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ORIGINAL PAPER

Monitoring of oxolinic acid residues in tilapia flesh (*Oreochromis niloticus*) using a microbiological screening technique and an LC-UV confirmatory method

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Abstract
A relevant analytical strategy was developed by combining a microbiological screening and an LC-UV chromatographic method for the identification and the quantification of oxolinic acid (OXO) in tilapia (*Oreochromis niloticus*) flesh. The sensitivity, accuracy and specificity of the test were 100% for OXO. The detection capacity (CCß) of the screening test was 0.75 times the maximum residue limits for OXO (100 µg kg^{-1}). The performance parameters of the LC-UV method were satisfactory in terms of linearity within the range of 2.5 to 1000 µg kg^{-1} ($R_2 = 0.99$), precision (< 23%), accuracy (− 20% to + 10%), selectivity and specificity. The limit of quantification (LOQ) and detection (LOD) was 5 and 2.5 µg kg^{-1} respectively. The withdrawal of OXO in tilapia is estimated for 8 days after treatment of six successive days with a dose of 12 mg kg^{-1} body weight per day. The strategy used in this study is simple, inexpensive and practical for the control of oxolinic acid residues in fishery products and foodstuffs.

Keywords LC-UV · Microbiological screening · *Oreochromis niloticus* · Oxolinic acid · Residues

Introduction

Contrary to other Mediterranean countries, Algeria is distinguished by its poor fishing capacity. Despite the large width of its coast (1600 km), the tonnage of fish products was estimated at 510.012 tonnes in 2018 [1]. The coverage of the fish deficit in Algeria cannot be ensured by maritime fishing due to the narrowness of its continental shelf, the oldness of its fishing fleet and the steepness of its coast. The food ration is below 5 kg/inhabitant/year compared to 20.5 kg/inhab/year of the world average [1]. The popularisation of marine and continental aquaculture, as well as the introduction of new fish species on the national market, seem to be adequate solutions to remedy this lack and improve the fish food ration. Among fish species, Nile tilapia (*Oreochromis niloticus*) seems to be a species of choice to promote aquaculture and provide it with sustainable development. This species is characterised by an easy and rapid reproduction in captivity, a basic diet at the lowest level of the trophic chain (phytoplankton and detritus), it can be produced with a cheap cost and displays interesting taste and nutritional properties [2].

Tilapiculture is an intensive farming mode requiring the use of antimicrobial chemotherapy to limit the economic impact of bacterial ichtyopathologies usually caused by *Aeromonas* spp., Enterobacteriaceae., *Enterococcus* spp., *Micrococcus* spp., Moraxellaceae, *Plesiomonas* spp., *Pseudomonas* spp., *Staphylococcus* spp., *Streptococcus* spp., *Vibrio* spp. [3–5]. Antibiotics are also used as a preventive

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measure during critical phases (early stages, fish transfers) or as growth promoters like flavomycin, florfenicol or oxytetracycline [6, 7].

Among the fluoroquinolones used in aquaculture worldwide, oxolinic acid (OXO) remains relevant in Algeria, despite the emergence of its resistance. It is used in the treatment of Gram-negative infections in aquaculture. OXO acts through its ability to cross the bacterial wall through porins and inhibit the action of DNA gyrase and topoisomerase IV (the most sensitive enzyme in Gram-negative bacteria) [7].

Since the early 1980s, a restrictive European legislation on veterinary medicinal products has been implemented to limit as far as possible the use of medicinal substances in the treatment of animals for human consumption. Maximum residue limits (MRLs) compatible with public health have been set at the European level for all active substances (European Regulation n° 37/2010) [8].

In Europe, Canada, the USA and Japan, the list of antibiotics authorised for use in aquaculture is very limited and is restricted to oxytetracycline, florfenicol, sarafloxacin, erythromycin, sulphonamides (trimethoprim, ormethoprim) and some fluoroquinolones [9, 10]. Furthermore, the largest producers and exporters of aquaculture products are in Asia (China, India, Vietnam, Indonesia, Bangladesh, Thailand, Philippines), Latin America (Chile and Brazil) and Africa (Egypt) where antibiotic therapy is used inappropriately and some prohibited substances in EU are still in use such as chloramphenicol and nitrofurans [11–13]. The Algerian context is also disastrous, as drug residue regulations are nonexistent and the laboratories are poorly equipped and sophisticated to survey the risk of the use of antibiotics [14]. In Algeria, good animal husbandry and veterinary practices are anarchic and the official control remains elementary. The availability of veterinary drugs at affordable costs leads to their abusive usage by the local fish farmers. These practices may lead to the direct presence of antibiotic residues in edible parts of fish and present a proven public health chemical hazard [13]. The persistence of antibiotics in the marine ecosystem induces the development of resistance in resident organisms; consequently, the resistance can be horizontally transferred to pathogenic and commensal human bacteria [13, 14]. This occurrence of new strains of highly antibiotic-resistant bacteria is described as cross-resistance to antibiotics being generated in the aquaculture sector, which raises concerns and worries of human therapists [10, 13, 15]. Antibiotic residues can also cause technological risks in the food production process and have a negative environmental impact [14].

Generally, antimicrobial residue monitoring procedures are divided into screening and confirmatory methods. Analytical techniques can be of a microbiological, immunochemical or physicochemical nature [11]. Screening methods are simple and qualitative ones used to reveal the presence of several analytes in a large number of samples, while confirmatory methods are applied for the identification and quantification of any specific antimicrobial residue that is positive in the screening tests. The microbiological tests (bioassays) are based on the principle of inhibition of bacterial growth by residues. The immunochemical procedures (biosensors, multi arrays, radioimmunoassays and the enzyme-linked immunosorbent assay "ELISA") are employed for the identification of specific antimicrobial residues or for the recognition of structurally similar metabolites through antibody/antigen/enzyme interaction [16]. Physico-chemical methods require expensive equipment and skilled personnel and are usually based on chromatographic separation methods such as high-performance thin layer chromatography (HPTLC) [17], HPLC coupled with UV [18] detection or mass spectrometry [19].

In view of this delicate situation in Algeria, our work was undertaken with the objective of developing an analytical strategy to detect and quantify oxolinic acid residues in tilapia culture products harvested from the Fat-steppes farm (Western Algeria). This analytical approach is based on a first qualitative microbiological screening test of a large number of samples. The positive ones are secondly confirmed and quantified by a quantitative method (LC-UV).

Materials and methods

Experimental design of tilapia contamination with oxolinic acid

Animal management

The experiment of tilapia contamination with oxolinic acid was carried out in triplicate on healthy male tilapia (n = 70/tank) weighing on average 92.4 g ± 25.4 g, belonging to the same population and originating from the Fat-Steppes farm, (Wilaya of Saida, Algeria). The fish were divided into two batches (treated & control), each consisting of three cylindrical plastic tanks (1 m^3). Adequate equipment has been installed on all aquariums (filter, oxygenator and submersible heater) to ensure a level of dissolved oxygen level between 5 and 10 ppm, a pH of 7 and to stabilise the water temperature between 29 and 30 °C. Daily cleaning was also performed and the photoperiod was natural and corresponded to 15 h of light and 9 h of darkness on average. All animal studies were performed according to the protocols approved by the Oran university's ethics committee. Before the onset of drug administration, tilapias were acclimated for 1 week during which they were fed antibiotic-free commercial feed.

Preparation and distribution of medicated feed

The medicated feed was prepared according to Julinta et al. [20] protocol. The veterinary oxolinic acid "Oxomid 20®" (VIRBAC, France) drug which is a 20% premix antibiotic powder, was first mixed with the pellets of the commercial tilapia feed to obtain a homogeneous distribution, at a concentration of 2.4 g of oxolinic acid per kg of medicated feed (i.e. 12 g of Oxomid per 988 g of feed). Subsequently, 1.5% cod liver oil was added. The mixture was made 1 week before the start of the experiment and stored at 4 °C protected from light and humidity.

During the six consecutive days of treatment, tilapias were fed by a manual distribution of the feed, twice a day, in order to simulate real rearing practices. To correspond to the recommended therapeutic dose of 12 mg kg^{-1} body weight of fish/day, a proportion of 0.50% of the biomass was distributed.

Experimental design and sampling

To control oxolinic acid residues from tilapia's flesh in both control and treated batches, samples of tilapia (*O. niloticus*) were daily taken from the first day (12 h post drug intake) until the 14 day after the first drug intake (Fig. 1). Five fish (n = 5) were randomly sampled from each tank and for each kinetic point: 12 h, 24 h, 2nd day, 3rd day, 4th day, 5th day, 6th day (cessation of treatment), 7th day, 8th day, 9th day, 10th day, 11th day and the 14th day. Fishes sampled from the same aquarium were anaesthetized using *Benzocaïne (Sigma-Aldrich*, ref. E1501) (200 mg/l of water). Their flesh (skin and muscle in natural proportion) were collected, crushed and pooled. Each pooled sample was analysed in triplicate (n = 3).

To evaluate the stability of oxolinic acid during refrigerated storage of feed, three samples of medicated feed were also collected before, during and after the experiment. All the samples of flesh and feed were preserved at − 20 °C until LC-UV analysis.

Optimisation of the analytical strategy

Reagents

All antibiotic standards were purchased from Sigma-Aldrich (St-Louis, MO, USA): oxolinic acid (75905), ciprofloxacin (17859), enrofloxacin (33699), flumequine (F7016), chlortetracycline (C4881), doxycycline (D9891), tetracycline (T7660) and oxytetracycline (O5875).

Bacillus subtilis spore suspension (Strain BGA containing 10^7 spore mL^{-1}.10649-ampoule of 2 mL), Test Agar pH 6 (1.10663) and sodium hydroxide were purchased from Merck (Darmstadt, Germany). Acetonitrile and ammonium hydroxide were purchased from Acros Organics (New Jersey, USA). Acetone and methanol were purchased from Biosolve (Valkenswaard, the Nederlands) and Prolabo (Pennsylvania, USA) respectively. The 12.7 mm diameter blank discs (0905A0 0005) were provided by Fiers (Belgium). Lomefloxacin (98079-51-7) was supplied by Sigma-Aldrich (St. Louis, MO, USA), 98% formic acid was provided by Acros Organics (New Jersey, USA), 98% ammonium acetate was provided by Merck (Darmstadt, Germany), and HPLC-grade water was provided by BDH (Poole, England).

Acrodisc 13 mm filters with a 0.2 µm membrane, 1 mL Hamilton syringes, SPE cartridges (SDB-RPS) and a Vac-Master were used during purification.

Preparation of solutions and the microbiological media

For the preparation of stock solutions concentrated at 1 mg mL^{-1}, tetracyclines (chlortetracycline, doxycycline, tetracycline and oxytetracycline) were directly dissolved in methanol. On the other hand, fluoroquinolones (oxolinic acid, ciprofloxacin, enrofloxacin and flumequine) were dissolved in a small volume of NH_4OH 2 M before methanol addition to facilitate the dissolution.

Chlortetracycline and tetracycline solutions were stored in the freezer (− 20 °C) and the other solutions at 4 °C.

For the calibration curve, ten solutions of oxolinic acid were used corresponding to 0 to 1000 µg kg^{-1} in fish, i.e. 0 to 10 × LMR, knowing that the LMR of oxolinic acid is 100 µg kg^{-1} in fish flesh (EU regulation, n°37/2010) [8].

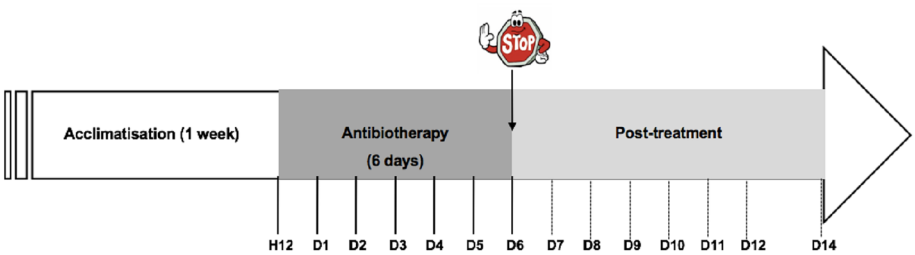

Fig. 1 Scheme of the oxolinic acid contamination experiment and sampling. H12, D1-D14: sampling points, H: hours, D: day, Dose of oxolinic acid antibiotherapy: 12 mg kg^{-1} body weight

Lomefloxacin was chosen as the internal standard for UV detection. It was prepared in a pool containing 30 µL of the stock solution (1 mg mL^{-1}) of each compound with the addition of 9.94 mL of formic acid at pH 2.5.

For the extraction procedure, a solution of ammonium hydroxide 1 M, a buffer solution (pH 4) of ammonium acetate 5 mM and a formic acid solution (pH 2.5) were prepared.

For the SPE column elution solution, a mixture of methanol-ammonium hydroxide 1 M (75:25, v/v) was made.

The medium pH 6 for screening was prepared from a mixture of pH 6 culture broth (200 mL), 2.66 mL of glucose solution 6% and 200 µL of Bacillus subtilis spore suspension.

Microbiological screening protocol

The protocol of Dang et al. [21] was optimised, validated and adapted for the microbiological screening of eight antibiotics residues in tilapia flesh, 4 Fluoroquinolones (oxolinic acid, ciprofloxacin, enrofloxacin and flumequine) and 4 Tetracyclines (chlortetracycline, doxycycline, tetracycline and oxytetracycline). In a 15 mL centrifuge tube, 4 g of fish flesh including skin in natural proportion) were mixed with 5 mL of acetonitrile: acetone (70:30) mixture. After shaking for 15 min in a rotary shaker and centrifuging for 15 min at 3000 rpm at 20 °C, the upper phase was transferred to a new tube. The extract was evaporated to dryness under a stream of nitrogen at 40 °C and then reconstituted in 200 µL of methanol. The mixture was vortexed and centrifuged for 10 min at 3000 rpm at 20 °C. Finally, 50 µL of the upper phase of the extract was loaded onto each of the two cellulose discs (disc 1 and disc 2) that were previously placed on the agar plate (medium pH 6). Twenty µL of NaOH 1% were added to the second disc only (Fig. 2). Petri dishes were left for 1 h at room temperature before being incubated for 18 h at 30 °C.

For the positive control, oxolinic acid and oxytetracycline were tested and chosen to represent the two antibiotic families of interest (fluoroquinolones and tetracyclines respectively). 50 µL of their intermediate standard solution (20 µg mL^{-1}) were inoculated onto each of the two cellulose discs placed in the Petri dish. For the negative control, 50 µL of methanol was loaded on each disc.

The positive result consists of the formation of a zone of inhibition ≥ 1.5 mm wide in at least one of the two loaded discs.

Validation of the microbiological screening

The microbiological screening method, for the eight antibiotics of interest, was validated in terms of sensitivity, specificity, accuracy and detection capability (CCβ) according to the recommendations of the European Commission decision 2002/675/EC [22] as well as the guidelines for the validation of screening methods for residues of veterinary medicines [23]. For this purpose, a series of 20 blank samples spiked with concentrations decreasing from the MRL, as well as 20 blank samples of control (uncontaminated tilapia) were analysed.

The detection capability (CCβ/Critical Concentration ß) corresponds to a concentration where at least 19 of the 20 samples tested give a positive result (inhibition zone width ≥ 1.5 mm) which ensures a ß-risk (risk of false-negative) ≤ 5%.

The 20 blank samples are considered as true negatives and the 20 fortified samples are categorized as true positives.

For the calculation of the performance parameters, three formulas were employed as shown in Table 1 [23]: $\text{Sensitivity}(\%) = \left(N^+/TP\right) \times 100\%$, $\text{Specificity}(\%) = (N^-/TN) \times 100\%$ and $\text{Accuracy}(\%) = \left[N/(TP + TN)\right] \times 100\% \left(N{:}N^+ + N^-\right)$

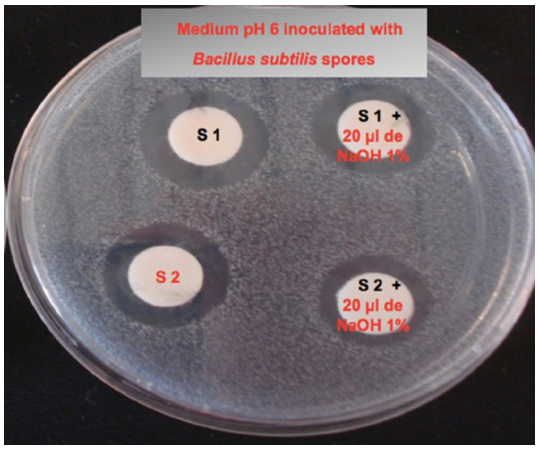

Fig. 2 Illustration of the one plate microbiological screening test. S1: 50 µL of sample 1 extract loaded, S2: 50 µL of sample 2 extract loaded

Table 1 Determination of the accuracy, specificity and sensitivity of the microbiological

Result of the test	True positive sample (TP) (fortified samples)	True Negative Sample (TN) (blanks)
Positive	N^+ (True Positive)	FP (False Positive)
Negative	FN (False Negative)	N^- (True Negative)

TP number of fortified samples analysed, *TN* number of blank samples analysed, N^+ number of samples detected as positive, among the N^+, N^- number of samples detected as negative, among the N^-, *FP* number of samples detected as positive, among the N^-, *FN* number of samples detected as negative, among the N^+

LC-UV protocol

The protocol of Danyi et al. [24] was optimised for UV detection of oxolinic acid in tilapia flesh. For the unknown samples, 1 g ± 0.05 g of homogenised fish flesh sample was supplemented with 100 µL of lomefloxacin (internal standard) in 50 mL centrifuge tubes and kept at room temperature for 15 min. For the calibration curve points (CC ×) 1 g ± 0.05 g of flesh was weighed from the blanks (uncontaminated tilapia) and mixed with 100 µL of the internal standard and 100 µL of the oxolinic acid intermediate solution at concentrations corresponding to 0–1000 µg kg^{-1} in fish flesh.

Ten mL of acetonitrile was added to the tube, and the mixture was vortexed, shaken for 15 min on a rotary shaker and centrifuged for 10 min at 4000 rpm at room temperature. The supernatant was transferred to a new Falcon tube and 3 mL of hexane was added. The mixture was then vortexed for 2 min and centrifuged for 5 min at 4000 rpm at room temperature. The lower phase was recovered and evaporated under nitrogen at 37 °C. The dry residue was reconstituted in 2 mL of ammonium acetate buffer (5 mM, pH 4). The mixture was vortexed for 15 s and then sonicated for 15 min.

After conditioning the SPE column (SDB-RPS) using 2 × 1 mL methanol, 2 × 1 mL water and 2 × 1 mL ammonium acetate buffer, the extract was transferred to the column and the tube was rinsed with 1 mL of ammonium acetate buffer and poured onto the column.

The column was then dried by centrifugation for 5 min at 4000 rpm. The analyte was eluted with 4 mL of methanol-ammonium hydroxide 1 M (75:25, v/v). The eluate was evaporated under nitrogen at 37 °C and the dry residue was reconstituted in 300 µL water–formic acid (pH 2.5), filtered through an Acrodisc® filter (0.20 µm) and finally loaded into vials with inserts which were sealed and stored at 4 °C until injection.

The analysis was carried out on a Spectra System P4000 HPLC chain (Thermo Fisher), coupled to an AS3000 automatic injector and detector of the diode array (UV6000LP) type. The injection volume was 20 µL on a Varian Chromsep SS 150 * 2 mm (L* ID) Polaris 3 C18-A column placed in an oven at 40 °C. The UV detection wavelength was 260 nm. The run time was 20 min and the flow rate was 0.4 mL min. XCalibur software was used to control the instruments and quantify the results. XCalibur automatically calculates the equation (linear regression) of the dose (oxolinic acid concentration) response curve (ratio of oxolinic acid peak area to internal standard peak area). The software also automatically calculates from the parameters of the linear regression, the oxolinic acid concentrations in the unknown samples. The operator checks that the peaks are correctly integrated and that the calculated concentrations of the unknown samples are within the working range of the calibration curve.

The elution gradient programme used is shown in Table 2.

Validation of the LC-UV method

The optimised quantitative LC-UV-MS method was validated in terms of trueness, precision (repeatability and reproducibility), linearity, specificity, selectivity, the limit of quantification (LOQ) and limit of detection (LOD) according to the European Commission Decision 2002/657/EC [22] (this European regulation was in force while this study was carried out).

In our study, five concentrations were evaluated (MRL/4, MRL/2, MRL, 1.5 × MRL and 2 × MRL) in triplicate on the flesh matrix to study trueness.

The results obtained must be within a range of − 20 to + 10% of the expected concentration (Decision 2002/657/EC) [22] for the concentration range in which the samples fall (≥ 10 ppb).

Repeatability and reproducibility were assessed in triplicate (n = 3) on a series of spiked samples at different concentrations (MRL/2, 1 × MRL, and 2 × MRL). The precision was expressed as a coefficient of variation (CV or RSD). The RSD value is set at 23% for the reproducibility CV and 15% for the repeatability CV (2/3 of the reproducibility CV value) for the concentrations range of this study [22].

The relative retention time (RTT), represents the ratio of the retention time (RT) of the oxolinic acid (OXO) to the RT of its internal standard (the reference). RRT of the OXO in the samples must not deviate from the average RRT of the reference OXO (calibration curve) by more than ± 2.5%.

Statistical analysis

The statistical analysis was carried out with the SAS system using a GLM (General Linear Model) and quadratic regression. Firstly, the influence of the experimental parameters of oxolinic acid contamination on the results of the screening and chromatographic analysis was evaluated. The experimental parameters taken into consideration were: sampling time, batch, fish weight, dissolved O_2, water pH, nitrite level, nitrate level and ammonia level.

In a second step, the comparison between the control and the contaminated tilapia batches was analysed. In all

Table 2 Oxolinic acid gradient elution program for LC-UV analysis

Time (min)	Acid formic solution pH 2.5 (%)	Acetonitrile (%)
0.00	90	10
1.00	90	10
12.00	20	80
14.00	0	100
14.20	90	10
20.00	90	10

cases, descriptive statistics (means ± standard deviations) were used to describe all of the results. Three specimens (n = 3) were analyzed for each parameter. The results were statistically interpreted by analysis of variance (parametric test Anova 1). The difference between the means concentrations of oxolinic residues was determined by Tukey's test ($P < 0.05$).

Finally, the correlation between analytical methods and between both results (screening & chromatography) was tested by calculating the linear correlation coefficient according to the Pearson test.

Results

Microbiological screening

Results of the detection capacity of the microbiological test (CCβ)

The detection capacity of the method was lower than the MRLs for each of the eight tested antibiotics (Table 3). The CCβ varied between 0.15 and 0.65 times the MRL for tetracyclines and it fluctuated between 0.13 and 0.8 times the MRL for fluoroquinolones.

Validation of the microbiological screening

The results of the performance parameters of the microbiological screening are shown in Table 4. The optimised method demonstrated the specificity of 100%, a sensitivity of at least 95% and an accuracy of at least 97.5% for enrofloxacin, flumequine and doxytetracycline. For the other antibiotics and in particular oxolinic acid, all performances were 100%.

Validation of the LC-UV method

The results shown in Table 5, demonstrate that the calculated trueness values for the five oxolinic acid concentrations in tilapia flesh are within the tolerated range (− 20 to + 10%). For the precision results under reproducibility conditions, all the calculated values of variance (RSD) are less than 23%.

The limit of quantification (LOQ) was 5 µg kg^{-1} (MRL/20) corresponding to the first point of the calibration curve (excluding 0) and the limit of detection (LOD) was 2.5 µg kg^{-1} (LOQ/2).

The linearity was checked on the basis of the coefficients of determination R2 and calculated by linear regression. The average result obtained for three calibration curves is $R_2 = 0.99$ for the flesh matrix (Fig. 3).

The relative retention time (RRT) of oxolinic acid fluctuated between 6.95 and 7.46 min, and that of the internal standard between 9.28 and 9.73 min. All calculated values are within the tolerated deviation of 2.5%.

The chromatogram illustrated on Fig. 4 reveals the selectivity of the method LC-UV by distinguishing between the peak and the retention time of the oxolinic acid (OXO) and the internal standard (IS).

Analysis of samples from tilapia treated with oxolinic acid

Microbiological screening

The results of the microbiological screening of the tilapia flesh samples contaminated with oxolinic acid are shown in

Table 3 Determination of the detection capacity (CCβ) of the microbial screening

Antibiotic	MRL* (µg kg^{-1})	Tested Concentration (µg kg^{-1})	Width of the zone of inhibition (mm) (n = 20)		Detection capacity CCβ expressed as a fraction of MRL
			Disc 1	Disc 2 (+ NaOH)	
Fluoroquinolones					
Oxolinic acid	100	75	2.03 ± 0.32	1.90 ± 0.39	0.75
Ciprofloxacin	100	80	0	1.82 ± 0.29	0.8
Enrofloxacin	100	30	0.60 ± 0.17	1.85 ± 0.19	0.3
Fluméquine	600	75	1.71 ± 0.15	0	0.13
Tetracyclines					
Oxytetracycline	100	65	1.59 ± 0.06	0	0.65
Doxytetracycline	100	15	1.86 ± 0.20	0.4 ± 0.37	0.15
Chlortetracycline	100	15	1.98 ± 0.33	0	0.15
Tetracycline	100	35	1.69 ± 0.16	0	0.3
Blanks		0	0	0	0

Mean and standard deviation (n = 20) are calculated for each antibiotic

*European regulation (EU) n° 37/2010 [8]

Table 4 Performance parameters of the microbiological screening method

Antibiotic	MRL* (µg kg^{-1})	Detection capacity CCβ (fraction of MRL)	Number of Positive N$^+$	Number of Negative **** N$^-$	Method performance		
					Specificity (%)	Sensibility (%)	Accuracy (%)
Fluoroquinolones							
Oxonolic acid	100	0.7	20	20	100	100	100
Ciprofloxacin**	100	0.8	20	20	100	100	100
Enrofloxacin**	100	0.3	19	20	100	95	97.5
Flumequine	600	0.1	19	20	100	95	97.5
Tetracyclines							
Oxytétracycline	100	0.6	20	20	100	100	100
Doxytétracycline***	100	0.1	19	20	100	95	97.5
Chlortétracycline	100	0.1	20	20	100	100	100
Tétracycline	100	0.3	20	20	100	100	100

*European regulation (UE) n° 37/2010 [8], **100 µg kg^{-1} for the sum of ciprofloxacin and enrofloxacin, ***MRL for meet, ****Measured on 20 blank samples

Table 5 Trueness and precision of the LC-UV method (n = 3)

Spiked concentration (µg kg^{-1})	Calculated concentrations (µg kg^{-1})		R.S.D % (Precision*)	Trueness % **
	Mean	SD		
25	26	5.7	21.9	+3.7
50	51	7.2	14.1	+1.8
100	103	2.8	2.8	+2.7
150	143	20.0	14.0	− 4.7
200	196	13.0	6.6	− 1.9

*Precision determined under reproducibility conditions R.D.S (%) < 23% [22], **Trueness interval: − 20% to + 10% [22]

Fig. 5. The average results of the microbiological screening of tilapia flesh from the three batches of contaminated fish (Fig. 5) showed a constant response of about 12 mm from the first sampling (12 h) after the start of antibiotherapy which persisted until the 7th day of the experiment (first day after the treatment cessation).

This saturation of the response is explained by the very high levels of oxolinic acid in the tilapia flesh, at which the dose–response relationship of the microbiological test is not proportional any more. The response started to decrease from day 2 after the treatment cessation and fell below the threshold of positivity of the test (width of the inhibition zone = 1.5 mm) on the 5th day after the treatment cessation (day 11).

The calculation of the average width of the inhibition zones of the 70 samples treated in each batch and which were inoculated on the two discs, reveals that the values obtained are very close (disc 1 containing just the extract and disc 2 containing the extract and 20 µl of 1% NaOH in addition). The average width of the inhibition zones was 10.1 ± 0.9 mm for disc 1 and 9.5 ± 0.9 mm for the second disc. These values are not significantly different ($P < 0.05$) between the widths of the two discs. In this fact, only one

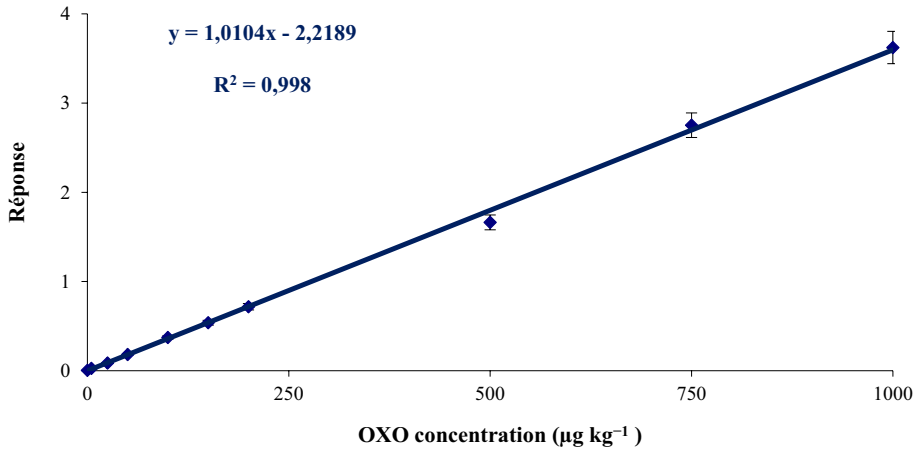

Fig. 3 Average calibration curve made from tilapia flesh spiked with increasing concentrations of oxolinic acid (n = 3). Response = Area of analyte/area of internal standard (IS)

disc could be used for the specific microbiological screening of oxolinic acid on a pH6 medium seeded with *Bacillus subtilis*.

Quantification of oxolinic acid concentrations in tilapia flesh by LC-UV

Twelve hours after the oral intake of the medicated feed, the average concentration of oxolinic acid in flesh was high

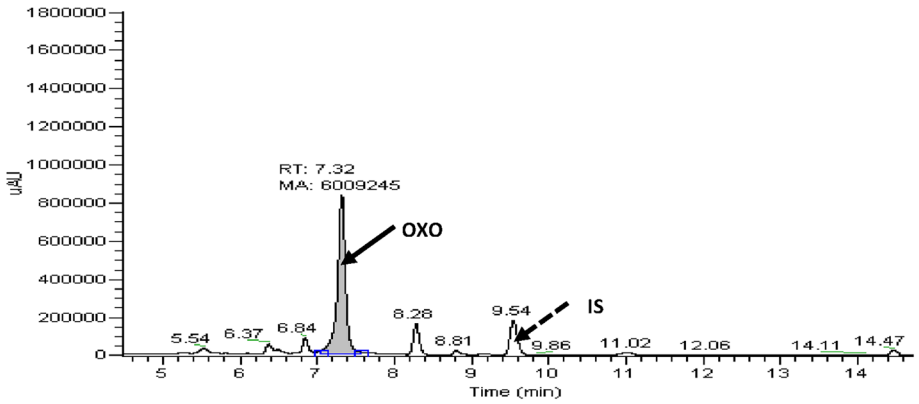

Fig. 4 Chromatogram obtained during HPLC–UV analysis of tilapia flesh containing oxolinic acid at a concentration of 50 µg kg.$^{-1}$

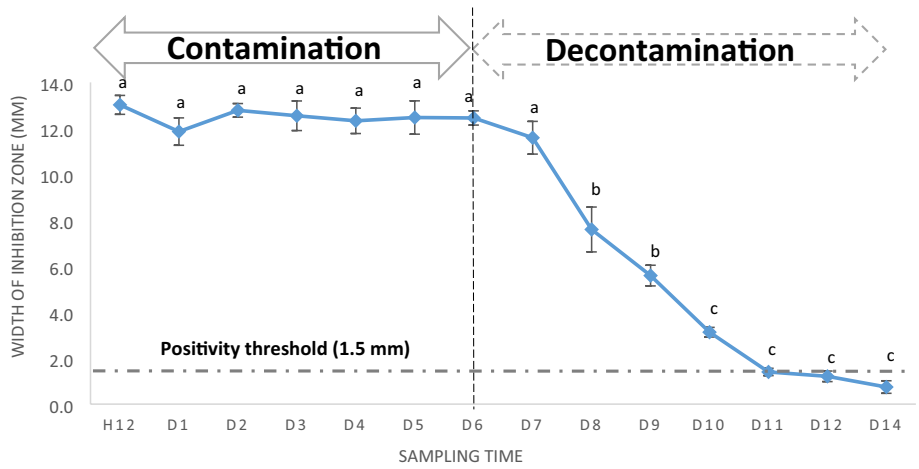

Fig. 5 Results of the microbiological screening of tilapia flesh of the oxolinic acid contamination experiment. Mean ± standard deviation (n = 5). Different letters/numbers on the same curve indicate a statistically significant difference (Tukey test, $p < 0.05$)

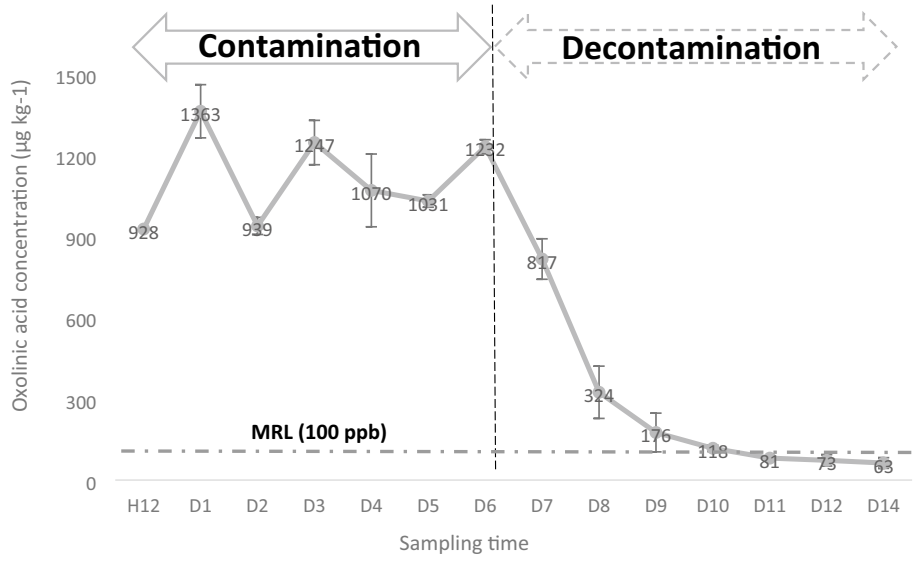

Fig. 6 Oxolinic acid concentration (µg kg^{-1}), determined by LC-UV, in the flesh of tilapia from the OXO contamination experiment at various sampling times. Mean ± standard deviation (n = 5). Different letters/numbers on the same curve indicate a statistically significant difference (Tukey test, $p < 0.05$)

(928 ± 98 µg kg^{-1}) (Fig. 6). This concentration increased rapidly to reach a peak of 1363 ± 32 µg kg^{-1} after 24 h of the antibiotherapy. The OXO average concentration in the three treated batches remained statistically stable ($P < 0.05$) and fluctuated between 939 ± 82 µg kg^{-1} and 1247 ± 134 µg kg^{-1} within the remaining 4 days of the treatment. In the decontamination phase (after treatment cessation), the average concentration drops rapidly and significantly ($P < 0.05$) from the first day of decontamination (817 ± 97 µg kg^{-1}) and continued to fall until it crossed the MRL threshold on day 11 (day 5 of treatment cessation) to reach 81 ± 21 µg/kg. Finally, a plateau was reached below the MRL threshold at the last two sampling points D12 and D14 (63 ± 20 µg kg^{-1}).

The comparison between both results allows us to demonstrate that the envisaged analytical strategy, microbiological screening followed by identification and quantification by LC-UV, operates well for the monitoring of oxolinic acid residues in tilapia flesh (*Oreochromis niloticus*). In fact, a result below the microbial screening threshold is well below the MRL when the analysis is performed by HPLC–UV and vice versa (Fig. 7).

Pearson test analysis of the obtained results by both analytical methods (screening & LC-UV) showed a significant and positive correlation between them ($r = 0.62$).

Discussion

The presence of veterinary drug residues in foodstuffs represents a proven chemical risk for the consumer [12, 15, 16]. Scientific research has made several efforts in developing analytical technics that allow the detection of antibiotic residues in particular [4]. Quantitative analysis of antibiotic residues follows a well planned strategy that usually begins with a simple non-specific microbiological screen. This is probably followed by a specific enzymatic or immunological test as a post-screen. Lastly, the confirmation and the quantification is done by liquid chromatography [25].

For qualitative monitoring based on microbiological screening purposes only, the European Commission Decision 2002/657/EC [22] states that a screening test is validated when its detection capacity (CCß) is below the MRL value of the antibiotic of interest. The interpretation of the results is translated as compliant for negative results and suspected non-compliant for positive results.

In addition, guidelines for the validation of screening methods for veterinary drugs of the CRL (Community Reference Laboratories Residues), have been published to standardise the monitoring protocols [23].

In this context, for our study on oxolinic acid residues (quinolone frequently used in tilapiculture) in tilapia flesh, we adapted an analytical methodology based on microbiological screening using a single pH 6 agar plate inoculated with *Bacillus subtilis* combined with a quantitative confirmation by LC-UV.

As a matter of fact, microbiological screening is of great analytical thanks to its affordability, simplicity to perform as well as quickness in time execution. The use of this method goes back several decades ago, and the progress that has been made led to variations in the matrices, the number and shape of the culture agar plates (Petri dishes or ampoules), the pH of the plates, the bacterial strain and the extraction protocol [25–30]. In general, the most requested strains are *Yersinia ruckeri* [26], *E. coli* [27], *Geobacillus stearothermophilus* often used in ampoules for the Premi® Test [28, 29] or the Delvotest® SP-NT [28], *Klebsiella pneumoniae* [26], *Bacillus cereus* [30] and *Bacillus subtilis* [31].

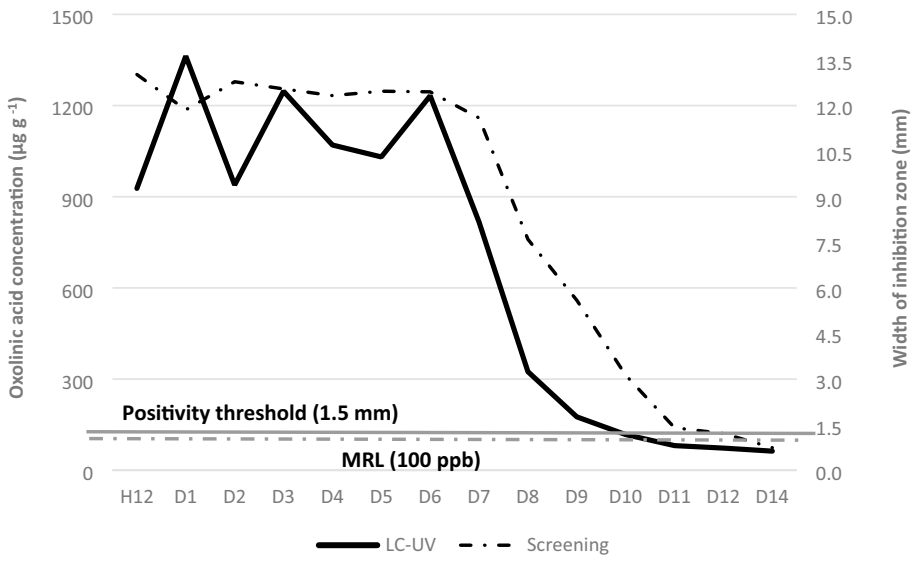

Fig. 7 Correlation between the results of screening and LC-UV analysis

The strain used for our microbial screening "*Bacillus subtilis*" has the particularity of being sensitive to several antibiotics, especially fluoroquinolones and tetracyclines [25, 26, 31]. It is the strain of choice for most European reference tests (Belgian Kidney test, New Dutch Kidney test and EU 4-plate test).

The obtained results in our experiment revealed that the screening test based on the inhibition of the growth of *Bacillus subtilis* inoculated in Agar pH6 and incubated at 32 °C for 18 h is conclusive.

The optimization of the extraction protocol [21] during the reconstruction of the extract in 200 mL instead of 250 mL initially, considerably improves the sensitivity of the method. The detection capacity (CCß) was improved from 0.95 (Data not shown) to 0.75 LMR fraction.

The performance parameters (accuracy, sensitivity and specificity) of the method for the detection of two families of antibiotics residues, namely fluoroquinolones and tetracyclines, are satisfactory. All the values of the detection capacities of the eight antibiotics of interest are lower than their respective MRLs.

The performance parameters of the technique are outstanding for all the studied antibiotics. Sensitivity is a minimum of 95%, accuracy a minimum of 97.5% and specificity at 100%. Especially for oxolinic acid, all tested parameters (accuracy, sensitivity and specificity) are at 100%. The detection capacity (CCß) was 75 µg kg^{-1}, which is lower than MRL (100 µg/kg).

The obtained results are in good agreement with those shown by Dang et al. [21, 25].

Our results are also in agreement with the results of Stead et al. [28] who demonstrated that the pre-extraction of antibiotic residues with a solvent mixture (acetonitrile/acetone) improved the detection limits of the Premi® Test screening test compared to the direct application of pork juice screened by the same test.

In the microbial screening of aquaculture products (especially crustaceans), the inhibitory effect of lysozyme [21] could interfere with the antibacterial effect and thus false the result (false positive) by widening the inhibition zone. Reconstituting the antibiotic extract in methanol should inactivate lysozymes [25].

The obtained results during the validation of the screening method and the analysis of contaminated tilapia samples show that the diffusion of antibiotics in the agar is influenced by the medium pH. Depending on the nature of the antibiotic, alkalising the medium (pH6 agar) by adding 20 µl of 1% NaOH, widens or narrows the zone of inhibition. It turns out that fluoroquinolones, being strong acids, are in the ionised form ($COO^- + H^+$) and diffuse better in this alkalinised medium. This is why the widths of the inhibition zone for enrofloxacin and ciprofloxacin are larger in the presence of NaOH (2nd disc).

On the other hand, tetracyclines (alkaline antibiotics) are in their non-ionised form, in this case, their diffusion is limited or inhibited and the width of the inhibition zone of the second disc, in the presence of NaOH, is small or zero.

The parameter of NaOH can be a distinguishing factor between the two families of the studied antibiotics.

As far as oxolinic acid is concerned, the 20 µl of 1% NaOH added to the second cellulose disc has no significant effect. Its nature as a weak acid with a pKa = 6.9 (almost neutral) limits its ionisation. In this case, a single disc loaded with the antibiotic extract can be used for the analysis. Consequently, the results are in accordance with those of Dang et al. [21, 25].

The optimisation of the HPLC–UV quantitative chromatographic analysis is relevant for the quantification of oxolinic acid residues in tilapia flesh. It was optimised and evaluated in terms of trueness, precision, the limit of detection (LOD), the limit of quantification (LOQ), linearity, specificity and selectivity according to the recommendations of the Commission Decision 2002/657/EC [22]. The obtained performance parameters for the analysis of tilapia flesh are satisfactory.

In comparison with extraction and purification protocols in various matrices, the technique used in the present work is pertinent with a fairly good handling time [19, 32, 33]. Depending on the detection mode used for the liquid chromatography, the OXO is detectable after a retention time of about 5 min [32] in fluorescence detection, 7 min by UV system (in the present study) and up to 9 min by mass spectrometry [33]. The developed technique demonstrated good analytical performance. The linearity of the dose–response relationship is correct for the flesh matrix. This result is consistent with the result of Peris-Vicente et al. [32] describing a method for the detection of oxolinic acid residues in edible tissues by HPLC coupled with fluorescence detection.

In our study, the elimination kinetics of oxolinic acid from flesh demonstrated charged samples throughout the contamination phase (6 days of treatment). The peak of 1363 ± 32 µg kg^{-1} is reached after 24 h of the antibiotherapy. Otherwise, throughout the decontamination phase, the concentrations of oxolinic acid decreased significantly ($P < 0.05$) and fell quickly below the tolerance level of 100 µg kg^{-1} (MRL) on the 11th day from the experiment beginning (day 5 of treatment cessation) by reaching 81 ± 21 µg/kg. This finding is in agreement with the elimination kinetics of oxolinic acid from the muscle matrix [32, 34].

Nevertheless, it is difficult to compare with the majority of scientific publications that generally address the pharmacokinetic or depletion aspect of oxolinic acid in other marine fish species. In addition, our main focus is divergent and directed towards the analytical methodology.

Among the research conducted on tilapia, Dang et al. [25] proposed a comprehensive analytical strategy with an

Enzyme-linked immunosorbent assay (ELISA) as an intermediate post-screening for the direct monitoring of a range of antibiotic residues in fish (including tilapia) and shrimp sold in Vietnam. In the same spirit, Phommachanh et al. [35] adopted an analytical strategy based on the Clean meat test (CMT) for the detection of antibiotic residues in tilapia samples sold in Laos. The distinction between antibiotic groups was made by the European Six Plate Test (ESPT) and the residue level of enrofloxacin and ciprofloxacin in the positive samples was quantified by the LC-UV method. Morshdy et al. [31] have adopted and described a similar approach by using *Bacillus subtilis* as a strain for the microbiological inhibition test followed by an LC-UV confirmatory method but for the surveillance of tetracycline residues in tilapia (*Oreochromis niloticus*) and catfish (*Claria gariepinus*) sold in Egypt.

Paschoal et al. [36] reported an LC-ESI-MS-MS QToF analysis of fluoroquinolones on blank fillets of tilapia (*Oreochromis niloticus*). They developed the same analytical protocol (extraction and purification) as the one applied in the present study. An LC–MS/MS or LC-UV methods were also directly applied to control antibiotics residues of chloramphenicol, nitrofuran metabolites [37] and tetracycline [18] in tilapia fish (*Oreochromis niloticus*) from Egyptian and Indonesian markets respectively.

Feng et al. [38] reported that florfenicol was rapidly eliminated by the 3rd day after the first drug administration of 10 mg kg^{-1} in tilapia (*O. niloticus*) reared at 28 °C. Abraham et al. [39] reported that depletion of oxytetracycline residues in tilapia (*O. niloticus*) flesh would require 23 days to fall below 10 µg g^{-1} (MRL = 200 ng g^{-1}) when the prescribed therapeutic dose of 80 mg kg^{-1} biomass day^{-1} was tenfold increased and distributed for 10 and 20 consecutive days.

Regarding the oxolinic acid studies, our results are consistent with the study of Haugland et al. [40] on the pharmacokinetics of oxolinic acid and flumequine in lumpfish (*Cyclopterus lumpus* L.) using LC–MS/MS following a single oral administration of 25 mg kg^{-1} by fish. The concentration of these antibiotics dropped to 0.42 ± 0.13 µg g^{-1} and 0.26 ± 0.19 µg g^{-1} by 48 h post-treatment. Likewise for the study conducted by Rosa et al. [41, 42] on the tissue depletion of oxolinic acid residues by UHPLC-MS/MS in farmed European sea bass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) after oral administration of 6 and 12 mg kg^{-1} of medicated feed. Oxo traces below the MRL were found up to day 14 after medication. Chen et al. [34] also demonstrated that depletion of oxolinic acid in cobia (*Rachycentron canadum*), treated with a dose of 60 mg kg^{-1} day^{-1} for 5 days, required 10 days to ensure that OXO levels were below 50 ng g^{-1}.

On the basis of our findings, the required withdrawal period of tilapia treatment with 12 mg kg^{-1} oxolinic is a minimum of 5 days. This finding is consistent with previous studies on the same pharmaceutical molecule on other species than tilapia, where the minimum withdrawal time is 6 days [34, 41, 42]. Knowing that the pharmaceutical company (Virbac) recommends a withdrawal period of 6 days for oxolinic acid (Oxomid 20®) used in aquaculture.

The microbiological screening can be used for the qualitative assessment of a large number of samples in the control of fishery or aquaculture products, but chemical confirmation is necessary to decide on the lot conformity. A confirmatory analysis is also necessary for studies that are carried out to determine the withdrawal period.

Regarding the efficiency of the screening-confirmation analytical strategy, our results show an excellent correlation between screening and confirmation results since the positivity threshold and the threshold of acceptability (MRL), respectively, are reached within the same time frame as in the oxolinic acid contamination of tilapia experiment.

From a quantitative point of view, the screening and confirmation differ. The microbiological screening showed a persistent saturation of the response from the first day of treatment which only started to decline in the decontamination phase to reach the threshold of positivity (1.5 mm width of the inhibition zone) on day 8 after cessation of treatment. This saturation indicates very high levels of oxolinic acid in tilapia flesh but not proportionally hence, making it difficult to estimate actual concentration values in treated flesh samples.

Chromatographic confirmation allowed a quantitative measurement in µg/kg which allows better monitoring of the elimination kinetics and determines the oxolinic acid withdrawal.

Ideally, Chen et al. [34], recommended a 30% safety margin in addition to the earliest slaughter date for the determination of the withdrawal period, when all residual concentrations are below the MRL. This corresponded to 70 µg kg^{-1} rather than 100 µg kg^{-1} in the case of oxolinic acid. According to our results, a waiting period of at least 12 days is required for the oxolinic acid concentration to fall below 70 µg kg^{-1}.

Conclusion

The analytical strategy developed for the analysis of oxolinic acid residues in tilapia is highly relevant. The performance characteristics of both methods are acceptable for the analysis of oxolinic acid in fish flesh. In practice, both methods will be easily transposable to other matrices less complex than fish. The microbiological screening will be effective for the quality control in Algeria of fishery and aquaculture

products and can be a tool for routine self-checking in quality management systems (HACCP).

The LC-UV chromatographic analysis will be used as a basis for the quantification of fluoroquinolone residues in foodstuffs of animal origin and the protocol will be optimised to develop further confirmatory methods for other families of antibiotics.

Our results can serve as a scientific basis for legislation and veterinary inspection services to better regulate fish sales in local markets.

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Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

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