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Optimization of a pressurized liquid extraction method by experimental design methodologies for the determination of fluoroquinolone residues in infant foods by liquid chromatography

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ABSTRACT

In the present study, we have developed a method based on pressurized liquid extraction (PLE) and liquid chromatography with fluorescence detection (LC-FLD) for the determination of residues of fluoroquinolones (FQs) in infant food products. PLE extraction has been optimized by the application of experimental design methodologies. Initially, a fractional factorial design (FFD) was used to screen the significance of four extraction parameters: solvent composition, temperature, pressure and number of cycles. The most significant factors, identified by ANOVA analysis, were the solvent composition, temperature and pressure, which were further optimized with the aid of a face centred design (FCD) and the desirability function. The optimized operating PLE conditions were as follows: ACN/*o*-phosphoric acid 50 mM pH 3.0 (80:20, v/v), 80 °C, 2000 psi and three extraction cycles of 5 min. Under these conditions, recoveries of the target FQs varied between 69% and 107% with RSDs below 9%. The whole method was validated according to the Commission Decision 2002/657/EC guidelines. The proposed method has been successfully applied to the analysis of different infant food products bought in local supermarkets and pharmacies. The results showed the presence of residues of enrofloxacin in a non-compliant baby food sample corresponding to a chicken-based formulation, which were also confirmed and quantified by LC–MS/MS analysis.

1. Introduction

During the last decades, increased awareness about the risks associated to globalization of the food production has resulted in the development of new and more stringent food regulations in many countries [1,2]. This is particularly true for the presence of veterinary drug residues in animal-food products. The antimicrobials are among the pharmacologically active substances most frequently used to control and prevent infectious diseases in livestock production.

The emergence of bacterial antibiotic-resistance [3], a worldwide problem particularly pressing in developing countries [4], has been linked to the overuse of antimicrobials in clinical and veterinary practices. In addition, the development of new antimicrobials has declined in the past 20–30 years due to the high costs, risk and time associated with animal and in vitro studies [5]. As a consequence, consumers and authorities have become more concerned about the presence of residual antibiotics in the food supply and their negative impact on human health. To this respect, different actions have been taken by international regulatory bodies, such as the European Union [6], the US Food and Drug Administration (FDA) [7] and the Codex Alimentarius [8], setting maximum residue levels (MRLs) for different antimicrobials in several raw foods and prohibiting their use for growth promotion within the EU since January 2006 [9].

Furthermore, according to the current EU legislation, the "zero tolerance" principle should be applied to those pharmaceuticals and foodstuffs for which MRLs have not been established [10,11]. That is the case of many processed foods, among which are infant foods. This type of foods should observe the highest safety standards, due to the potential higher sensitivity of infants to toxicants. The recent food scandal in China dealing with the adulteration of infant formulations with melamine has focused the attention of the public health authorities on baby food safety.

Although European Commission Directives 2006/141/EC [12] and 2005/856/EC [13] have set maximum levels for pesticides and mycotoxins in processed fruit, vegetables or cereal-based baby foods and infant formulae, there is no current legislation regarding the tolerance levels of veterinary antimicrobials in infant foods.

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As a consequence, the application of the "zero tolerance" concept requires the development of analytical methods sensitive enough to determine the presence of these pharmaceuticals at very low concentration levels.

So far, only a few works dealing with LC determination of antimicrobials residues in infant foods have been reported in the literature [14–16]. Two of them deal with the determination of fluoroquinolones (FQs), based on sample deproteinization and defattening prior to SPE clean-up with Strata X cartridges [14] or molecularly imprinted polymers (MIPs) [15]. The main drawbacks of these methods are the extensive sample manipulation, and the lack of automation.

The growing concern about food safety and the constant increase in the number of samples to be analyzed in the food monitoring programmes, demand faster and high-throughput techniques amenable for automated sample handling. In this sense, pressurized liquid extraction (PLE) is becoming increasingly important as sample preparation technique in food analysis, combining the benefits of high-throughput, automation and favourable environmental impact due to the low solvent consumption, compared to conventional extraction techniques (e.g. Soxhlet), even after the recent improvements incorporated in the Soxhlet extractors [17,18].

Thus, pressurized hot water extraction (PHWE) has been successfully applied to the extraction of 14 sulfonamides (SAs) from raw meat and infant foods without the need of further clean-up steps [16]. The optimization of the extraction parameters, namely, temperature, pressure, extraction time and number of cycles, was achieved by changing one variable at a time (OVAT approach), whilst keeping the others constant. This kind of optimization is often time-consuming and true optimum conditions cannot be guaranteed, especially when these parameters are not completely independent. An alternative to the OVAT approach is the application of experimental design methodologies that allow the simultaneous variation of all studied experimental parameters, with the possibility to detect interactions between them [19,20].

The aim of this work was to develop an analytical method based on PLE extraction, followed by liquid chromatography with fluorescence detection (LC-FLD) for the determination of some of the most widely used veterinary FQs in different types of infant foods. Chemometric optimization of the PLE extraction conditions (e.g. composition of the extraction solvent, temperature, pressure and number of cycles) was carried out by using an experimental design methodology, consisting of a fractional factorial design (FFD), complemented with a face centred design (FCD) [21,22] to optimize the extraction conditions with a minimum number of experiments. Compared to previously developed SPE or MISPE methodologies [14,15], the present method allows to efficiently extract the target FQs from the infant food matrices with a significant reduction in the sample treatment time, mainly due to method automation and the elimination of a further SPE clean-up step. Validation of the PLE-LC-FLD method under the optimized extraction conditions has been performed according to the Commission Decision 2002/657/EC [23]. LC–MS/MS confirmatory analysis of a non-compliant baby food sample is also presented.

2. Experimental

2.1. Reagents and materials

Ciprofloxacin hydrochloride (99.8%) (CIPRO) and enrofloxacin (99.7%) (ENRO) were a gift of Bayer AG (Leverkusen, Germany) and sarafloxacine hydrochloride (90%) (SARA) was a gift from Fort Dodge Veterinaria S.A. (Girona, Spain). Danofloxacin methanesulfonate (75.1%) (DANO) was supplied by Pfizer S.A. (Groton, CT, USA) and norfloxacin (98%) (NOR) from Sigma–Aldrich (St. Louis, MO, USA). Lomefloxacin hydrochloride (LOME) from Sigma–Aldrich (St. Louis, MO, USA) was used as internal standard. Chemical structures of the FQs included in this study are shown in Fig. 1. Methanol (MeOH) and acetonitrile (ACN) of HPLC grade were provided by SDS (Pepyn, France) and *o*-phosphoric acid (HPLC grade 85%) was from Fluka (Buchs, Switzerland). Trichloroacetic acid (TCA) from Riedel de Haën (Seelze, Germany) and sodium hydroxide from Merck (Darmstadt, Germany) were of analytical grade. A C_{18} bulk sorbent (Fluka, Buchs, Switzerland) and a diatomaceous earth (Isolute HM-N, Symta, Madrid, Spain) were tested as matrix dispersants for PLE extraction. Water was purified with a Milli-Q system (Millipore, Bedford, MA, USA). All solutions prepared for HPLC were passed through a 0.45 μm nylon filter before use.

Five different brands of powdered milk-based infant formulae (PIF) and eight brands of poultry-based baby foods (in the form of purée) were obtained from local pharmacies and supermarkets.

2.2. Instrumentation

A laboratory-blender (Turbomix plus 300, Moulinex, Spain) was used for infant food samples homogenization. Samples of PIF were homogenized using a laboratory mixer (2 L capacity) from Filtra Vibración (Barcelona, Spain). The pH of buffer solutions was adjusted with a pH meter GLP 22 from Crison (Barcelona, Spain). A Dionex ASE 200 apparatus (Sunnyvale, CA, USA) equipped with 5 mL stainless steel extraction cells and glass–fiber filters was used for PLE extraction.

2.3. Standard solutions

FQs individual stock solutions were prepared in methanol at a concentration of 100 $\mu g\,mL^{-1}$, taking into account the purity of the standards. These solutions were stored at 4 °C in the dark for no longer than 1 month. For method validation, seven-point matrix-matched calibration curves were obtained by spiking extracts of blank PIF and baby food samples (previously tested not to contain the FQs included in the study) with the target FQs in the range from 5 to 1000 $ng\,mL^{-1}$ (equivalent concentration in the samples between 25 and 5000 $\mu g\,kg^{-1}$).

2.4. Preparation of spiked samples

For the recovery experiments, composite blank PIF samples were obtained by mixing with the aid of a laboratory mixer (Filtra Vibración, Barcelona, Spain) three different infant formula brands from the Spanish market that were tested not to contain the antimicrobials at the method detection limits. Before spiking, portions of the composite blank PIF samples (100 g) were soaked in 500 mL of acetone and sonicated for 10 min. Then, samples were fortified with the FQs mixed standard solution, at four concentration levels in the range 35–158 $\mu g\,kg^{-1}$. LOME at a concentration of 150 $\mu g\,kg^{-1}$, was added as internal standard. The samples were equilibrated by shaking, in the dark, for 90 min and afterwards evaporated to dryness at 30 °C, using a rotary evaporator (Büchi Labortechnik, Flawil, Switzerland). Finally, spiked PIF samples were dried in an oven at 35 °C for 8 h and stored in a desiccator, protected from light until use.

On the other hand, portions of 100 g of infant food samples were homogenized and directly spiked, at four concentration levels with FQs mixed standard solutions in the range from 40 to 193 $\mu g\,kg^{-1}$ and 150 $\mu g\,kg^{-1}$ of LOME. The samples were stored at −20 °C until use.

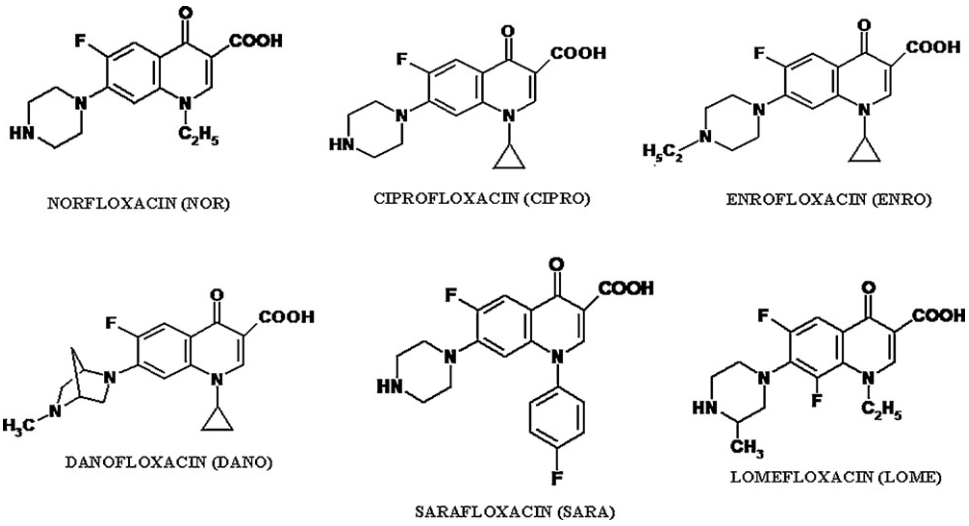

Fig. 1. Chemical structure and acronyms of the studied FQs: norfloxacin (NOR), ciprofloxacin (CIPRO), enrofloxacin (ENRO), danofloxacin (DANO) and sarafloxacin (SARA). Lomefloxacin (LOME) has been used as internal standard.

Table 1
Optimized PLE conditions for the extraction of FQs from PIF and baby food samples.

Extraction solvent	ACN/*o*-phosphoric acid 50 mM, pH 3.0 (80:20, v/v)
Temperature (°C)	80
Pressure (psi)	2000
Heat-up time (min)	5
Extraction time (min)	5
Flush volume (%)	50
Number of cycles	3
Purge time (min)	1
Cell volume (mL)	5
Total extraction time (min)[a]	21
Total solvent used (mL)[a]	13

[a] Per sample.

2.5. Sample extraction by PLE

One-gram subsamples were accurately weighed and then, thoroughly mixed and blended with 1.0 g of diatomaceous earth until a complete dispersion was obtained. The 5 mL stainless steel extraction cells, containing glass–fiber filters were filled with this mixture. The remaining empty space was filled with more dispersing agent. The optimum PLE extraction conditions are shown in Table 1.

At the end of each extraction, a total extract volume of approximately 13 mL was obtained. The extracts were made up to a final volume of 15 mL, but infant formula extracts required the addition of 1 mL of TCA methanolic solution (20%, v/v), to facilitate the precipitation of proteins, which are present to a large extent (typical values of 10–15%) in the analyzed samples. Then, the extracts were cooled at −20 °C for 30 min to allow complete precipitation of fat and other matrix co-extracted compounds, due to the reduced solubility at low temperature. After centrifugation at 14,000 × *g*, and 4 °C for 5 min, 3 mL aliquots of the raw extracts were evaporated to dryness using a Turbovap LV evaporator (Zymark, Hoptkinton, USA). The residue was reconstituted in 1 mL of *o*-phosphoric acid (25 mM, pH 3.0)/ACN (83:17, v/v) and then, filtered through glass-fiber filters before injection (10 μL) into the LC system.

2.6. Experimental design software

The experimental designs, response surfaces and desirability functions were generated using the statistical packages Design Expert 7.1 (Stat-Ease, MN, USA) and Statgraphics Centurion XV (StatPoint, Inc., VI, USA).

2.7. LC-FLD instrumentation and chromatographic conditions

The chromatographic system consisted of a HP 1100 HPLC from Agilent Technologies (Palo Alto, CA, USA), equipped with a quaternary pump, on-line degasser, autosampler, automatic injector and a fluorescence detector (FLD).

LC separation was carried out on an AQUA™ C_{18} analytical column (250 mm × 4.6 mm, 5 μm) preceded by a C_{18} guard column (4.0 mm × 3.0 mm, 5 μm), both from Phenomenex (Torrance, CA, USA). The analytical separation was performed using gradient elution, combining solvent A (*o*-phosphoric acid 25 mM, pH 3.0), solvent B (ACN) and solvent C (MeOH), as follows: 17% B and 0% C (8 min, 1 mL min^{-1}); 17–54% B and 0–12% C (8 min, 1.8 mL min^{-1}). Afterwards, the initial conditions were maintained for 6 min at 1 mL min^{-1}, prior to the next injection. The fluorescence excitation/emission wavelengths were set at 280/440 nm, respectively.

2.8. LC–MS/MS instrumentation and chromatographic conditions

LC–MS/MS confirmatory analysis was carried out by an Official Laboratory according to a previously developed methodology [24] by using a Waters Acquity UPLC system (Chicago, IL, USA) coupled to a Quattro Premier triple quadrupole mass spectrometer from Micromass (Manchester, UK), equipped with an electrospray interface (ESI) and operated in positive ESI mode.

Separation was carried out on an Acquity C_{18} UPLC BEH column (100 mm × 2.1 mm, 1.7 μm) from Waters (Milford, MA, USA) at 40 °C. The chromatographic separation was performed under gradient conditions combining a 0.2% formic acid solution (solvent A) and ACN containing 0.1% formic acid (solvent B), as follows: 5–25% B (4 min, 0.3 mL min^{-1}), 25–80% B (3.5 min, 0.3 mL min^{-1}), 80% B (1 min, 0.3 mL min^{-1}), 5% B (0.5 min, 0.3 mL min^{-1}) and 5% B (3 min, 0.3 mL min^{-1}). The ESI source was operated in positive ionization mode at the following working conditions: capillary voltage 3.5 kV, source block and desolvation temperatures of 120 and 400 °C, respectively, desolvation and nebulizer gas flow rates of 650 and 50 L h^{-1}, respectively, argon pressure in the collision cell 4×10^{-3} mbar.

Data acquisition for quantification was performed in the multiple reaction monitoring (MRM) mode, following two transitions for

ENRO, namely 360.3 > 316.8 and 360.3 > 245 that correspond to the fragment ions $[MH–CO_2]^+$ and $[MH–CO_2–NC_4H_9]^+$, respectively.

3. Results and discussion

3.1. Optimization of the PLE extraction conditions using an experimental design

An experimental design has been applied for the optimization of the PLE parameters that influence FQs extraction from PIF samples including, solvent composition, temperature, pressure and number of cycles.

Previous works [25–27] have shown that the nature of the extraction solvent, specially the type of organic solvent and the acidity (% *o*-phosphoric acid), has a remarkable effect on FQs recovery from different food matrices. Based on our previous results, MeOH provides lower antimicrobial recoveries than ACN [25]. Therefore, a mixture of ACN and *o*-phosphoric acid (50 mM, pH 3.0) was initially selected for the experiments. On the other hand, the use of a C_{18} sorbent as dispersing agent did not offer significant advantages, in terms of cost, recovery or cleanness of the extracts than those obtained with diatomaceous earth. In addition, the elevated fat content of the PIF samples makes C_{18} sorbent recycling difficult for further use. Thus, diatomaceous earth in a mass ratio 1:1 was selected as dispersing agent for further studies.

Optimization was carried out using blank PIF samples, spiked with 150 $\mu g\,kg^{-1}$ of each of the target FQs. Two different experimental designs were applied: (1) a fractional factorial design (FFD) has been used to evaluate the significance of the different parameters affecting the PLE extraction process (e.g. solvent composition, temperature, pressure and number of extraction cycles); and (2) a face centred design (FCD), together with a desirability function (*D*), has been applied to optimize the values of the significant parameters to achieve the highest global recovery for all the FQs. The optimized PLE conditions were further applied for method development and validation.

3.1.1. Screening design. Fractional factorial design (FFD)

Four PLE parameters were considered in the FFD design: extraction solvent composition (ACN), referred to the percentage of ACN (% v/v) in a mixture of ACN/*o*-phosphoric acid (50 mM), temperature (*T*), pressure (*P*) and number of extraction cycles (*C*).

A fractional factorial design was defined by an experimental domain constituted by a central point and two levels corresponding to the maximum and the minimum values for each extraction parameter, selected based on previous results [25]. Although FQs have been reported to be stable to heating [28] temperatures higher than 80 °C resulted in extremely dirty extracts, due to the co-extraction of a large fraction of matrix components. Therefore, the highest extraction temperature level was set to 80 °C. The recovery of each antimicrobial was chosen as the target response.

The experimental domain and the resulting FFD design matrix are shown in Table 2. The design consisted of 8 experiments performed by duplicate and 5 replicates for the central point. The results, in terms of average recoveries, are collected in Table 2.

Analysis of variance (ANOVA) and model residuals were used to check the adequacy of the mathematical models. Statistical evaluation of the results was attained at the 5% significance level. The ANOVA results showed that the data do not fit to a linear model and that it was necessary to include some interactions and curvature terms (second-order effects) in the models. Nevertheless, factorial designs fail when second-order effects are significant and, a response surface methodology should be applied [29].

Fig. 2 shows standardized Pareto charts displaying the effect (*t* value) of each extraction parameter on the recovery of the FQs included in the study. Those variables showing *t* values higher than the critical *t* value were considered statistically significant. Thus, the nature of the extraction solvent was the most significant parameter affecting the antimicrobial extraction yields. Higher percentages of ACN and lower percentages of o-phosphoric acid in the extraction solvent resulted in lower FQs recoveries. FQs behave as zwitterions at neutral pH (pKa_1 = 5.5–6.0, pKa_2 = 7.5–8.5) and the extraction recoveries, as it is shown in Table 2, were significantly improved, in the presence of *o*-phosphoric acid (e.g. % ACN in the solvent mixture between 50% and 75%), probably due to their higher solubility in the cationic form.

The extraction temperature usually has significant effect on PLE yields [30]. According to Fig. 2, the extraction temperature is the second parameter statistically significant on the recovery of most of the FQs tested (except SARA). Nevertheless, the effect was not the same in all cases being more important for CIPRO, DANO and ENRO than for the other antimicrobials. In all cases temperature has a positive effect on the antibiotic extraction, e.g. recovery increases at higher temperatures. The viscosity of the solvent decreases with increasing temperature, thereby increasing its ability to wet the matrix, and therefore to solubilize the analytes, but also higher temperatures help to disrupt analyte–matrix interactions, thus enhancing FQs extraction efficiency [31].

Although several studies have shown that pressure has little, or none effect, on PLE extraction and it is only required to maintain the extractant in the liquid phase, in this case, pressure has shown a positive effect (statistically significant) for CIPRO, DANO and ENRO. Higher PLE pressures facilitate solvent penetration into the sample matrix, which favours the disruption of the analyte–matrix interactions yielding higher antibiotic recoveries.

Finally, the number of extraction cycles has a significant effect on the extraction of NOR and SARA, being positive for NOR and negative for SARA.

In conclusion, the most significant parameters affecting PLE of the selected FQs from PIF samples were the extraction solvent nature (% ACN/*o*-phosphoric acid (50 mM), v/v), temperature and pressure. These parameters were considered for further optimization using a response surface methodology by a face centred design (FCD).

3.1.2. Optimization of the significant variables. Face centred design (FCD)

A face centred design (FCD) is a special case of a central composite design used to simultaneously optimize the levels of the variables of a response surface to attain the best system performance [21]. Central composite designs (CCD) are composed by a two-level full factorial design (points of the vertices of a cube), a star design in which experimental points are at a certain distance from its centre, and a central point. The name "face centred" derives from the fact that the location of the star points corresponds to the centre of the faces of the cube. A FCD combined with an overall desirability function (*D*) was applied for the optimization of the three most significant variables of the PLE method, namely the % ACN/*o*-phosphoric acid (50 mM) (v/v), extraction temperature and pressure. The number of extraction cycles was set at three, as a compromise between the total extraction time and the recovery of the analytes.

The FCD design matrix is shown in Table 3. The experimental domain was defined taking into account the results obtained previously applying the FFD design. A total of 14 experiments were performed in duplicate plus a central point (5 replicates). The average recoveries collected in Table 3 show that, for all the FQs tested, the best extraction yields were obtained using ACN/*o*-phosphoric acid (50 mM), 80:20 (v/v), 80 °C and 2000 psi.

Nevertheless as various responses (recovery of each FQ) had to be optimized simultaneously, a multi-criteria methodology based

Table 2
Experimental domain and FFD design matrix.

Parameter	Code	Level		
		Minimum	Central	Maximum
ACN (%)	ACN	50	75	100
Temperature (°C)	*T*	60	70	80
Pressure (psi)	*P*	1000	1500	2000
Number of cycles	*C*	2	3	4

Experiment	ACN	*T*	*P*	*C*	Recovery (%)					
					NOR	CIPRO	LOME	DANO	ENRO	SARA
1	50	60	1000	4	55	56	60	71	72	66
2	50	60	2000	2	46	63	70	71	105	80
3	50	80	2000	4	87	94	85	93	108	83
4	50	80	1000	2	62	76	86	90	89	77
5	75	70	1500	3	74	72	107	109	108	99
6	100	60	1000	2	0	8	0	7	6	53
7	100	60	2000	4	15	16	21	40	28	21
8	100	80	1000	4	23	22	22	53	48	25
9	100	80	2000	2	0	53	25	87	68	53

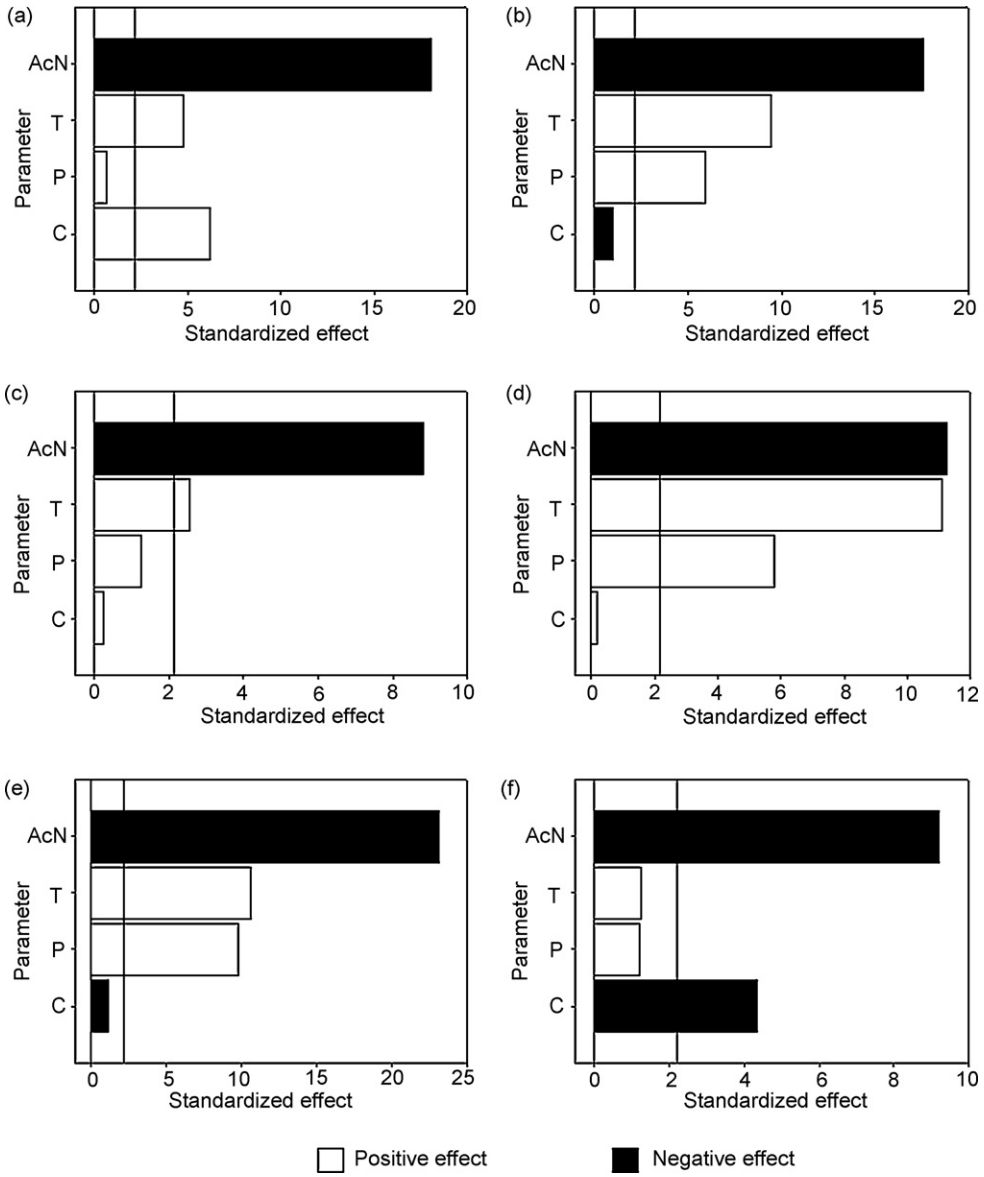

Fig. 2. Standardized main effects Pareto charts for the FFD design. (a) NOR, (b) CIPRO, (c) LOME, (d) DANO, (e) ENRO and (f) SARA. ACN: %ACN; *T*: temperature; *P*: pressure; *C*: number of extraction cycles.

Table 3
Experimental domain and FCD design matrix.

Parameter		Code		Level					
				Minimum		Central		Maximum	
ACN (%)		ACN		50		65		80	
Temperature (°C)		*T*		70		75		80	
Pressure (psi)		*P*		1500		1750		2000	
Experiment	ACN	*T*	*P*	Recovery (%)					
				NOR	CIPRO	LOME	DANO	ENRO	SARA
1	50	70	1500	91	78	65	69	72	54
2	50	80	1500	107	90	53	87	90	99
3	50	75	1750	42	42	34	39	37	36
4	50	70	2000	62	56	33	26	46	13
5	50	80	2000	52	49	42	44	43	33
6	65	75	1500	11	10	23	34	35	36
7	65	70	1750	90	76	68	80	85	66
8	65	75	1750	35	45	18	43	46	40
9	65	80	1750	98	96	81	82	94	72
10	65	75	2000	53	54	43	48	50	36
11	80	70	1500	77	95	90	96	101	92
12	80	80	1500	18	46	23	57	58	54
13	80	75	1750	71	90	39	94	102	100
14	80	70	2000	72	97	60	94	100	93
15	80	80	2000	103	109	85	104	111	109

on the desirability function was applied. This procedure is based on constructing a desirability function for each individual response (d_i), defined as partial desirability function, whose values range from 0 (undesirable recovery) to 1 (completely acceptable recovery). Then, with the individual desirabilities, an overall desirability function (D) is obtained, defined as the weighed geometric mean of the individual desirability functions. Thus, the simultaneous optimization process is reduced to find the levels of the parameters that provide the maximum overall desirability and hence, the best analytical response.

Fig. 3 shows the contour plots for the overall desirability function (D) in the experimental domain studied. The results demonstrate, once again, that the conditions providing the maximum desirability values ($D > 0.9$) are located in a relatively narrow region where the three factors considered are set at their highest values. Thus, the optimum PLE conditions, assuring quantitative extraction (recoveries >90%, Table 3) for all FQs in the PIF samples were ACN/*o*-phosphoric acid (50 mM), 80:20 (v/v), 80 °C and 2000 psi. These conditions were subsequently applied to the PLE of the target analytes from poultry-based baby food samples,

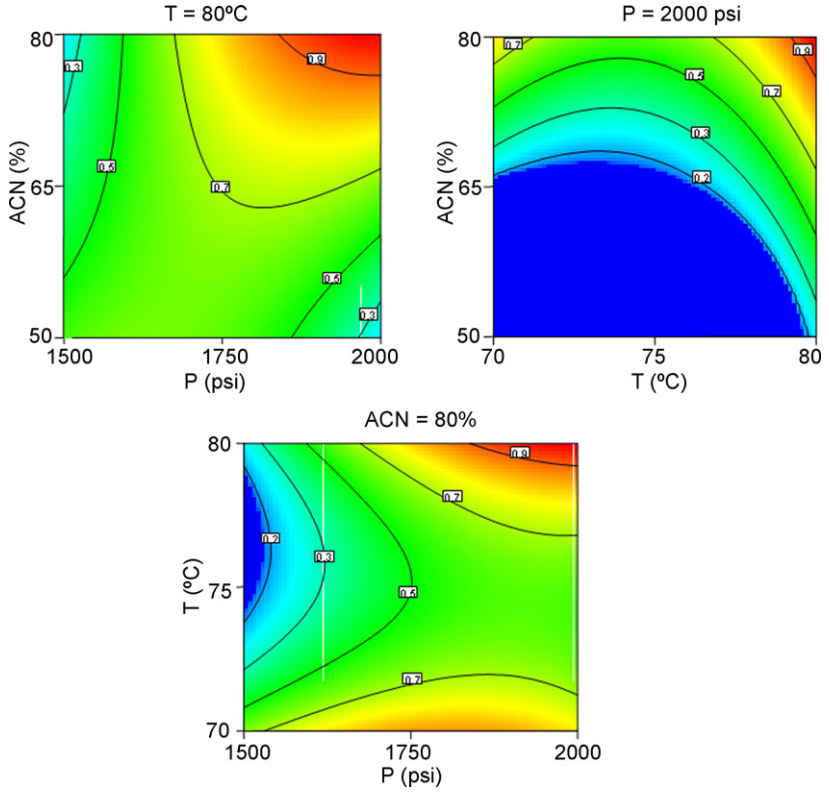

Fig. 3. Contour plots of the overall desirability function (D) of the PLE method for the extraction of FQs from PIF samples.

obtaining recoveries >80% for all the FQs tested, except ENRO (68%).

3.2. PLE-LC-FLD method validation

The method was validated, using spiked PIF and baby food samples, in terms of specificity, linearity, precision (repeatability and within-laboratory reproducibility), accuracy, decision limit (CC_α) and detection capability (CC_β), according to the European Commission Decision 2002/657/EC guidelines [23].

The specificity of the proposed method was evaluated by the analysis of twenty blank PIF and baby food samples. The absence of any chromatographic peaks at the same retention time of the target antibiotics indicated that the proposed method is free of matrix interferences. Fig. 4 shows the chromatograms corresponding to extracts of blank PIF and baby food samples, as well as extracts of PIF and baby food samples, fortified with the target FQs.

Linearity was evaluated using matrix-matched standards ($n = 7$) consisting in blank PIF and baby food extracts spiked with each of the target FQs in the range from 5 to 1000 ng mL^{-1} (equivalent concentration in the samples of 25–5000 μg kg^{-1}). The calibration curves were obtained by plotting the peak area ratio of FQ/internal standard versus FQ concentrations. Good linearity ($r > 0.999$) was observed for all the FQs, within the studied concentration range.

Since neither MRL nor MRPL values have been established for the target FQs in the analyzed matrices, the accuracy and repeatability were determined from the analysis of PIF and baby food samples, spiked at three concentration levels (in the range 35–110 μg kg^{-1}), above the quantification limit (LOQ) of each FQ in each matrix. Mean recoveries ranged between 94% and 108% in PIF samples with RSDs < 7%, whereas in baby food samples recoveries were between 93% and 107% with RSDs < 6%, except for ENRO (69–73%, RSDs < 6%, Table 4).

To determine the within-laboratory reproducibility, PIF and baby food samples were spiked with the target antibiotics at four concentration levels in the range 35–193 μg kg^{-1}. The internal standard LOME was added at a concentration of 150 μg kg^{-1}. The samples were extracted and analyzed by triplicate on three different days. The results, summarized in Table 4, showed that for PIF samples mean recoveries ranged between 95% and 105%, with RSDs < 8%, whereas in baby food samples, mean recoveries were between 93% and 107% with RSDs < 7%, with the only exception of ENRO (69–71%, RSDs < 7%). These results confirm the good reproducibility of the method.

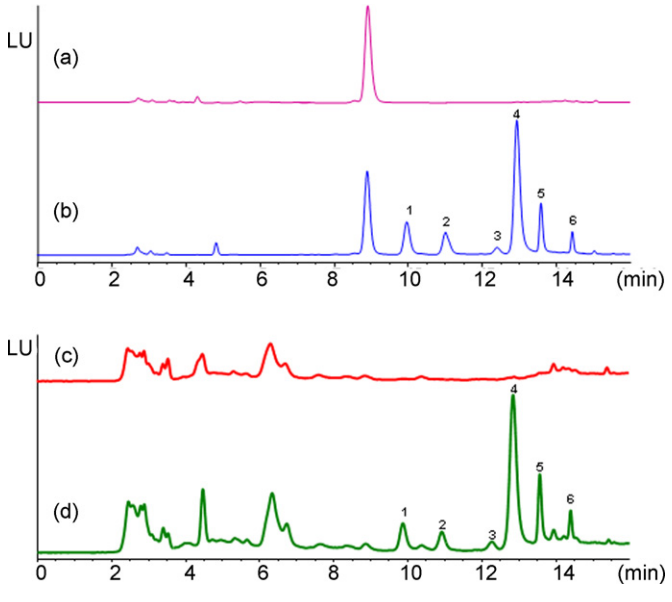

Fig. 4. Representative LC-FLD chromatograms of (a) an extract of blank PIF; (b) an extract of blank PIF, spiked with 150 μg kg^{-1} of FQs; (c) an extract of a baby food blank sample and (d) a baby food blank extract, spiked with 150 μg kg^{-1} of the FQs. (1) NOR; (2) CIPRO; (3) LOME; (4) DANO; (5) ENRO and (6) SARA. Chromatographic conditions are described in Section 2.7.

On the other hand, the decision limit (CC_α) and detection capability (CC_β) were also calculated. According to Commission Decision 2002/657/EC for those substances which do not have MRL or MRPL, the CC_α is defined as the lowest concentration level at which a method can discriminate with an error probability of 1% that a particular analyte is present in a sample. By contrast, the CC_β is the lowest concentration at which a method is able to detect truly contaminated samples with an error probability of 5%. In other words, CC_α is a parameter used to prevent false positive results, whereas CC_β should prevent false negative results.

Commission Decision 2002/657/EC indicates two different approaches for the calculation of CC_α and CC_β. The first involves the analysis of twenty blank samples from which the average noise level around the retention time of the analytes is calculated. The second one relies on the calculation of the *y*-intercept of a calibration curve obtained with samples fortified at different concentration levels above and below the MRLs or MRPLs.

Many analysts have encountered problems with the application of the Decision 2002/657/EC guidelines, in particular with the CC_α and CC_β concepts for banned or unauthorized substances, due to miss-interpretations of the confusing terminology. Moreover, depending on the utilized approach and the calibration curve shape, sometimes for banned or unauthorized substances very low or even negative CC_α and CC_β values are obtained that cannot be checked with spiked samples. In other cases, especially when high-resolution mass spectrometers are used, another problem is the absence of measurable noise in many blank samples that leads to meaningless CC_α values of zero.

This situation has partially solved with the publication in 2004 of the document SANCO 2004/2726rev1 [32], which provides guidance for the implementation of Decision 2002/657/EC. Moreover, recently a few papers have been published giving recommendations to understand and apply these definitions in the residues of control laboratories [33,34].

Thus, CC_α and CC_β values were calculated by applying the calibration curve procedure described in Commission Decision 2002/657/EC [23]. For that purpose, and following the recommendations of the document SANCO 2004/2726rev1 [32] for substances with no permitted limit, the calibration curves were prepared with spiked samples fortified at and above the minimum required performance level (mrpl), considered as the LOQ of each FQ in each matrix.

Following the rules of Decision 2002/657/EC, using the calibration curve approach, calculation of CC_α and CC_β has to be made as follows: the CC_α value is the concentration corresponding to the *y*-intercept plus 2.33 times the within-laboratory standard deviation of the lowest calibration level; whereas, the CC_β value is the concentration at the CC_α plus 1.64 times the within-laboratory standard deviation at the CC_α level. Table 5 summarizes the obtained CC_α and CC_β values obtained for the target FQs in the infant food samples.

The stability of the FQs in the extracts of baby food samples was checked at different temperatures. The samples were spiked with 70 μg kg^{-1} of NOR, CIPRO, DANO and ENRO and 90 μg kg^{-1} of SARA. After PLE the extracts were kept in the dark at −20 °C, 4 °C and +20 °C and analyzed after 0, 7, 14, 21 and 28 days. The results showed that no significant decrease in the peak area was observed over the tested period for CIPRO, DANO and SARA at none of the storage temperatures selected in the study. NOR and ENRO were stable for 21 days, but after that time an increase in the peak areas of both FQs was observed probably due to matrix evolution.

Table 4
Method accuracy and precision.

	PIF samples					Baby food samples				
	NOR	CIPRO	DANO	ENRO	SARA	NOR	CIPRO	DANO	ENRO	SARA
Repeatability (intra-day)										
Spiked level ($\mu g\,kg^{-1}$)	35–70	35–70	35–70	35–70	45–90	40–80	55–110	40–80	40–80	50–100
Recovery (%)	98–102	98–104	94–103	95–104	96–108	90–102	98–103	97–100	69–73	97–99
RSD (%) ($n=5$)	4–6	6–7	4	4–7	3–5	4–6	1–4	2–3	4–6	3
Reproducibility (inter-day)										
Spiked level ($\mu g\,kg^{-1}$)	35–123	35–123	35–123	35–123	45–158	40–140	55–193	40–140	40–140	50–175
Recovery (%)	98–105	95–102	100–103	96–101	97–104	93–105	97–101	98–103	69–71	98–107
RSD (%) ($n=9$)	5–6	5–8	5–7	4–9	4–7	5–6	3–5	3–5	5–7	2–7

Table 5
LOQ, CC_α and CC_β values obtained for the target FQs in the infant foods.

Analyte	PIF samples			Baby food samples		
	LOQ ($\mu g\,kg^{-1}$)	CC_α ($\mu g\,kg^{-1}$)	CC_β ($\mu g\,kg^{-1}$)	LOQ ($\mu g\,kg^{-1}$)	CC_α ($\mu g\,kg^{-1}$)	CC_β ($\mu g\,kg^{-1}$)
NOR	15	14	24	24	14	23
CIPRO	13	12	20	19	11	18
DANO	12	15	25	17	13	22
ENRO	14	11	18	19	11	19
SARA	22	10	17	29	19	32

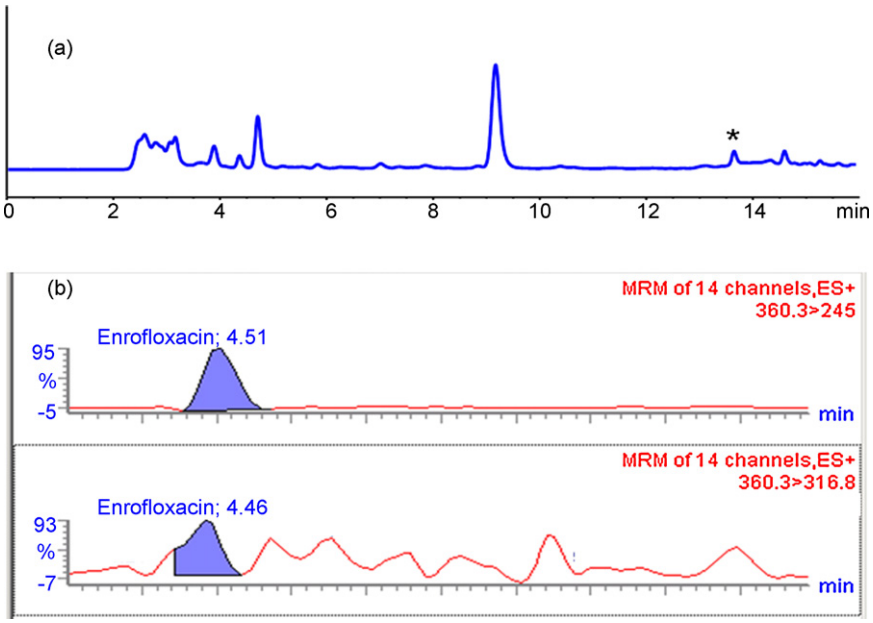

Fig. 5. (a) LC-FLD and (b) LC–MS/MS chromatograms of an ENRO-incurred baby food sample. The peak marked with an asterisk (*) in chromatogram (a) indicates the suspected positive for ENRO. Chromatographic conditions are described in Sections 2.7 and 2.8.

3.3. Analysis of infant food samples

The method has been applied to the detection of the incidental presence of FQs residues in 22 PIF and poultry-based baby food samples bought in local supermarkets and pharmacies in Madrid. The results showed that one chicken-based baby food sample was non-compliant and residues of ENRO were detected (average value 3.0 $\mu g\,kg^{-1}$). Fig. 5 shows the LC-FLD and LC–MS/MS chromatograms of this incurred chicken-based baby food sample. Confirmation was carried out following the rules of the Commission Decision 2002/657/EC by comparison of the signal intensity ratios of the two transitions of ENRO (360.3 > 245 and 360.3 > 316.8 quantification and confirmation, respectively) with those obtained using fortified extracts. ENRO ion ratio of the incurred sample was within 20% of the ENRO ion ratio in the matrix-matched standards, and thus within the guidelines of confirmation laid down in the Decision 2002/657/EC. In addition, the retention time for ENRO in the incurred sample was identical, within instrumental variation, to the retention time of ENRO in the matrix-matched standards.

Therefore, bearing in mind the zero tolerance policy to veterinary drug residues in infant foods, this baby food sample would not be acceptable for infant feeding and more efforts should be made by regulatory authorities and producers to achieve infant foods free of antibiotic residues.

4. Conclusions

The study shows that the application of an experimental design methodology is an efficient tool for the simultaneous optimization of the PLE parameters that ensure the highest recoveries of FQs from PIF and poultry-based baby food samples. The composition of the extraction solvent (% ACN/*o*-phosphoric acid 50 mM in the solvent

mixture) and the extraction temperature are the most statistically significant parameters that influenced the recovery of all the FQs tested.

The methodology has been exhaustively validated according to the guidelines of the Commission Decision 2002/657/EC and it has been successfully applied to the analysis of different types of commercially available PIF and poultry-based baby food samples, detecting the presence of residues of ENRO in a non-compliant sample which was further confirmed and quantified by LC–MS/MS analysis.

The benefits of the proposed PLE-LC-FLD method include: high extraction efficiency (69–107%) with low solvent volumes (13 mL per sample) and reduced extraction time (around 20 min per sample), compared to Soxhlet extraction, which require larger solvent volumes (50–200 mL) and longer extraction times (typically several hours). In spite of the complexity of the analyzed matrices, sample extracts do not require additional SPE clean-up before LC analysis, as Soxhlet or microwave-assisted extraction generally do, with the corresponding reduction of the analysis time. Repeatability and reproducibility values (RSDs < 9%) are far superior to those normally achieved with Soxhlet extraction, due to the reduction of sample handling favoured by method automation and avoiding the need of evaporating large solvent volumes after sample extraction. In conclusion, the optimized method could be easily implemented as a routine procedure in Food Safety Laboratories for the screening of residues of FQs in infant foods.

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