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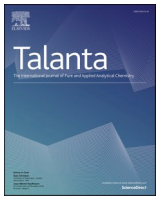

Analytical method for the determination of usually prescribed antibiotics in human nails using UHPLC-MS/MS. Comparison of the efficiency of two extraction techniques

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ABSTRACT

Antibiotics are a group of drugs used for the treatment of bacterial diseases. They are used in both human and veterinary medicine and, although they are not permitted, they are sometimes used as growth promoters. The present research compares two extraction techniques: ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) in order to evaluate their efficiency in the determination of 17 usually prescribed antibiotics in human nails. The extraction parameters were optimized using multivariate techniques. Once both techniques were compared, MAE was selected as optimal due to its greater experimental practicability together with the better extraction efficiencies it provides. Target analytes were detected and quantified by ultra-high performance liquid chromatography with tandem mass spectrometry detection (UHPLC-MS/MS). The run time was 20 min. The methodology was then successfully validated, obtaining acceptable analytical parameters according to the guide used. Limits of detection were between 0.3 and 3 ng g^{-1} and limits of quantification were in the range from 1.0 to 4.0 ng g^{-1}. Recovery percentages ranged from 87.5% to 114.2%, and precision (in terms of standard deviation) was less than 15% in all cases. Finally, the optimized method was applied to nails taken from 10 volunteers and the results revealed the presence of one or more antibiotics in all the samples examined. The most commonly found antibiotic was sulfamethoxazole, followed by danofloxacin and levofloxacin. The results demonstrated, on the one hand, the presence of these compounds in the human body and, on the other hand, the suitability of nails as a non-invasive biomarker of exposure.

1. Introduction

Pharmaceuticals, and in particular antibiotics, are produced and consumed daily throughout the world. Once ingested, these compounds are conjugated through the liver and eliminated mainly in the urine. A non-conjugated active part is also eliminated, reaching wastewater treatment plants, which are often unable to eliminate them completely, entering the environment and being considered emerging pollutants [1,2]. Antibiotics are a group of drugs used for the treatment of bacterial diseases, and are known as agents capable of killing bacteria or inhibiting their growth by different mechanisms [3]. Since Alexander Fleming discovered penicillin in 1928, antibiotics have contributed to the treatment of bacterial infections, reducing their prevalence and the risk of death of the sick [4]. They are used in both human and veterinary medicine and, although not permitted, are sometimes used as growth promoters [5]. This group of drugs is used more and more every year. In 2012, the consumption of antibiotics in non-hospitalized patients reached 3400 t in European countries. France with 719 t, Great Britain with 415 t or Spain with 321 t stands out notably [6]. Due to the massive and continuous use of antibiotics, they are constantly entering the environment and are therefore considered pseudo-persistent contaminants. Although these compounds have a short half-life, their continuous entry means that they are ubiquitous [6]. They cause environmental effects such as decreased microbial diversity, immunity of pathogenic

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bacteria or destabilization of activated sludge in wastewater treatment plants [5,7].

As for adverse effects in humans, there is the possibility of developing allergic reactions or inducing antibiotic resistance [4,8]. The presence and spread of bacterial resistance to antibiotics in the environment is nowadays considered a serious global health problem [9]. Excessive consumption of these drugs, as well as animal-derived products such as milk or meat, involves the consumption of low doses of antibiotics over a long period of time, which significantly increases the risk of developing global antimicrobial resistance and decreases the efficacy of antibiotics [10]. For that reason, the European Union determined maximum residue limits (MRLs) for veterinary drugs in animal products [3]. In this context, it is necessary to monitor the presence of antibiotics in human and environmental samples to study their effect on health. The development of new low-cost and high-recovery analytical methods, where the sample treatment procedure plays a fundamental role, is crucial at present, since the concentration of these drugs in real samples is very low and they have a high matrix effect [10].

Human biomonitoring studies (HBM) make it possible to determine the total exposure to a mixture of contaminants in human matrices such as blood, urine, breast milk, saliva, hair or nails, taking into account lifestyle habits [11]. HBM is especially useful because it can help find new chemical exposures, identify the most vulnerable groups, study exposure patterns in the general population or in specific groups, and associate environmental exposures with health risks [12]. Keratinized human matrices, such as hair and nails, allow the study of the accumulation of substances over long periods of time, making possible the retrospective investigation of past drug use [13–15]. On the one hand, hair has been used for decades in forensic and toxicological sciences for routine analysis [16]. On the other hand, nails have recently been used as an alternative to hair in forensic applications, such as in post-mortem cases or when hair is not available (e.g. in case of hyalopecia or after chemotherapy treatment) [17]. The incorporation of substances takes place through diffusion of blood supply during nail growth [18]. This is continuous as well as bidirectional (nails grow 3 mm/month in the hands and 1.5 mm/month in the feet versus 1 cm/month in the hair) [19].

Some studies have used human fingernails as an analytical matrix for the determination of different compounds in the organism. For example, Soleymani et al. studied the presence of heavy metals in the nails of petrochemical workers [20]; Zeng et al. determined PAHs and their hydroxylated metabolites in this matrix [21]; Martín-Pozo et al. investigated the accumulation of endocrine-disrupting UV filters in human nails [22], and Zhe et al. developed a method for the simultaneous determination of diacetylpolyamines in nails of cancer patients [23]. However, to our knowledge, there is no multi-residue method to determine the presence of different antibiotic families in human nails.

The objective of the present study was to develop and validate a selective, sensitive and accurate multiclass UHPLC-MS/MS method capable of determining the presence of 17 antibiotics, widely used in human and veterinary medicine, in human nail samples. The method involves a sample treatment consisting of microwave-assisted extraction with high recoveries. The method has been applied to samples collected from 10 volunteers.

2. Materials and methods

2.1. Chemicals and reagents

Analytical grade standards of analytes: Nalidixic acid (NAL), sulfamethoxazole (SMX), trimethoprim (TMP), flumequine (FLU), oxolinic acid (OXO), norfloxacin (NOR), marbofloxacin (MAR), difloxacin (DIF), moxifloxacin (MOX), erythromycin (ERY) and clarithromycin (CLA) were supplied by Sigma Aldrich (Madrid, Spain). Cinchophen (CIN), penicillin V (PEN-V), danofloxacin (DAN), enrofloxacin (ENR), levofloxacin (LEVO) and oxacillin (OXA) were purchased from Alfa Aesar (Massachusetts, MA, USA) and ciprofloxacin (CIP) was bought at Supelco (Bellfonte, Pennsylvania, USA).

Ultrapure water was purified by a Milli-Q Plus® purification system (Millipore, Madrid, Spain), and solvents used such as LC-MS methanol (MeOH) and acetonitrile (ACN) were obtained from Prolabo Chemicals (Barcelona, Spain), ethyl acetate (EtOAc) from Honeywell (Madrid, Spain), and acetone from Panreac (Barcelona, Spain). Formic acid (98–100%) used as additive in the aqueous phase and sodium hydroxide (NaOH) (>98%, pellets) were obtained from Sigma-Aldrich. Finally, hydrochloric acid (6 N) was obtained from Panreac. The chemical structures of the different compounds under study are shown in supplementary material (Table S1).

2.2. Instrumentation and software

Freeze-drying of the samples was carried out with a ScanVac CoolSafe™ freeze-dyer (Lynge, Denmark). For pulverisation of the samples, a ball mill Retsch® MM 301 (Biometa, Asturias, Spain) was used. An IKA vortex shaker (Staufen, Germany), a balance Mettler-Toledo AND GX400 (Columbus, OH, USA) and a sample concentrator (Stuart) were also used. Two types of centrifuges were employed, a Spectrafuge 24D Labnet Ultracentrifuge (New Jersey, USA) and a Hettich Universal 320 centrifuge (Tuttingen, Germany) adapted for Eppendorf tubes. For sample digestions, an ultrasound probe 400 W digital sonifier (Branson Ultrasonic Corporation) with a 0.5-inch (12.7 mm) probe operating at a frequency of 20 kHz and a microwave Milestone's ETHOS SEL extraction Labstation (Sheldon, CT, USA), operating at 2455 MHz with a maximum power of 1000 W, were used.

Chromatographic analyses were done with an Acquity™ I-Class chromatography system (Waters, Manchester, UK), equipped with an Acquity sample manager and an Acquity UPLC™ binary solvent manager was used. A Waters UPLC® HSS T3 chromatographic column (2.1 × 100 mm i. d., 1.8 μm particle size) and an Acquity UPLC® BEH C18 column (50 mm × 2.1 mm i. d., 1.7 μm particle size) were tested for analyte separation. The chromatographic system was coupled to a Xevo TQ-XS triple quadrupole mass spectrometer (Waters) using Z-spray™ as the ionization source (ESI) for analyte detection. Data processing was performed with MassLynx V 4.1 SCN 803 (Waters, Manchester, UK) and the statistical package Statgraphics version 16.0 (Virginia, USA).

2.3. Sample collection, storage and pre-treatment

Ten volunteers from the province of Granada (Spain), aged between 14 and 60 years, collected nail samples at home. All volunteers were informed of the study and gave their prior consent. Sample collection was carried out over a period of 3 months during which the subjects cut their fingernails and toenails and stored them together in polypropylene jars. The samples were stored at room temperature and in the dark. Since antibiotics are widely used in both human and veterinary medicine, it is very difficult to find an analyte-free matrix, with the added drawback of limited sample quantity. All samples were analyzed to ensure the presence or absence of the analytes under study. A sample from a volunteer with low amounts of antibiotics or below the detection limits of the method was used for calibration and validation of the method.

To avoid external contamination (e.g., dust particles, nail polish or other compounds adhering to the nail surface) prior to the analyte extraction, the samples were cleaned with a 1% Triton X-100 solution in an ultrasonic bath twice for 15 min, followed by three ultrasonic baths with Milli-Q water to remove detergent residues. Subsequently, to remove possible nail polish, they were washed with acetone in an ultrasonic bath and finally with MeOH for 15 min. After washing, the nails were freeze-dried for 24 h to remove moisture and then pulverized in a ball mill for 10 min at 30 Hz. In this way, the particle size is decreased while increasing the contact surface between the analytes under study and the solvents, improving the extraction efficiency. Samples ready for extraction were stored at room temperature in the dark until analysis.

Table 1
Transitions and parameters used for MS/MS analysis.

Compound	t_R (min)	MRM Transitions	CV (V)	CE (V)	Ratio (a/b)
TMP	3.16	291.1 → 230.0[a]	76	18	27.6
		291.1 → 93.0[b]	76	30	
MAR	3.38	363.1 → 320.0[a]	18	12	1.5
		363.1 → 72.0[b]	18	18	
LEVO	4.37	362.0 → 318.1[a]	30	16	1.3
		362.0 → 261.1[b]	30	24	
NOR	4.89	320.2 → 302.0[a]	20	16	1.3
		320.2 → 276.1[b]	20	14	
CIP	5.49	332.1 → 314.0[a]	26	16	1.1
		332.1 → 231.0[b]	26	32	
SMX	5.6	253.9 → 155.9[a]	20	14	1.7
		253.9 → 92.0[b]	20	26	
DAN	6.24	358.1 → 340.0[a]	40	18	4.0
		358.1 → 314.1[b]	40	36	
ENR	6.03	360.1 → 316.1[a]	18	16	2.0
		360.1 → 245.1[b]	18	22	
DIF	7.1	399.9 → 356.1[a]	42	16	1.3
		399.9 → 299.0[b]	42	26	
MOX	9.79	402.0 → 384.1[a]	34	22	1.2
		402.0 → 358.1[b]	34	18	
NAL	11.07	233.0 → 215.0[a]	20	12	1.8
		233.0 → 187.0[b]	20	22	
FLU	11.22	261.9 → 244.0[a]	14	18	1.9
		261.9 → 202.0[b]	14	28	
OXO	11.23	261.9 → 244.0[a]	24	14	303
		261.9 → 215.9[b]	24	24	
ERY	11.27	734.4 → 158.1[a]	18	24	1.8
		734.4 → 576.3[b]	18	14	
PEN-V	11.48	351.1 → 229.0[a]	54	26	1.4
		351.1 → 333.0[b]	54	14	
OXA	11.74	402.0 → 144.0[a]	60	20	3.9
		402.0 → 384.1[b]	60	14	
CLA	11.81	748.4 → 158.1[a]	28	24	2.7
		748.4 → 590.3[b]	28	14	
CIN	11.91	250.1 → 128.0[a]	40	30	2.0
		250.1 → 222.1[b]	40	26	

Spectrometric conditions	
Source temperature: 158.9 °C	Impactor voltage: 2.9 kV
Desolvation temperature: 600 °C	Cone gas flow: 150-147 L h^{-1}
Desolvation gas flow: 1000-989 L h^{-1}	Nebulizer gas pressure: 7.00–6.94 bar
Cone/desolvation gas: N_2 (≥99.995%)	Collision gas: Ar (≥99.999%)
Dwell time: 25 ms	Inter-scan delay: 3 ms

t_R: retention time; CV: cone voltage; CE: collision energy.
[a] Transition for quantification.
[b] Transition for confirmation.

2.4. Preparation of fortified samples

Optimization of the extraction procedure, calibration and method validation were performed with spiked samples. Briefly, 50 μL of a methanolic solution containing the target analytes and the surrogate (cinchophen) were added to 0.05 g of nail blank sample, obtaining a final concentration of 100 ng g^{-1}. After fortification, the samples were kept in the dark at room temperature for 24 h to ensure contact between the analytes and the matrix and evaporation of the solvent. In addition, for calibration and validation, different concentration levels (1–100 ng g^{-1}) were used for all analytes, excluding the internal standard, whose concentration was kept constant at 100 ng g^{-1}. At this point, the spiked nail samples were ready for extraction procedures.

2.5. Basic procedures

Microwave-assisted extraction (MAE). 0.05 g of lyophilized nail was placed in a microwave vessel. A volume of 1.75 mL of MeOH/ACN (50/50 v/v) was added and vortexed for 30 s to ensure contact of the extraction solvent with the entire sample. Ten vessels were processed at the same time in the apparatus, which operates at 88 °C for 1 min, with a 10 min preheating ramp and 5 min subsequent ventilation to lower the temperature (<45 °C). The total digestion time was 16 min. The power of the equipment was 1000 W and the pressure was automatically controlled. Two extraction cycles were performed. The supernatants were centrifuged for 5 min at 3000 rpm and transferred to a glass tube in which they were evaporated to dryness under a stream of N_2 for 1 h (note that 36 extracts are evaporated at the same time). The dried extracts were dissolved in a final volume of 200 μL of the initial mobile phase. They were then centrifuged for 10 min at 13,000 rpm and transferred to the vial to be injected directly into the LC-MS system. Taking into account the times independent of the researcher, i.e., those required by the equipment, the extraction and reconstitution of the extracts takes approximately 1 h and 50 min.

Ultrasound-assisted extraction (UAE). 0.05 g of freeze-dried nails were weighed into a 10 mL glass tube and 2.75 mL of extraction solvent MeOH/ACN (75/25 v/v) was added. It was vortexed for 30 s and placed in a water bath along with the ultrasound probe. Up to 13 samples were processed at the same time. The probe was run at 70% amplitude for 60 min. Three extraction cycles were performed and, in between, the bath water was changed to avoid overheating. After digestion, the tubes were centrifuged for 5 min at 3000 rpm and the supernatant was removed and mixed after each cycle. Finally, the extract was dried under a stream of N_2 for 2.5 h (36 extracts are evaporated at the same time) and the residue was dissolved in the initial mobile phase in the same way as in the previous case. In this case, the total time used for digestion of the samples, as well as for reconstitution of the extracts, is 3 h and 50 min.

Liquid chromatographic-mass spectrometric analysis. Chromatographic separation was performed using a Waters Acquity HSS T3 column (2.1 × 100 mm i. d., 1.8 μm) maintained at 40 °C and a mobile phase consisting of water (solvent A) and methanol (solvent B), both acidified with formic acid (0.1% v/v). The flow rate was set at 0.3 mL min^{-1} and the injection volume was 2 μL. The gradient program was as follows: 0.0–1.0 min, 20% B, 1.0–6.0 min, 20–25% B, 6.0–8.0 min, 25–30% B, 8.0–9.0 min, 30–50% B, 9.0–14.0 min, 50–100% B, 14.0–16.0 min, 100% B, 16.0–16.1 min, back to 20% B. The total run time was 20 min.

For MS, positive ionization and multiple reaction monitoring (MRM) mode were used to identify and quantify all target analytes in order to improve specificity and sensitivity. Two different transitions were monitored for each analyte. The most abundant one was used for quantification and the other one for identification. In addition, to improve the selectivity of the method, time intervals were selected to measure the different transitions. The instrumental parameters for MS analysis are shown in Table 1.

2.6. Factorial design 3^2

Response surface 3^2 factorial experimental designs were performed for the simultaneous optimization of two significant variables in the three-level extraction procedure, where neither the maximum nor the minimum is desired. Three central points were performed for a total of 12 experiments. The number of extraction cycles was evaluated along with the solvent volume, as well as the modifiable parameters of the extraction equipment, which for the ultrasound probe were time and amplitude, and for the microwaves, time and temperature. Mathematical model equations were used for extraction enhancement and analysis of variance (ANOVA) and correlation coefficients (R^2) detailed the statistical significance of linear, quadratic or cross interactions between variables.

2.7. Validity requirements

Quality Assurance/Quality Control procedures were carried out to study the validity of the method according to the US Food and Drugs Administration (FDA) guidance on validation of bioanalytical methods [24]. For the background contamination study, experimental blanks were performed and processed in the same way as the samples. All target analytes were either not detected or detected but not quantified (<LOQ). In addition, to study contamination and instrumental variability, a

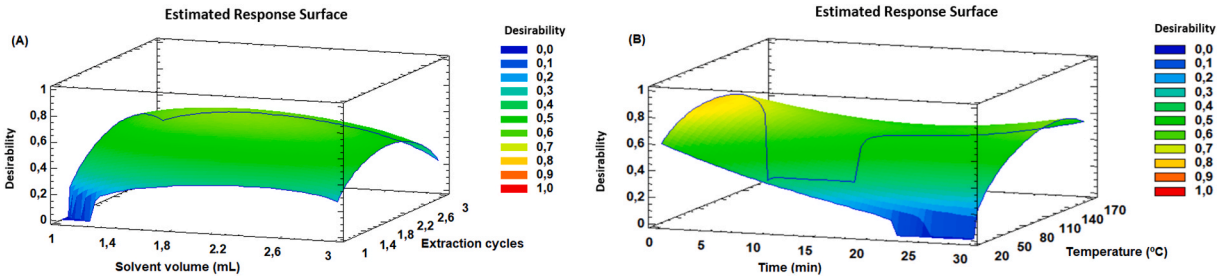

Fig. 1. Response surface 3^2: solvent volume and number of extraction cycles for MAE (a) and response surface 3^2: time and temperature for MAE.

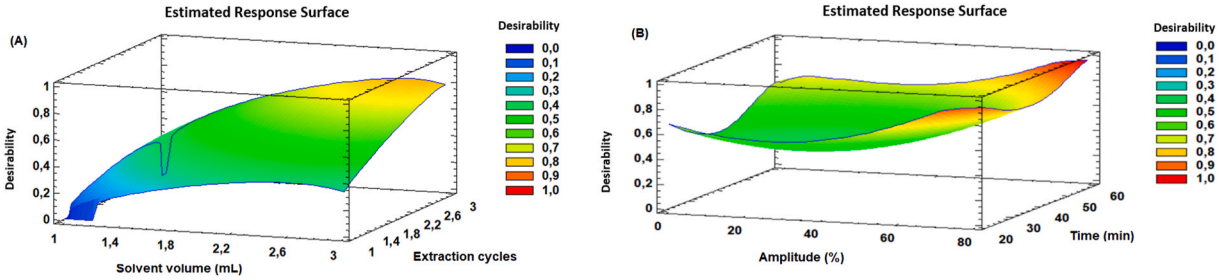

Fig. 2. Response surface 3^2: solvent volume and number of extraction cycles for UAE (a) and response surface 3^2: time amplitude for UAE (b).

standard was prepared in the initial mobile phase at a concentration of 100 ng mL^{-1} and injected every 10 analyses. A calibration curve with five or six concentration levels was performed for each analyte to relate the signal obtained to the amount of the compound. Linearity was evaluated with the R^2 coefficients. All samples were analyzed in triplicate and injected three times in the equipment.

3. Results and discussion

3.1. Liquid chromatography-tandem mass spectrometry analysis

The optimization of the method started with the comparison of two different chromatographic columns, an Acquity UPLC® HSS T3 column (100 mm × 2.1 mm i. d., 1.8 μm particle size) and an Acquity UPLC® BEH C18 column (50 mm × 2.1 mm i. d., 1.7 μm particle size). Although both gave good results, the first showed better peak quality in terms of width and shape for most compounds. The chromatographic separation was optimized using a 100 ng mL^{-1} standard mixture containing all the compounds studied. For the mobile phase, the combination of an aqueous phase (phase A) with the two modifiers usually used in liquid chromatography MeOH and ACN (phase B) was tested. It was observed that the presence of the latter produced a total loss of signal for some of the compounds studied, so its use was discarded. Finally, to improve the ionization of the compounds at the interface, the addition of formic acid in a proportion of 0.1% (v/v) only in phase A, or in both phases, was evaluated; proving that when it was in both phases it improved the signal corresponding to the analytes in a very notable way.

3.2. Optimization of microwave-assisted extraction

As general variables that may affect the MAE process, solvent volume, number of cycles and different parameters affecting the digestion, such as microwave time and temperature, were studied by two factorial experimental designs (3^2). In addition, the extraction solvent and extraction pH were optimized in this case by the one-factor-at-a-time method. The power of the equipment was set at 1000 W, which is the maximum power it can reach. MeOH, ACN, mixtures of both (25/75, 50/50 and 75/25, v/v), EtOAc and a mixture of acetone/hexane (50/50 v/v) were tested to cover a wide range of polarities, according to the literature consulted [22,25,26]. Fig. S1, in the supplementary material, shows how the use of acetonitrile causes the disappearance of the analytical signal for most of the quinolones studied. The 50/50 (v/v) mixture of ACN with MeOH gives better signals for all compounds than both solvents separately or any of the other mixtures studied. In addition, the acetone-hexane mixture also did not give good extraction results and EtOAc improved extraction efficiencies only in the NAL, FLU, DIF and MOX cases. The effect of extraction volume and number of extraction cycles was also studied using a 3^2 experimental design. One to three extraction cycles and 1–3 mL of the solvent selected as optimum (MeOH/ACN 50/50 v/v) were evaluated. The matrix with the experimental conditions is detailed in the supplementary material (Table S2). Three levels of each variable were evaluated in 12 runs. The response surface obtained is shown in Fig. 1A. It can be seen how, with a desirability of 64%, the optimum volume of extraction solvent is 1.75 mL and two cycles should be performed.

Another experiment consisted of modifying the pH of the extraction solvent by preparing solutions 0.1 mol L^{-1} of HCl and NaOH in the solvent and subjecting the samples to digestion. Data obtained are shown in supplementary material (Fig. S2). It was observed that the basic medium clearly impaired the extraction of all analytes, whereas the acidic medium improved recoveries (in particular for quinolones), but eliminated the signal of two compounds, ERY and CLA. Therefore, in compromise with all analytes it was decided not to add acid in the extraction solvent. Finally, the effect of irradiation time and temperature was also tested using a 3^2 experimental design. Three levels of each variable were studied in 12 experiments, as shown in supplementary information (Table S3). The response surface (Fig. 1B) indicates that 1 min and 88 °C give the best extraction efficiency with 74% desirability. The Pareto plots of the target analytes selected for experiment analysis are shown in supplementary information (Fig. S3). These plots indicate that temperature positively affects the extraction efficiencies, whereas time depends on the analyte, and their combination (parameter AB) negatively influences the extraction efficiency. Probably, high temperatures for prolonged times result in degradation of compounds, which would explain the differences in responses over time among some of them. The ANOVA analysis provides coefficients of determination (R^2) around 60% and P_{lof} values > 5%. Therefore, the model used is satisfactory at 95% confidence level.

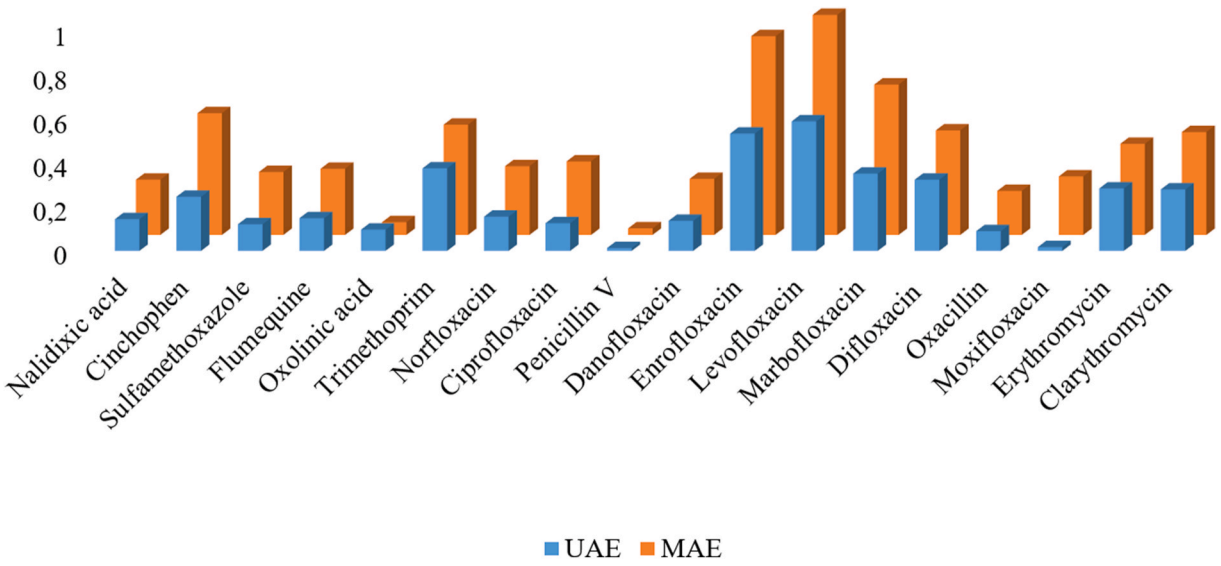

Fig. 3. Comparison of extraction techniques: MAE and UAE. Signal responses are plotted.

Table 2
Matrix calibration parameters. Accuracy, recovery and precision.

	n	b ($g\ ng^{-1}$)	R^2 %	LOD ($ng\ g^{-1}$)	LOQ ($ng\ g^{-1}$)	Recovery study, %R (RSD, %, n = 15)		
						LOQ ($ng\ g^{-1}$)	50 ($ng\ g^{-1}$)	100 ($ng\ g^{-1}$)
CIP	5	$3.99\ 10^{-3}$	93.1	1	4.0	95.8 (7.6)	99.2 (12.4)	98.2 (10.2)
CLA	6	$4.03\ 10^{-2}$	99.4	0.3	1.0	103.2 (10.7)	101.0 (10.0)	99.9 (2.4)
DAN	5	$2.98\ 10^{-3}$	96.9	1.5	5.0	108.3 (3.0)	99.3 (5.6)	100.4 (10.6)
DIF	6	$3.32\ 10^{-3}$	98.9	3	9.0	87.5 (12.6)	95.3 (13.0)	98.9 (12.2)
ENR	5	$6.33\ 10^{-3}$	96.3	0.9	4.0	96.2 (14.4)	111.3 (7.3)	97.9 (9.8)
ERY	5	$1.39\ 10^{-3}$	97.9	0.9	4.0	99.6 (7.1)	106.3 (12.0)	98.3 (2.4)
FLU	5	$3.02\ 10^{-2}$	98.1	0.8	3.0	102.6 (7.4)	106.8 (12.0)	98.3 (2.4)
LEVO	5	$8.66\ 10^{-3}$	96.7	0.8	3.0	101.8 (11.3)	114.2 (4.5)	98.1 (7.5)
MAR	5	$6.42\ 10^{-4}$	97.5	3	9.0	108.3 (11.6)	91.4 (8.0)	102.1 (5.2)
MOX	5	$1.97\ 10^{-3}$	97.1	0.9	4.0	91.0 (9.4)	111.1 (3.8)	98.5 (8.6)
NAL	5	$6.14\ 10^{-2}$	99.4	0.7	3.0	99.9 (13.2)	107.9 (9.7)	90.9 (5.3)
NOR	5	$2.65\ 10^{-3}$	90.1	2	8.0	112.1 (8.3)	97.4 (13.9)	96.4 (6.5)
OXA	5	$5.75\ 10^{-4}$	99.4	1.5	5.0	104.1 (5.5)	97.3 (9.1)	92.0 (2.5)
OXO	5	$1.87\ 10^{-2}$	99.6	0.7	3.0	109.4 (13.1)	103.2 (5.8)	92.5 (7.1)
PEN-V	6	$5.54\ 10^{-3}$	99.5	0.4	1.0	106.9 (6.2)	95.3 (5.1)	93.5 (5.1)
SMX	5	$1.15\ 10^{-2}$	99.7	0.9	4.0	111.5 (10.9)	92.1 (5.8)	98.8 (11.1)
TMP	5	$1.93\ 10^{-3}$	99.3	0.9	4.0	113.0 (10.6)	105.8 (11.0)	94.2 (6.5)

Linear dynamic range: LOQ-100 ng g^{-1}; n: calibration levels; b: slope of matrix calibration; $\%R^2$: determination coefficient; LOQ: limit of quantification; LOD: limit of detection; RSD: Relative Standard Deviation.

3.3. Optimization of ultrasound-assisted extraction

In the same way that the different variables that influence the extraction procedure with MAE were optimized, a study of these parameters was carried out using the UAE technique. In this case, the specific parameters of the extraction equipment are the ultrasound amplitude and the extraction time. The extraction volume, cycles, amplitude and extraction time were optimized using factorial designs, whereas the extraction solvent and pH were evaluated using a one-factor-at-a-time approach. First, the same solvents as in the previous section were studied, using 2 mL of each one and subjecting them to a single digestion for 20 min at an amplitude of 30. The results are shown as supplementary material (Fig. S4). As shown in the figure, with the MeOH/ACN mixture (75/25 v/v) the best extraction yields are achieved for most analytes than pure MeOH. In contrast, the worst results were obtained with non-polar solvents.

As a second step, a response surface experimental design was performed to simultaneously evaluate the required volume of solvent and the number of extraction cycles, with the same conditions as in the case of MAE, shown in Table S2. Fig. 2A presents the response surface obtained for this experiment.

The optimal values were 2.75 mL of extraction solvent with 3 consecutive extraction cycles. The desirability of the experiment was 82% with Plof values > 5%, with a confidence level of 95%.

As in the case of MAE, a test was carried out in which the pH of the extraction medium was modified by preparing solutions of 0.1 mol L^{-1} of HCl and NaOH in the extraction mixture. Consistent with the results obtained with MAE, it was found that, on the one hand, the basic medium affected negatively in all cases while, on the other hand, the acidic medium mainly benefited the quinolone signal but eliminated ERY and CLA, as can be seen in supplementary information (Fig. S5). For this reason, it was also decided in this case not to change the pH value of the extraction solvent.

Finally, the parameters involved in the ultrasound equipment were studied. A response surface design of experiments was performed, whose matrix is shown in supplementary material (Table S4), consisting of 12 experiments where there were three levels of each variable: amplitude 10, 40 and 70% and time, 1, 15.5 and 30 min. The results showed that, with a desirability of 80%, the optimum results were 30.8% amplitude and 30 min. In terms of time, the desirability was maximum and, for this

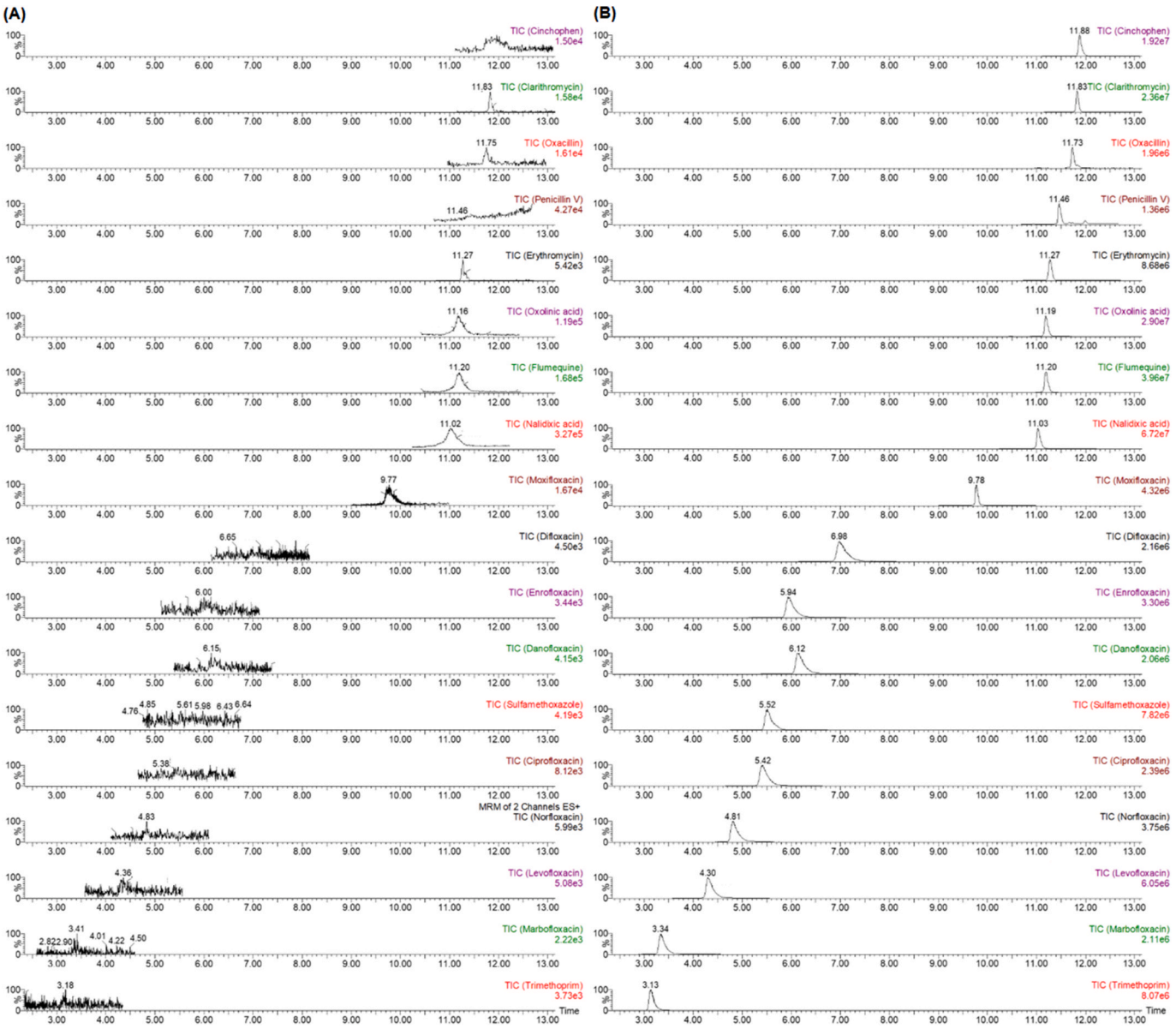

Fig. 4. Comparison of a chromatogram of the blank (a) and a spiked nail matrix (100 ng g^{-1}) (b). MRM of two transitions in ESI + mode.

reason, it was decided to perform a second experimental design with a longer digestion time, up to 60 min. The matrix with the experimental conditions is also shown as supplementary material (Table S5). In this case, the maximum response surface was reached at 70% amplitude and 60 min, again the maximum time level evaluated, as can be seen in Fig. 2B. Longer times were not tested because they would excessively lengthen the process, which is not desirable. The confidence level was 95% and the Plof values were >5%. Pareto plots, shown in supplementary information (Fig. S6), demonstrate that amplitude is not significant, whereas time positively affects extraction efficiencies. Analysis of variance showed coefficients of determination greater than 18.8–77.1%.

3.4. Comparison of extraction techniques

Extraction efficiencies. Fig. 3 shows the comparison of the two extraction techniques under the optimized final conditions. The results show significant differences. Thus, MAE provides much higher recoveries, which means that temperature control in the extraction is very important. Moreover, it should be noted that this is a common behaviour for all the analytes studied, indicating that the extraction efficiency depends on the technique, and not so much on the rest of the variables studied, such as the extraction solvent, the amount of solvent or the number of extraction cycles. Furthermore, although the conditions to which the samples are subjected with MAE are more drastic compared to UAE, nevertheless, the resulting extracts are apparently very clean, transparent and similar to those obtained with UAE, but significantly improving the extraction yield.

Practicability issues and extraction conditions. The in-depth study and optimization of the two extraction techniques leads to the conclusion that the efficiency of MAE is significantly higher for all target analytes. Moreover, the volume used (1.75 mL per extraction cycle) versus 2.75 mL in UAE, implies that a total of 3.75 mL in MAE and 8.25 mL in UAE are used. On the other hand, if the time necessary for the digestion is taken into account, MAE requires 32 min, while UAE takes 3 h. Based on the principles of Green Chemistry and feasibility, it was decided to use MAE conditions for routine analysis of the application of the method on volunteer samples.

Table 3
Application of the method to nail samples. Concentration in ng g^{-1}. Relative standard deviation in parentheses.

	CIP	CLA	DAN	DIF	ENR	ERY	FLU	LEVO	MAR	MOX	NAL	NOR	OXA	OXO	PEN	SMX	TMP
S1	D	2.9 (0.9)	D	ND	D	ND	D	D	6.0 (4.5)	ND	D	85.0 (7.2)	8.9 (8.9)	D	ND	D > LDR	ND
S2	ND	2.1 (0.8)	ND	ND	ND	ND	ND	ND	ND	ND	49.0 (7.8)	ND	ND	ND	ND	25.7 (6.1)	7.7 (2.3)
S3	D	ND	ND	ND	ND	ND	D	ND	D	ND	D	23.7 (4.7)	5.0 (4.2)	ND	D	34.2 (4.0)	ND
S4	9.3 (0.4)	ND	5.5 (5.7)	ND	D	ND	4.6 (5.4)	11.3 (7.9)	ND	ND	4.3 (6.8)	22.0 (4.3)	5.0 (7.8)	D	ND	81.4 (8.4)	ND
S5	34.6 (4.9)	2.3 (0.2)	100.0 (11.8)	17.8 (3.2)	90.0 (1.7)	ND	9.1 (2.6)	85.6 (10.6)	ND	ND	D	51.0 (2.2)	ND	D	ND	D > LDR	D
S6	ND	ND	D	ND	ND	ND	ND	ND	ND	ND	ND	10.7 (1.6)	D	ND	ND	35.4 (2.3)	ND
S7	13.7 (9.6)	ND	ND	ND	ND	ND	3.0 (2.0)	ND	ND	ND	ND	ND	D	4.7 (1.9)	ND	18.2 (6.3)	14.5 (6.0)
S8	ND	4.8 (11.9)	D	D	D	D	8.0 (1.4)	22.3 (3.6)	ND	ND	4.9 (1.6)	25.0 (6.7)	9.7 (4.5)	ND	ND	6.3 (12.3)	8.9 (3.4)
S9	2.3 (10.8)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1.4 (5.4)	D
S10	25.1 (1.3)	ND	ND	ND	ND	ND	8.2 (2.8)	ND	12.0 (2.9)	D	30.3 (1.8)	D	9.1 (3.1)	7.5 (9.2)	ND	15.4 (11.3)	4.1 (5.2)

D: detected (> LOD but < LOQ); ND: non detected (<LOD).

3.5. Analytical performance (calibration curves)

The matrix effect was evaluated by comparing calibration curves prepared in pure solvent (mobile phase) with those prepared using blank human nail. The slopes of both calibration curves were compared with a *t-Student* test. First, the variances estimated as $s^2_{y/x}$ were compared using the *F-Snedecor* test. Next, the *t-Student* test was applied. Since the calculated *t* was slightly higher (from 2.15 for CLA to 2.93 for PEN) than that tabulated for a 95% confidence level ($t_{tab,\ n=45} = 2.00/t_{tab,\ n=54} = 2.01$), statistical differences were found between the slopes of both calibration curves in all cases, indicating the presence of slightly significant matrix effects. Consequently, a blank matrix standard calibration at 5 or 6 concentration levels (from LOQ to 100 ng g^{-1}) was prepared for quantification. Each level was prepared in triplicate and injected into the system three times. Cinchophen was used as a surrogate at a final sample concentration of 100 ng g^{-1}. Calibration curves were obtained by plotting the antibiotic/peak area ratio of the surrogate as a function of antibiotic concentration. The calibration parameters are summarized in Table 2.

3.6. Method validation

Antibiotic validation was carried out according to FDA guideline for Bioanalytical Method Validation as indicated in *Section 2.7*. The method was validated in terms of linearity, sensitivity, selectivity and accuracy (trueness and precision).

Linearity. This was checked by means of the coefficients of determination (%R^2) and the p-values of the lack of fit test (% P_{lof}). The R^2 values obtained were between 90.1 and 99.7 and the P_{lof} values were >5% in all cases. This means good linearity within the established ranges.

Selectivity. This was evaluated by analysis of the blank samples. Fig. 4 compares the chromatogram of the blank (a) with its corresponding enriched nail matrix containing all target antibiotics (100 ng g^{-1}) (b). No interferences from endogenous compounds are observed in the retention time of the analytes, demonstrating the selectivity of the method.

Sensitivity. LODs and LOQs were determined experimentally analyzing decreasing concentrations of the antibiotics in the blank samples, and allowed checking the sensitivity of the proposed method. Additionally, for confirmation, they were also calculated according to $3{\cdot}s_0$ and $10{\cdot}s_0$, for LOD and LOQ respectively, where s_0 is the standard deviation of the blank. The LODs were in the range of 0.3–3 ng g^{-1}, while the LOQs were between 1.0 and 9.0 ng g^{-1}. The results are also shown in Table 2.

Accuracy. It was evaluated by performing a recovery assay using nail samples spiked at three concentration levels: LOQ, 50 and 100 ng g^{-1} (low, intermediate and high). Intra- and inter-day precision, expressed as relative standard deviation (%RSD), was calculated by analyzing six replicates of each level per day, and repeated for three consecutive days. Trueness was estimated as recovery rates (%R) by comparing the concentration determined by interpolation from the standard calibration curve with the concentration previously added to the matrix. The results obtained are summarized in Table 2.

3.7. Biomonitoring of antibiotics in human fingernails

Once the proposed method was successfully validated, it was applied to nail samples from 10 subjects who were previously informed of the study to be performed. These volunteers were men (40%), women (60%) and children (two in total). Table 3 shows the results obtained for the samples tested, which were analyzed in triplicate, as well as the standard deviation obtained. In addition, Fig. 5 illustrates a chromatogram of a positive sample, belonging to volunteer 5.

Among the results obtained, it should be noted that all the antibiotics studied were detected in at least one of the samples. The most common antibiotic was SMX, which was detected in 100% of the volunteers at concentrations between 6.3 ng g^{-1} and above the upper limit of the linear dynamic range studied in two cases. On the other hand, NOR and OXA were detected in 80% (8/10) of the samples and quantified in 70% and 60% respectively, in a concentration range of 10.7–85.0 ng g^{-1} for NOR and 5.0–9.7 ng g^{-1} for OXA, followed by the quinolones CIP and FLU, detected in 70% of the samples (7/10) and quantified in 50% at concentrations ranging from 8.3 to 34.6 ng g^{-1} for the former and 3.0–9.1 ng g^{-1} for the latter. DAN, NAL and OXO were detected in 6/10 volunteers, and quantified in 40% of cases or less. Concentrations ranged from 5.5 to 100 ng g^{-1} for DAN and 4.3–49 ng g^{-1} for NAL, while OXO was found in notably lower concentrations (4.7–5.5 ng g^{-1}). Finally, the rest of the target analytes were detected in 50% or less of the samples and at low concentrations or even below the detection limit, although in the case of LEVO and ENR two samples were found with concentrations of 85.6 and 90.0 ng g^{-1}, which coincidentally corresponded to volunteer number 5, which turned out to be the subject with the highest concentration of practically all the antibiotics studied and who was known from her medical history to be undergoing numerous antibiotic treatments as part of her cancer treatment.

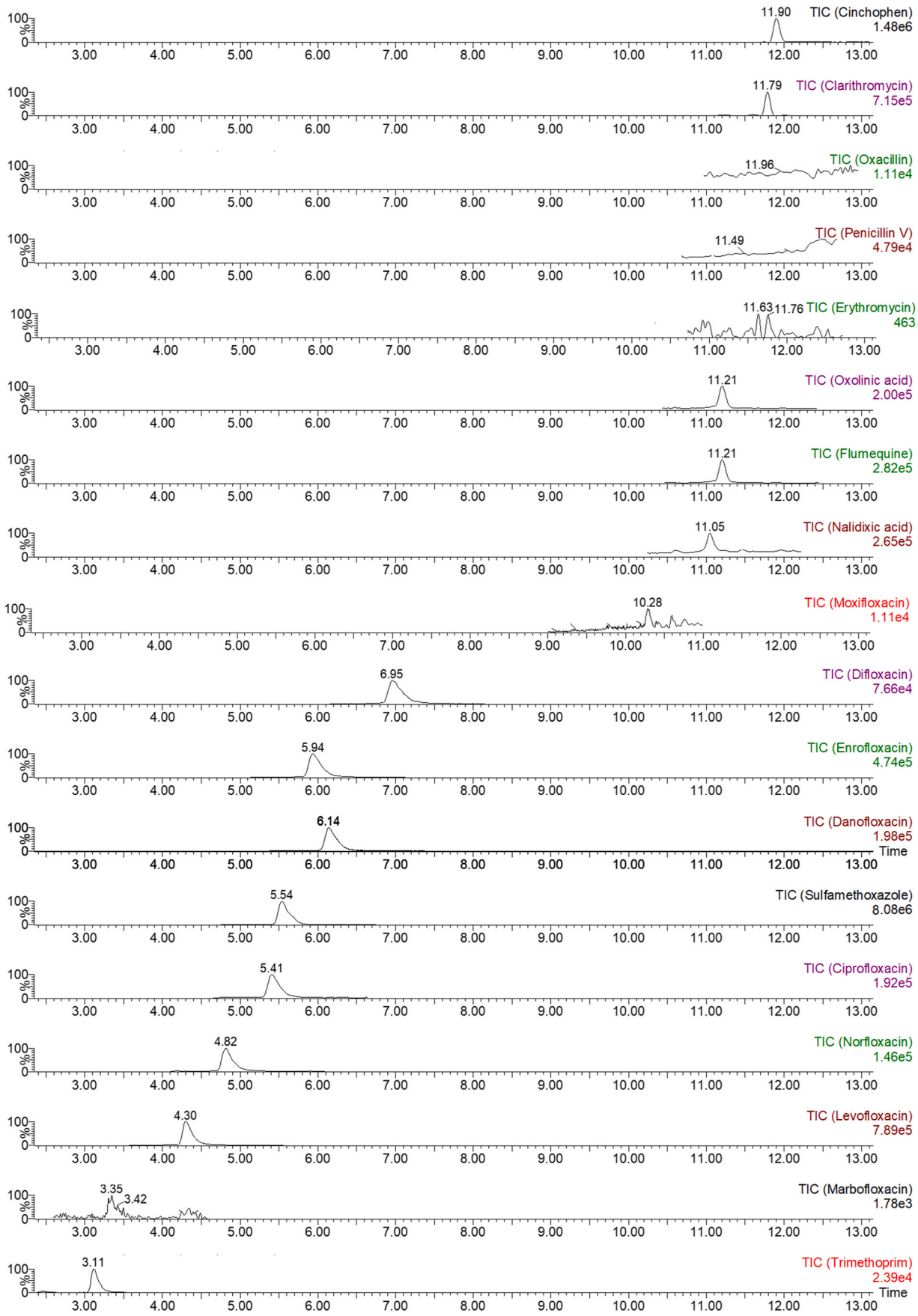

Fig. 5. Chromatogram of a contaminated sample (volunteer 5). MRM of two transitions in ESI + mode.

From the results obtained, it has been possible to formulate some observations. SMX was found in all samples, but in contrast, TMP was only detected in 50% of the subjects, despite the fact that they are antibiotics that are used together due to their synergistic effects. This suggests that TMP is metabolized more and/or accumulates less in the body. The fact that SMX is the most common antibiotic in nails is not surprising, as it (along with TMP) is widely used to treat highly prevalent infections and pneumonias. They are also considered first-line antibiotics in veterinary medicine, so the route of exposure for humans is not only through direct consumption, but their use in animal care may also contribute to their presence. This is also the case for some quinolones widely used in the veterinary field, such as DAN, DIF, ENR, FLU, MAR, NOR and OXO, which have also been detected but at lower concentrations. This is consistent with the intended use, since in the case of quinolones, the European Medicines Agency (EMA) classifies them in category B, meaning that they are relevant in human medicine and should be limited and used only when no other antibiotic is effective, to reduce health risks [27]. It is worth mentioning the kinship relationships of some of the participants: volunteers 1 and 2 are father and daughter, 3 and 6 are sisters, and 8 and 9 are fathers. There seems to be no apparent relationship between the presence of antibiotics or their concentration with the parentage, age, or sex of the volunteers, which is to be expected since the main exposure to these compounds is through prescription consumption. Further research should be conducted with a significant population sample to investigate this aspect.

Information to date on the presence of antibiotics in human nails is limited, and although other studies have considered hair in addition to nails due to their similarity in structural composition, this field also needs to be studied further. Alves et al. used these matrices to evaluate the presence of organophosphorus flame retardants, demonstrating that the concentrations found were higher in nails than in hair [27]. Although the nail is a matrix used in toxicology and for monitoring illegal drug use, its use is not as widespread in biomonitoring exposure. In this regard, there is a gap in the scientific literature. Several compounds have been studied in nails. For example, Kuwayma et al. used the nail for the evaluation of an antihistamine [14], Liu et al. focused on perfluoroalkyl compounds [28] and Lemos et al. investigated on methadone, an illicit drug [29]. Other studies have evaluated the presence in the nail of compounds present in personal care products with endocrine disrupting behaviour, such as triclosan and triclocarban [30], bisphenols and parabens [22] and UV filters [31], demonstrating that these compounds bioaccumulated in the nail during its growth and were found in higher concentrations than in other biological matrices. Li et al. investigated uric acid in nails and found that the concentration in men was higher than in women and propose this matrix as an alternative for non-invasive assessment of systemic diseases [32].

All these studies have shown that human fingernail as a matrix is a good biomarker of exposure, but their results are not comparable with those of the present study because they are different compounds. However, they all share an important idea that nail as a matrix has numerous advantages, such as allowing assessment over a long period of time, providing retrospective information on the consumption of certain compounds and facilitating sampling (as it is non-invasive), transport and storage [28,33].

Antibiotics have been extensively studied and a significant amount of research has been directed towards biomonitoring their exposure by analysing other biological samples. By far the most studied sample has been urine (non-invasive and easy to collect), but being a method of excretion in the body, it allows measuring short-term exposure, unlike fingernails, which could be a reservoir. In addition, some of these studies have tried to link antibiotic exposure to some diseases such as altered gut microbiota [34,35], obesity, mental disorders, allergic diseases [36] and mental health [37], reflecting the concern about exposure to these compounds and the harm they may cause over a medium to long period of time, and the importance of biomonitoring when exposure is prolonged, either through drugs or dietary intake.

As proposals for the future, further research on nails is needed to obtain a more general view of this matrix and to have more experience with the analysis. First, the variety of substances analyzed is still limited, as for pharmaceuticals only sedatives have been studied [38]. Second, in order to assess the accumulation of antibiotics in the human body considering a single route of entry (oral), investigations on drug consumption profiles in volunteers or even in patients with established treatments should be performed to know the behavioural profile and to perform case-control investigations. In general, more studies using nails as a matrix are therefore needed to reach more solid conclusions about their applications.

4. Conclusions

A multiclass UHPLC-MS/MS method for the analysis of antibiotics in human nail samples has been optimized and validated. The proposed method has allowed the simultaneous determination of 17 antibiotics belonging to different families. To our knowledge, no previous study has analyzed antibiotic accumulation in nail samples. Two extraction techniques were optimized and compared, and the results showed that with MAE superior efficacy and viability were obtained. The MAE extraction procedure consisted of two extraction cycles with methanol at 88 °C for 1 min. The analytical performance parameters were good in terms of linearity, sensitivity, selectivity and accuracy (trueness and precision), in accordance with FDA guidelines for bioanalytical methods. Finally, the method was applied to samples obtained from 10 volunteers. Although the number of volunteers used is too limited, and they all come from the same geographical area, to draw reliable epidemiological conclusions, the results have shown that SMX was present in all samples, while the other antibiotics under study were detected in at least one of the samples analyzed. This high frequency of antibiotic detection demonstrates the risk to human health and the threat posed by the development of bacterial resistance. The presence of antibiotics in our volunteers can be considered a serious fact, since to our knowledge; many of them had not consumed antibiotics in the last 2 years. The present study reveals the bioaccumulation of these compounds in the human body and the suitability of nails as biomarkers of exposure as they are deposited through the bloodstream into the matrix which acts as a reservoir. Their use for future research in biomonitoring of emerging contaminants in the human body and exposure is of great interest.

Credit author statement

MdCGR: Formal analysis, investigation, methodology, writing-original draft, writing-review & editing; LEM: investigation, methodology; LMP: Conceptualization, data curation, writing- original draft, writing-review & editing; AZG: Conceptualization, formal analysis, investigation, supervision, writing-review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2023.124687.

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