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Enantioselective Monitoring of Biodegradation of Ketamine and Its Metabolite Norketamine by Liquid Chromatography

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Abstract: Ketamine (K) and its main metabolite, norketamine (NK), are chiral compounds that have been found in effluents from wastewater treatment plants (WWTPs) and aquatic environments. Little is known about their enantioselective biodegradation during sewage treatment; however, this information is pivotal for risk assessment, the evaluation of WWTP performance and wastewater epidemiological studies. The aim of this study was to investigate the biodegradation pattern of the enantiomers of K by activated sludge (AS) from a WWTP. For that, an enantioselective liquid chromatography with diode array detection (LC-DAD) method was developed and validated to quantify the enantiomers of K and NK. Both K and NK enantiomers were separated, in the same chromatographic run, using a Lux® 3 µm cellulose-4 analytical column under isocratic elution mode. The method was demonstrated to be linear (r^2 > 0.99) and precise (<11.3%). Accuracy ranged between 85.9 and 113.6% and recovery ranged between 50.1 and 86.9%. The limit of quantification was 1.25 µg/mL for the enantiomers of NK and 2.5 µg/mL for K. The method was applied to monitor the biodegradation assay of the enantiomers of K by AS for 14 days. K was poorly biodegraded, less than 15% for both enantiomers, and enantioselectivity in the biodegradation was not observed. The metabolite NK and other possible degradation products were not detected. This work reports, for the first time, the behavior of both enantiomers of K in biodegradation studies.

Keywords: ketamine; norketamine; enantioseparation; enantioselectivity; biodegradation assays

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1. Introduction

The Organization for Economic Co-operation and Development (OECD) recommends governments to take a collective, life cycle approach for managing pharmaceuticals in the environment. The recommendations encourage the uptake of new monitoring methods to better understand and predict the risks and prioritize substances [1]. Enantioselective studies on biodegradation assays are important tools to investigate changes of the enantiomeric fraction (EF) during wastewater treatment and to predict the release of the enantiomers in aquatic ecosystems [2–5]. Enantioselective biodegradation has already been reported for some classes of pharmaceuticals such as beta-blockers, antidepressants and their metabolites, anticoagulants, non-steroidal anti-inflammatory drugs (NSAIDs), antibiotics and illicit drugs [6–10]. Racemization has also been reported, for instance, Maia et al. showed the formation of the (*R*)-enantiomer during the biodegradation of levofloxacin by activated sludge (AS) [11]. Furthermore, some studies have emphasized differences in

the enantioselective biodegradation process among wastewater treatment plants (WWTPs) due to the diverse microbial community [5].

Ketamine (K) is a chiral pharmaceutical behaving as a non-competitive *N*-methyl-D-aspartate (NMDA) receptor antagonist by binding to the phencyclidine (PCP) site [12–14]. It is used for different clinical purposes including anesthesia, pain management and for treatment-resistant depression [15]. However, K is also misused in recreational environments by adolescents and young adults for its hallucinogenic properties [16]. Commercially, K is available as a racemate (*R*,*S*), though pure enantiomeric forms such as (*S*)-K (esketamine) have been approved by the US Food and Drug Administration as a nasal spray for use in treatment-resistant depression. On 19 December 2019, (*S*)-K nasal spray was also approved for the same indication in Europe [17]. The (*S*)-K enantiomer has greater affinity for NMDA receptors than (*R*)-K [18,19]. However, studies have shown detrimental side effects induced by (*R*,*S*)-K and (*S*)-K, suggesting that (*R*)-K can be safer for humans [20,21]. In addition, recent studies in rodent models of depression demonstrated that (*R*)-K has greater potency and longer-lasting antidepressant effects than (*S*)-K; however, the precise molecular mechanisms underlying the antidepressant actions of (*R*)-K remain unknown [22]. Norketamine (NK) is its main chiral active metabolite [15]. NK enantiomers bind to the PCP site, though with less affinity than K, evidencing anesthetic potential, particularly in (*S*)-NK [21,23].

K can be introduced into the aquatic environment from a variety of sources, following the same pattern as other pharmaceuticals, such as hospitals and veterinary facilities, drug manufacturers, domestic sewages and mainly by the incapability of the WWTPs to completely eliminate pharmaceutical residues [24]. The source of K residues in the environment is mostly from human and animal excretion, but direct disposal (bedside wasting) can also be a contributing factor [25]. Over the last few years, some reports have been carried out to understand the occurrence of K and NK, the environmental fate, and possible negative effects in aquatic living organisms [26,27]. In fact, some studies have shown that K is refractory in aquatic environments and cannot be completely removed by natural purification processes or by conventional WWTPs [28–31]. A recent study showed high concentrations of K (up to 1.100 μg/L) in the sewage of a Chinese urban area [32]. Another study showed its detection in surface water (up to 9.53 μg/L) and wastewater (up to 138 μg/L) during a music festival [33]. Negative removal values of K were also observed in the Plaszow WWTP, confirming its recalcitrance [24]. However, these studies ignore the enantiomers and consider K as a unique molecular entity. Knowledge about the EF of chiral pharmaceuticals during sewage treatment is essential for different purposes, including the evaluation of the performance of the WWTP treatment process, risk assessment and wastewater epidemiological studies (e.g., distinction between direct disposal or consumption) [34–37].

Thus, the development of an enantioselective analytical method to quantify both enantiomers of K and NK is imperative for environmental monitoring studies and biodegradation assays. The aim of this work was to develop and validate an enantioselective LC method and quantify the enantiomers of K and NK in biodegradation studies by AS.

2. Materials and Methods

2.1. Chemicals and Standards

Methanol (MeOH), HPLC grade, was purchased from Fisher Scientific UK (Leicestershire, United Kingdom); ethanol (EtOH) was acquired from ChemLab NV (Zedelgem, Belgium); acetonitrile (ACN) from CARLO ERBA Reagents (Val-de-Reuil, France). Ammonium acetate was obtained from Sigma-Aldrich (Steinheim, Germany); diethylamine (DEA) and triethylamine (TEA) were acquired from Sigma Aldrich (St Louis, MO, USA). Ultrapure water (UPW) was obtained from SG Ultra Clear UV plus equipment. All solvents of the mobile phase were previously filtered using glass microfiber filters with particle retention 0.7 μm, purchased from VWR (Leuven, Belgium).

Standards of K and NK were acquired from Sigma Aldrich (St Louis, MO, USA). Individual standard stock solutions were prepared at 1 mg/mL in MeOH and stored in amber bottles at −20 °C. Working standard solutions were freshly prepared and obtained by dilution of stock solutions in appropriate solvent depending on the study, and minimal salts medium (MM) was used for biodegradation assays.

The MM was prepared with the following reagents: di-sodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$), magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), ammonium sulfate ($(NH_4)_2SO_4$), sodium hydroxide (NaOH), potassium dihydrogen phosphate (KH_2PO_4) and ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), which were purchased from Merck (Darmstadt, Germany); ethylenediaminetetraacetic acid disodium salt hydrate ($Na_2EDTA_2 \cdot 2H_2O$) and calcium chloride ($CaCl_2$), were acquired from PanReac AppliChem ITW Reagents (Darmstadt, Germany); sodium sulfate (Na_2SO_4) and cupric sulfate ($CuSO_4$), were acquired from José Manuel Gomes dos Santos, LDA (Odivelas, Portugal); zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$), was purchased from Riedel-de Haën (Seelze, Germany); and sodium molybdate dihydrate (Na_2MoO_4) and sulfuric acid (H_2SO_4) 95–97%, were acquired from Sigma Aldrich (St Louis, MO, USA). The sodium acetate used as an external source of feed was acquired from Merck (Darmstadt, Germany).

Biodegradation assays were performed in an Infors HT Ecotron incubator (Fisher Scientific, Portugal) at 25 °C under dark and light conditions. Optical density (OD) was measured using a UV/Vis spectrometer (ATI Unicam, Leeds, England). Centrifuge Heraeus Biofuge Pico (Hanau, Germany), centrifuge Refrigerated Heraeus Biofuge 1.0R (Hanau, Germany), magnetic stirrer multipoint VARIOMAG® (Darmstadt, Germany) and an autoclave were also used.

2.2. Enantioseparation

The development and validation analytical method for the enantioseparation of K and NK was performed in a LaChrom Merck Hitachi HPLC system with Diode Array Detector (LC-DAD), a pump (L-7100), an autosampler (L-7200), a DAD (L-7455) and an interface system (D-7000) from Merck Hitachi. Data acquisition software was HPLC System Manager HSMP-7000, Version 3.0. The chiral analytical column used was the Lux® 3 µm Cellulose-4 (150 × 4.6 mm) coupled to a SecurityGuard™, Guard Cartridge (4 × 3.0 mm), both obtained from Phenomenex® (Torrance, CA, USA).

2.3. Enantioseparation Optimization and Method Validation for Quantification of Ketamine and Its Metabolite Norketamine

Optimization experiments for K and NK separation were performed in isocratic mode at room temperature (RT). Flow rate of 0.5 and 1.1 mL/min and sample injection volume was set at 10 µL. Different mobile phase compositions consisting of 20 mM ammonium acetate in UPW with or without 0.1% of DEA combined with EtOH or ACN in reverse elution mode were investigated to achieve resolution of the enantiomers. DAD detector was set at 220 nm.

The optimized analytical chromatographic conditions consisted of 20 mM ammonium acetate in UPW with 0.1% of DEA combined with ACN (70:30, *v*/*v*) as mobile phase, DAD detector set at 220 nm, flow rate of 1 mL/min and injection volume of 10 µL.

The optimized method was validated according to the International Conference on Harmonization guidelines (ICH) [38] and according to previous studies [11,39]. Selectivity, linearity and range, accuracy, recovery, intra- and inter-day precision, and detection (LOD) and quantification limits (LOQ) were determined. Selectivity was verified by comparing the chromatograms of standards of K and NK prepared in MeOH with those prepared in MM with AS, to assess the matrix interferences. The linearity was evaluated using calibration curves performed in MM at six calibration standard mixtures, each one in triplicate. The enantiomeric concentrations were 2.5, 5, 10, 15, 20 and 25 µg/mL for K, and 1.25, 2.5, 5, 7.5, 10 and 12.5 µg/mL for NK. Calibration curves were obtained by linear regression. Spiked samples were used for determination of LOD and LOQ with the signal/noise ratio of 3 for LOD and 10 for LOQ. Accuracy and intra- and inter-day precision were determined

by analysis in triplicate of three quality control standard solutions (QC) in MM, at three different concentrations within the linearity range. The enantiomeric concentrations were 4, 12.5 and 17.5 μg/mL for K and 2, 6 and 8.5 μg/mL for NK. Precision was expressed as the relative standard deviation (% RSD) percentage of the triplicate measurements, and the accuracy of the method was evaluated as the percentage of agreement between the method results and the nominal amount of compound added. Blank samples of MM inoculated with AS were fortified at the three QC concentrations and used for recovery assays. AS was spiked with K and NK in MM at the three QC levels and shaken for 2 h at 110 rpm. The AS samples were centrifuged at 13,000 rpm for 20 min (centrifuge Heraeus Biofuge Pico), and the supernatants were collected. The recovery was calculated comparing the peak areas K and NK obtained in the supernatants with standards in MM with the same concentration of the corresponding QC level.

2.4. Biodegradation Assay

The AS inoculum used in the biodegradation assay was obtained from the secondary/biological treatment aerated tanks of a municipal WWTP (Ponte de Moreira, Maia, Portugal), which receives domestic and industrial wastewater. The AS was collected in glass amber flasks and transported in a styrofoam box with ice and cold accumulators until properly conditioned at 4 °C until usage.

The MM used in the biodegradation assay was prepared with the following composition (per 1 L of UPW): $Na_2HPO_4{\cdot}2H_2O$, 2.67 g; KH_2PO_4, 1.4 g; $MgSO_4{\cdot}7H_2O$, 0.2 g; $(NH_4)_2SO_4$, 0.5 g with 10 mL of a trace elements solution. The trace elements solution was prepared in the following composition (per 1 L of UPW): $ZnSO_4{\cdot}7H_2O$, 0.4 g; $Na_2EDTA_2{\cdot}2H_2O$, 12 g; $CuSO_4$, 0.1 g; NaOH, 2 g; $FeSO_4{\cdot}7H_2O$, 2 g; Na_2MoO_4, 0.1 g; Na_2SO_4, 10 g; $CaCl_2$, 1 g; H_2SO_4 95–97%, 0.5 mL. The sodium acetate stock solution was prepared in MM at a final concentration of 1 M. The MM and the sodium acetate solution were previously autoclaved to avoid bacterial growth, and manipulated by flame (sterile environment). The stock solution of K racemate was prepared at final concentration of 1 mg/mL in MM.

The AS inoculum was washed with MM before being used in the biodegradation assay, through 3 wash cycles: centrifuge at 4000 rpm for 10 min at 4 °C (Heraeus Biofuge 1.0R), discard the supernatant and resuspend the pellet in MM. After washing, an aliquot of the AS inoculum was autoclaved to perform the assay with the dead cells.

The biodegradation assay was performed in batch mode using 100 mL covered flasks containing 25 mL of MM inoculated with AS, at an optical density (OD) of ca. 0.3 at 600 nm measured using a UV/Vis spectrophotometer. The K racemate stock solution was added to obtain an initial concentration of 40 μg/mL in the flasks of assay. Biodegradation assay was assessed under non-natural light (glass transparent flask under 6000 Lux) and dark (coated flasks with aluminum foil) conditions; in the presence or absence of sodium acetate as an extra carbon source at an initial concentration of 200 μg/mL; and with AS or dead cells (Supplementary material, Table S1).

All experiments were performed in triplicate (except experiments with dead cells and control that were performed in duplicate), using glass flasks of a volume fourfold the medium volume to guarantee the aeration of the cultures. The cultures were incubated at 25 °C with shaking at 110 rpm. Control assays without inoculation, under light and dark conditions, were also included. The assay was monitored during 14 days by the LC-DAD validated method. The sample preparation procedure was based on previous studies [9–11]. Briefly, 1 mL of each sample assay was collected with sterile syringe (every 2 days), centrifuged at 13,000 rpm (centrifuge Heraeus Biofuge Pico) for 20 min, and the supernatant was collected and stored in amber glass vials at −20 °C until LC-DAD analysis. Growth was monitored by measuring the OD of the cultures at 600 nm previously to centrifugation.

3. Results and Discussion

3.1. Enantioseparation Optimization for Ketamine and Its Metabolite Norketamine

The enantioseparation of K and NK was performed using the commercial analytical column Lux® 3 µm Cellulose-4 (150 × 4.6 mm), a polysaccharide-based chiral stationary phase (CSP), cellulose tris(4-chloro-3-methylphenylcarbamate), in a reversed elution mode. For the enantioseparation optimization, different mobile phase compositions and flow rates were evaluated (Table 1).

Table 1. Experimental conditions of analytical optimization of K and NK.

Equipment	Mobile Phase	Composition (*v*/*v*)	Flow Rate (mL/min)
LC-DAD	20 mM Ammonium Acetate with 0.1% DEA: ACN	73/27	1.1
		65/35	1.0
		67/33	
		70/30	
		73/27	
		74/26	
		75/25	
		70/30	0.5
		67/33	
		65/35	
	20 mM Ammonium Acetate with 0.1% DEA: EtOH	50/50	
		60/40	
		70/30	
	20 mM Ammonium Acetate: ACN	70/30	

First, conditions were attempted with 20 mM ammonium acetate in UPW with 0.1% of DEA and EtOH (50:50, *v*/*v*), a flow rate of 0.5 mL/min, an injection volume of 10 µL, detection at 220 nm and at room temperature (RT). EtOH was selected to replace the use of classic hazardous polar organic solvents. However, enantioseparation was not observed under these conditions, even with different proportions (Table 1). Thus, EtOH was replaced by ACN, and different proportions were tested. Additionally, flow rates between 0.5 and 1.1 mL/min were tested. Good enantioseparation and enantioresolution were observed using 20 mM ammonium acetate in UPW (with 0.1% of DEA) and ACN (70:30, *v*/*v*) at 0.5 mL/min, yet at longer retention times (Figure 1).

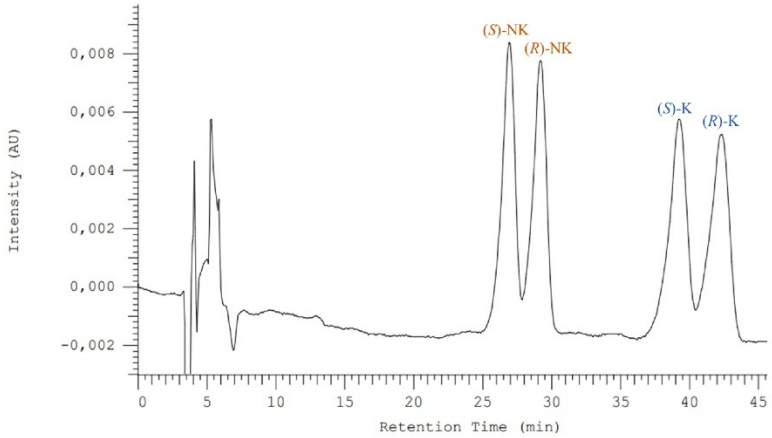

Figure 1. Chromatogram of enantioseparation optimization of K and NK (at 50 µg/mL in MeOH) in the analytical Lux® 3 µm Cellulose-4 column by LC-DAD at RT. Mobile phase: 20 mM ammonium acetate in UPW (with 0.1% of DEA) and ACN (70:30, *v*/*v*); flow rate: 0.5 mL/min; injection volume: 10 µL; detection: 220 nm.

Thus, to reduce the retention time of the analytes while maintaining enantioseparation, different flow rates (0.5 and 1 mL/min) and proportions (v/v) of this mobile phase were tested (namely, 75:25, 74:26 and 73:27). Figure 2 shows the chromatogram with 20 mM ammonium acetate in UPW (with 0.1% of DEA) and ACN (75:25, v/v) as a mobile phase. However, despite the good enantioselectivity and resolution, the analysis time was higher than 30 min.

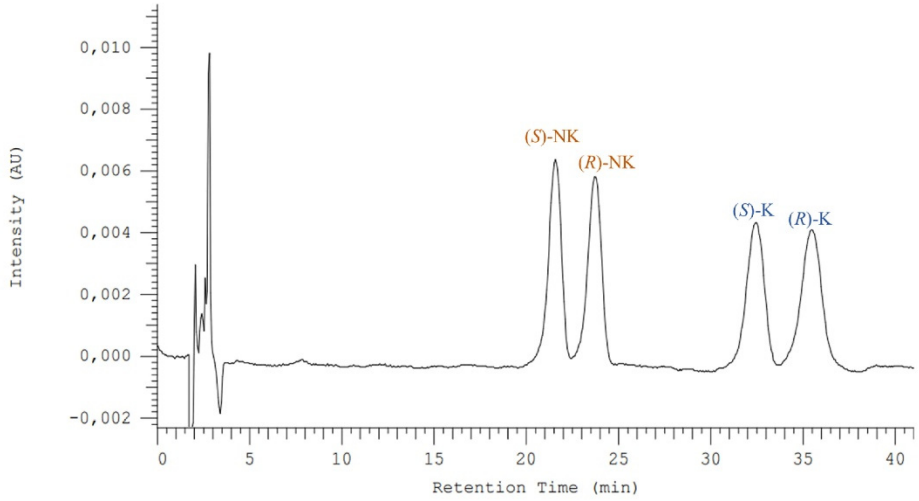

Figure 2. Chromatogram of enantioseparation of K and NK (50 µg/mL in MeOH) in the analytical Lux® 3 µm Cellulose-4 column by LC-DAD at RT. Mobile phase: 20 mM ammonium acetate in UPW (0.1% DEA) and ACN (75:25, v/v); flow-rate: 1 mL/min; injection volume: 10 µL; detector: 220 nm.

The optimized conditions were established with 20 mM ammonium acetate in UPW (with 0.1% of DEA) and ACN (70:30, *v/v*) as the mobile phase, a flow rate of 1 mL/min and an injection volume of 10 µL. Under these conditions, good enantioseparation and lower retention times were achieved. These conditions were selected for further validation of the method. The order of elution was determined according to a previous study reported elsewhere [40]. The retention times for NK and K enantiomers were 13, 14, 19 and 20 min for (*S*)-NK, (*R*)-NK, (*S*)-K and (*R*)-K, respectively (Figure 3).

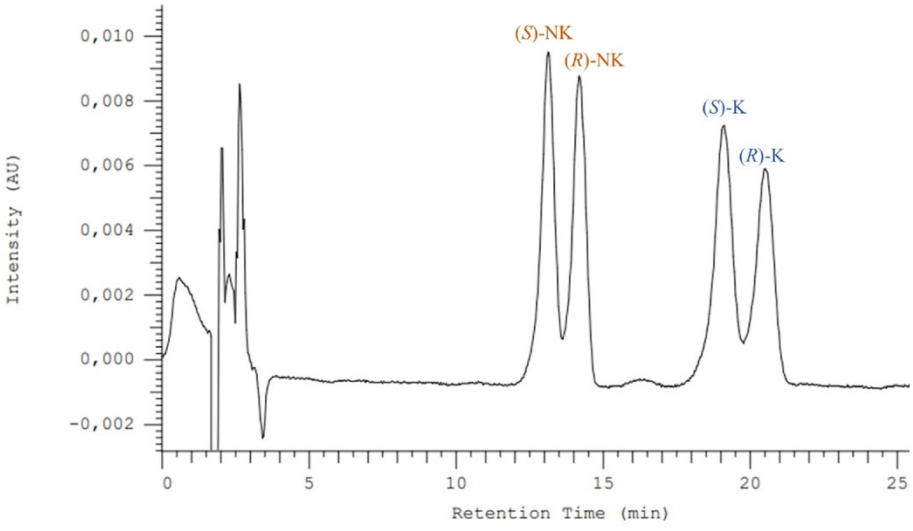

Figure 3. Chromatogram of the enantioseparation of K and NK (at 50 µg/mL in MeOH) in the analytical Lux® 3 µm Cellulose-4 column by LC-DAD at room temperature. Mobile phase: 20 mM ammonium acetate in UPW (0.1% DEA) and ACN (70:30, v/v); flow rate: 1 mL/min; injection volume: 10 µL; detection: 220 nm.

3.2. Validation of the Method for Quantification of Ketamine and Norketamine

The selectivity of the method was verified by comparison of the chromatograms with standards of K and NK in MeOH, MM (blank matrix) and spiked blank matrix with AS in the presence and in the absence of sodium acetate after 2 h of incubation. The nominal racemate concentrations used for K and NK were 50 and 25 µg/mL, respectively. The matrix was composed of several ionizable salts present in the MM that did not interfere with the chromatographic parameters.

The linearity was assessed for both NK and K enantiomers, which ranged from their LOQ to 12.5 for NK and 25 µg/mL for K (Table 2) and was analyzed by linear regression. The concentration range, linear regression equation and correlation coefficient ($r^2 > 0.99$) obtained for each enantiomer (Table 2) presented values in accordance with the requirements specified by the international guidelines [38].

Table 2. Linearity, LOD and LOQ for K and NK enantiomers.

Enantiomer	Linearity			Limits	
	Range (µg/mL)	Linear Regression	Correlation Coefficient (r^2)	LOD (µg/mL)	LOQ (µg/mL)
(*R*)-NK	1.25–12.5	$y = 5384.7x - 1847$	0.9908	0.5	1.25
(*S*)-NK	1.25–12.5	$y = 6038.7x - 1608.4$	0.9918	0.5	1.25
(*R*)-K	2.5–25	$y = 5127.9x + 493.42$	0.9998	0.5	2.5
(*S*)-K	2.5–25	$y = 5896.1x - 843.12$	0.9994	0.5	2.5

Accuracy, intra- and inter-day precisions and recovery were determined at three different QC standard solutions with enantiomeric concentrations of 2, 6 and 8.5 µg/mL for NK and 4, 12.5 and 17.5 µg/mL for K (Table 3).

Table 3. Accuracy, intra- and inter-day precision and recovery obtained for K and NK enantiomers.

Enantiomer	Concentration (µg/mL)	Validation Parameters			
		Accuracy (%)	Recovery (%)	Intra-Day Precision (%RSD)	Inter-Day Precision (%RSD)
(*R*)-NK	2	113.6	75.4	6.1	6.7
	6	94.8	81.9	8.1	8.4
	8.5	106.9	67.9	4.5	4.8
(*S*)-NK	2	105.4	69.5	6.3	8.8
	6	101.2	76.2	8.7	8.8
	8.5	105.9	64.0	5.9	6.1
(*R*)-K	4	86.6	61.0	9.4	10.5
	12.5	104.1	86.9	2.3	3.4
	17.5	102.3	71.1	1.8	2.7
(*S*)-K	4	85.9	50.1	10.4	11.3
	12.5	104.1	79.3	2.8	2.9
	17.5	103.6	69.4	3.6	3.8

Accuracy for NK ranged from 94.8 to 113.6% and for K from 85.9 and 104.1% (Table 3). Intra- and inter-day precisions were lower than 10.4 and 11.3%, respectively (Table 3). Precision values obtained agreed with those demanded by the ICH (up to 20% for LOQ and 15% for QC). Recovery varied between 50.1 and 86.9%. LOD was 0.5 µg/mL for both enantiomers of K and NK, while LOQ was 1.25 µg/mL for NK enantiomers and 2.5 µg/mL for K enantiomers (Table 2). These values proved to be suitable for the monitoring of the target enantiomers during the biodegradation assays.

3.3. Enantioselective Biodegradation Assays

The enantioselective biodegradation of K racemate was monitored using the validated LC-DAD method. The biodegradation assays were performed with K racemate at an initial concentration of 40 µg/mL in the MM inoculated with AS. The capacity of the AS to degrade the compound was investigated in the presence and in the absence of sodium acetate as an external carbon and energy source, under dark and light conditions. The abiotic and dead cells experiments were performed under the same conditions. The AS was obtained from the aerated tanks of a municipal WWTP selected due to its high microbial diversity. The biodegradation assay was monitored for 14 days (Figure 4). To construct the biodegradation patterns of K at the different experimental conditions, the concentration of K enantiomers was monitored by the enantioselective validated LC method and converted into degradation% according to the following formula:

$$\% \, D_t = 100 - \left[\frac{C_t}{C_0} \times 100 \right], \quad (1)$$

where % D_t is the degradation percentage at day t; C_t is the concentration at day t; and C_0 is the initial concentration (day 0).

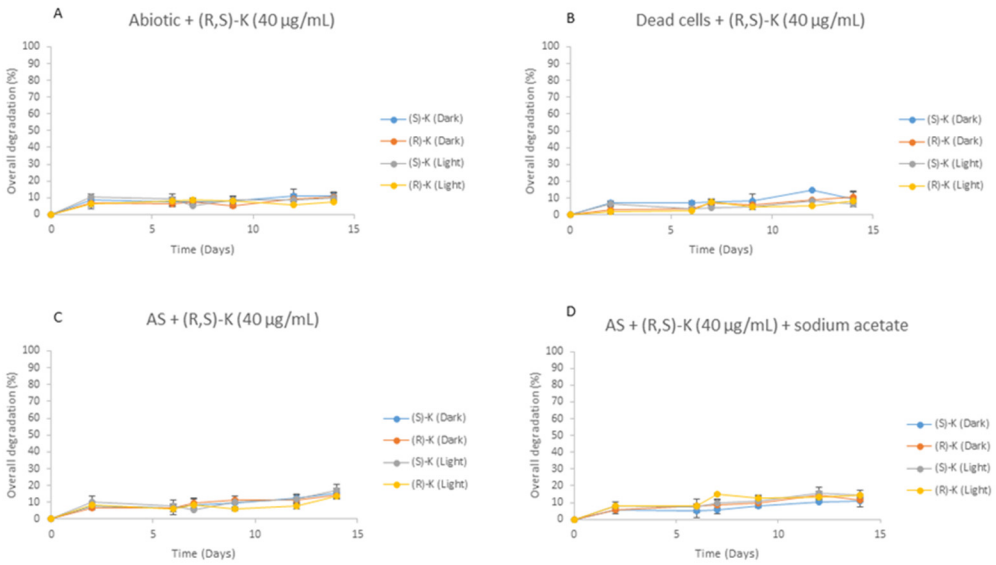

Figure 4. Monitoring of the enantioselective biodegradation assays with K racemate (at 40 µg/mL) during 14 days with: (**A**) abiotic; (**B**) dead cells; (**C**) AS; (**D**) AS supplemented with sodium acetate. (Blue and orange lines—assays performed in the dark; grey and yellow lines—assays performed in the light.)

Abiotic degradation or changes in the EF were not observed in both dark and light conditions (Figure 4). These results suggest that K is photostable, and that the abiotic process seems not to contribute to the degradation of K during sewage treatment. In both assays, the formation of unknown transformation products and NK was not observed. Regarding biotic assays, a slight reduction in K enantiomers (~28%) was observed in both dark and light conditions. Nevertheless, no enantioselective processes or differences between dark and light conditions were found. The biodegradation patterns in abiotic and biotic conditions (dark and light) are quite similar.

These results are of high concern. In fact, these data are in accordance with other monitoring reports that have shown not only the occurrence of K in aquatic environments but also its recalcitrance [29–31,33,41]. High concentrations of K ranging from 0.1 to 10 µg/L have been detected in wastewater and effluent samples [42–44], and it is ubiquitous in the receiving surface waters [27,30,45,46]. In addition, a recent study showed the presence of K in drinking water ranging from 0.00014 to 0.00112 µg/L [41]; however, K was also

previously reported at 0.015 µg/L [47], demonstrating its ubiquity in water samples. K is a biological active substance and negative effects on non-target exposed organisms might occur.

Recently, the impact of K on *Caenorhabditis elegans* physiological functions at environmentally relevant concentrations and an eco-risk assessment in surface waters were studied [31]. The authors observed that concentrations higher than 0.5 µg/L induced a negative impact on the feeding rate, locomotion, gustation, olfaction and vulva [31]. Another recent work showed that K tended to accumulate in fish tissues [29]. Further, K and NK enantiomers reveal different potencies and thus the risk posed by the occurrence of these chiral persistent substances to aquatic organisms should be evaluated. A recent study conducted by our research group showed a decrease in the percentage of growth of the protozoan *Tetrahymena thermophila* and mortality of the crustacea *Daphnia magna* exposed to K and NK [40]. In that study, enantiotoxicity and different susceptibilities of the organisms were observed.

4. Conclusions

An enantioselective LC-DAD method was developed and validated to quantify the enantiomers of K and NK. The analytical method was demonstrated to be precise and accurate and can be adapted to follow the enantioselective process in other environmental matrices. The method was successfully applied to follow a 14-day enantioselective biodegradation assay of K by AS.

The biodegradation studies regarding the racemate of K in different conditions, with and without the addition of sodium acetate in dark and light conditions, showed that K is poorly degraded and photostable, which supports its persistence reported in the aquatic environment. The metabolite NK was not detected in these assays and both enantiomers of K showed similar behavior. This study was conducted in a well-controlled aquatic medium and the results may not fully reflect the fate of K in the aquatic environment, but it can be a prediction of its persistence in the environment and to call attention to the importance of more ecotoxicological studies enrolling K, its main metabolite NK and its enantiomers. Additionally, these studies emphasized the importance of enantioselective biodegradation assays to understand the nature, source and toxicological impact of chiral substances in the environment.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/chemosensors9090242/s1, Table S1: Experimental conditions of biodegradation assay of K racemate in AS.

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