbents (pH range 0-14)[297]. Studies comparing the performance of C18 and polymeric sorbents, found that for ibuprofen, generally the recoveries obtained from different sorbents was similar[72,299] Kot-Wasik et al compared three C18 sorbents to Strata-X. The results showed that comparable recoveries between the C18 and polymeric sorbents, therefore either can be used. The use of polymeric sorbents is generally seen when ibuprofen is analysed with other pharmaceuticals and not as a single compound[27,29,57,72,147,212,298,299].

The drawbacks of off-line SPE in time consumption and possible analyte loss during elution and elution drying, has not reduced its popularity as a simple, economical and flexible technique compared to the complexity of on-line SPE[297].

2.8 Internal Standard Calibration

Internal standard calibration uses a compound with external reference standards and the samples of study. It compensates for possible effects of errors from sample pre-treatment or instrumentation conditions. The chosen compound must have similar structure, physico-chemical properties and behaviour in the analytical method, but separate quantification and elution is possible and it is absent from the sample of study. Regression analysis is performed for a calibration curve, prepared by plotting the ratio of analyte signal to internal standard signal as a function of analyte concentration.

Analyte concentration in sample solutions is calculated using the obtained linear equation [300].

In this study, internal calibration is used to compensate for any losses during the sample pre-treatment and processing in SPE and the possible variances in analyte migration time common to CE[300,301]. Benzoic acid (BA) was chosen as the internal standard due to its classification in the same class of compounds (carboxylic acids) and similar pKa (4.19) as IBU, 1OH, 2OH and CBX[302,303]. BA therefore shares similarity in structure (Figure 18) and behaviour in the HPLC mobile phase (neutral at acidic pH) and CE background electrolyte (ionized to anionic form at basic pH) and is absent in the samples of study. It is recommended as an CE internal standard for acidic compounds to prevent effects from interactions[301]. The SPE extracts of standard and sample solutions were spiked with the same amount of BA during reconstitution for HPLC and CE analysis.

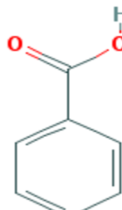


Figure 18. Structure of benzoic acid[303]

2.9 Method Validation

Laboratories must demonstrate that methods of analysis are suitable for their intended application by method validation[304,305]. Several bodies such as the International Conference on Harmonization (ICH), the Association of Official Analytical Chemists International (AOAC) and International Standards Organization (ISO/IEC 17025: 2005) provide guidance for this process[304–310]. Validation parameters should be selected upon consideration of the physico-chemical properties and molecular structure of the analyte(s); concentration range in the sample; sample matrix; possible interferences; analytical purpose (quantitative or qualitative); required limits of detection and quantification; required precision and accuracy; required robustness; instrument capabilities; applicable legislation[305,309–311]. For this study, six parameters were chosen considering research purpose of the developed methods.

2.9.1 Specificity

Specificity is the ability to identify the target analyte(s) in the presence of other components such as impurities, degradation products and matrix. It may be difficult due to sample matrix complexity, low analyte concentration, large number of analytes, similarity between the analyte and matrix or the analytes[304,309–311].

2.9.2 Linearity

Linearity is the ability to obtain results directly proportional to analyte concentration across the method range. A minimum of five standard concentrations evenly spanning 80-120% of the expected sample concentration range is recommended. A minimum number of three replicate injections is required for each concentration, however five to six injections are recommended[304,309,310].

A two-fold evaluation is performed to assess the degree of linearity of the results. The first is visual inspection of response plotted as a function of analyte concentration. The second is statistical treatment of the results by linear regression (method of least squares) to generate a calibration curve and linear equation. The data may require previous mathematical transformation. The regression parameters of correlation coefficient (r), y-intercept (a), slope of regression line (b) and residual sum of squares are also provided. Standard deviation (SD) or variance (CV) of the results of the standard solution series should be assessed before construction of the calibration curve using the Hartley's F_{max} test (significance level of $\alpha = 0.05$) to determine any significant statistical difference. If there is no statistical difference, the calibration curve can be plotted[311].

A correlation coefficient (r) $\geq$ 0.99 is accepted as good linearity. However, it is recommended to verify linearity by another method such as plotting the residuals (the deviation of the measured data points) versus the concentration. If the data fits the regression model, then the residuals will be randomly distributed around zero [292,304].

2.9.3 Range

The range is the interval between the lower and upper analyte concentrations expected in the sample, that give acceptable precision, accuracy and linearity. It is expressed in the same units as the measured results from the limit of quantification to the maximum standard solution concentration used for calibration[304,310].

2.9.4 Precision

Method precision is the closeness of agreement between a measurement series attained from multiple sampling from the same homogenous sample and is expressed as SD or CV of that series[304,310]. Three components can be considered:

a. Repeatability (intra-assay precision): expresses the closeness between consecutive measurements under the same operating conditions (instrument, analyst, day, standard and reagent batch). At least six replications at 100% of test concentration

or nine determinations covering the specified method range, are recommended. The Dixon's Q test (significance level of $\alpha = 0.05$) is applied to detect outliers. Variance in results is assessed using the Hartley's F_{max} test (significance level of $\alpha = 0.05$) to determine any significant statistical difference[311].

b. Intermediate precision: evaluates the possible effects for normal variations within a laboratory such as different days, analysts, equipment, manufacturers and batches of standards and reagents to reveal potential problems with method.
c. Reproducibility: evaluates precision between laboratories through collaborative studies for method standardization, interlaboratory and proficiency tests.

2.9.5 Limit of Detection

The limit of detection (LOD) is the minimum amount of analyte that can be reliably detected. It can be determined as follows[304,310]:

a. Visual Inspection: analysis of samples of known analyte concentrations (standard in solvent or matrix) to establish the minimum level for confident detected.
b. Signal to Noise ratio: determined by comparison of measured sample signals of known low analyte concentration with signals of blank samples to establish the minimum concentration that gives a ratio of 3.
c. Standard Deviation of the Blank: determined from the average and standard deviation from 10 independent measurements of 10 independently prepared blank samples to obtain the magnitude of the analytical background response.
d. Standard Deviation of the Response based on the Calibration Curve Slope: a calibration curve is prepared using the measured responses (at least five replicate injections are recommended) of the three lowest analyte concentrations used to determine linearity. LOD is determined as the mean of the LOD determined from residual standard deviation of regression line and the LOD determined from standard deviation of the y-intercept using:

$$\text{LOD from residual standard deviation} = \frac{3.3\text{residual SD}}{b} \quad \text{Equation 21}$$

$$\text{LOD from standard deviation of the y-intercept} = \frac{3.3 \text{ y-intercept SD}}{b} \quad \text{Equation 22}$$

The accuracy of the calculated LOD is accepted if 10 times the value is greater than the lowest standard concentration (condition 1) and if the value is smaller than the lowest standard concentration (condition 2). If the LOD fails to meet condition

1, the concentrations in the standard solutions were too high and should be remade with lower concentrations. If condition 2 is failed, the concentrations were too low and should be remade with higher concentrations[311].

2.9.6 Limit of Quantification

The limit of quantification (LOQ) is the minimum amount of analyte that can be reliably quantified with appropriate precision and accuracy[304,310]. LOQ can be determined from the same approaches used LOD. The acceptance criteria for signal to noise ratio is 10:1 or LOQ can be determined from the standard deviation of the response based on the calibration curve slope using the calculated LOD value:

$$LOQ = 3 \times LOD \qquad \text{Equation 23}$$

2.9.7 Accuracy

Accuracy is the closeness of agreement between the accepted value taken as the conventional accepted reference value and the measured value. It can be assessed using several approaches including extracting analyte from a matrix and comparing the measured response that from the reference material dissolved in pure solvent to determine the amount recovered. Assessment should cover the method range by selecting concentrations close to the LOQ, the middle and high concentration of the calibration curve. A minimum of three concentrations with a minimum of three replicates (nine determinations) is recommended. The parameter is reported as percent recovery by the assay of the known added amount of analyte or as the difference between the mean and accepted true value with confidence intervals[304,310].

2.9.8 Robustness

The parameter is the capacity to withstand small, deliberate variations in the method parameters and maintain reliability during routine use[304,310]. This is considered during development phase.

2.10 Statistical Analysis

2.10.1 Student's t-test

Student's t test compares the means of two series of results and calculated as:

$$t = \frac{\left(x_{1m} - x_{2m}\right)}{\sqrt{\left(n_1 - 1\right)SD_1^2 + \left(n_2 - 1\right)SD_2^2}} \sqrt{\frac{n_1 n_2 \left(n_1 + n_2 - 2\right)}{n_1 + n_2}} \qquad \text{Equation 24}$$

Where x_{1m} and x_{2m} are the means for both series of results, SD_1 and SD_2 are the standard deviations. The calculated t (t_{cal}) is compared to the applicable critical value

(t_{crit}) in the table of critical values of student's t test (Annex 1a), at significance level (α = 0.05) and the number of degrees of freedom ($f = n_1+n_2 - 2$). If $t_{cal} < t_{crit}$, there is no statistically significant difference between the means of the compared series of results is accepted. If it exceeds, there is a statistical difference[290,292,311].

2.10.2 Hartley's F_{max} Test

The Hartley's F_{max} test compares the standard deviations or variances of two or more series of results using:

$$F_{max} = \frac{CV^2_{max}}{CV^2_{min}} \quad \text{Equation 25}$$

Where CV^2_{min} and CV^2_{max} are the smallest and largest coefficients of variance. The F_{max} value is compared to the applicable critical value (F_{maxo}) in the table of critical values of Hartley's F_{max} test (Annex 1b), at selected significance level (usually α = 0.05), calculated number of degrees of freedom equivalent to the number of results ($f = n-1$) and the number of compared series. If $F_{max} < F_{maxo}$, there is no statistically significant difference between the standard deviations of the compared series of results. If it exceeds, there is a statistical difference[311,312].

2.10.3 Dixon's-Q Test

The Dixon's-Q test is used to detect outliers within a series of results. The results are arranged in increasing order smallest value (x_1) to largest value value (x_n) and the parameters of range R, Q_1 and Q_n calculated from the following equations:

$$R = x_n - x_1 \quad \text{Equation 26}$$

$$Q_1 = \frac{x_2 - x_1}{R} \quad \text{Equation 27}$$

$$Qn = \frac{x_n - xn_{-1}}{R} \quad \text{Equation 28}$$

Q_1 and Q_n are compared to the applicable critical value (Q_{crit}) in the table of critical values of Dixon's Q test (Annex 1c), at the selected significance level (usually α = 0.05) and the number of degrees of freedom equivalent to the number of results ($f = n$). If Q_1 and Q_n do not exceed Q_{crit}, there is no outlier within the results. If they exceed, an outlier is present[290,292,311].

3. Materials and Methods

3.1 Reagents and Materials

The reagents and materials used at each university is presented as follows:

3.1.1 Reagents

Universidade do Algarve (UAlg)

HPLC

Acetonitrile (≥99.8% HiPerSolv CHROMANORM) was purchased from Fisher Scientific (Loughborough, UK). Sulphuric acid (96%), ammonium chloride and glacial acetic acid were purchased from Panreac Applichem (Barcelona, Spain). Methanol (≥99.8% HiPerSolv CHROMANORM) and ammonium acetate (Hipersolv for HPLC) were purchased from VWR International (Carnaxide, Portugal).

Synthetic Wastewater

Sodium acetate (99%), iron sulphate heptahydrate, potassium chloride, calcium chloride, manganese chloride tetrahydrate, monobasic potassium phosphate, ethylenediaminetetraacetic acid, sodium hydroxide, potassium hydroxide and dibasic potassium phosphate were purchased from VWR International (Carnaxide, Portugal). Zinc sulphate heptahydrate was purchased from Applichem Panreac Applichem (Panreac Quiminica SLU, Barcelona, Spain). Colbalt chloride hexahydrate, ammonium heptamolybdate tetrahydrate and copper sulphate pentahydrate was purchased from Riedel-de-Haen (Honeywell Specialty Chemicals Seelze, Germany).

The organic solvents and ammonium acetate were HPLC grade. All other reagents were analytical grade. Milli-q water from a Merck Millipore Elix10 system (Merck Millipore, Madrid, Spain) was used for all reagent and standard preparation.

Universidade de São Paulo (USP)

HPLC

Methanol (Chromasolv, ≥99.9%), acetonitrile (Chromasolv, ≥99.9%), glacial acetic acid, formic acid (≥ 96%) were purchased from Sigma Aldrich Chemie Gmbh (Munich, Germany).

CE

Sodium tetraborate decahydrate and acetonitrile (Chromasolv, ≥99.9%) were purchased from Sigma Aldrich Chemie Gmbh (Munich, Germany). Sodium dodecyl

sulphate was obtained from Sigma Aldrich (St. Louis, MO, USA). Tetrahydrofuran (THF) was procured from Tedia Company Inc. (Fairfield, OH, USA). Cetrimonium bromide was purchased from Sigma Aldrich (Milwaukee, MI, USA).

Synthetic Wastewater

Sodium acetate was procured from Anidol Products (São Paulo, Brazil). Magnesium sulphate heptahydrate was acquired from Cromato Productos Quimico (São Paulo, Brazil). Potassium chloride, iron sulphate heptahydrate and ammonium heptamolybdate tetrahydrate were obtained from Lab Synth Products (São Paulo, Brazil). Ammonium chloride and benzoic acid were purchased from Carlo Erba Do Brazil (São Paulo, Brazil). Ethylenediaminetetraacetic acid and dibasic potassium phosphate were purchased from Mallindrodt (St. Louis, MO, USA. Monobasic potassium phosphate was purchased from F. Maia Ind e Comerico (São Paulo, Brazil). Zinc sulphate heptahydrate was procured from J.T Baker Chemical Co (NJ, USA). Calcium chloride and potassium hydroxide were obtained from Cinetica Quimica Ltd (São Paulo, Brazil). Manganese chloride tetrahydrate and colbalt chloride hexahydrate were purchased from Vetec Quimica Fina LTDA (Rio de Janiero, Brazil). Copper sulphate pentahydrate was purchased from Regen Industris Quimicas (Rio De Janiero, Brazil). Potassium hydroxide and sodium hydroxide were purchased from Merck Chemicals Ltd (São Paulo, Brazil).

SPE

Concentrated hydrochloric acid (HCl) was purchased from Lab Synth Products (Diadema, São Paulo, Brazil).

The organic solvents were HPLC grade, all other reagents were analytical grade. Milli-Q water from a Merck Millipore Milli-Q system (Merck Millipore, São Paulo, Brazil) was used for all reagent and standard preparation.

3.1.1.1 Reference Standards

Ibuprofen (certified reference material, purity 99.7%), 1-hydroxyibuprofen (pharmaceutical impurity standard, purity 99.7%), 2-hydroxyibuprofen (VETRANAL analytical standard, purity 99.3%), carboxyibuprofen (VETRANAL analytical standard, purity 99.0%) were purchased from Sigma Aldrich (Sintra, Portugal).

3.1.2 Materials

3.1.2.1 SPE

Strata C18e (200 mg, 6mL) SPE cartridges were obtained from Phenomenex (Torrance, CA, USA).

3.1.2.2 Characterization of Reactor Influent and Effluent

COD, phosphorus, total nitrogen and ammonia were measured using LANGE commercially available kits supplied by HACH (Carnaxide, Portugal). The kit specifications are given in Table 6.

Table 6. Parameters and kits used for characterization of the SBR influent and effluent

Parameter measured	Kit	Concentration Range* (mg/L)
Ammonium (NH_4-N)	LCK 304	0.015–2.0
COD	LCK 514	100-2000
Nitrate (NO_3-N)	Powder pillows, cadmium reduction method – 8039	0.3-30.0
Phosphorus (PO_4^{3-})	USEPA Phosver3® (ascorbic acid) method 8048	0.02-2.50

*Indicated by manufacturer

3.1.3 Experimental Preparation

3.1.3.1 Synthetic Wastewater Preparation

Synthetic wastewater was prepared using two media to simulate typical characteristics of sewage wastewater. Media A was composed of sodium acetate (63 mM), magnesium sulphate heptahydrate (3.6mM) and potassium chloride (4.7 mM). Media B was composed of ammonium chloride (35.4 mM), dibasic potassium phosphate (4.2 mM), monobasic potassium phosphate (2.1 mM) and 10 mL/L trace element solution[164]. The trace element solution was prepared using ethylenediaminetetraacetic acid (50.0 g/L), zinc sulphate heptahydrate (22.0 g/L), calcium chloride, calcium chloride (5.54 g/L), manganese chloride tetrahydrate (5.06 g/L), iron sulphate heptahydrate (4.99 g/L), ammonium heptamolybdate tetrahydrate (1.10 g/L), copper sulphate pentahydrate (1.57 g/L), colbalt chloride hexahydrate (1.61 g/L) and adjusted to pH 6 using potassium hydroxide[313]. Finally, 150 mL of each media was mixed with 1300 mL of tap water[164].

3.1.3.2 Preparation of Reference Standards and Internal Standard

Individual compound primary stock solutions were prepared by weight at UAlg and USP, in methanol and acetonitrile respectively to solubilize the compounds according to the organic solvent tested in experiments. These solutions were stored at -20^{0}C for at least 3 months use, due to stability in organic solvent[29,87]. A series of reference standard solutions for this study were prepared from these individual primary stock solutions as represented in Figure 19 and described as follows for the work at each university

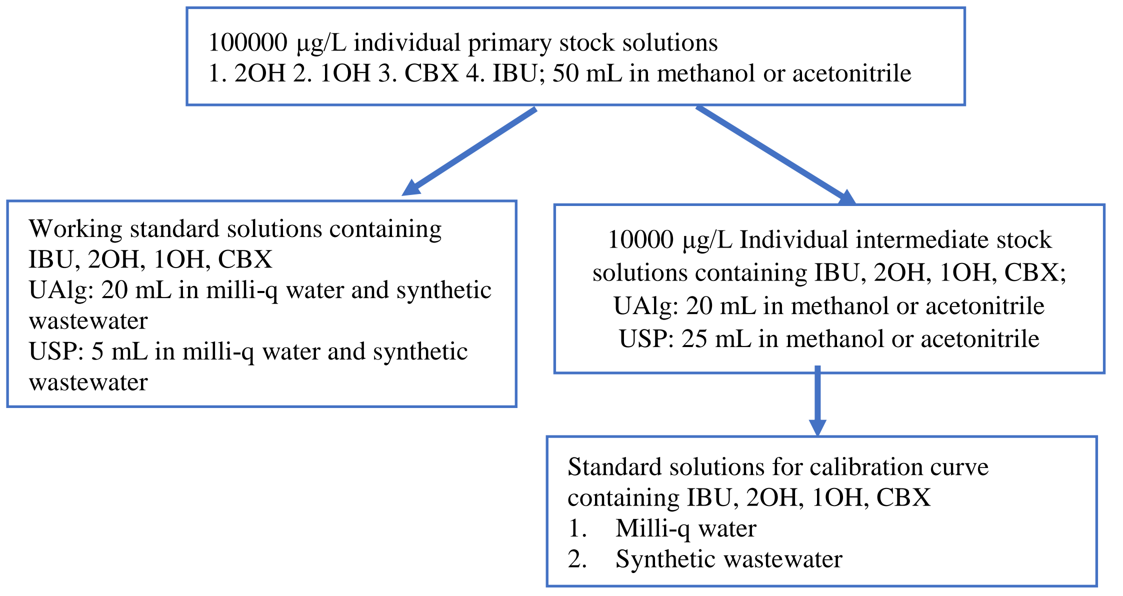


Figure 19. Schematic representation of reference standard solution preparation

UAlg

Working standard solutions (0.5 – 10000 µg/L) were used in method development and optimization experiments to determine compound retention time and selectivity, peak resolution and smallest concentration detectable on the HPLCs. Working standard solutions were used to prepare a calibration curve of range 50 – 500 µg/L. The concentrations for all solutions, corrected for purity as provided by the manufacturer, are shown in Tables 7 and 8.

Table 7. Actual concentrations of primary and intermediate standard stock solution and working standard solution

Compound	Weight (g)	Primary stock solution concentration (µg/L)	Intermediate stock solution concentration (µg/L)	Working standard solution concentration (µg/L)
IBU	0.0050	99700	9970	9970
1OH	0.0050	99700	9970	9970
2OH	0.0050	99300	9930	9930
CBX	0.0050	99000	9900	9900

Table 8. Preparation and actual concentrations of working standard solutions used in method development, optimization, calibration curve

Standard Solution No.	Volume from individual intermediate stock solution (mL)	Theoretical concentration (µg/L)	Actual concentration (µg/L)			
			IBU	1OH	2OH	CBX
2	10	5000	4985	4950	4965	4985
3	4	2000	1994	1980	1986	1994
4	2	1000	997	990	993	997
5	1	500	499	495	497	499
6	0.2	100	98	99	99	98
7	0.1	50	50	50	50	50
8	0.05	25	25	25	25	25
9	0.02	10	10	10	10	10
10	0.002	1	1	1	1	1
11	0.001	0.5	0.5	0.5	05	0.5

USP

A series of reference standard solutions were prepared as shown in Figure 19. The actual concentrations for all solutions, corrected for purity as provided by the manufacturer, are given in Tables 9, 10 and 11.

Table 9. Preparation of primary and intermediate standard stock solutions

Compound	Weight (g)	Actual Primary stock solution concentration (µg/L)	Intermediate stock solution concentration (µg/L)
IBU	0.00500	99700	9970
1OH	0.00501	99900	9990
2OH	0.00500	99300	9930
CBX	0.00500	99899	9990

Table 10. Preparation of standard solution used in method development and optimization

Standard Solution No.	Volume from primary stock solution (µL)	Theoretical concentration (µg/L)	Actual concentration (µg/L)			
			IBU	1OH	2OH	CBX
1	100	2000	1994	1998	1986	1980
2	200	4000	3996	3988	3972	3960
3	300	6000	5994	5982	5958	5940
4	400	8000	7992	7976	7944	7920
5	500	10000	9990	9970	9930	9900
6	600	12000	11988	11964	11916	11880
7	700	14000	13986	13958	13902	13860

Table 11. Preparation of standard solution series for calibration curve

Standard Solution No.	Volume from primary stock solution (µL)	Theoretical concentration (µg/L)	Actual concentration (µg/L)			
			IBU	1OH	2OH	CBX
1	7.5	15	15.0	14.9	14.9	15.0
2	12.5	25	24.9	24.8	24.8	25.0
3	25.0	50	49.9	49.5	49.7	49.9
4	37.5	75	74.8	74.3	74.5	74.9
5	50.0	100	99.7	99.9	99.3	99.9
6	62.5	125	124.6	124.6	124.1	124.9

Internal standard benzoic acid was prepared with final concentration of 100000 µg/L by weighing 0.0100 g in 100 mL volumetric flask and making to volume in milli-q water. 250 µL of this solution was added to standard solutions for a final concentration of 5000 µg/L.

3.2 Solid Phase Extraction

SPE was performed with Strata C18e 200 mg/6mL cartridges using a vacuum manifold system connected to Fanem dia pump (Figure 20A). The procedure is described in Figure 20B. The procedure was adapted from a common method used in several papers)[22,27,29,72,147,212,240,298,299].

A

B

SPE Step	**Procedure**
Cartridge Conditioning	Cartridges were conditioned using 3 mL of methanol, followed by 3 mL of milli-q water and 3 mL of milli-q water at pH 2.
Sample Pretreatment	pH of standard and sample solutions was adjusted to 2 using concentrated HCl
Loading	100 and 200 mL of standard and sample solutions was loaded at 3 mL/min and passed through the cartridges
Washing and drying	The cartridges were rinsed with 3 mL of milli-q water and then dried for 10 min on the vacuum manifold
Elution	The compounds were then eluted using 1 mL of methanol
Eluent drying	The eluents were dried using a speed vacuum concentrator at room temperature for 2.5 hours at level 5.1 vacuum.
Reconstitution	25uL benzoic acid (final concentration of 5000 µg/L) and 475 µL of milli-q water were added to reconstitute the extracts to final volume of 500 µL. The extracts were then divided for HPLC and CE analysis.

C

Figure 20. Solid Phase Extraction: (A) SPE equipment used and (B) Speed vac concentrator (C) procedure performed

The performance of the procedure was tested by spiking triplicate preparations of milli-q water with the reference standards at concentration of 50 µg/L and then processing through the C18 cartridges. Two volumes (100 and 200 mL) were tested. The preconcentration factor was determined as the ratio of volume loaded to volume eluted. Blanks (non-spiked milli-q water) processed through SPE were spiked with reference standards at 5000 and 10000 µg/L for comparison of recovery to the processed milli-q water preparations. The concentrations accounted for preconcentration factor of 100 and 200 respectively. Blanks were also processed and analysed to determine any possible interferences from the SPE sorbent. The extracts were dried using a Thermo Scientific Savant SPD 1010 speed vacuum concentrator (Figure 20C). The procedure was used with the HPLC and MEKC methods to the analysis of samples taken from the SBRs (Section 3.9).

3.3 Configuration and Operation of Sequencing Batch Reactors

3.3.1 SBR set-up

Two SBRs, were constructed by adapting materials available in the laboratory, with a total height of 35 cm and internal diameter of 6 cm. The working volume of 763 mL based on the maximum influent height at 27 cm (Figure 21). The height to internal diameter ratio was 5.8. Synthetic wastewater (influent) was introduced via two tubes at the bottom of the SBRs. Effluent was withdrawn at a height of 13 cm from the bottom. Both systems were operated in cycles using an automatic timer (Carlo Gavazzi DMB51 multifunction timer) to control the pumps for influent addition, aeration and effluent withdrawal. Aeration was performed at the bottom of the reactor using a tube connected to a fine bubble aerator, except during influent addition, settling and effluent withdrawal. The flow rate for reactor 1 was 194 L/min and 90 L/h for reactor 2.

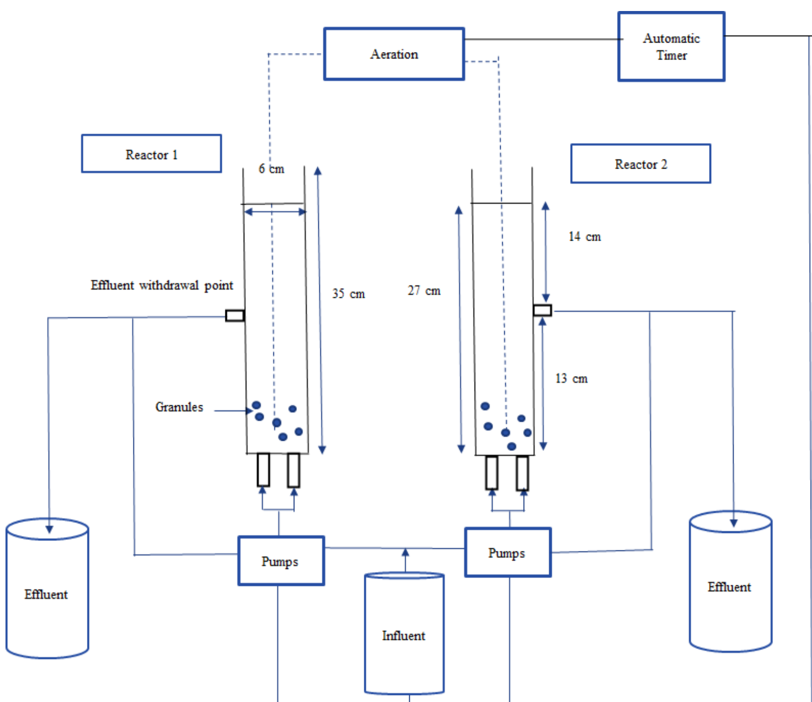


Figure 21. Schematic representation of the two SBRs

Inoculum for SBR Start-up

Aerobic granules (10.3 g/L total suspended solids) were generously supplied by the Águas de Portugal Nereda municipal wastewater treatment plant in Frielas, Lisbon, was used as inoculum (2 g/L, total suspended solids) for start-up of the SBRs. The diameter of 20 granules was measured using SteREO Lumar Flourescence

Stereomicroscope (Carl Zeiss, Göttingen, Germany) with amplification of 13x using AxioVision software.

Determination of Total Suspended Solids

The total suspended solids of the granules was measured to determine the volume to be placed in each reactor for start-up using a laboratory protocol from the Faculdade de Ciêncas e Tecnologia (Mestrado Integrado em Engenharia do Ambiente)[314]. An empty Whatman filter (110 mm, pore size) was weighed and placed in a Buchner funnel attached to a filtration apparatus. The granule suspension was gently shaken for proper homogenization and 100 mL measured into a beaker. 3 mL was removed from the beaker using a pipette with a cut-off tip and evenly applied to the empty filter. The filter was washed three times (5 mL distilled water) under vacuum, removed using tweezers to a watch glass and dried at 110^{0}C for 1 hr. The dried filter was weighed. The value of total suspended solids was calculated as:

$$\frac{\text{weight of filter +solid (mg)} - \text{weight of empty filter (mg)}}{\text{volume added to filter (L)}} \qquad \text{Equation 29}$$

3.3.2 Operation

The reactors were operated for 2 months at room temperature (23 ± 2^{0}C) in successive cycles of 168 min (2.8 h, 8.6 cycles daily). Reactor 1 was operated in an aerobic cycle[156] while reactor 2 was operated in the typical anaerobic/aerobic cycle of aerobic granular sludge reactors and wastewater treatment plants [156,166,174–182,189–191]. The phases and respective duration are presented in Table12. An additional contact time of 42 min was programmed for reactor 2 to prolong contact of influent with the granules due to the short feeding time. In each cycle, 395 mL was withdrawn, which accounted for 52% of the reactor volume (calculated using height of the withdrawal point of 13 cm).

Table 12. Phases and duration of reactor operating cycle

<table>
<tr><th rowspan="2">Phases</th><th>Reactor 1 (aerobic)</th><th>Reactor 2 (anaerobic/aerobic)</th></tr>
<tr><th colspan="2">Time (min)</th></tr>
<tr><td>Influent feeding (no aeration)</td><td></td><td></td></tr>
<tr><td>a. Filling from hallway point</td><td>2</td><td>2</td></tr>
<tr><td>b. Additional contact time</td><td></td><td>42</td></tr>
<tr><td>Aeration</td><td>162</td><td>120</td></tr>
<tr><td>Settling (no aeration)</td><td colspan="2">1</td></tr>
<tr><td>Effluent withdrawal</td><td colspan="2">3</td></tr>
<tr><td>Total cycle length</td><td>168</td><td>168</td></tr>
</table>

Hydraulic retention time was calculated as: $\frac{\text{total cycle length}}{\text{volume withdrawn/total working volume}}$ Equation 30

The daily influent loading rate was calculated as:

$$\text{Influent loading rate} = \frac{\text{Total volume of influent added daily}}{\text{Surface area of clarifier/ cylinder}} \quad \text{Equation 31}$$

Dissolved oxygen (mg/L) and pH was monitored by offline instrumentation but not controlled (Section 3.6). COD (mgO_2/L), phosphorus (mg PO_4^{3-}/L), total nitrogen (mg NO_{3-}-N/L) and ammonia (mg NH_3-N/L) were determined on days 14, 17, 20, 21 and 27 using the commercial kits. Sludge volume index was determined by measuring the time taken to settle to the bottom of the reactor after cessation of aeration.

3.3.3 Introduction of Ibuprofen and Samples

The SBRs were dosed with ibuprofen twice, first with 50 µg/L on day 21 of operation and with 100 µg/L on day 27. Samples (T0 to T6) were withdrawn 7 times from both reactors respectively, during one cycle at times corresponding to t= 0, 30, 60, 90, 120, 150, 168 min, to determine presence and concentration of ibuprofen the metabolites. The first and last samples corresponded respectively to introduction of influent and effluent withdrawal. Samples were filtered using sterile syringe filters (0.2 µm pore size) to remove biomass and bacteria. Controls were prepared as ibuprofen was introduced to the reactors, by spiking synthetic wastewater (Control SW) and milli-q water (Control W) respectively in the same dosage. The controls were allowed to stand for the length of the same cycle during which sampling was performed, to assess if degradation occurred by other mechanisms. Quantification of acetate in influent and effluent was performed using the Varian HPLC equipped with a UV/Vis detector at wavelength 210 nm, at room temperature and a flow rate of 0.5 mL/min. The mobile phase was 0.0025M sulphuric acid using a Rezex RFQ-Fast Acid H+ (8%) column supplied by Phenomenex (Torrance, CA, USA).

3.4 High Performance Liquid Chromatography

3.4.1 Instrumentation

UAlg

Preliminary experimentation was performed first using a Varian 380-LC HPLC (Varian Inc, Palo Alto, Ca, USA)) equipped with diode array detector (UV/Vis filters) controlled by Clarity (Figure 20), followed an Agilent 1220 Infinity series HPLC equipped with DAD (lamps with monochromator) operated using Chemstation OpenLab Chromatographic Data System (CDS) software (Agilent Technologies,

Santa Clara, CA, USA) (Figure 21). The Varian HPLC offered the capacity to use isocratic or gradient modes. Agilent HPLC could only be used in the isocratic mode.

Separations were performed at room temperature as both instruments were not equipped with ovens. An Xbridge® reverse phase C18 column (hybrid particle, 250 mm length, 4.6 mm i.d, 5.0 µm particle size) attached to a Xbridge C18 guard column (20 mm length, 4.6 mm i.d, 5.0 µm particle size) (Waters Corporation, Milford MA, USA). The injection volume was 20 µL and performed manually. A wavelength scan was performed on the Varian HPLC at 220, 221, 223 and 225 nm, to determine the wavelength at which ibuprofen showed the highest absorbance. Subsequent experiments were subsequently performed at the selected wavelength (221 nm).

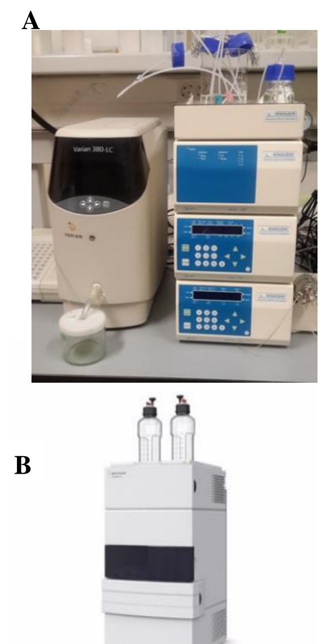


Figure 22. A) Varian 380-LC HPLC (B) Agilent 1220 Infinity Series HPLC

USP

Method development and analysis of samples were performed using a Shimadzu Prominence-i LC-2030 HPLC system coupled to UV/Vis DAD (deuterium lamp with monochromator) controlled by Lab Solutions Analysis Data Software (Shimadzu Corporation, Kyoto, Japan) (Figure 22), capable of operation in gradient and isocratic modes. Chromatographic separations were performed using a Shimpack C18 reverse phase column (VP-ODS, 250 mm length, 4.6 mm i.d, 4.6 µm particle size) (Shimadzu Corporation, Kyoto, Japan). A Shimadzu Shimpack guard column (GVP-ODS, 10 mm length, 4.6 mm i.d., 5 µm particle size) was attached. The injection volume was 20 µL and was performed using an auto-injection module.

Figure 23. Shimadzu Prominence-i LC-2030 HPLC

3.4.2 Method Development and Optimization

3.4.2.1 UAlg: Method Development

Method development was initiated at UAlg. Several mobile phases in literature used solvent A as water adjusted to pH 3 or ammonium acetate buffer and solvent B as methanol or acetonitrile to successfully separate and analyse ibuprofen and the metabolites[86,147,223]. The tested mobile phases are listed in Table 13. Preliminary assessments were performed to determine retention time and retention factor (k) of each compound, resolution between the four compounds and smallest concentration detectable, identification of critical peak pair. Experimental design described in Section 3.7.1, was used to optimize retention factor ($1<k<10$ or $0.5< k <20$) and resolution (≥1.5) of the four compounds once a mobile phase was identified.

Table 13. Mobile phases tested during method development at UAlg

Mobile Phase		Composition	Chromatographic mode	Test flow rate (mL/min)	Reference
Solvent A	Solvent B				
Water with 0.1% glacial acetic acid	Methanol	65:35, (v/v)	Isocratic[223]	0.8	
Water, pH 3 adjusted using glacial acetic acid	Acetonitrile	50:50 (v/v)	Isocratic	2	86
Water	Methanol	60:40, (v/v)	Isocratic	0.8	147
10 mM ammonium acetate, pH 4	Methanol (1) Acetonitrile		Isocratic	1	142
10 mM ammonium acetate, pH 4.5	Acetonitrile	60:40, (v/v) 65:35, (v/v) 70:30, (v/v)	Isocratic	1	
10 mM ammonium acetate, pH 5	Acetonitrile	60:40, (v/v) 65:35, (v/v) 70:30, (v/v)	Isocratic	1	
25 mM ammonium acetate, pH 4	(1) Methanol (2) Acetonitrile		Isocratic	0.8 1	142
25 mM ammonium acetate, pH 4.5	Acetonitrile		Isocratic	1	
25 mM ammonium acetate, pH 5	Acetonitrile		Isocratic	1	

[1]Varian 380-LC HPLC [2]Agilent 1220 HPLC

3.4.2.2 USP: Method Development and Optimization

Method development was continued and finalized at USP using a Shimadzu Prominence-I LC 2030 HPLC. Preliminary assessments of the retention time and retention factor of each compound, resolution between the peaks and verification of the critical peak pair were carried for each mobile phase listed in Table 14 by

performing a gradient separation from 5 - 100% B in 15 min using a flow rate of 2 mL/min at a temperature of 30 ^{0}C, at 221 nm. Retention time for each compound was confirmed by injecting solutions of individual compound. The initial chromatograms were evaluated to determine if isocratic separation was possible. The mobile phase with the best results on evaluated criteria (water (solvent A) and acetonitrile (solvent B) both acidified with formic acid, 0.01% (v/v)) was selected for further experimentation to optimize the gradient separation focusing on retention factor (1<k*<10), resolution between the compounds, particularly for the critical peak pair (≥1.5), peak efficiency and signal to noise ratio of the four compounds, at two temperatures (25^{0}C and 30^{0}C). Signal to noise ratio was evaluated at two wavelengths (221 and 230 nm) and the wavelength with the higher ratio was selected for experimental analysis.

Table 14. Mobile phases tested during method development at USP

Mobile Phase		Composition	Chromatographic mode	Test flow rate (mL/min)	Reference
Solvent A	Solvent B				
Water	Acetonitrile	Both acidified with 0.01% formic acid (v/v)	Gradient	2	[30]
Water	Acetonitrile	Both acidified with acetic acid, 3% (v/v)	Gradient	2	
Water	Acetonitrile	Both acidified with acetic acid, 1% (v/v)	Gradient	2	
Water	Acetonitrile	Both acidified with formic acid, 0.1% (v/v)	Gradient	2	
Water, pH 3	(1) Methanol (2) Acetonitrile	adjusted to pH 3 with glacial acetic acid	Gradient	2	[86]
Water, pH 3	Acetonitrile	Adjusted to pH 3 with phosphoric acid			

3.4.2.3 Preparation of Mobile Phases

The buffers for the mobile phases were prepared on a weight basis using volumetric flasks and diluting to volume with milli-q water. The solvents acidified with formic acid and acetic acid were prepared on a volume by volume basis by adding the appropriate volume to volumetric flasks and diluting to volume with water or acetonitrile respectively. The pH of the ammonium acetate buffers and water, pH 3 was adjusted using glacial acetic acid. All solvents were vacuum filtered using 0.22 µm polypropylene membrane filters to remove impurities and then sonicated for 10 min to remove excess gas that could interfere with the system.

3.4.3 Identification and Quantification of Ibuprofen and Metabolites

Sample analysis was carried out on the Shimadzu HPLC System using the method optimized after development work. The details of the optimized method and gradient programme are given in Tables 15 and 16.

Table 15. Optimized chromatographic conditions for separation of Ibuprofen and metabolites

Chromatographic parameter	Optimized condition
Mobile phase	Solvent A: water acidified with formic acid, 0.01% (v/v) Solvent B: acetonitrile acidified with formic acid, 0.01% (v/v)
Flow rate (mL/min)	2 mL/min
Temperature (^{0}C)	30
Injection volume (µL)	20
Wavelength (nm)	230

Table 16. Optimized gradient programme for separation of Ibuprofen and metabolites

Time (min)	% Solvent B
0	25
12	80
12.5	25
16	25

3.5 Capillary Electrophoresis

3.5.1 Instrumentation

Method development and analysis of samples was performed using a Beckman Coulter ProteomeLab PA800 capillary electrophoresis system coupled to a DAD detector (UV/Vis filters) controlled by 32 Karat^{TM} 8.0 software. (Figure 24)

Figure 24. Beckman Coulter ProteomeLab PA800 CE System

Chromatographic separations were performed using an unfused and uncoated silica capillary (75 µm i.d) (Polymicro Technologies, Phoenix, AZ, USA) at wavelength of 214 nm and room temperature. Standard and sample solutions were injected hydrodynamically for 3 s towards the cathode. Preliminary assessment was performed using a capillary of total length 50.5 cm, length to detector of 40 cm and applied voltage of 25 kV. To reduce the migration time, the total length of the capillary was decreased to 40 cm and the length to the detector to 29.5 cm, subsequent experiments, method development and sample analysis were performed with these dimensions.

3.5.2 Method Development and Optimization

Preliminary assessment was performed using 10 mM borate (pH 9) as BGE to determine compound migration time and effective mobilities and the critical peak pair. Effective mobility curves were plotted as a function of pH and a response function used to identify the best pH for separation. Peak resolution and migration time of the compounds were examined in two different background electrolytes, acetate buffer at pH 5 (compounds in neutral form) and borate buffer at pH 9 (compounds in anionic form). Four factors critical to CE separation: buffer concentrations (10, 20, 25, 30 and 50 mM), addition of micelles (SDS, cetrimonium bromide), organic solvent (acetonitrile, methanol, tetrahydrofuran) and separation voltage (20, 22, 24, 25 kV), were examined to determine their influence on migration time, critical resolution and peak efficiency (Table 17). Polarity of hydrodynamic injection mode (normal and reverse) was also assessed.

Table 17. BGE and conditions examined for CE method development

Electrolyte	Buffer concentration (mM)	Micelle	Micelle concentration (mM)	Organic Solvent	Organic Solvent concentration (%)	Separation voltage (kV)
Acetate buffer, pH 5	20, 25, 30, 50	-	-	-	-	20, 22, 24, 25
Borate buffer, pH 9	10, 15, 20, 25, 30	SDS CTAB	20, 30, 40 0.2	Acetonitrile Methanol Tetrahydrofuran	10, 20 10 5, 10, 15, 20	20, 25

A wavelength scan (200, 214, 254 and 280 nm) was performed after selection of best electrolyte for separation to determine the wavelength of highest absorbance of the compounds and signal to noise ratio. This wavelength was subsequently used for further work. Experimental design described in Section 3.7.2 was used to optimize and resolution (≥1.5) of the four compounds once a BGE was identified.

3.5.3 Stacking

After selection of the electrolyte, most suited for separation of the compounds, experiments were performed to investigate enhancement via on-line preconcentration using field amplified sample stacking. The conditions examined for optimization of peak height were placement of the milli-q water plug (before and after sample), length of injection of water plug (t=0, 2, 3, 5, 7, 10, 12, 16, 18, 20, 30s), length of injection time for the sample (t= 3s, 6s), addition of organic solvent to the water plug in the same ratio as the electrolyte.

3.5.4 Background Electrolyte Preparation

Acetate Buffer, pH 5

The buffer was prepared on a weight basis by weighing the appropriate amount of glacial acetic acid to obtain the desired final concentration in a volumetric flask. The pH was adjusted using 0.5M sodium hydroxide.

Borate Buffer, pH 9

The buffer was prepared on a weight basis by weighing the appropriate amount of sodium tetraborate decahydrate and additive and by adding the appropriate volume of organic solvent in a volumetric flask to obtain the desired final concentration, then filtered. The pH of borate solutions did not require adjustment.

3.5.5 Identification and Quantification of Ibuprofen and Metabolites

The samples were analysed using the MEKC method developed and optimized for the separation of the compounds. The details are given in Table 18.

Table 18. Optimized MEKC conditions for separation of Ibuprofen and metabolites

MEKC parameter	Optimized condition
BGE composition	
Borate concentration (mM)	15
SDS concentration (mM)	40 mM
% THF	15
Runtime (min)	15 min
Separation voltage (kV)	20
Temperature (^{0}C)	25
Injection mode	Hydrodynamic
On-line preconcentration	Water plug containing 15% THF injected after sample
Water injection (time, pressure)	16 s, 0.5psi
Sample injection (time, pressure	3 s, 0.5 psi
Wavelength (nm)	200
Capillary dimensions	
Total length (cm)	40
Length to detector (cm)	29.2

3.6 Supporting Instruments, Equipment, Materials

Supporting instruments and equipment for experimental procedures for weighing, preparation of solutions, measurement and adjustment of pH, sonication of mobile phases are shown below in Table 19.

Table 19. Supporting instruments and equipment

Instrument	Model	Manufacturer	Capacity	Use
Balance[1]	ECN611-2271	VWR, Carnaxide, Portugal	310 g	Weighing of reagents
Balance[1]	440-35N	Kern & Sohn, GmbH, Germany	400 g	Weighing of reagents
Balance, analytical[2]	AB204-S	Mettler Toledo, Columbus, OH, USA	210 g	Weighing of reagents
Balance, semi-micro[2]	AUW 220D	Shimadzu, Kyoto, Japan	220 g	Weighing of reagents
Multi water analyzer: dissolved oxygen	CD650	Eutech instruments, Landsmeer, Netherlands	600% DO saturation 90.00 mg/L DO concentration	DO monitoring of SBR
Graduated pipettes		Pyrex, NY, USA	1 mL, 2 mL, 5 mL, 10 mL	Preparation of standard solutions
pH meter[1]	GLP 21	Crison Instruments, Barcelona, Spain		Adjustment of solution pH, pH monitoring of SBR
pH meter[2]	DM-20	Digimed, São Paulo, Brazil	-2-20	Adjustment of solution pH
Membrane filters[1]	hydrophilic polypropylene,	Pall Life Sciences, MI, USA	0.2 µm	Filtration of solutions
Micropipette		Eppendorf, NY, USA	1-10 µL, 20-200 µL, 10-1000 µL	Preparation of standard solutions and reagent solutions
Oven[1]	ED53	Binder, Germany	Max 300^0C	Drying filter
Syringe membrane filter[1]	Polyethersulfone membrane	VWR, Carnaxide, Portugal	0.2 µm	Filtration of solutions
Syringe filter[2]	Captiva, Polytetrafluoroethylene	Agilent Technologies, CA, USA	0.2 µm	Filtration of solutions
Ultrasonic bath[1,2]	2510E-DTH	Branson, CT, USA	2.8 L	Sonication of mobile phase
Volumetric flasks[1,2]	Pyrex Normax Labbox	5, 10, 20, 25, 50, 1000 mL		Preparation of standard solutions and reagent solutions
Whatman filter paper[12]	Silicone treated filter paper	Whatman, plc, GE Healthcare Services, Buckinghamshire, UK	110 nn, pore size	Filtration of solutions TSS

[1]UAlg [2]USP

3.7 Experimental Design

3.7.1 HPLC: Optimization of Critical Resolution

UAlg

A full factorial design of 2^2 was employed to evaluate the factors critical to the separation of the critical peak pair (2OH and CBX) and their interaction. The factors considered were %solvent B (methanol) and mobile phase pH at two levels (-1, +1). The experiments were performed in mobile phase of 10 mM ammonium acetate buffer (solvent A) and methanol (solvent B). The mobile phase, factors and values, listed in Table 20, were selected after exploratory runs to determine the conditions with best potential for separation of the critical peak pair and analysis time.

Table 20. Selected factor affecting resolution of critical pair and levels

Level	Factor	
	pH	% Solvent B
High (+1)	4.5	40
Low (-1)	4.0	35

The factorial design, which consists of four experiments is given in Table 21.

Table 21. Full factorial Design 2^2 employed for investigating resolution of critical peak pair

Experiment No.	Factor	
	pH	% Solvent B
1	-1	-1
2	1	-1
3	-1	+1
4	+1	+1

The resulting chromatograms was ranked with the response functions CRS, CRF, CEF, R_{sum}, R_{cp} and RP to determine the most optimum conditions for separation.

3.7.2 MEKC: Optimization of Critical Resolution and Migration time

A full factorial design of 2^3 was employed to optimize the MEKC method, evaluating the factors critical to the separation of the critical peak pair (2OH and 1OH), compound migration time and their interaction (response variables). The factors considered were borate concentration, SDS concentration and %THF at two levels (-1, +1). The experiments were performed for the borate buffer. The electrolyte composition, factors and their values, listed in Table 22, were selected after exploratory runs to determine the conditions with best potential for separation of the critical peak pair. The factorial design, which consists of 8 experiments is given in Table 23.

Table 22. Selected factor affecting resolution of critical pair and levels

Level	Factor		
	Borate concentration (mM)	SDS concentration (mM)	%THF
High (+1)	20	40	15
Low (-1)	15	20	10

Table 23. Full factorial Design 2^3 employed for investigating resolution of critical peak pair

Experiment No.	Factor		
	Borate concentration (mM)	SDS concentration (mM)	%THF
1	-1	-1	-1
2	+1	-1	-1
3	-1	+1	-1
4	+1	+1	-1
5	-1	-1	+1
6	+1	-1	+1
7	-1	+1	+1
8	+1	+1	+1

The resulting electropherograms was ranked with the response functions CRS, CRF, CEF, R_{sum}, R_{cp} and RP to determine the most optimum conditions for separation.

3.8 Method Validation

The validation of both methods were conducted in solvent (milli-q water) according to the ICH Guideline Q2 (R1)[304,310] and AOAC Guidelines for Standard Method Perfomance Requirements[308].

Specificity was established through identification of the separation conditions best suited to achieve resolution ≥ 1.5 and injection of individual reference standards to confirm migration and retention time for each compound. Linearity was assessed using six standard solutions ranging from 15-125 µg/L prepared in solvent (water). The range was chosen to analyse the concentrations dosed to the SBRs (50 and 100 µg/L). Replicate injections of each concentration was performed (5 injections were used in HPLC, 6 injections were used for CE). The Hartley's F_{max} test (significance level α 0.05) was applied to determine any statistically significant difference in the variances of the standard solution series. Calibration curves were constructed using linear regression analysis. Residual plots were prepared to confirm linearity. Calibration curves were also prepared in sample matrix (synthetic wastewater). Student's t-test was applied to compare the y-intercepts of the solvent and matrix calibration curves to assess matrix effect and any statistically significant difference between the two curves. If there was statistically significant difference, the remaining

the remaining validation parameters and sample analysis would be performed using the matrix calibration curve.

LOD was determined from a calibration curve prepared from the replicate measurements of the three lowest concentrations (15, 25 and 50 µg/L). LOD was calculated as the mean of the LODs determined from the residual standard deviation and slope of the solvent calibration curve. The accuracy of LOD was assessed by two conditions (LOD < 15 µg/L, condition 1; 10 x LOD > 15µg/L, condition 2). LOQ was obtained from LOD. Range was determined using LOQ and the highest standard concentration.

Precision was assessed through repeatability (intra-day) and intermediate precision (extra-day). Repeatability was calculated by fifteen independent analyses of three sample solutions for HPLC and eighteen analyses for CE. Synthetic wastewater was at three concentrations 25, 50 and 100 µg/L respectively. The Dixon's Q-test (significance level α 0.05) was applied to check for outliers in the results. The Hartley's F_{max} test (significance level α 0.05) was then applied to evaluate statistical significant difference between the variances of the results. Intermediate precision was obtained by repeating injections on a second day.

Recoveries were evaluated by comparison of concentrations obtained after SPE with initial fortification in synthetic wastewater for three concentrations 25, 50 and 100 µg/L. Blank (non-spiked samples) were analysed and amounts found were subtracted from those of the spiked samples. Analyte quantification for linearity and recovery was performed using internal standard (benzoic acid). Robustness was studied during method development and optimization for the effect on peak resolution by varying HPLC (flow rate, temperature, mobile phase, detection wavelength) and MEKC experimental conditions (borate and SDS concentration, separation voltage, % THF), particularly for the critical peak pair.

3.9 Application of HPLC and MEKC Methods to SBR Samples

Both methods were applied for the analysis of synthetic wastewater SBR samples, synthetic wastewater and milli-q water controls. Off-line SPE was used for both methods as described in Section 3.3.3, to preconcentrate analyte concentrations to method sensitivity limits. 200 mL of sample and calibration curve standard solutions were processed using the Strata C18e cartridges. Analyte concentration was assessed using solvent calibration curves.

4. Results and Discussion

4.1 Overview of Thesis Experimental Plan and Work

Águas do Algarve seeks to improve local urban wastewater treatment by installing an aerobic granule WWTP. The company is interested in the treatment's capacity to degrade priority emerging environmental pollutants frequently detected in WWTPs such as pharmaceuticals. The granules show resistance and adaptability to toxic compounds and operating cycles are malleable to produce desired effluent conditions (Section 1.6). The NSAID, ibuprofen is a good choice for study due to its global popularity and persistence in conventional WWTP influent and effluent, surface and ground waters, along with its metabolites (1-hydroxyibuprofen, 2-hydroxyibuprofen and carboxyibuprofen), despite high removal efficiency by aerobic biodegradation (Section 1.4 and 1.5).

Two aerobic granule SBRs were constructed and operated using synthetic wastewater at laboratory scale in anaerobic/aerobic and aerobic cycles respectively (Section 3.3.1- 3.3.2). The SBRs were dosed with ibuprofen at environmentally realistic concentrations of 50 (day 21) and 100 µg/L (day 27) to investigate their efficiency and type of operating conditions on the degradation of ibuprofen and its metabolites. Synthetic wastewater and milli-q water controls were prepared at the same time as ibuprofen introduction and in the same dosage, but not passed through the SBRs. Samples were taken from each reactor at several timepoints for analysis with the controls (Section 3.3.3). SBR performance was monitored by several parameters.

HPLC (Section 2.2) and CE (Section 2.3), coupled with UV/Vis, were chosen to develop analytical methods for the compounds of study. Several chromatographic and CE methods have been developed for successful environmental analysis of ibuprofen with other pharmaceuticals but few include the metabolites (Section 2.1). HPLC development and optimization were initiated at UAlg and completed along with validation at USP (Section 3.4, 3.7, 3.8). CE development, optimization and validation was performed at USP (Section 3.5, 3.7, 3.8). Off-line SPE was used to preconcentrate analyte concentrations to method detection limits (Section 3.2). On-line preconcentration was additionally used for CE (Section 3.5.3). After method validation was performed, both methods were applied to analyse the SBR samples (Section 3.9). The results are discussed in the following sections.

4.2 SPE

An SPE procedure successfully used in previous literature was selected for use in this study[22,27,29,72,147,212,240,298,299]. The performance of the procedure was evaluated using Strata C18e cartridges as described in Section 3.2. Milli-q water, spiked with reference standards at 50 µg/L with pH adjusted to 2 using concentrated HCl were processed. Two volumes (100 and 200 mL) were studied. Analytes were eluted using methanol. To allow compatibility of the same SPE extract for both HPLC and MEKC analysis, eluents were dried and reconstituted in milli-q water (solvent). Recoveries were compared for SPE sorbent matrix effect to blank (milli-q water) spiked post SPE with reference standards at 5000 and 10000 µg/L respectively (accounting for preconcentration factor of 100 and 200). The extracts were analysed by HPLC using an internal standard calibration curve (2000 – 14000 µg/L, Table 10). The recoveries are displayed in Table 24.

Table 24. Mean SPE recoveries in milli-q water, 95% confidence interval, Strata C18e cartridges (n=3)

Compound	100 mL		200 mL	
	Mean % recovery	RSD %	Mean % recovery	RSD %
2OH	147 ± 8.1	4.9	154 ± 46	26
CBX	161 ± 13	6.7	152 ± 11	6.5
1OH	165 ± 7.3	3.9	155 ± 9.8	5.6
IBU	66 ± 32	43	88 ± 49	49

The recoveries for 2OH, 1OH and CBX were significantly higher while IBU recovery was lower than the AOAC recommended 110%[308]. These results were unexpected as previous studies that applied this sorbent to ibuprofen, obtained recoveries no larger than 103%[72,299]. These recoveries could be due to matrix effect from the SPE sorbent, highlighted by comparison of the recoveries of the blanks spiked post matrix at 5,000 and 10,000 µg/L, also calculated using the calibration curve, that are closer to the AOAC limit (Table 25). Volumetric errors from the micropipettes used to prepare the solution, could account for the values over 110%. Blank matrix processed through the SPE was analysed, however no peaks indicating possible interferences were observed that could account for the high recoveries.

Table 25. Recoveries calculated from calibration curve for blank matrix spiked post SPE

Compound	% recovery obtained for 5,000 µg/L Preconcentration factor of 100	% recovery obtained for 10,000 µg/L Preconcentration factor of 200
2OH	103	112
CBX	111	112
1OH	103	112
IBU	115	119

Due to limited numbers of SPE cartridges and the inability of the supplier to provide more C18 cartridges or the polymer sorbents for several months, investigation of these results or comparison of recoveries obtained from other SPE sorbents could not be performed. The SPE procedure was still used for method validation and sample analysis as the reactors were dosed at environmentally realistic concentrations (50 μg/L), which required preconcentration to analyse at method detection levels. The volume of 200 mL was chosen for the higher preconcentration factor (Annex 2).

4.3 HPLC

4.3.1 Method Development and Optimization

UAlg

Method development was initiated at UAlg. Conditions were selected after assessing compound physico-chemical properties and expected behaviour under experiment conditions and literature review [27,86,142,147,223](Table 1). The Agilent 1220 HPLC (isocratic elution) was used for most experiments. This HPLC had greater sensitivity for analyte detection in the environmentally realistic concentrations dosed to the SBRs, as its UV/Vis detector used a monochromator to provide the UV wavelength in contrast to the UV/Vis filters of the Varian HPLC detector. Reference standard solutions prepared in solvent (milli-q water) were used to perform the experiments (Table 8).

Mobile phases (Table 13) were tested to determine reproducibility of compound separation. Preliminary experiments ascertained influential factors and their levels for experimental design. Peaks were not visible in the mobile phases that employed water or acidified water as solvent A and methanol as solvent B. The compounds are in neutral form and hydrophobic, therefore they would be retained very strongly to the stationary phase, resulting in an extremely long retention time particularly in methanol[257]. Peaks were finally observed using water adjusted to pH 3 with acetic acid (solvent A) and acetonitrile (solvent B), 50:50 (v/v). The compounds are neutral in this pH. A wavelength scan (220, 221, 223 and 225 nm) confirmed the highest absorbance was observed at 221 nm in keeping with published literature[148,223,225]. 2OH and CBX were identified as the critical peak pair. Varying pH, type of organic solvent and % solvent B that would most influence the selectivity of the compounds could not produce separation of 2OH and CBX using water, pH 3 as solvent A. Methanol increased the selectivity of the compounds to the column

stationary phase and increased retention time, particularly for ibuprofen, which as the most hydrophobic of the compounds[257]. Acetonitrile decreased both retention and runtimes without changing selectivity (some chromatograms are provided in Annex 3). Solvent A was therefore switched to ammonium acetate buffer to change selectivity based on the ionic nature of the analytes.

Buffer concentration (10 and 25 mM), buffer pH (4, 4.5, 5), solvent B (methanol, acetonitrile) and % solvent B (30, 35, 40%) were evaluated. Increasing buffer concentration above 10 mM increased retention time particularly for ibuprofen, without influencing critical resolution. pH 5, which ionized the analytes, caused peak distortions regardless of organic solvent and percentage used. 2OH and CBX coeluted in acetonitrile regardless of buffer concentration, pH and % solvent B. Methanol in contrast gave selectivity that produce critical pair separation but gave long runtimes. The conditions that gave the best potential for critical pair separation were identified as 10 mM buffer, pH 4 and 4.5 with methanol (solvent B, 35, 40%). Flowrates higher than 1 mL/min resulted in system pressure exceeding recommended limits due to mobile phase viscosity with methanol[257].

Full factorial design (Section 3.7.1). showed the best separation was obtained in 10 mM ammonium acetate buffer pH 4.5: methanol (65:35 v/v), as displayed in Figure 25. This was confirmed by the calculated response functions (Annex 3). Attempts were made to prepare a calibration curve in the working range of 50 – 500 µg/L in preparation for evaluating performance of the SPE procedure. However, the reproducibility of replicate injections and injection volume was poor due to a problem with the HPLC injector. Linearity could not be established before the end of thesis work at UAlg, therefore method development was transferred when the thesis period shifted to USP.

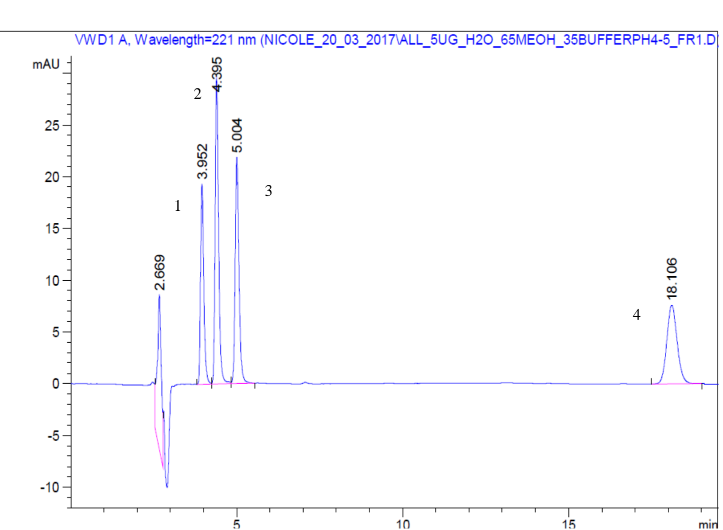


Figure 25. UAlg HPLC optimized conditions: 10 mM ammonium acetate buffer pH 4.5: methanol (65:35 v/v)

Peaks 1: CBX, 2: 2OH, 3: 1OH, 4: IBU

USP

The separation conditions developed at UAlg could not be reproduced at USP, possibly due to differences in the HPLC columns used. Both stationary phases were C18, however the manufacturers were different and the UAlg column was a chemically modified hybrid. The order of peak elution as different with CBX to elute first on UAlg column and 2OH eluting first on the USP column.

Exploratory linear gradients were performed using water acidified with formic acid, acetic acid and phosphoric acid respectively (solvent A) and methanol and acetonitrile (solvent B) as solvent A (Table 14). Water and acetonitrile, both acidified to pH 3 with phosphoric acid, produced broad peak shapes and compounds were difficult to identify from the baseline. The compound peaks were not visible in the chromatograms obtained with 3% acetic acid, 1% formic acid and water pH 3 as solvent A. A significant negative baseline drift was observed with acidified solvent A. This phenomenon occurs when solvent B absorbs at a lower wavelength than solvent A and can create problems in chromatographic integration. Solvent B was therefore acidified in the same proportion as solvent A for subsequent experiments to correct this problem. The positive baseline drift is observed as solvent B has stronger absorbance then solvent A. However this does not affect chromatographic quality[255].

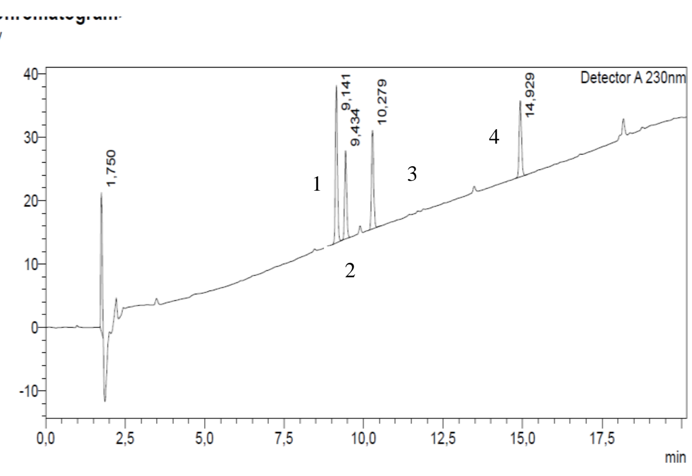


Figure 26. Exploratory gradient for compound separation in water and acetonitrile, both with 0.01% formic acid, v/v

Peaks 1: 2OH, 2: CBX, 3: 1OH, 4: IBU

Peak detection and compound separation was achieved with water (solvent A) and acetonitrile (solvent B), both acidified with 0.01% formic acid as shown in Figure 26 with the order of elution. Selectivity is based on individual compound hydrophobicity. At gradient start, low % B causes stronger mobile phase polarity. Hydrophobic compounds are therefore strongly attracted through hydrophobic interactions due to bonds formed with hydrophobic hydrocarbons of the C18 stationary phase, increasing retention time. The strength of this interaction varies according to individual molecular structure[256,257]. The retention times for each compound was confirmed. k* was 2.6 (equation 4). Compound

separation by isocratic elution was possible (Section 2.2.1, equation 1) as $\Delta tr / t_G$ value was intermediate (0.30). Isocratic elution was tested at 53% B (equation 2), but 2OH and CBX coeluted. Experiments were therefore continued in this mobile phase with gradient elution. The compounds are neutral in this acidic mobile phase.

Optimization of Separation Conditions

Gradient separation conditions and range were optimized by increasing starting %B and reducing final %B while maintaining resolution ≥ 1.5. The best temperature and detection wavelength were determined to 30^0C and 230 nm according to resolution and signal to noise ratio. The optimized conditions, displayed in Figure 27, were linear gradient 25-80% in 16 min at 30^0C, flow rate of 2 mL/min with k* of 4.9.

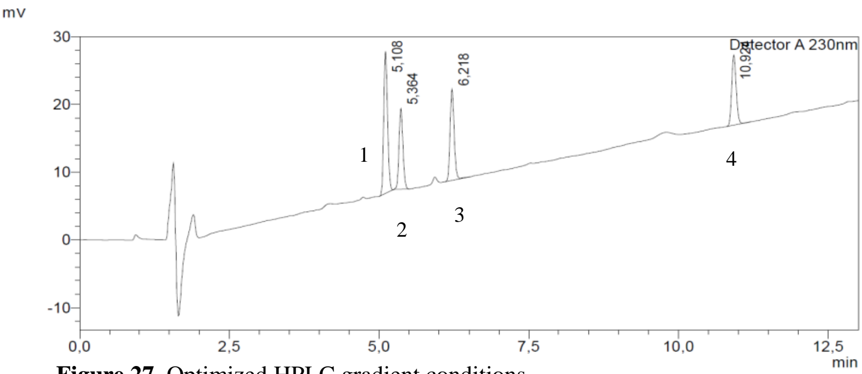


Figure 27. Optimized HPLC gradient conditions
water (solvent A), acetonitrile (solvent B), both acidified with 0.01% formic acid;
Peaks 1: 2OH, 2: CBX, 3: 1OH, 4: IBU

Figure 28 shows the final optimized conditions with confirmation of the suitability of benzoic acid as internal standard as it did not coelute with the target analytes. Table 26 shows the chromatographic quality parameter results. Analysis in synthetic wastewater showed no interferences with compound elution and similar retention times and resolution were observed (Annex 3).

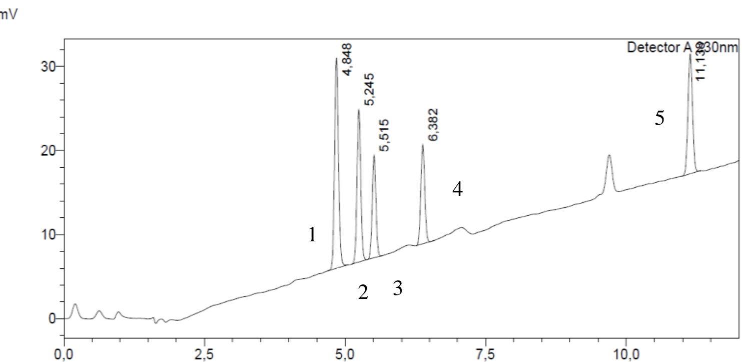


Figure 28. HPLC: Verification of benzoic acid suitability as internal standard

water (solvent A), acetonitrile (solvent B), both acidified with 0.01% formic acid; Peaks 1: BA, 2: 2OH, 3: CBX, 4: 1OH, 5: IBU

Table 26. Chromatographic quality parameters for optimized HPLC method

Compound	Retention time	Rs	Column Efficiency*	Tailing Factor
Benzoic acid	4.848		18419	1.1
2OH	5.248	2.8	23146	1.1
CBX	5.515	2.0	25505	1.0
1OH	6.382	6.1	30722	1.0
IBU	11.138	31.1	76849	1.0

*N ≥17500 is recommended for 25 cm column[257]

4.3.2 Validation

Validation was performed in solvent (milli-q water) as described in Section 3.8. The results are shown in Table 27.

Table 27. Validation results for HPLC method in milli-q water

Compound	Linear equation	r^2	r	LOD µg/L	LOQ µg/L	Range µg/L	Precision	
							Repeatability %CV	Intermediate Precision
1OH	y=0.0341x -0.0017	0.9766	0.9882	3.2	9.5	9.5 - 125	1.0	0.05
2OH	y=0.041x-0.0032	0.9795	0.9897	3.8	11	11 - 125	9.4	0.04
CBX	y=0.0347x-0.0018	0.9803	0.9901	4.3	13	13 - 125	1.6	0.04
IBU	y=0.0234x-0.0052	0.7477	0.8647	7.5	23	23 - 125	2.5	0.04

The individual compound retention times and peak resolution confirmed specificity. Linearity was assessed for a working range of 15 –125 µg/L, based on the concentrations dosed to the SBR (50 and 100 µg/L). Two calibration curves were prepared in solvent (milli-q water) and matrix (synthetic wastewater) respectively (Table 11). SPE used to provide a preconcentration factor of 200 bringing detection to the range of 3000 – 25,000 µg/L. The variances of the standard solution series were evaluated for statistically significant difference using Hartley's-F_{max} test as described in Section 2.10.2. 2OH, CBX and IBU passed, however 1OH did not (F_{max} values are shown in Annex 1b).

The calibration charts were nevertheless plotted. The linear equation and regression parameters for each compound are shown in Table 27. The 2OH curve is shown in Figure 29 and the remaining curves are given in Annex 4a. Residual plots were prepared to confirm linearity, particularly for IBU (Annex 4b). The plots for all compounds plots show random distribution for the first four standard concentrations indicating good linearity, however there is a sharp

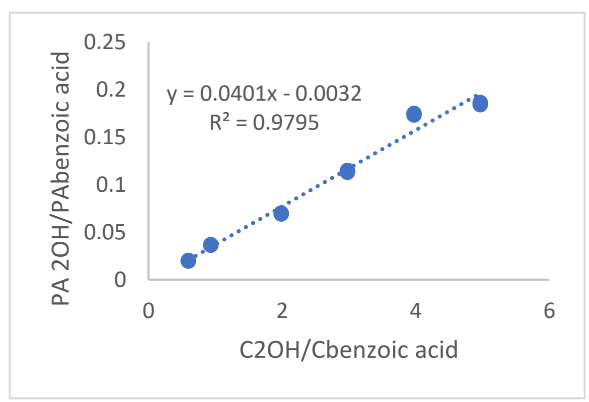


Figure 29. HPLC calibration curve for 2OH in water

increase in standard deviation from concentration 4 to concentration 5 (100 µg/L), followed by a decrease to concentration 6 (125µg/L). This could be due to matrix effect from the SPE sorbent or volumetric errors during sample preparation[292].

LOD was determined using the residual standard deviation and deviation of the y-intercept obtained from a calibration curve of the three lowest concentrations. The value which is the ratio of compound concentration to concentration of BA, was converted to concentration by multiplying with BA concentration (5000 µg/L) and then dividing by the preconcentration factor of 200. The LODs passed the acceptance tests for correct calculation (Annex 4c). LOQ was calculated from LOD, however the IBU LOQ is higher than the minimum calibration concentration. The range was determined from LOQ to the maximum calibration standard used.

Repeatability was assessed using solutions of 25, 50 and 100 µg/L. Outliers were evaluated using the Dixon's Q-test (Values given in Annex 1c). 2OH was the only compound that did not pass. The variances of the standard solution series were evaluated for statistical significant difference using the Hartley's F_{max} test. IBU and 2OH failed the test (Annex 1b). determined from LOQ to the maximum calibration standard used. Repeatability still calculated as the average %CV, although some of the compounds failed the statistical tests. Repeatability is acceptable for all compounds according to AOAC recommendations (<21 % for 10 ppb, <15% 100 ppb)[308]. The solutions were injected on a second day for intermediate precision, which was calculated as SD of all 30 measurements.

Method recovery, shown in Table 28, was determined by calculating the concentrations obtained in three spiked solutions processed through SPE (25, 50 and 100 µg/L) from the calibration curve. 2OH is the only compound with overall recoveries close to AOAC recommendations (60 - 115% for 10 ppb, 80-110% for 100 ppb)[308]. 1OH and CBX had good recoveries for 50 and 100 µg/L and high for 25 µg/L. IBU had good recoveries for 50 µg/L and high recoveries for 25 and 100 µg/L. This indicates possible matrix effects from the sorbent or sample preparation.

Table 28. HPLC: Recoveries obtained for the compounds, n=1

Concentration, µg/L	25	50	100
Compound		% Recovery	
2OH	75	94	99
1OH	124	103	105
IBU	130	110	127
CBX	122	106	102

Comparison of the Solvent and Matrix Matched Calibration Curves

Statistically significant variance in the synthetic wastewater standard solution series was assessed using the Hartley's F_{max} test. Only 1OH passed the test (Annex 1). The calibration charts were nevertheless plotted for each compound. 2OH is shown in Figure 30. The curves for the remaining compounds are given in Annex 4a. The regression parameters are given in Table 29. Residual plots were prepared to confirm linearity (Annex 4b). For CBX and 1OH, the residuals appear to be randomly distributed. However, for IBU and 2OH, standard deviation problems are visible.

Table 29. Regression parameters for synthetic wastewater calibration curves

Compound	Linear equation	R^2	r
1OH	y=0.0342x + 0.0004	0.9893	0.9946
2OH	y=0.0391x - 0.0034	0.9927	0.9964
CBX	y=0.0342x + 0.0002	0.9910	0.9955
IBU	y=0.0264x - 0.0068	0.9238	0.9611

The y-intercepts of the two curves were compared using student's t-test (significance level $\alpha = 0.05$) to assess for statistically significant difference, as described in Section 2.10.1. t_{cal} were 0.28 (2OH), 1.85 (1OH), 0.06 (IBU) and 0.09 (CBX). There was no statistically significant difference between the curves as their t values are lower than $t_{cri\ f=58} = 2.005$.

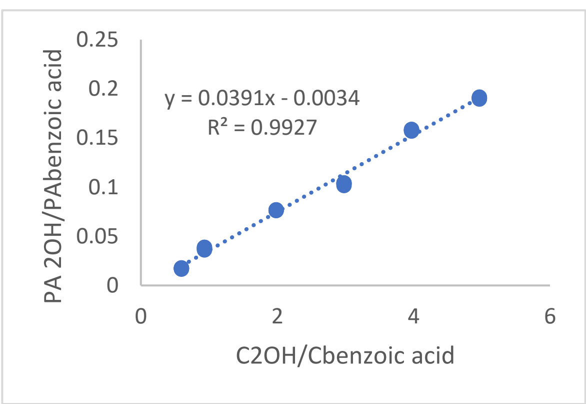


Figure 30. HPLC calibration curve for 2OH in synthetic wastewater

4.4 Capillary Electrophoresis

4.4.1 Method Development and Optimization

Preliminary analysis was performed using 10 mM borate buffer (pH 9) as BGE. Individual migration times were identified and effective mobilities from pH 0 to 12 were calculated. The response function had its maximum value at pH 5. The effective mobility curves for each compound and response function were plotted as a function of pH (Figure 31). Experiments in acetate buffer were performed to confirm the suitability of this pH. Buffer concentration, hydrodynamic injection mode and separation voltage were tested (Table 17). Figure 32 shows the best conditions (50

mM, normal polarity, 20kV) identified for compound separation and order of elution. The isomers 2OH and 1OH were identified the critical peak pair.

During the experiments, high migration times and broad peak shape were observed for CBX. Additionally, the peak did not appear in some experiments performed to verify reproducibility of the separation conditions. This behaviour can be explained by the conversion of the acidic compounds to anions at this pH. As a double anion, due to the deprotonation of its carboxylic acid groups, CBX has the slowest effective mobility and migrates against the EOF, towards the anode. The EOF and ionic strength of the buffer is too low to overcome this effect, due to high buffer concentration (50 mM). CBX may therefore either migrate slowly to the detector or its peak is not visible on the electropherogram as it exists the capillary towards the anode[236,258,267]. Reducing the capillary length improved migration time but did not correct its problem of erratic non-detection. With this evaluation, it was decided to assess the separation of the compounds using borate buffer (pH 9), which is recommended to create conditions for faster effective mobilities for the four anions[246].

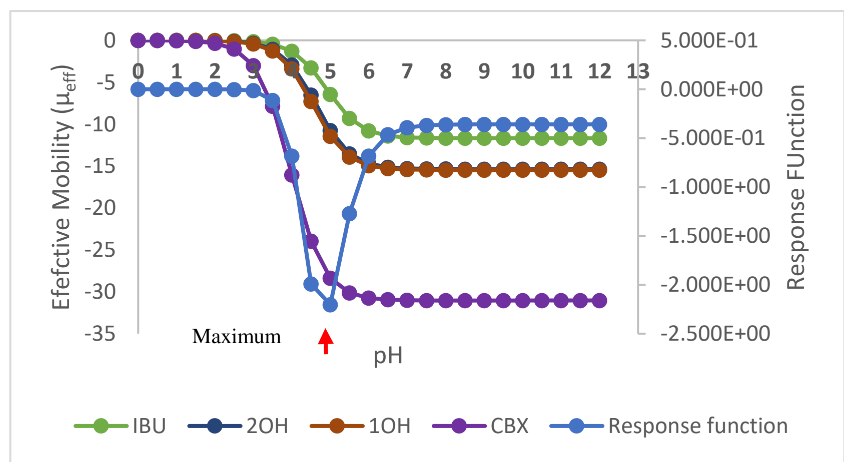


Figure 31. Graph of effective mobilities and response function vs pH

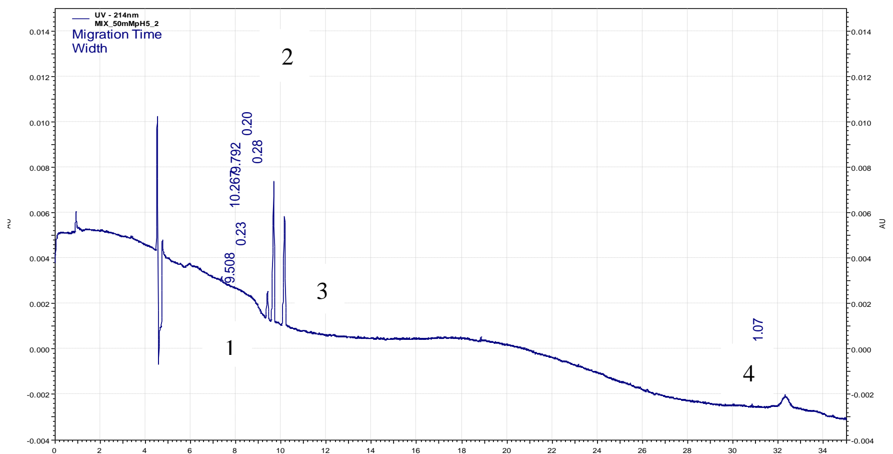


Figure 32. Electropherogram of separation of 2OH, 1OH, IBU and CBX in acetate buffer, pH 5

Peaks 1: 2OH, 2: 1OH, 3: IBU, 4: CBX

Testing of Separation Factors in Borate Buffer

Buffer concentration (10, 15, 20, 25 and 30 mM), surfactant type (SDS, CTAB), surfactant concentration (20, 30 and 40 MM for SDS; 0.2 mM for CTAB), organic solvent and percentage of organic solvent (methanol–10%, acetonitrile –10, 20%, THF–5, 10, 15 and 20%), and separation voltage (20 and 25 kV) were examined to identify possible separation conditions (Table 17). Critical pair separation was difficult to achieve. Initially, one factor was varied with the others kept at constant level due to unavailability of literature on the separation of these compounds. Migration time increased proportionally to buffer concentration and percentage of organic solvent, but did not change selectivity. Varying organic solvent and addition of only SDS and cetrimonium bromide did not produce separation. SDS concentration above 40 mM increased buffer viscosity and the likelihood of high current and Joule effects. Unexplainable, peaks were not observed with CTAB. Their mobilities may have been too fast for detection at that voltage. 20kV was chosen as the separation voltage for subsequent experiments as higher voltages increased current and potential for Joule effects while lower voltages increased migration and analysis times.

Separation of the critical pair was finally obtained under MEKC conditions with 20 mM borate, 20 mM SDS and 10% THF. The elution order was 2OH, 1OH, IBU and CBX. Concentration controlled selectivity by decreasing electrolyte ionic strength and EOF, leading to changes in electrophoretic mobilities of the analytes[270]. Increasing buffer concentration above 20 mM lengthened the migration time of CBX and analysis time with slight improvement to critical resolution.

SDS changes the selectivity of these hydrophobic compounds by increasing their solubility and reducing hydrophobic interactions for decreased mobility that separates the isomers [238,274,275]. This was reflected in the higher migration times observed with SDS addition compared with those in borate buffer only. As an anionic surfactant, SDS micelles electrostatically migrates towards the anode. Yet, normal polarity can be used for separation as EOF velocity prevails to carry the micelles towards the cathode and detector, albeit with slow migration (Figure 33)[260,275].

In MEKC, organic solvents change selectivity by the dual effect of decreasing EOF by increasing viscosity and modifying the partition coefficient of the hydrophobic compounds between the micelles and electrolyte[260,272]. Consequently, both separation and migration of the compounds increase. Methanol and acetonitrile

are more commonly used, however the aprotic THF was selected for its potential to create better selectivity between the two isomers. THF has been applied to CE to successful separation of quinolones which have similar pKas and functional groups (carboxylic acid) as the compounds of this study [315]. Research has shown the better capacity of THF to disrupt hydrogen bonds and interact with OH groups in comparison to methanol[316].The presence of the two methyl groups at the 2^0carbon atom in 2OH, creates a crowded space for more steric hindrance of hydrogen bond formation between the OH group and THF, compared to the position of the OH group in 1OH. This results in weaker hydrogen bonding between 2OH and THF promoting the selectivity difference between the two isomers [317]. It was observed that increasing THF above 15% resulted in a high migration time of CBX and resulting analysis time.

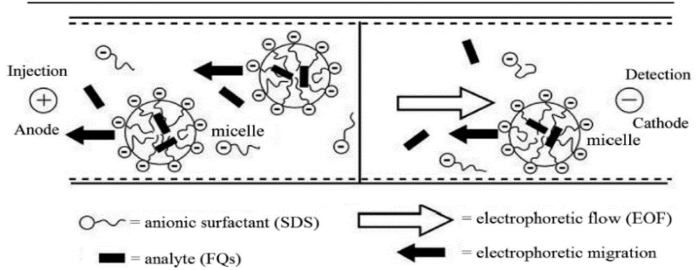


Figure 33. Representation of MEKC separation with SDS[275]

Optimization of Separation Conditions and Evaluation of Response Functions

Full factorial experimental design (Tables 22 and 23) was performed to optimize critical resolution with buffer concentration, % THF and SDS concentration. The levels were set from the preliminary experiments to give separation and avoid high current and Joule effects. The results are shown in Figure 34. It was observed that increasing buffer concentration, SDS concentration and %THF improved resolution of the critical pair and overall peak resolution. The calculated response functions (Section 2.6) are displayed with the runtime in Table 30. The quality of separation (low CRS, low CEF, high CRF, high R_{sum} and high R_{cp}) was evaluated.

Table 30. Response function results for the 2^3 factorial design

Electrolyte	CRS	CRF	CEF	R_{sum}	R_{cp}	RP	Runtime (min)
1	10	60	408	38	1.0	2	7.0
2	11	57	308	37	1.1	2	7.5
3	2.0	66	7.8	39	1.8	4	7.0
4	3.6	68	7.8	43	2.2	4	8.5
5	59	72	32	54	1.5	2	9.5
6	27	69	47	52	1.4	2	10
7	5.3	77	10	56	2.7	4	11
8	5.8	79	11	60	3.5	4	12.5

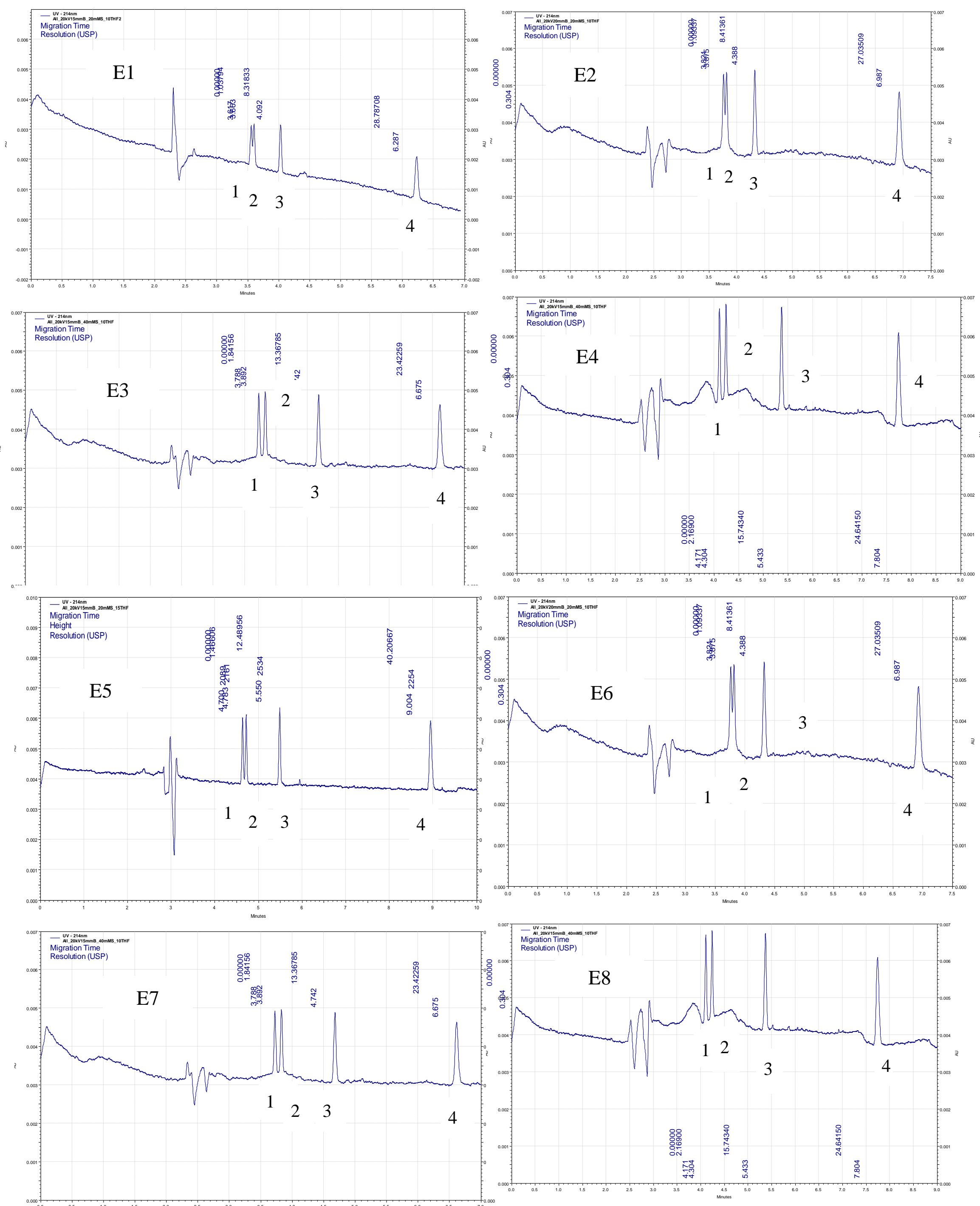


Figure 34. 2^3 factorial designed electropherograms   Peaks 1: 2OH, 2: 1OH, 3: IBU, 4: CBX

The CRS and CEF ranks experiment 3 as the best condition followed by 4, 7 and 8. Experiments 8 followed by 7 has the highest CRF, R_{sum} and R_{cp}. Experiments 3, 4, 7 and 8 have all peaks separated. Evaluating all response functions, the conditions of experiment 3 (15 mM borate, 40MM SDS, 10% THF) were selected due to complete separation of all four compounds, critical resolution of 1.8 and short runtime. Figure 35 shows the initial optimized conditions including the suitability of benzoic acid as internal standard as it did not coelute with the target analytes.

Analysis in synthetic wastewater showed no interferences with the elution of each compound and similar retention times and resolution (Annex 5).

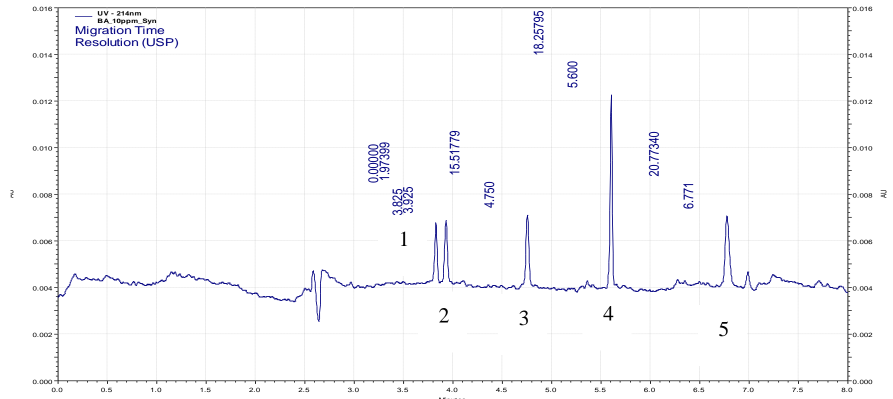


Figure 35. Electropherogram showing the compounds with internal standard benzoic acid 15 mM borate, 40mM SDS, 10% THF; Peaks 1: 2OH, 2: 1OH, 3: IBU, 4: BA, 5: CBX

In subsequent analysis, critical resolution (≥ 1.5) was no longer achievable with these conditions. The separation conditions of experiment 7, 15 mM borate, 40 mM SDS and 15% THF, were therefore selected for the largest critical pair resolution and positive evaluation with all response functions. This was used to perform method validation. This effect is possibly to the modifications of the capillary wall surface that occur in CE analysis due to the BGE and sample[258,260]. This buffer in combination with SDS was frequently used for NSAID separation as earlier discussed. Problems with changing conditions were not reported. Some researchers condition the capillary in between runs to avoid this problem, however attempts to return to the experiment 3 conditions by conditioning of the capillary or changing the capillary were not successful. Figure 36 shows the final optimized separation conditions achieved with stacking (water plug injected 16s after the sample). The migration times are given in Table 31. An analytical run was performed using 15 mM borate, 40 mM SDS with 20 % acetonitrile, the latter is equivalent to 10% THF. Methanol was not tested as longer analytical times are usually observed [233]. A longer runtime (9.5 min) and lower critical resolution (1.7) was observed in comparison to the optimized conditions (Annex 5). Additionally, baseline separation between the critical pair was not achieved.

Table 31. Chromatography quality parameters for optimized MEKC method

Compound	Migration time	Rs	Column Efficiency	Tailing Factor
2OH	4.646		41581	1.0
1OH	4.808	1.8	41138	0.9
IBU	6.254	12.6	29844	0.9
Benzoic acid	7.621	9.5	43468	1.0
CBX	9.821	13.1	37547	1.1

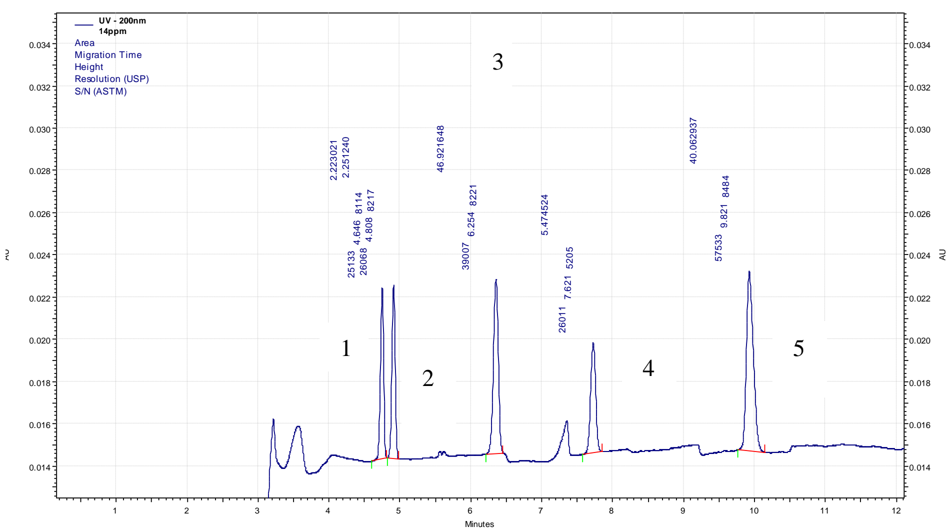


Figure 36. MEKC separation using 15 mM borate, 40 mM SDS, 15% THF

Peaks 1: 2OH, 2: 1OH, 3: IBU, 4: BA, 5: CBX

4.4.2 Stacking

On-line preconcentration was investigated as described in Section 3.5.3 using field amplified sample stacking with the electrolyte conditions of 15 mM borate, 40mM SDS and 10% THF. The placement of the milli-q water plug (before and after sample), length of injection of water plug (t=0, 2, 3, 5, 7, 10, 12, 16, 18, 20, 30s), length of injection time for the sample (t= 3s, 6s), addition of organic solvent to the water plug in the same ratio as the electrolyte were examined. The solvent (milli-q water) has lower conductivity than the BGE. The technique was combined with an element of field-amplified sample injection by injection of a water plug into the capillary[318]. The compound peaks were not visible in experiments where the sample was injected electrokinetically. The injection of the water plug before the sample gave better peak heights (Figure 37). Injecting the water plug after the sample, tended to cause distortion in the CBX peak and smaller heights. Increasing sample injection time resulted in decreased critical resolution. In subsequent analysis, the separation conditions were changed to 15 mM borate, 40 mM SDS and 15% THF as shown in Figure 36. The water plug was modified to include 15%THF to improve

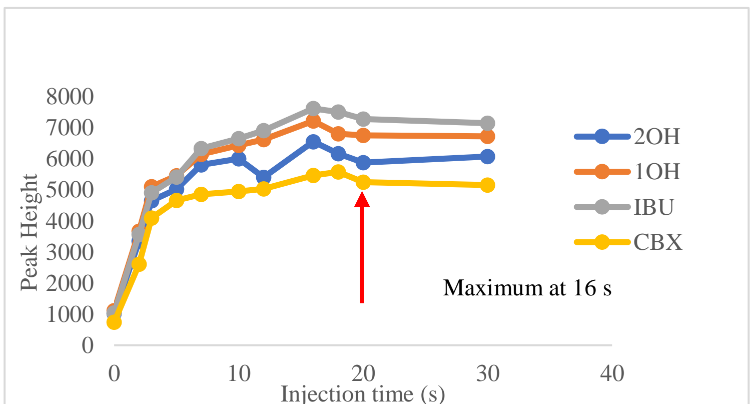


Figure 37. Peak heights obtained with injection of the water plug before the sample

peak shape. Higher peak shapes were now obtained with the water plug injected after the sample for 16s, influenced by the addition of the organic solvent to the water plug. The common practice is to inject the plug before the sample, followed by injection of anions in reverse polarity and finally switching to normal polarity for separation[284–286]. By injecting water after the sample, the anions which migrate to the anode, stack at the back of the sample/electrolyte zone. This is opposite to the effect seen in Figure 14. The strength of the BGE EOF carries the anions to the cathode and detector. A wavelength scan performed after the stacking experiments showing the highest compound absorbance at 200 nm.

4.4.3 Method Validation

Validation was performed in the solvent (milli-q water) as described in Section 3.8. The results are shown in Table 32.

Table 32. Validation parameters for MEKC curve in solvent

Compound	Linear equation	r^2	r	LOD µg/L	LOQ µg/L	Range µg/L	Precision	
							Repeatability %CV	Intermediate Precision
1OH	y=0.05 9+ 0.0024	0.9600	0.9798	3.3	9.9	9.9 - 125	1.8	0.1
2OH	y=0.0569 + 0.0023	0.9680	0.9838	3.9	12	12 - 125	3.5	0.1
CBX	y=0.149x + 0.0096	0.9660	09829	5.1	15	15 - 125	2.7	0.2
IBU	y=0.053x - 0.0066	0.6341	0.7962	15	45	45 - 125	19	0.9

Specificity was obtained from the separation of all four compounds and confirmation of their individual migration times in water (Figure 36). The critical pair resolution decreased to 1.7, which was lower than that observed in method optimization. Additionally, the migration time for each compound changed. However, the obtained resolution (≥ 1.5) was acceptable.

Linearity was assessed for a working range of 15 –125 µg/L, based on the concentrations dosed to the SBRs (50 and 100 µg/L). Two calibration curves were prepared in solvent (milli-q water) and matrix (synthetic wastewater) respectively (Table 11). SPE provided a preconcentration factor of 200 bringing detection to the range of 3000 – 25,000 µg/L. Variances in the standard solution series was evaluated for statistically significant difference using Hartley's F_{max} test (Section 2.10.2). 1BU was the only compound that passed the test (F_{max} values are shown in Annex 1b). The calibration charts were nevertheless plotted. The linear equation and regression parameters for each compound are shown in Table 32. The curve for 2OH is shown in

Figure 38 and the remaining curves are given in Annex 6a. Residual plots were prepared to assess linearity, particularly for IBU (Annex 6b). The plots show that standard deviation appears to increase with concentration. This could be due to matrix effect or volumetric errors during sample preparation[292].

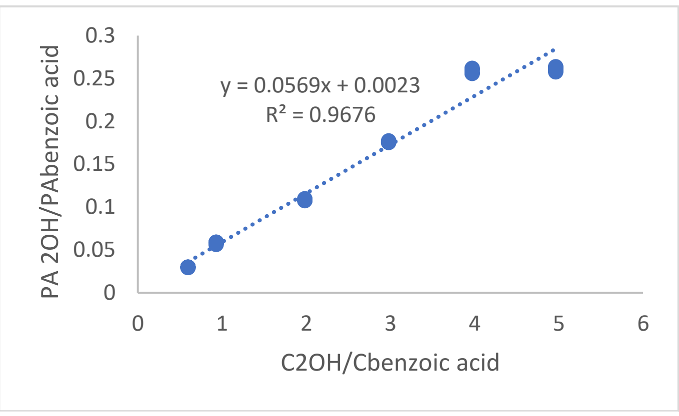


Figure 38. MEKC calibration curve prepared for 2OH in milli-q water

LOD was determined using the residual standard deviation and deviation of the y-intercept obtained from a calibration curve of the three lowest concentrations. The value which is the ratio of compound concentration to concentration of BA, was converted to concentration by multiplying with BA concentration (5000 µg/L) and then dividing by the preconcentration factor of 200. The LODs passed the acceptance tests for correct calculation (Annex 6c), except for IBU, whose LOD was the same value as the minimum concentration. LOQ was calculated from LOD with CBX and IBU LOQs higher than the minimum calibration concentration. The range was determined as LOD concentration to the maximum calibration standard.

Repeatability was obtained using solutions of 25, 50 and 100 µg/L. Outliers were assessed using the Dixon's Q-test (Values given in Annex 1c). IBU and CBX passed the test, 2OH and 1OH did not. Variances in the standard solution series was evaluated for statistically significance difference using the Hartley's F_{max} test. 1OH passed the test, 2OH, IBU and CBX did not (Annex 1b). Repeatability was still calculated as the average %CV and was acceptable for three compounds except IBU according to AOAC recommendations for analyte concentration(<21 % for 10 ppb, <15% 100 ppb) [308]. The solutions were injected on a second day to obtain intermediate precision, which was calculated as SD of all 36 measurements.

Method recovery was determined by calculating the concentrations obtained in three spiked solutions processed through SPE (25, 50 and 100 µg/L) from the calibration curve. The results are shown below in Table 33. 2OH, 1OH and CBX had overall reasonable recoveries according to AOAC recommendations (60 - 115% for 10 ppb, 80-110% for 100 ppb)[308]. IBU had significantly high results which indicates possible matrix effects from the sorbent or sample preparation including pre-treatment.

Table 33 Recoveries obtained for the compounds in MEKC, n=1

Concentration, μg/L	25	50	100
Compound		% Recovery	
2OH	104	103	105
1OH	116	103	115
IBU	211	134	1111
CBX	112	107	107

Comparison of the Solvent and Matrix Matched Calibration Curves

Statistically significant variance in the synthetic wastewater standards was assessed using the Hartley's F_{max} test. Only 1OH passed the test (Annex 1b). The calibration charts were nevertheless plotted. 2OH is shown in Figure 39. The curves for the remaining compounds are given in Annex 4. The regression parameters are given in Table 34. Residual plots were prepared to confirm linearity, especially for IBU (Annex 6). For 2OH and 1OH, the residuals appear to be randomly distributed, however with great variation in the standard deviations. A problem of increasing standard deviation is visible for IBU and CBU.

Table 34. Regression parameters for synthetic wastewater calibration curve

Compound	Linear equation	R^2	r
1OH	y=0.0505x + 0.0142	0.9714	0.9855
2OH	y=0.0457x + 0.0122	0.9798	0.9898
CBX	y=0.18242 - 00817	0.9838	0.9918
IBU	y=0.1195x - 0. 817	0.3224	0.5678

Comparison of the y-intercept of the two curves using student's t-test (significance level $\alpha = 0.05$) as described in Section 2.10.1 showed statistically significant difference between the curves. t_{cal} values were 10.4 (2OH), 9.1 (1OH), 3.3 (IBU) and 12.5 (CBX), which are higher than $t_{cri\ f=70} = 1.994$.

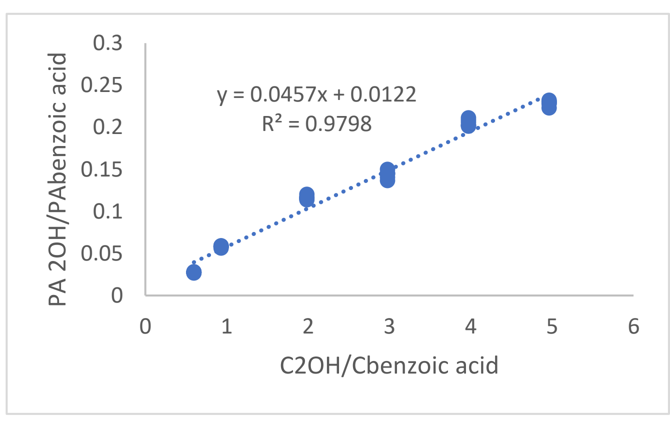


Figure 39. CE calibration curve for 2OH in synthetic wastewater

4.5 Application of HPLC and MEKC Methods to Real Samples

Despite, the poor validation results, both methods were applied to analyse samples taken from the aerobic SBRs as described in Section 3.3.3. The methods were used to assess the system's ability to degrade ibuprofen or if ibuprofen and its metabolites were present in quantifiable amounts. The reactors were dosed with 50 and

100 µg/L on day 21 and 27 respectively. Synthetic wastewater and milli-q water controls were used to evaluate if degradation occurred due to the system or other means. The controls were prepared at the same time as the reactor influent containing ibuprofen and dosed in the same concentration tested. The controls were not passed through the reactor, but allowed to stand for the length of the cycle during which sampling was performed. Analysis was performed only for the 50 µg/L samples due to limited number of SPE cartridges.

A total of 16 samples was analysed. The solvent calibration curve was used to analyse the samples for both methods, although there was statistically significant difference between the solvent and matrix curves for the MEKC method. This was performed due problems with method robustness that limited analysis time for submission of data to thesis deadline. In MEKC, CBX was the only compound detected in method limits, in two samples (Annex 5 shows an electropherogram of a sample where no compound was detected). These were found in sample T5 taken from the aerobic reactor corresponding to 150 min and in the synthetic wastewater control. 2OH, 1OH and IBU were not detected in any samples. However, the concentrations calculated using the solvent calibration curve for both samples were extremely high and exceeded method range. This could be the result of several factors. As discussed in Section 1.5, CBX tends to be the prevalent metabolite in ibuprofen degradation[20,27–33]. Degradation could have occurred in the granules to concentrations below method detection limits. Additionally, linearity ($r < 0.99$) for CBX calibration curve was poor, which would influence calculated concentrations.

In HPLC, no peaks corresponding to CBX were detected in any sample including the control and T5 aerobic. 1OH was the only compound detected. Figure 40 shows the difference in the compounds detected in each method. The 1OH concentrations calculated for the synthetic wastewater control, water control and the sampling time points are illustrated on Figure 41. Calculated 1OH values were significantly high and exceeded method range. The anaerobic reactor has the lowest concentration at the end of the time cycle. While the aerobic reactor has the highest concentration during aeration and at the end of the cycle. This SBR therefore seems less efficient at removing at least one metabolite. Both SBRs appear to degrade IBU, 2OH and CBX in both anaerobic/aerobic and aerobic operating cycles. This has great potential as generally ibuprofen degradation requires highly aerobic conditions and contrasts with the study of Zhao et al, which observed only 34 – 45% removal[204].

Ibuprofen was not detected in the synthetic wastewater and milli-q water controls by either method. It has been observed that metabolites have been detected in WWTP influent, indicating the degradation of the compound before entry to WWTP[32,87,102]. It may be possible that this same effect occurred in the controls, however repetition of the experiments is required for verification. It is possible that some degradation could have occurred during transport of the samples for analysis to Brazil. The samples were lyophilized but were detained for one month by the national customs agency. The delay under transport and storage conditions may have compromised results.

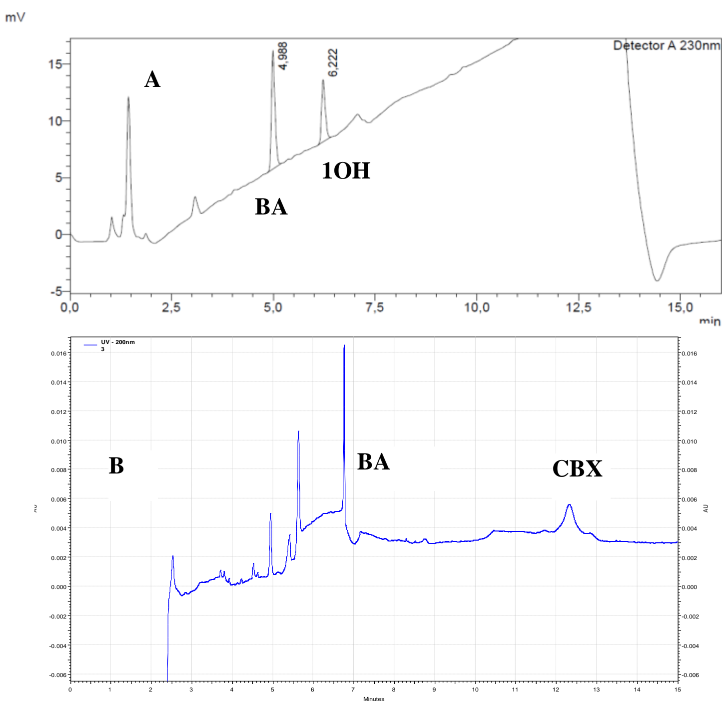


Figure 40. Comparison of peaks detected in the synthetic wastewater control A. HPLC B. MEKC

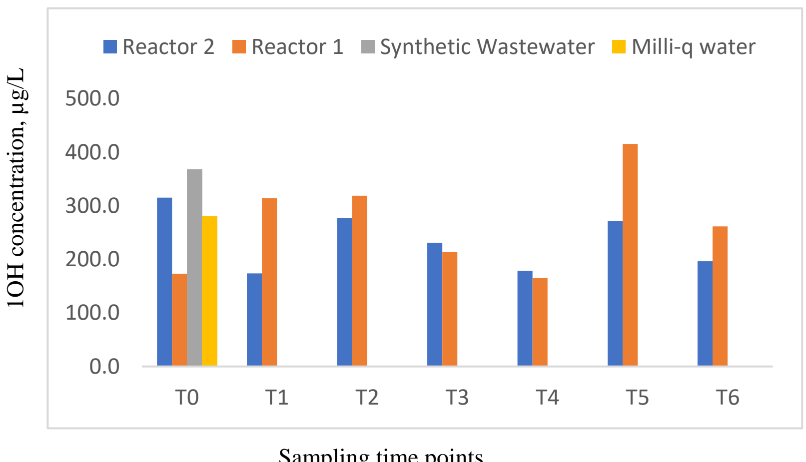


Figure 41. Determined 1OH concentrations using HPLC method

4.6 Comparison of the Methods and Robustness

The methods detected different analytes, therefore instead of student's t test, they were compared on validation parameters. Both methods gave selectivity and separation of the compounds. Analysis time was also comparable. However over time, the conditions in both methods show signs of problems with robustness. Robustness was a larger problem with CE. Much time was spent trying to reproduce separation conditions. CBX peak distortion was observed in some analytical runs and decreasing resolution of the critical peak pair (Figure 42). For HPLC, a problem appeared when analysing the low concentrations of the calibration curve, which could account for the repeatability results for 2OH. The resolution between benzoic acid and the first compound peak, 2OH decreased below the recommended limit to 1.2 (Figure 43). This peak distortion affected precision results. Both methods experienced a 1-2% variation in retention time, which is common given daily lab work. However, this may have contributed to distortion of the CBX peak. For HPLC, a significant difference in compound peak height was observed in 50 µg/L standard preparation for the SPE trials compared with the solvent calibration curve (Annex 2).

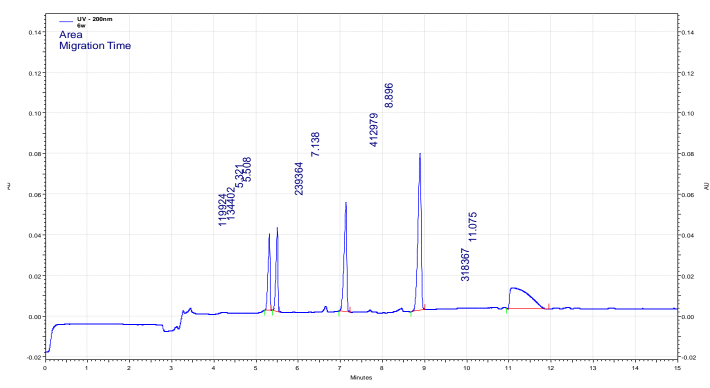


Figure 42. CE experiment optimized conditions used for method

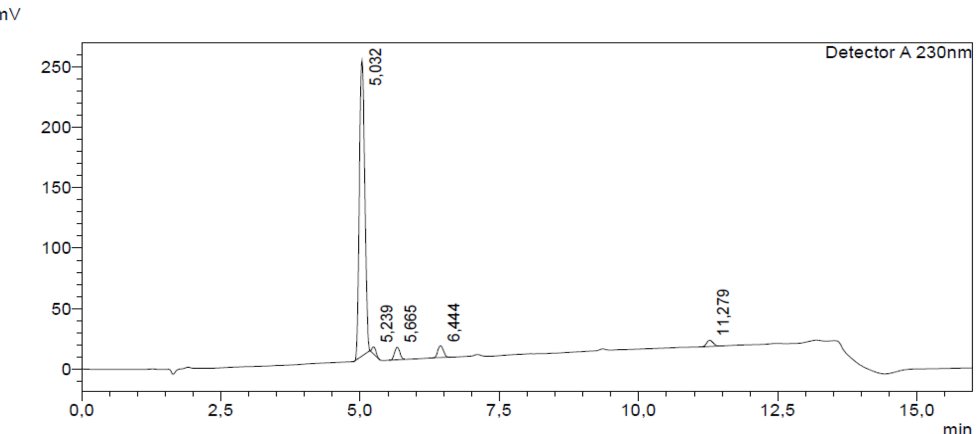


Figure 43. Chromatogram of benzoic acid merged with 2OH in standard concentration 15 µg/L

The validation results obtained for each method are compared in Table 35. Recoveries were highly variable between both methods, concentrations tested and compounds. For HPLC, recoveries within AOAC guidelines were achieved for 2OH, 1OH and IBU had both high and low recoveries while CBX had very low recoveries. In contrast, 2OH and 1OH had recoveries within AOAC guidelines in the MEKC method, while the majority for CBX recoveries were low. IBU recoveries were unrealistically high. HPLC gives better linearity and intermediate precision for 2OH 1OH and CBX. IBU has poorer linearity in HPLC compared with MEKC, but the former provides better intermediate precision. Method sensitivity (LOD and LOQ) for all compounds is higher in HPLC than MEKC, despite the use of both off-line and on-line preconcentration for MEKC. This is most likely due to the observed deterioration in experimental conditions. Repeatability for 1OH, CBX and IBU was superior in HPLC, while 2OH has best results in MEKC. Both methods experienced failure of some standard solution results to meet the statistical tests for repeatability and linearity. Further statistical treatment observed that the methods would pass the statistical tests only with the reduction of the working range to 15 – 100 µg/L and reduction in the number of replicates. SPE, HPLC and MEKC validation will have to be repeated to achieve methods properly suited for their intended application.

4.7 Comparison to Published Literature

A comparison of the validation results, experimental conditions and application of the HPLC and MEKC methods to four published studies that investigated these compounds are given in Tables 35 and 36[27,29,30,142]. These studies investigated the biodegradation of ibuprofen and its metabolites and detection in aquatic matrices.

The SPE procedure used in this study was adapted from Paíga et al.[29] and several authors[22,27,29,72,147,212,240,298,299]. Polymer sorbents were used as the compounds were analysed with other pharmaceuticals with the exception of Ferrando-Climent et al[27]. Validation recoveries were published only by Ferrando-Climent and Paíga. Most of the recoveries observed by Ferrando-Climent and Paíga using Oasis HLB and Strata-X polymer SPE cartridges were close to AOAC guidelines and met the United States Environemtal Protection Agency recommendations for LC-MS/MS methods[319].

The four methods couple LC analysis to mass spectrometry detectors, in contrast to the UV/Vis detectors used, to enhance method sensitivity and selectivity. The HPLC mobile phase of this study is similar to Boix et al with the substitution of acetonitrile as solvent B[30]. Ferrando-Climent and Paiga assessed the possibility of

matrix effect arising from differences in the sample matrix and calibration standard solution matrix. Ferrando-Climent observed no matrix effect while Paiga noted ion enhancement for ibuprofen. Both applied internal standard calibration to correct for this effect.

Ferrando-Climent, Larsson and Paiga published results of validation for linearity, LOD, LOQ and precision. Linearity, expressed in these papers as r^2, was established, with all values > 0.99. Precision (repeatability and intermediate precision) were comparable to those obtained in this study for HPLC and MEKC. Ferrando-Climent, and Paiga had greater method sensitivity than the HPLC and MEKC methods, with LOD and LOQs in ng/L levels. However, the methods of this study had lower LODs and LOQs than Larsson et al. due to the selected working range.

Table 35. Comparison of validation results of HPLC, MEKC and other methods developed for compounds of study

Study	r^2	LOD µg/L	LOQ µg/L	Calibration curve range µg/L	Precision		% Recoveries, %RSD
					Repeatability %CV	Intermediate Precision	
1OH							
HPLC[a]	0.9766	3.2	9.5	15-125	1.0 (n=5)	0.05 (n=5)	124; 103; 105 (n=1)
MEKC[a]	0.9600	3.3	9.9	15 - 125	1.8 (n=6)	0.1 (n=6)	114; 110; 130 (n=1)
Ferrando-Climent et al., 2012[27]	0.9975	0.02232[b] 0.01723[c] 0.01664[d]	0.0742[b] 0.0572[c] 0.0554[d]	0.1 -100	1.61 (n=5)	0.16 (n=5)	87.6 (5.8%); 79.0 (6.9%); 75.0 (3.4%) (n=3)
Larsson et al., 2014[142]	0.9982	28.2	93.9	250-2000	<9	<9	Not provided
Paíga et al., 2015 [29,,e]	0.9974	0.0039	0.0118	0.1-1000 Linear range: 5-100	4.48 (n=6)	2.70 (n=6)	85.2 (2.8%); 80.5 (2.5%); 83.7 (2.8%) (n=3)
2OH							
HPLC[1]	0.9795	3.8	11	15-125	9.4 (n=5)	0.04 (n=5)	75; 94; 99 (n=1)
MEKC[1]	0.9680	3.9	12	15 - 125	3.5 (n=6)	0.1 (n=6)	104; 103; 105 (n=1)
Ferrando-Climent et al., 2012	0.9979	0.0210[b] 0.0163[c] 0.0101	0.0699[b] 0.0545[c] 0.0336[d]	0.1 -100	2.12 (n=5)	12.61 (n=5)	41.1 (16.2%); 70.8 (2.7%); 119.8 (0.8%) (n=3)
Larsson et al., 2014	0.9995	21.0[d]	69.9	250-2000	<9	<9	Not provided
CBX							
HPLC[1]	0.9803	4.3	13	13 -125	1.6 (n=5)	0.04 (n=5)	122; 106; 102 (n=1)
MEKC[1]	0.9660	5.1	15	15 - 125	2.7 (n=6)	0.2 (n=6)	11; 107; 10 (n=1)
Ferrando-Climent et al., 2012	0.9909	0.0234[b] 0.0232[c] 0.0085[d]	0.0781[b] 0.0073[c] 0.0285[d]	0.1 -100	3.22 (n=5)	14.80 (n=5)	190 (19.0%); 74.5 (1.6%); 58.8 (4.4%) (n=3)
Larsson et al., 2014	0.9990	58.0	193.1	250-2000	<9	<9	Not provided
Paíga et al., 2015	0.9925	8.18	24.8	Linear range 100 - 1000	2.47 (n=6)	0.4 (n=6)	68.7 (5.8%); 68.2 (3.0%); 65.8 (4.3%)
IBU							
HPLC[1]	0.7477	7.5	23	23 -125	2.5 (n=5)	0.04 (n=5)	130; 110; 127 (n=1)
MEKC[1]	0.6341	15	45	45 - 125	19 (n=6)	0.9 (n=6)	211, 134, 1111 (n=1)
Ferrando-Climent et al., 2012	0.9998	0.0200[b] 0.0021[c] 0.0007[d]	0.0665[b] 0.0069[c] 0.0024[d]	0.1 -100	0.31 (n=5)	2.70 (n=5)	193.1 (2.0) 70.9 (12.4) 84.8 (0.1) (n=3)
Larsson et al., 2014	0.9996	44.2	147.2	250-2000	<9	<9	Not provided
Paíga et al., 2015	0.9957	0.00008	0.00026	Linear range 10 -100	2.59 (n=6)	8.10	86.5 (2.2%); 89.5 (4.3%); 89.0 (4.0%) (n=3)

[a]This study [b]WWTP influent [c] WWTP effluent [d] River water [e]Did not study 2OH

Table 36. Comparison of experimental conditions and extraction procedure of HPLC, MEKC and other methods applied to compounds of study

Study	Research	Sample matrix	Calibration standard solution solvent	Technique/Detector	Mobile Phase	Analysis time (min)	Extraction Method	Technique
HPLC[a]	Aerobic granule degradation and removal of IBU, 2OH, 1OH and	synthetic wastewater	milli-q water	HPLC/ UV/Vis	Water (solvent A), acetonitrile (solvent B), both acidified with 0.01% formic acid (v/v)	16	SPE Strata C18e (200 mg, 6 mL)	Section 3.2 adapted procedure from Paíga et al. 2017 and several authors[22,27,29,72,147,212,240,298,299]
MEKC[a]	CBX			MEKC/UV/Vis	15 mM borate, 40 mM SDS, 15% THF	15		
Ferrando-Climent et al., 2012	Biodegradation batch experiments of IBU in WWTP activated sludge	WWTP influent WWTP effluent river water	methanol-water (10:90, v/v)	UPLC-MS/MS, gradient elution, C18 column, internal standard calibration (ibuprofen-*d*3)	5 mM ammonium acetate pH 8(solvent A) Methanol (solvent B)	6.7	SPE Oasis HLB (60 mg, 3 mL)	Adapted procedure[79] Sample pretreatment: ethylenediaminetetraacetic acid disodium to adjust pH to 4.5. Conditioning solvents, methanol, HPLC-grade water Wash solvent: HPLC-grade water Drying: under vacuum 15 min Elution solvent: methanol Reconstitution solvent: Methanol-water (10:90, v/v)
Larsson et al., 2014	Identification of ibuprofen and metabolites with other NSAIDs in wastewater	WWTP influent WWTP effluent	0.1 M ammonium carbonate buffer, pH 9	LC-MS/MS, LC-MS, standard addition, isocratic	10 mM ammonium acetate pH 4 (solvent A) Methanol (solvent B); 40:60, v/v	14	Hollow fibre liquid phase microextraction:	Acceptor buffer 0.1 M ammonium carbonate solution with pH 9
Boix et. al., 2016[30, b]	Degradation of ibuprofen and other pharmaceuticals in activated sludge batch experiments	surface water Mineral media	Not described	UPLC-time-of-flight mass spectrometry, gradient	Water (solvent A), methanol (solvent B) both acidified with 0.01% formic acid	14 – 18	Oasis HLB, 60 mg	Not described
Paíga et al., 2015	Determination of NSAIDs in seawater	seawater	acetonitrile–ultra-pure water (30:70, v/v) by	UPLC-MS/MS, C18 column, internal standard calibration (ibuprofen-*d*3)	Ultra-pure water (solvent A), methanol (solvent B)	10.5	SPE Strata-X (200 mg, 3 mL)	Strata-X cartridges (200 mg) Conditioning solvents, methanol, ultra-grade water pH 2 Wash solvent: ultra-grade water Drying: under vacuum 1 hr Elution solvent: methanol Drying: nitrogen Reconstitution solvent: acetonitrile–ultra-pure water (30:70, v/v)

[a]This study [b]Did not provide validation data

4.8 Performance of Aerobic Granule SBRs

Two aerobic granule SBRs were operated in different conditions as described in Section 3.3.1 and 3.3.2. The SBRs were constructed by adapting material available in the laboratory, therefore the dimensions were smaller than those used in previous aerobic granule studies with pharmaceuticals [198–203]. The SBRs were dosed with ibuprofen at two concentrations (50 and 100 µg/L) to assess the ability of aerobic granules to biodegrade ibuprofen and the efficiency of removal. Identical pumps were initially used for both reactors, however due to failure, the reactor 2 pump was replaced. Hydraulic retention time was 5.4 hrs and influent loading rate was 0.120 m^3/m^2 per day. The measured pH ranged from 7.4 to 8.4. Dissolved oxygen was 8.26 mg/L.

SBR performance was evaluated by COD (Figure 44), phosphorus (Figure 45) and ammonia removal (Figure 46). COD removal was initially higher in reactor 1. However, removal in reactor 2 increases until the performance of reactors is comparable (approximately 90%). Under normal SBR operating conditions (reactor 2), most COD consumption by the microorganisms occur in the anaerobic phase. Early aerobic granule SBRs had a short feeding period as applied to the SBRs in this study, until research proved that longer feeding periods were required to maintain granule stability at full-scale operation[191]. Phosphorus removal, which occurs during the aerobic stage, was initially higher in reactor 1 but the removal in reactor 2 eventually improved and surpassed. However, the maximum observed removal of 14%. Low phosphorus removal is a consequence of competition between polyphosphate and glycogen accumulating organisms for COD uptake [162,201] Ammonia removal was higher in reactor 1 until day 27 when the removal in both reactors were comparable. Similar COD, phosphorus, nitrogen removal profiles were observed in previous aerobic granule SBR studies with pharmaceuticals.

A comparison of nitrogen concentration in the influent and effluent of both reactors is shown in Figure 47. Nitrogen effluent concentration was initially lower in reactor 1 at day 14. Subsequently, reactor 2 showed the best removal of nitrogen until days 21 and day 27 when nitrogen concentration was higher in the effluent of both reactors in comparison to the influent. This indicates the suppression of denitrification, causing nitrogen accumulation and could be the result of two factors. Firstly, the accumulative effects of the constant high dissolved oxygen in the reactors

which reduces granule nitrification ability. Ammonia and nitrogen removal is generally good in previous studies where dissolved oxygen was monitored but not controlled. However larger reactors were used than those employed in this study, which could a role in reducing this effect. Secondly, this occurred after the addition of ibuprofen to the SBRs. Amorim et al. and Shi et. al observed this effect in aerobic granule SBRs dosed with chiral pharmaceuticals and tetracycline respectively[198,201]. The addition of ibuprofen on day 21, also appeared to significantly reduce phosphorus removal. However, reactor performance recovered to normal levels, even with the addition of ibuprofen on day 27, indicating granule adjustment to ibuprofen. Confirmation of the effect of ibuprofen in producing these removal profiles is required by comparison to SBRs operated with no introduction of ibuprofen.

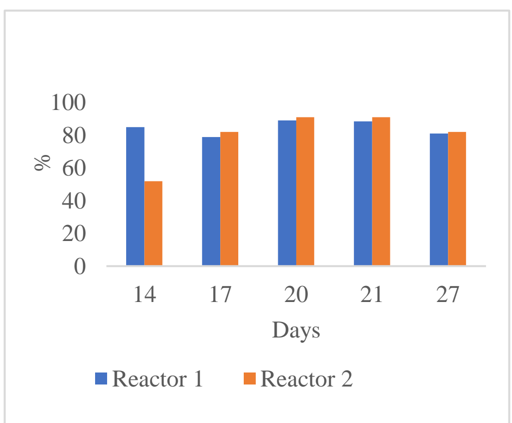


Figure 44. COD removal during 27 days of operation

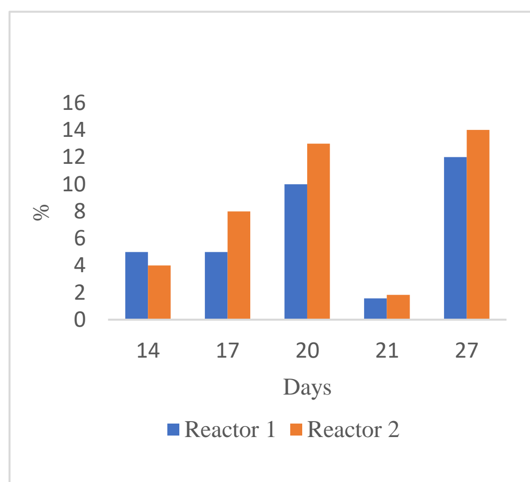


Figure 45. Phosphorus removal during 27 days of operation

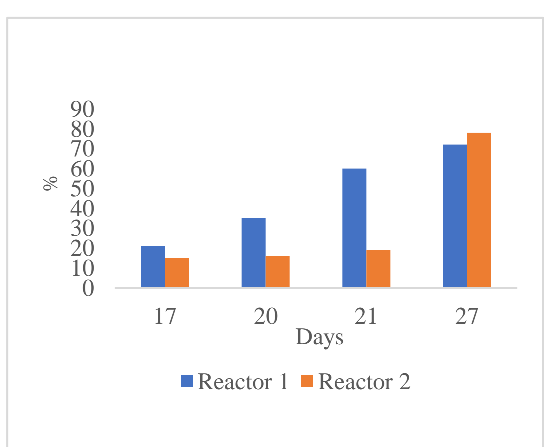


Figure 46. Ammonia removal during 27 days of operation

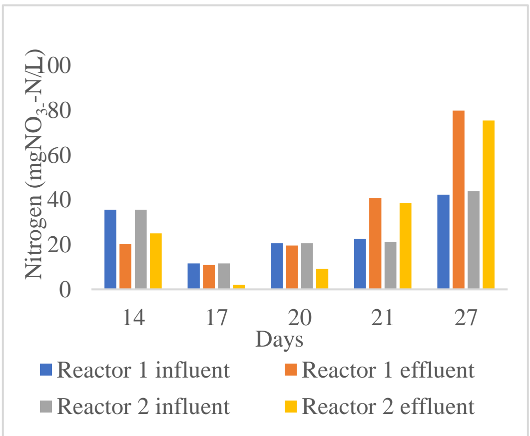


Figure 47. Nitrogen concentration in reactor influent and effluent during 27 days of operation

After one month of operation, filamentous bulking and slime in the aerobic granules was observed, indicating possible nutrient deficiency. Fungal growth was also observed in the influent containers. Acetate concentration in freshly prepared synthetic wastewater, influent and effluent respectively was analysed by HPLC. The results confirmed that acetate concentration of the influent introduced to the SBRs was low, therefore the granules were not receiving the carbon load needed to maintain stability and form. It is possible that degradation in the influent reservoir is occurring as the influent is stored in between cycles. The operations of both SBRs was halted until a solution could be implemented to avoid this problem.

Granule Measurements

The SBRs were started with aerobic granules (2 g/L, total suspended solids) from the Águas de Portugal Nereda® Frielas WWTP in Lisbon, Portugal. The TSS of the supply was measured at 10.3 g/L. After 17 days, the granule layer increased by 61%. The SVI_5 as 25 secs. The size of the granules ranged from 2 to 5 mm with the average size of 3 ±1.2 mm (Figure 48).

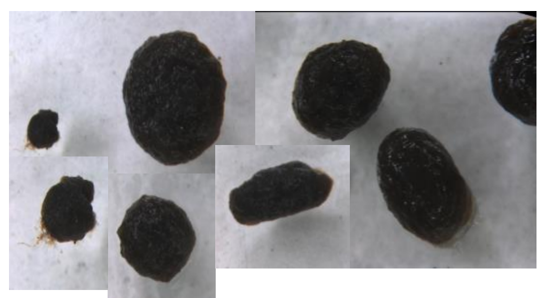


Figure 48. Aerobic granules used to as start-up inoculum for SBRs

5. Conclusions

Two aerobic granule SBRs were operated in synthetic wastewater at laboratory scale in anaerobic/aerobic and aerobic cycles respectively to assess the ability of aerobic granules to degrade ibuprofen and its dependence on operating cycle. Samples were taken from the reactors which were dosed with 50 and 100 μg/L of ibuprofen. Operation was halted due to acetate degradation reducing the carbon load to the granules arising from the growth of filamentous bulking and slime in the aerobic granules and fungi in the influent container.

A HPLC gradient method and a MEKC method using UV/Vis were developed and optimized for the separation and analysis of ibuprofen and its three metabolites in the SBR samples. Preconcentration of the environmentally realistic concentrations (μg/L) to ppm level was performed off-line using SPE for HPLC, while both off-line with SPE and on-line techniques with FASS were utilized for MEKC. Interestingly, a new technique for FASS was explored by combining elements of FASI with the injection of a water plug after the sample instead of the standard method of injecting the plug before the samples. Its simplicity in removing the need to switch polarity, could be further explored for a new technique for application to anion analysis and on-line preconcentration.

Validation was performed in solvent to determine selectivity, linearity, LOD, LOQ, range, precision, recovery and robustness applying ICH and AOAC principles. Achieving selectivity was difficult for 2OH and 1OH in CE and 2OH and CBX in HPLC but eventually the compounds could be separated. It was observed that although separation conditions could be achieved in CE, the conditions were not robust.

The results of the remaining parameters could not confirm the validity of the methods for their intended application. Highly variable recoveries were obtained in experiments to evaluate the SPE procedure and validation of both methods. Some results complying with AOAC guidelines (80-110%) and corresponded to published literature, while unrealistically high recoveries ranged from 119 – 1111% and was not consistent to a particular compound. Both methods had results for linearity and repeatability that failed to pass the Harley's F_{max} test and Dixon's Q-test indicating statistically significant difference in the variances of the standard solutions of the calibration curve and solutions used to assess repeatability and the presence of outliers. Linearity in the desired working range could not be confirmed. Calibration curves were still prepared and repeatability calculated due to limitations on time for thesis submission. Repeatability was acceptable (< 15%) in both methods except for IBU in the MEKC method. Calculated LOD passed all tests for accuracy of calculation. However,

LODs for IBU in both HPLC and MEKC and CBX in MEKC, were higher than the minimum standard concentration used for the calibration curves. Solvent and matrix calibration curves were compared using student's t-test for statistically significant difference and matrix effect. There was no significant difference between the curves for HPLC but the MEKC method failed to pass the test.

The methods were applied to the SBR samples. Each method detected a different analyte. MEKC detected CBX in contrast to HPLC, which detected 1OH. The concentrations of the other compounds were below method detection limits.

Despite the problems experienced, both methods are excellent starting points for further improvement and application for simple environmental analysis of ibuprofen and its metabolites. Some of the validation results were comparable to published literature. The methods show potential for application to ibuprofen environmental analysis once investigation to address experimental problems and revalidation is performed.

Limited research has been undertaken thus far to investigate the potential of aerobic granules as a wastewater treatment that can address the global problem of pharmaceuticals as emerging environmental pollutants. In literature review, no studies were identified that considered the metabolites, which can have similar or more toxic environmental effects, in addition to the parent compound.

6. Future Perspectives

Further work is required to verify that aerobic granules can degrade ibuprofen and its metabolites in both aerobic and anaerobic/aerobic operation and to confirm the suitability of the developed HPLC and MEKC methods for their intended application to the SBRs. The following recommendations are proposed for continuation of research:

Methods

1. **Re-evaluation of SPE Procedure:** The SPE procedure must be repeated for evaluation of suitability for preconcentration of these compounds, using at least 3 replicates per concentration. A comparison of the recoveries obtained using polymer SPE cartridges (Strata-X, Oasis HLB or Oasis MCX) should be performed. If the same recoveries are obtained, adjustment of the washing solvent by addition of organic solvent (starting at 5%) to remove possible interferents can be considered, Due to desired investigation at trace concentrations, the use of micropipettes cannot be avoided.
2. **Revalidation:** The validation must be performed again for all compounds to confirm linearity in the desired working range and acceptable repeatability with results that meet the applied statistical tests for acceptability of results. The obtained LOD and LOQ of both methods also require improvement of sensitivity to be for both methods to be comparable to other published methods in surface and ground waters (ng to low µg/L). The compounds may be present in analysed samples, but at concentrations below method limits. Revalidation will also be required for method transfer to UAlg.
3. **Reanalysis of Samples:** Both methods detected different analytes. Reanalysis of the 50 µg/L samples is required to verify the validity of these results. The 100 µg/L samples should also be analysed for comparison of detected compounds.

SBR

The reactors appear to degrade ibuprofen, however, experiments should be repeated to for verification due to unrealistically high results. A method of influent preparation and introduction is required to introduce the carbon source immediately before the cycle to the reactors. This should address the problem of competing organism growth in the influent containers. Confirmation of the effect of ibuprofen in producing the removal profiles observed in this study should be conducted by comparison to SBRs operated with no introduction of ibuprofen.

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8. Annexes

Annex 1. Statistical Tests and Critical Tables[311]

a. Student's t test: comparison of the means of two results

Critical Values, Student's *t* Test

f	α = 0.05	α = 0.01
1	12.706	63.567
2	4.303	9.925
3	3.182	5.841
4	2.776	4.604
5	2.571	4.032
6	2.447	3.707
7	2.365	3.499
8	2.306	3.355
9	2.262	3.250
10	2.228	3.169
11	2.201	3.106
12	2.179	3.055
13	2.160	3.012
14	2.149	2.977
15	2.131	2.947
16	2.120	2.921
17	2.110	2.898
18	2.101	2.878
19	2.093	2.861
20	2.086	2.845
22	2.074	2.819
24	2.064	2.797
26	2.056	2.779
28	2.048	2.763
30	2.042	2.750
35	2.030	2.716
40	2.021	2.706
45	2.014	2.690
50	2.009	2.678
60	2.000	2.660
70	1.994	2.648
80	1.990	2.639
100	1.984	2.626
∞	1.960	2.576

HPLC: t_{cal} values for comparison of calibration curves, t_{crit} $_{(f=58,\ \alpha = 0.05)}$ = 2.005

Compound	Calibration Curve	y-intercept	SD	Absolute value t_{cal}
1OH	solvent	0.0025	0.00627	1.836
	matrix	0.0142	0.00445	
2OH	solvent	0.0012	0.00537	0.280
	matrix	0.1225	0.00339	
CBX	solvent	-0.0658	0.02082	0.058
	matrix	-0.0818	0.13668	
IBU	solvent	0.0096	0.01444	0.085
	matrix	0.04401	0.00793	

MEKC: t_{cal} values for comparison of calibration curves, t_{crit} $_{(f=70,\ \alpha = 0.05)}$ = 1.994

Compound	Calibration Curve	y-intercept	SD	Absolute value t_{cal}
1OH	solvent	0.0025	0.00627	10.4
	matrix	0.0142	0.00445	
2OH	solvent	0.0012	0.00537	9.1
	matrix	0.1225	0.00339	
CBX	solvent	-0.0658	0.02082	3.3
	matrix	-0.0818	0.13668	
IBU	solvent	0.0096	0.01444	12.5
	matrix	0.04401	0.00793	

b. Hartley's F_{max} test for statistically significant variance in measurement series

Critical Values, Hartley's F_{max} Test for Significance Level $\alpha = 0.05$

f	k = 2	3	4	5	6	7	8	9	10	11
2	39.0	87.5	142	202	266	333	403	475	550	626
3	15.4	27.8	39.2	50.7	62.0	72.9	83.5	93.9	104	114
4	9.60	15.5	20.6	25.2	29.5	33.6	37.5	41.1	44.6	48.0
5	7.15	10.8	13.7	16.3	18.7	20.8	22.9	24.7	26.5	28.2
6	5.82	8.38	10.4	12.1	13.7	15.0	16.3	17.5	18.6	19.7
7	4.99	6.94	8.44	9.70	10.8	11.8	12.7	13.5	14.3	15.1
8	4.43	6.00	7.18	8.12	9.03	9.78	10.5	11.1	11.7	12.2
9	4.03	5.34	6.31	7.11	7.80	8.41	8.95	9.45	9.91	10.3
10	3.72	4.85	5.67	6.34	6.92	7.42	7.87	8.29	8.66	9.01
15	2.86	3.54	4.01	4.37	4.68	4.95	5.19	5.40	5.59	5.77
20	2.46	2.95	3.29	3.54	3.76	3.94	4.10	4.24	4.37	4.49
30	2.07	2.40	2.61	2.78	2.91	3.02	3.12	3.21	3.29	3.36
60	1.67	1.85	1.96	2.04	2.11	2.17	2.22	2.26	2.30	2.33
∞	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

HPLC: F_{max} values for Linearity and Precision

Compound	F_{max},		
	$F_{maxo\ (k=5,\ f=4,\ \alpha = 0.05)}$: 29.5		$F_{maxo\ (k=3,\ f=4,\ \alpha = 0.05)}$ 15.5
	Linearity HPLC solvent	Linearity HPLC matrix	Repeatability for solvent curve
1OH	31.9	20.7	2.6
2OH	7.9	105.6	236.5
CBX	6.5	35.4	2.8
IBU	10.8	958.4	35.8

MEKC: F_{max} values for Linearity and Precision

Compound	F_{max},		
	$F_{maxo\ (k=6,\ f=5,\ \alpha = 0.05)}$:18.7		$F_{maxo\ (k=3,\ f=4,\ \alpha = 0.05)}$ 10.8
	Linearity CE solvent	Linearity CE matrix	Repeatability for solvent curve
1OH	24.8	5.4	6.9
2OH	19.4	24.1	52.3
CBX	24.0	23.6	30.6
IBU	7.6	39.3	23.4

c. Dixon's Q-test for outliers in results

TABLE A.5
Critical Values (Q_{crit}) of Dixon's Q Test

f	α = 0.10	α = 0.05	α = 0.01
3	0.886	0.941	0.988
4	0.679	0.765	0.889
5	0.557	0.642	0.780
6	0.482	0.560	0.698
7	0.434	0.507	0.637
8	0.399	0.468	0.590
9	0.370	0.437	0.555
10	0.349	0.412	0.527

HPLC: Q_1, Q_n for the compounds in water for HPLC repeatability, n=5, $Q_{crt\ (\alpha = 0.05)}$ = 0.642

Compound	Q_1	Q_n
2OH	0529, 0.393, 0.198	0.059, 0.438, 0.758
1OH	0.280, 0.482, 0.144	0.440, 0.139, 0.316
IBU	0.062, 0.071, 0.393	0.375, 0.286, 0.159
CBX	0.165, 0.100, 0.530	0.140, 0.100, 0.061

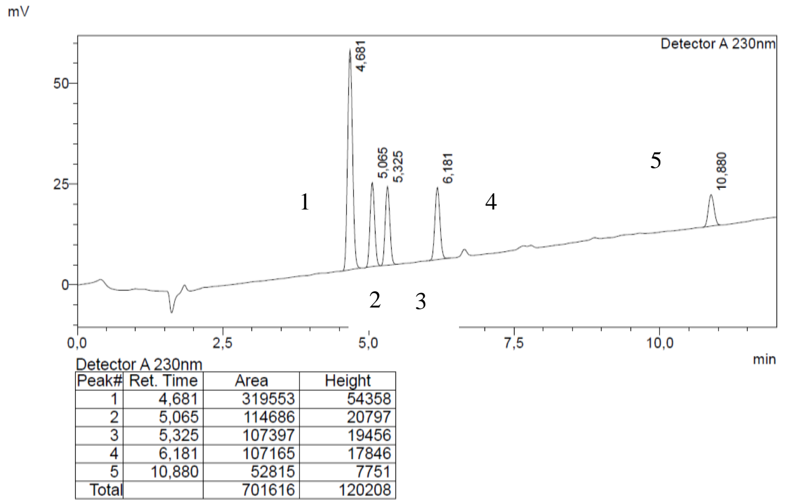
MEKC: Q1, Qn for the compounds in water for MEKC repeatability, n=6, $Q_{crt\ (\alpha = 0.05)}$ = 0.560

Compound	Q_1	Q_n
2OH	0.083, 0.067, 0.429	0.750, 0.200, 0.214
1OH	0.123, 0.098, 0.082	0.131, 0.617, 0.428
IBU	0.016, 0.137, 0.178	0.430, 0.528,0.467
CBX	0.355, 0.307, 0.133	0.249, 0.266, 0.200

d. Confidence Interval

$\text{Mean} \pm z \times \frac{SD}{\sqrt{n}}$ z value for 95% confidence interval = 1.96

Annex 2. SPE Results

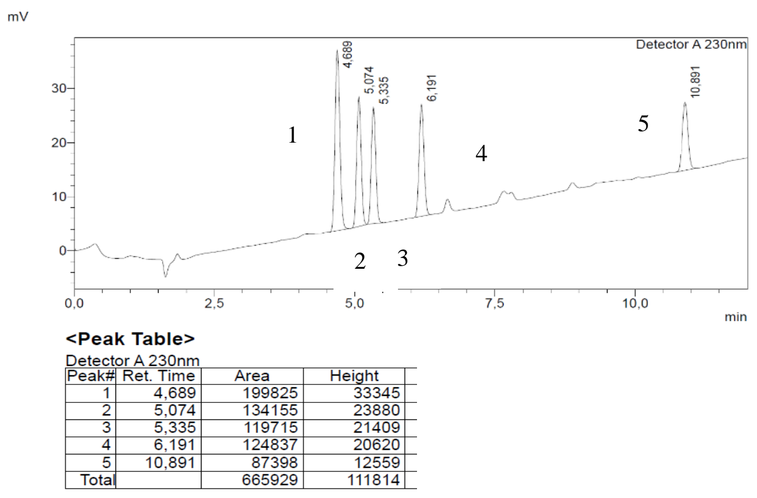


Detector A 230nm

Peak#	Ret. Time	Area	Height
1	4,681	319553	54358
2	5,065	114686	20797
3	5,325	107397	19456
4	6,181	107165	17846
5	10,880	52815	7751
Total		701616	120208

Chromatogram of SPE trial, preparation 3, 50 µg/L in 100 mL milli-q water

Peaks 1: BA, 2: 2OH, 3: CBX, 4: 1OH, 5: IBU



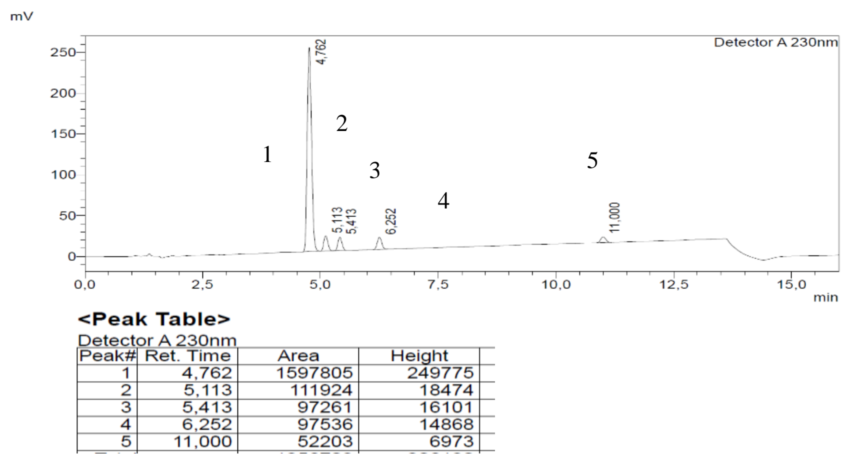
<Peak Table>

Detector A 230nm

Peak#	Ret. Time	Area	Height
1	4,689	199825	33345
2	5,074	134155	23880
3	5,335	119715	21409
4	6,191	124837	20620
5	10,891	87398	12559
Total		665929	111814

Chromatogram of SPE trial, preparation 3, 50 µg/L in 200 mL milli-q water

Peaks 1: BA, 2: 2OH, 3: CBX, 4: 1OH, 5: IBU



<Peak Table>

Detector A 230nm

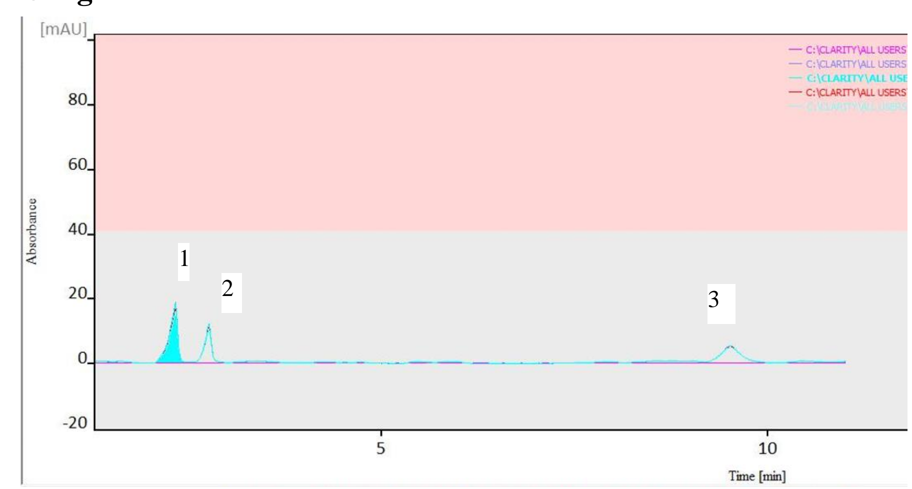
Peak#	Ret. Time	Area	Height
1	4,762	1597805	249775
2	5,113	111924	18474
3	5,413	97261	16101
4	6,252	97536	14868
5	11,000	52203	6973

Chromatogram of 50 µg/L method validation calibration curve standard processed by SPE

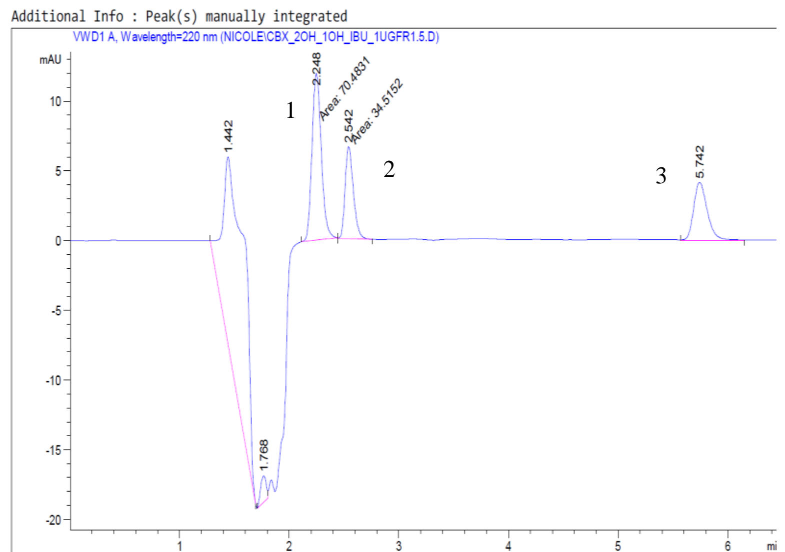
Peaks 1: BA, 2: 2OH, 3: CBX, 4: 1OH, 5: IBU

Annex 3. HPLC Method Development Calculations and Chromatograms

a. UAlg



Varian HPLC: Chromatogram showing compound peaks in water, pH 3 adjusted with acetic acid: acetonitrile, 50:50 (v/v). Peaks 1: 2OH and CBX, 2: 1OH, 3: IBU



Agilent HPLC: Chromatogram showing compound peaks in water, pH 3 adjusted with acetic acid: acetonitrile, 60:40 (v/v). Peaks 1: 2OH, CBX, 2: 1OH, 3: IBU

UAlg: Retention factor for mobile phases tested in experimental design

Mobile Phase	Retention factor, k			
	1OH	2OH	CBX	IBU
10 mM ammonium acetate buffer pH4: Methanol, (65:35)	1.1	0.83	0.7	7.4
10 mM ammonium acetate buffer pH4.5: Methanol, (65:35)	0.9	0.7	0.5	5.8
10 mM ammonium acetate buffer pH4: Methanol, (60:40)	1.8	1.3	1.2	13
10 mM ammonium acetate buffer pH4.5: Methanol, (60:40)	1.4	1.0	0.8	11

Response function results for the 2^2 factorial design

Mobile Phase	CRS	CRF	CEF	R_{sum}	R_{cp}	RP	Runtime (min)
1	-3705	28	42	42	1.5	2	23
2	14	39	12	40	2.5	4	19
3	100	6.3	113	50	1.4	2	38
4	24	23	19	50	3.1	4	33

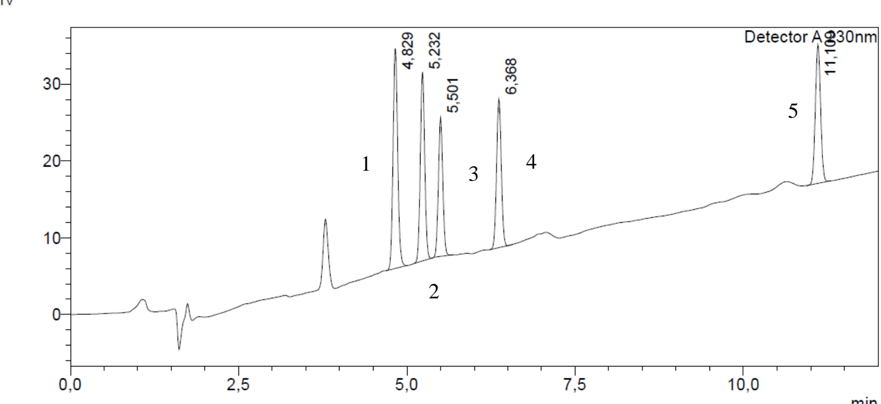
b. USP

$V_m = 0.0005 \text{ x } (4.6)^2 \text{x} 250 = 2.645$ mL

Gradient retention factor $k^* = (0.87 \text{ x } 15 \text{ X } 2)/ (2.645 \text{ x } 0.95 \text{ x } 4) = 2.6$

$\Delta tr/t_G = 4.539/15 = 0.30$

Isocratic $\%B \approx 6.3\ (t_{ravg} - t_D) - 2 = 6.3\ [10.010 - 2.645/2] = 52.7\%$

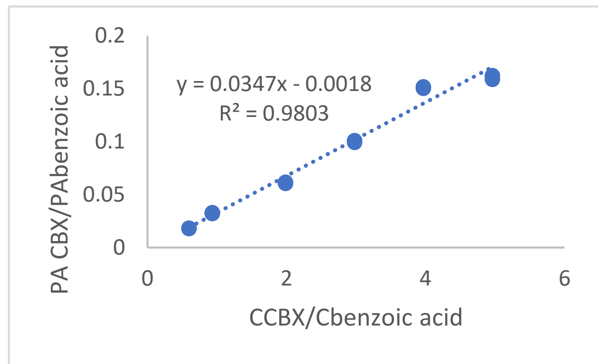


Chromatogram of compound separation in synthetic wastewater with optimized HPLC gradient conditions: water (solvent A), acetonitrile (solvent B), both acidified with 0.01% formic acid (v/v)

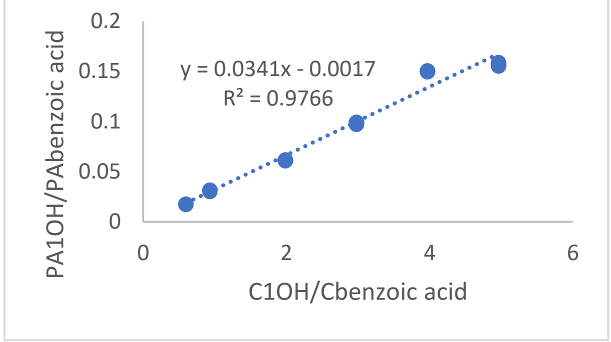
Peaks 1: BA, 2: 2OH, 3: CBX, 4: 1OH, 5:

Annex 4. HPLC Validation Graphs and Calculations

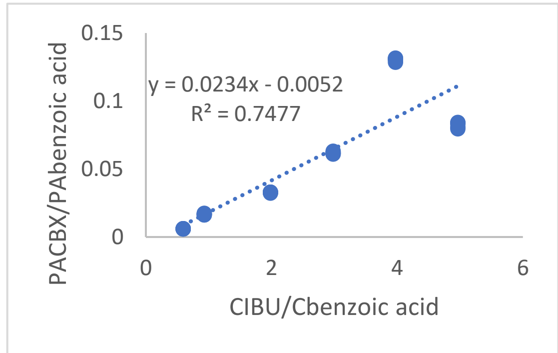
a. Linearity Calibration Curve



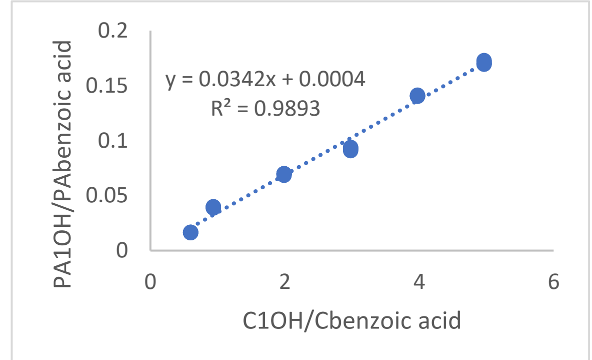
HPLC calibration curve for CBX in water



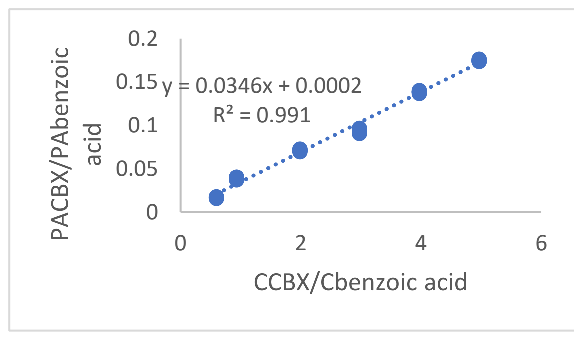
HPLC calibration curve for 1OH in water



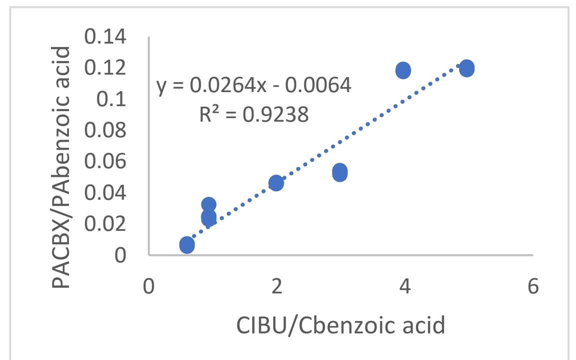
HPLC calibration curve for IBU in water



HPLC calibration curve for 1OH in synthetic wastewater

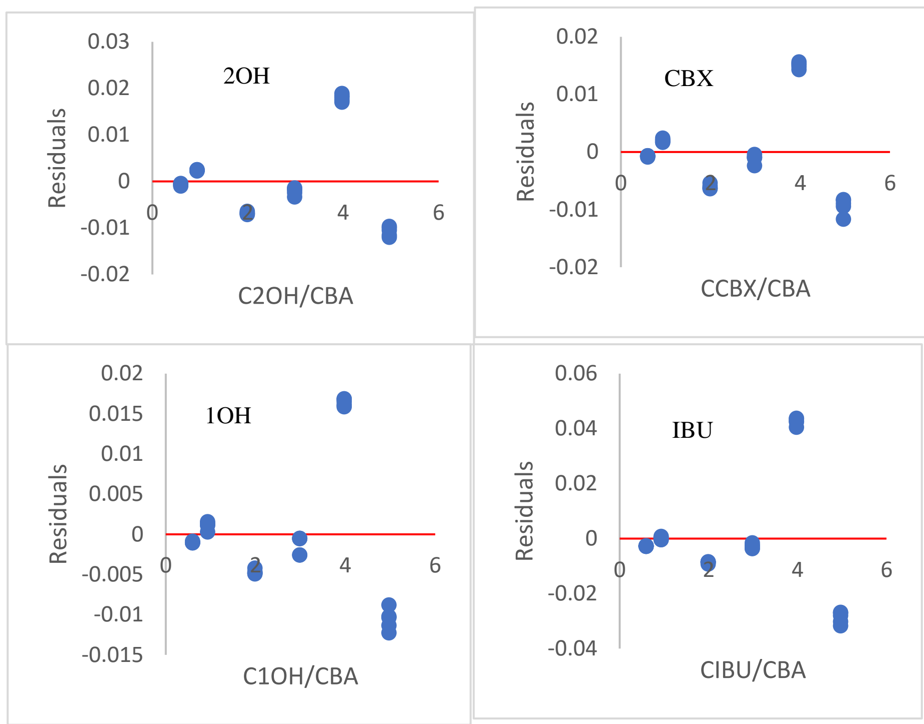


HPLC calibration curve for CBX in synthetic wastewater

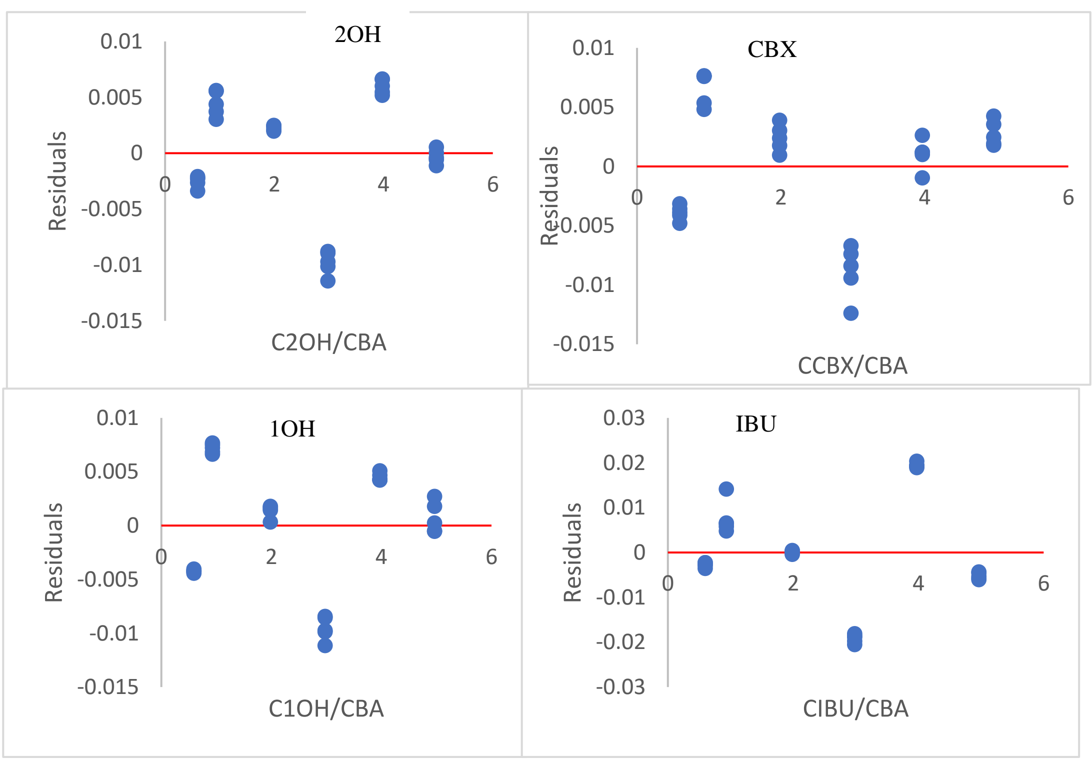


HPLC Calibration curve for IBU in synthetic wastewater

b. Residual Plots



HPLC residual plots for the compounds in water

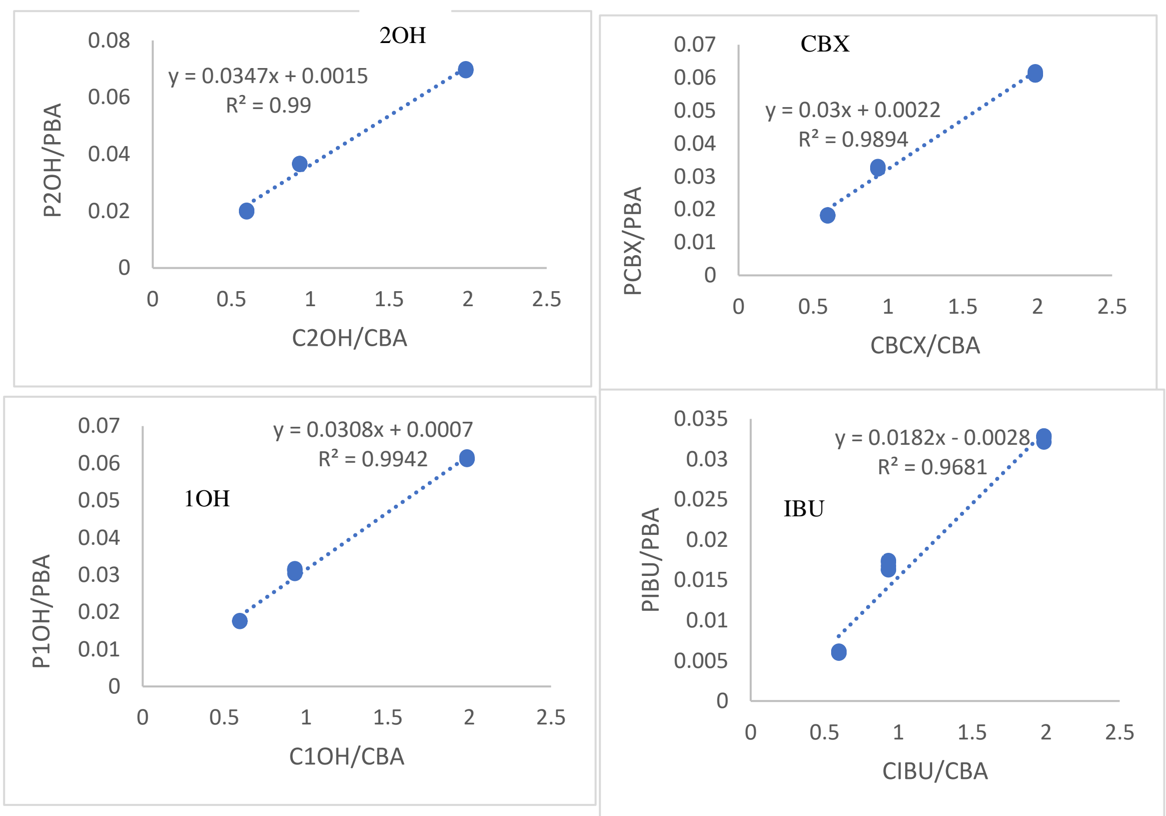


HPLC residuals plot for the four compounds in synthetic wastewater

c. LOD Conditions and Calibration Curves

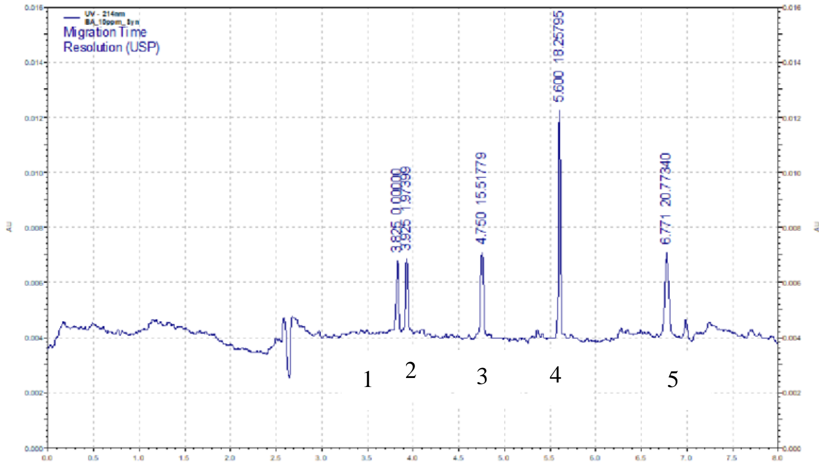
HPLC LOD acceptance conditions for accuracy of calculations

Compound	10 x LOD > 15 µg/L, c_{min}	LOD < 15 µg/L, c_{min}
1OH	32	3.2
2OH	38	3.8
CBX	43	4.3
IBU	75	7.5



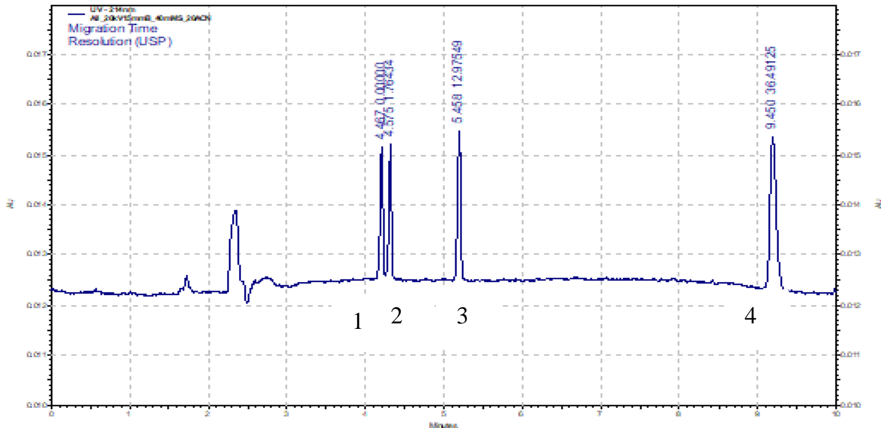
HPLC LOD Calibration curves for the four compounds in water

Annex 5. CE Electropherograms

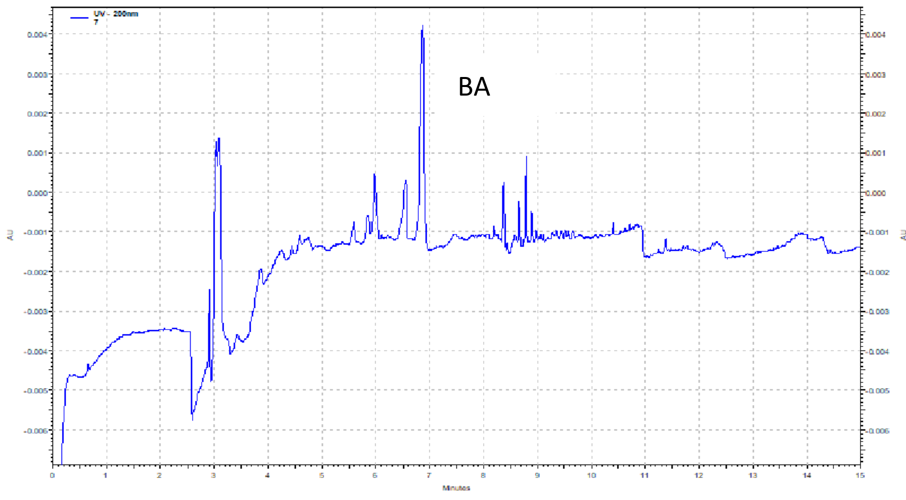


Electropherogram of compound separation in synthetic wastewater in optimized MEKC conditions

15 mM borate, 40 mM SDS, 10% THF; Peaks 1: 2OH, 2: 1OH,



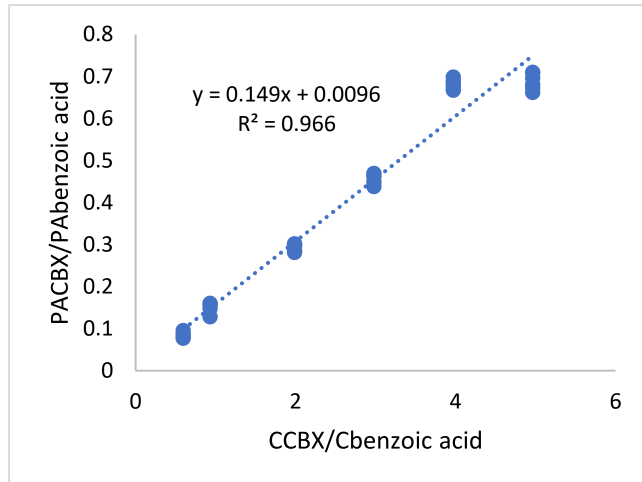
Electropherogram of separation observed in 15 mM borate, 40 mM SDS, 20% acetonitrile



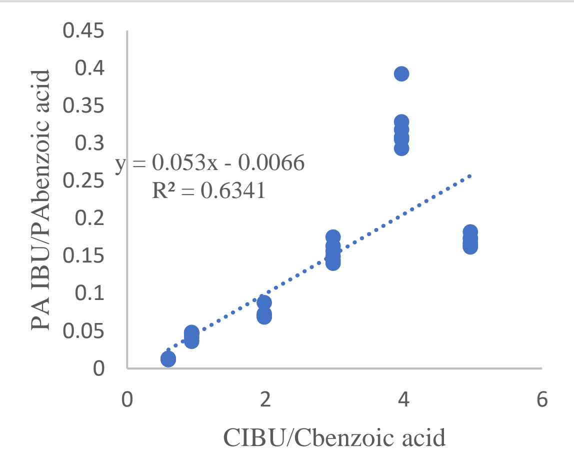
Electropherogram of sample T4 (120 min) taken from aerobic reactor: no compounds detected

Annex 6. CE Graphs and Validation Calculations

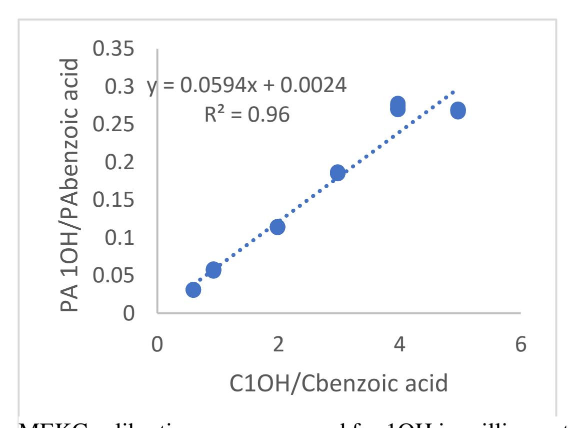
a. Linearity Calibration Curves



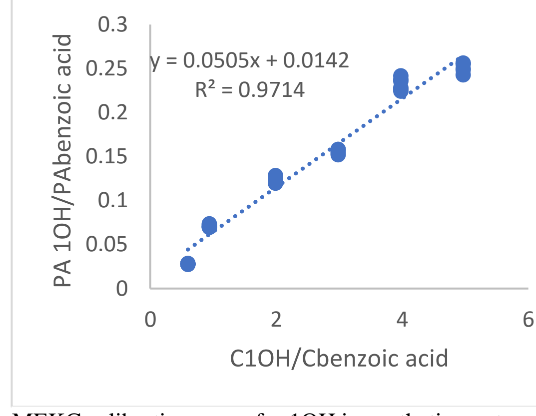
MEKC calibration curve for CBX in water



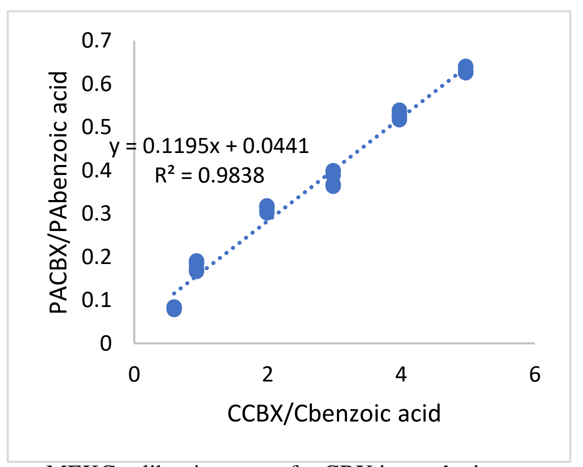
MEKC calibration curve prepared for IBU in milli-q water



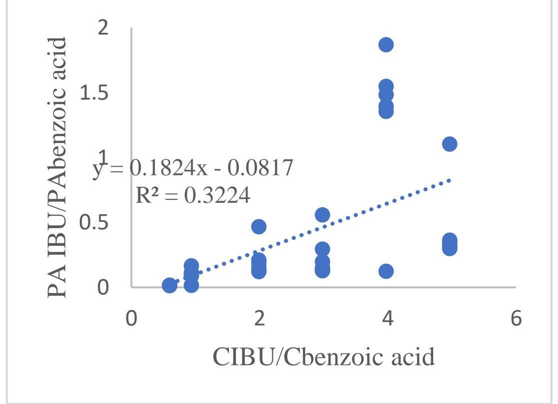
MEKC calibration curve prepared for 1OH in milli-q water



MEKC calibration curve for 1OH in synthetic wastewater

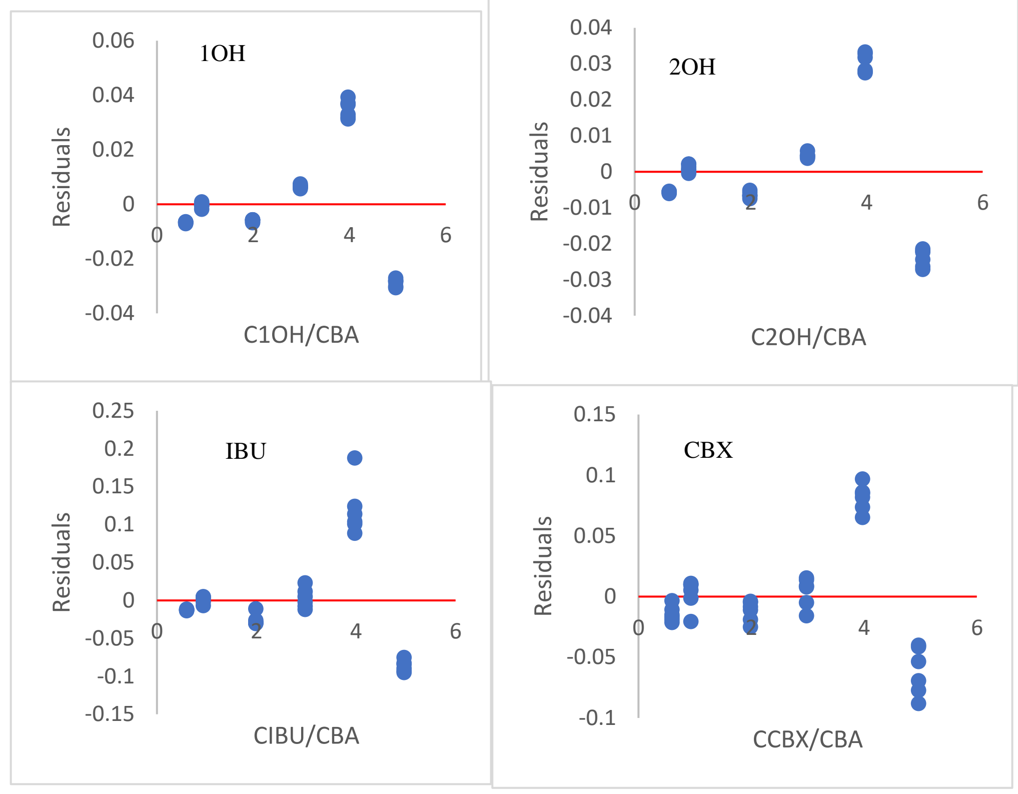


MEKC calibration curve for CBX in synthetic wastewater

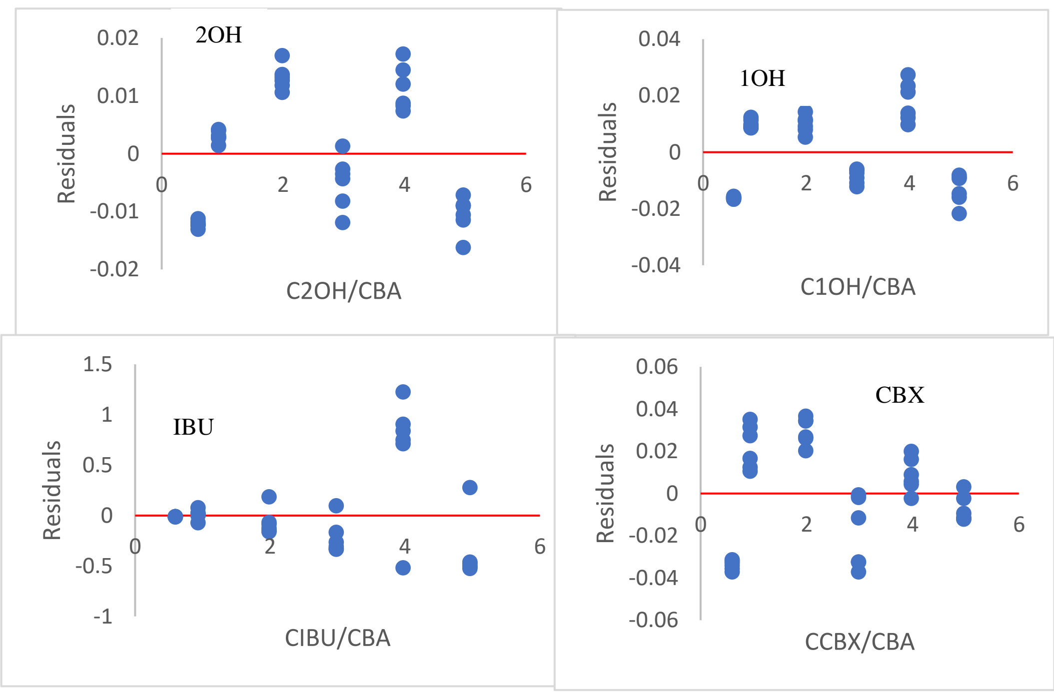


MEKC calibration curve prepared for IBU in synthetic wastewater

b. Residual Plots



MEKC residual plots for the four compounds in water

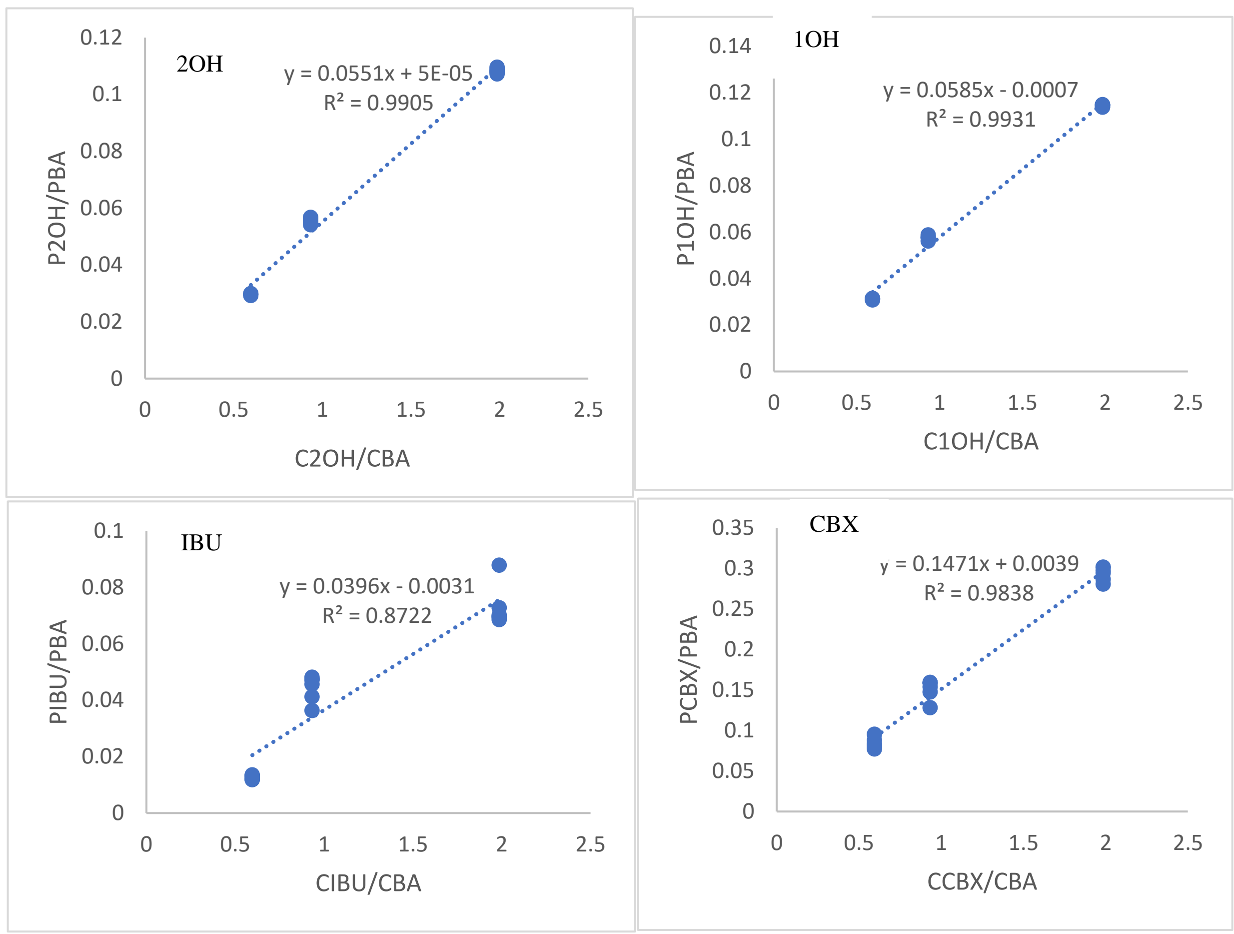


MEKC residual plots for the compounds in synthetic wastewater

c. LOD Conditions and Calibration Curves

CE LOD acceptance conditions for accuracy of calculations

Compound	10 x LOD > 15 µg/L, c_{min}	LOD < 15 µg/L, c_{min}
1OH	33	3.2
2OH	39	3.3
CBX	51	5.1
IBU	151	15



MEKC LOD calibration curves for the four compounds in water