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NOVEL SOLID PHASE EXTRACTION METHOD FOR THE ANALYSIS OF 5-NITROIMIDAZOLES AND METABOLITES IN MILK SAMPLES BY CAPILLARY ELECTROPHORESIS

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Abstract

A new sample treatment has been developed for the extraction of 5-nitroimidazole (5-NDZ) drugs in milk samples previous to their determination by micellar electrokinetic chromatography (MEKC). Fat removing and protein precipitation were simultaneously carried out by the addition of trichloroacetic acid (TCA) and subsequent centrifugation. Clean-up and off-line concentration were achieved by a novel solid-phase extraction (SPE) method employing mixed cation exchange (MCX) cartridges, obtaining an off-line concentration factor of 18. Analyses were performed in less than 18 min employing 20 mM phosphate buffer (pH 6.5) and 150 mM SDS as background electrolyte (BGE). During the separation procedure a temperature of 20 ºC and a voltage of 25 kV (normal mode) were applied. Due to sweeping effects, an on-line concentration was achieved for all the studied compounds and detection limits lower than 1.8 μg L^{-1} were obtained. This method has been successfully applied to milk samples of different origins, including raw ewe milk.

Keywords

Nitroimidazoles. Milk. SPE. Protein precipitation. Capillary electrophoresis.

1. Introduction

Milk consumption is much extended around world due to its nutritive components like saturated fat, proteins and calcium. Nevertheless, uncontrolled use of antibiotics in farms for treating veterinary diseases can turn this popular feed into a risk source for human's health. Allergic reactions in hypersensitive individuals or appearance of drug-resistant microorganisms could be the consequences (Samanidou & Nisyriou, 2008). In order to control the presence of antibiotic residues in animal products intended for human consumption, including milk, European Commission (EC) has established maximum residue limits (MRLs) of veterinary medicinal substances in foodstuffs of animal origin (Commission Regulation (EU), 2009). According to this Regulation, pharmacologycally active compounds are classified in two categories depending on if their use is allowed (these substances have an assigned MRL) or they are forbidden for veterinary use. Antibiotics like chloramphenicol, nitrofurans or 5-nitroimidazoles (5-NDZs) are not admitted as veterinary antimicrobials.

5-NDZs have a wide action spectrum, being very effective against anaerobic protozoans and bacteria. 5-NDZs are characterized by an imidazole cycle containing a NO_2 group in fifth position which is reduced inside cells. Subsequently the reduced agent causes strand breakage of microorganism DNA (Edwards, 1980). Chemical structures of NDZ compounds and their metabolites are shown in Figure 1. Although antibiotics belonging to 5-NDZ family, such as ornidazole (ORZ) or metronidazole (MNZ), are still used as human antimicrobials, they have been banned as veterinary drugs because of the adverse health effects of their residues. Some reports attribute genotoxic, carcinogenic and mutagenic properties to these compounds (Rodríguez Ferreiro et al., 2002). The prohibition of treating animal diseases with 5-NDZs has not been only established by the European Union (EU), also their use has been banished in other countries like United States (US) (Food Animal Residue Avoidance & Depletion Program, 2010) and China (USDA Foreign Agriculture Service, 2011). Also, the traditional addition of 5-NDZs to animal feed

as growth promoters involves the need of carrying out exhaustive controls with the aim of detecting this illegal practice. Since the beginning of January 2006, the use of antibiotics as growth promoters have been forbidden by EC Regulation No. 1831/2003. Because no MRLs have been set for banned veterinary antibiotics, European Community reference laboratories (CRLs) suggests a recommended level (RL) of 3 μg L^{-1} as the minimum level that analytical methods must reach to determine RNZ (ronidazole), MNZ and DMZ (dimetridazole) in all matrices (CRL Guidance Paper, 2007).

Several analytical methods have been proposed for the determination of these compounds in a quite wide variety of matrices such as eggs ([Daeseleire, De Ruyck & Van Renterghem, 2000] and [Cronly, Behan, Foley, Malone & Regan, 2009]), poultry meat ([Hurtaud-Pessel, Delépine & Laurentie, 2000] and [Mitrowska, Posyniak & Zmudzki, 2010]), swine tissues ([Xia, Li, Zhang, Ding, Jiang & Shen, 2007] and [Fraselle, Derop, Degroodt & Van Loco, 2007]), honey (Huang, Lin, & Yuan, 2011] or fish (Sorensen & Hansen, 2000). A few studies have been focused on the development of 5-NDZ methodologies for their determination in milk samples. For this matrix, the most employed analytical technique is liquid chromatography (LC) coupled to mass spectrometry (MS) ([Cronly et al., 2010] and [Toelgyesi, Sharma, Fekete, Fekete, Simon & Farkas, 2012]), although for screening purposes, optical biosensors have also been used (Thompson, Traynor, Fodey, Crooks, 2009). Milk is a very complex matrix and the determination of residual antibacterial presents several problems because some of them can bind easily to proteins and also it contains significant amounts of divalent and trivalent cations that form complexes with these compounds, increasing their retention in the matrix (Blasco, Torres & Pico, 2007). With the aim of removing analytical interferences from so complex matrix and/or to modify the nature of the matrix, some sample treatments have been proposed. Different procedures have been reported for 5-NDZ extraction from milk samples, such as an organic extraction with acetonitrile ([Cronly et al.] and [Thompson et al.]) or as a solid-phase extraction (SPE) method (Toelgyesi et

al.). Although good results have been obtained with the SPE method, it is time-consuming methodology because it requires a shaking-centrifugation step of 70 min prior to the sample clean-up with a Strata SDB (3 mL 200 mg) cartridge. In general, no long pretreatments are required when LC is employed as separation technique, especially when MS is used as detection system.

Capillary Electrophoresis (CE) has been successfully applied for monitoring other antibiotics in milk samples [(Bailón-Pérez, García-Campaña, del Olmo Iruela, Gámiz-Gracia & Cruces-Blanco, 2009) and (Lara, García-Campaña, Alés-Barrero, Bosque-Sendra & García-Ayuso, 2006)] but, as far as we know, the determination of 5-NDZs by CE has never been carried out before in this matrix. Important advantages such as speed of analysis, high efficiency, separation selectivity, small sample size capability, low reagent consumption and automation make CE a powerful analytical technique in food analysis (García-Campaña, Gámiz-Gracia, Lara, del Olmo Iruela & Cruces-Blanco, 2009). However, CE has an important limitation that is the low achieved sensitivity when UV/Vis detection is employed, as well as the poor selectivity when complex matrices are analyzed. Sensitivity restriction can be solved by using on-line stacking procedures or off-line concentration processes in sample treatment. Normally, 5-NDZ separation has been performed through LC or gas chromatography (GC) (Mahugo-Santana, Sosa-Ferrera, Torres-Padrón & Santana-Rodríguez, 2010) and very few methods based on CE have been previously reported; all of them limited to the determination of a maximum of three 5-NDZ drugs and metabolites.

Different modes of CE have been considered for the analyses of a wide range of samples containing 5-NDZs. Capillary zone electrophoresis (CZE) has been applied in human urine for MNZ determination (Jin, Li, Xu, & Dong, 2000), while MNZ, DMZ, RNZ, secnidazole and benzomylmetronidazole have been analyzed by CZE in artificial porcine muscle tissue (Lin, Su, Liao, Yang, Yang & Choi, 2012) by the addition of tetrabutylammonium bromide to the running

buffer for improving the separation. MEKC methodology has been applied for the determination of MNZ, DMZ and RNZ in pig liver tissue (Nozal, Arce, Simonet, Ríos & Valcárcel, a, 2006) and microemulsion electrokinetic chromatography (MEEKC) has been considered for MNZ, DMZ and RNZ separation (Nozal, Arce, Simonet, Ríos & Valcárcel, b, 2006). A two-dimension CE method has been applied for MNZ, DMZ and RNZ and tetracycline detection (Santos, Simonet, Ríos, & Valcárcel, 2007). Recently, we have published a new MEKC method for the simultaneous determination of nine 5-NDZ antibiotics and metabolites (metronidazole-OH (MNZ-OH), MNZ, dimetridazole-OH or HMMNI, RNZ, DMZ, ternidazole (TRZ), ORZ, ipronidazole-OH (IPZ-OH) and ipronidazole (IPZ)) in water samples by CE (Hernández-Mesa, Cruces-Blanco & García-Campaña, 2012). As far as we know this is the highest number of compounds of this family that has been simultaneously analyzed in a single run by CE.

In the present paper we propose a MEKC method with UV detection for the determination of six 5-NDZs (MNZ, DMZ, RNZ, IPZ, TRZ and ORZ) and three of their metabolites (MNZOH which is MNZ metabolite, IPZOH that is IPZ one, and HMMNI which is DMZ and RNZ metabolite, although both of them have different degradation pathways) in different milk samples, including ewe raw milk. To the best of our knowledge, this is the first CE method proposed for the analysis of these compounds in milk samples, providing a green alternative to LC, in terms of low solvent consumption and reduction of contaminant waste. To overcome the lack of sensitivity inherent to CE with UV detection, sweeping is proposed as a very useful on-line concentration strategy in MEKC (Quirino, Kim & Terabe, 2002). Sweeping is designed to focus the analytes into a narrow band within the capillary, thereby increasing the sample volume that can be injected without any loss of efficiency. It uses the interactions between an additive (i.e., a pseudostationary phase or micellar media) in the separation buffer and the sample in a matrix that is free of the employed additive and involves the accumulation of charged and neutral analytes by the pseudostationary phase that penetrates the sample zone and “sweeps” the analytes, producing a focusing effect.

On other hand a novel off-line sample treatment based on two steps has been developed for the concentration and clean-up of milk samples in order to overcome the lack of sensitivity in CE. Firstly, fat removing and protein precipitation are simultaneously carried out and then SPE by a mixed cation exchange (MCX) Oasis cartridge is employed for removing interferences and concentrating the analytes, obtaining limits of detection lower than 1.8 μg L^{-1}.

2. Experimental

2.1. Materials and reagents

All reagents were of analytical reagent grade, unless indicated otherwise, and solvents were HPLC grade. Ultrapure water (Milli-Q plus system, Millipore, Bedford, MA, US) was used throughout the work. Sodium hydroxide (NaOH), sodium dihydrogen phosphate, disodium hydrogen phosphate and ammonium hydroxide 30% were obtained from Panreac-Química (Madrid, Spain); methanol and acetic acid from VWR International (West Chester, PA, US). Acetonitrile (MeCN) and sodium dodecyl sulfate (SDS) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Formic acid, triethylamine (TEA) and trichloroacetic acid (TCA) were supplied by Merck (Darmstadt, Germany).

Analytical standards of MNZ (1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole), DMZ (1,2-dimethyl-5-nitroimidazole), RNZ (1-methyl-2-carbamoyloxymethyl-5-nitroimidazole), ORZ (1-(3-chloro-2-hydroxypropyl)-2-methyl-5-nitroimidazole), HMMNI (2-hydroxymethyl-1-methyl-5-nitroimidazole), MNZOH (1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole) and IPZOH (1-methyl-2-(2'-hydroxyisopropyl)-5-nitroimidazole) were supplied by Sigma-Aldrich (St. Louis, MO, USA), while IPZ (2-isopropyl-1-methyl-5-nitroimidazole) and TRZ (1-(3-hydroxypropyl)-2-methyl-5-nitroimidazole) hydrochloride by Witega (Berlin, Germany).

0.2 µm nylon membrane filters (Pall Corp, MI, US) were used for the filtration of milk samples, except for goat's raw milk samples for which 0.45 µm polyethersulfone membrane filters (VWR International, West Chester, PA, US) were used.

Extraction cartridges containing a mixed-mode polymeric sorbent with 30 µm of particle diameter (Oasis MCX 60 mg, 3 cc and 150 mg, 6 cc; Waters, Milford, MA, US) were tested in the SPE step.

2.2. Preparation of standards

Individual stock standard solutions of every 5-NDZ containing 1 g L^{-1} were prepared by dissolving each pure compound in MeCN. These solutions were stored in dark bottles at -20°C and warmed to room temperature before use. They were stable for at least 6 months. An intermediate standard solution of 100 mg L^{-1} was prepared by mixing aliquots of each individual stock solution and the mixture was diluted with MeCN. It was kept in a dark bottle at 4°C. It was stable for at least 3 months.

A working standard solution of 10 mg L^{-1} was prepared by the dilution of the intermediate standard solution with ultrapure water. It was stocked in a dark bottle at 4ºC and warmed to room temperature before use.

2.3. Instrumentation and equipment

CE experiments were carried out with an Agilent 7100 CE System (Agilent Technologies, Waldbron, Germany) equipped with a diode-array detector. Data were collected using the HP ChemStation (Version B.02.01) software. Separations were performed in an uncoated fused-silica capillary (61.5 cm x 50 µm i.d.) with an optical path length of 150 µm (bubble cell capillary from Agilent Technologies) and an effective length of 53 cm.

A pH-meter (Crison model pH 2000, Barcelona, Spain) with a resolution of ±0.01 pH unit, an evaporator with nitrogen (System EVA-EC from VLM GmbH, Bielefeld, Germany) and a vortex (Genie 2 model from Scientific Industries, Bohemia, USA) were also used. SPE procedure was carried out on a vacuum manifold system from Supelco (Bellefonte, PA, US).

2.4. Electrophoretic procedure

Before the first use, the new capillary was rinsed with 0.1 M NaOH at 20 ºC for 15 min and then with the background electrolyte (BGE) at 20 ºC for 15 min. At the beginning of each session, the capillary was rinsed at working temperature with the running buffer for 15 min. Before each run, the capillary was pre-washed at working temperature with the running buffer for 2 min. In all instances, a pressure of 1 bar was applied. At the end of the day, the capillary was cleaned at working temperature with deionized water for 5 min and afterwards with air for 5 min. A pressure of 5 bars was applied in the cleaning process.

A previous MEKC method developed by our research group (Hernández-Mesa et al.) was applied for 5-NDZ determination, including some modifications. The BGE consisted on an aqueous solution of 20 mM sodium phosphate (mixture of sodium dihydrogen phosphate and disodium hydrogen phosphate; pH 6.5) and 150 mM SDS. A voltage of 25 kV (normal mode) was applied for the electrophoretic separation using a ramp of 0 - 25 kV at the beginning in 0.5 min. The capillary temperature was kept constant at 20 ºC. Standard solutions and samples were prepared in a buffer similar to the running buffer but without micellar medium and they were hydrodynamically injected at 50 mbar for 25 s. All the 5-NDZs were monitored at a wavelength of 320 nm. A stable electrical current of 61 μA was observed.

2.5. Preparation of milk samples

A wide variety of milk samples were analyzed. Samples were selected considering different compositions, nature and, in some cases, the previous treatments that they suffered in industry

196 before their reception in our laboratory. Whole pasteurized cow milk and semi-skimmed goat milk
197 were acquired in a local supermarket (Granada, Spain); goat raw milk and ewe raw milk were
198 gently supplied by a local farm from Extremadura (La Serena, Spain).

199 A novel sample treatment for extraction, concentration and clean-up employing Oasis MCX
200 cartridges has been developed. Aliquots of 3.5 mL of milk samples were distributed in 15 mL
201 polypropylene centrifuge tubes and spiked with different levels of 5-NDZ antibiotics. To each
202 sample, 0.35 g of TCA was added and subsequently the mixture was shaken by vortex for a few
203 seconds. Afterwards, samples were centrifuged for 10 min at 9000 rpm. While fat remains at the
204 top of the solution, protein precipitation can be observed. Sample supernatant is taken using a
205 syringe with a coupled needle and filtered with a 0.2 µm nylon filter in order to remove suspended
206 matter. Filtered solution was discharged on the 150 mg Oasis MCX cartridge. Previously the
207 cartridge was sequentially conditioned with 1 mL of methanol and 1 mL of TCA 0.1 g/mL.
208 Following that, the sample was applied to the column at 1 mL min^{-1}. Thereafter, a washing step
209 was sequentially carried out with 1 mL formic acid, 1 mL of methanol and 1.5 mL of 5% methanol
210 and 2% ammonium hydroxide aqueous solution. In order to guarantee that no washing solution
211 remains in the cartridge, vacuum was applied for a few seconds until no drops falling down from
212 the cartridge were observed. Elution of 5-NDZ antibiotics was performed with 2 mL of methanol
213 solution containing 2% ammonium hydroxide. The recollected sample was evaporated to dryness
214 under a gentle nitrogen current, and then it was reconstituted with 200 µL of an aqueous solution
215 of 20 mM sodium phosphate (pH 6.5).

216 3. Results and discussion

217 3.1. Electrophoretic method

218 The MEKC method previously proposed in our research group for the determination of nine 5-
219 NDZs in water samples (Hernández-Mesa et al.) has been employed to evaluate the sample

220 treatment that has been developed for the analysis of these compounds in milk samples. The
221 main characteristic of MEKC procedure is the use of BGE that consist on a 20 mM sodium
222 phosphate aqueous solution (mixture of sodium dihydrogen phosphate and disodium hydrogen
223 phosphate) (pH 6.5) and 150 mM SDS. A voltage of 25 kV (normal mode) was applied for the
224 electrophoretic separation using a ramp of 0-25 kV at the beginning in 0.5 min. The temperature
225 of the capillary was kept constant at 20 ºC. At these conditions, a stable electrical current of 61
226 μA was obtained. All the 5-NDZs were monitored at a wavelength of 320 nm.

227 In the present method, some modifications were carried out respect to the ones previously fixed.
228 It was observed a slight negative effect in migration time reproducibility when the capillary was
229 rinsed with NaOH each day previously to its use or between analyses as it was proposed before.
230 For this reason the consequences of avoiding NaOH in this method were evaluated. Although
231 NaOH is necessary for the conditioning of a new capillary (for silanol groups activation), we
232 observed that the removal of further NaOH undergoes a decrease in the RSD of the migration
233 times. Taking into account this observation, a new conditioning procedure for each working day
234 and between analyses was selected. It consists on rinsing the capillary exclusively with the BGE.
235 Better precision in the migration times (RSDs from 5.7 - 14.9 % to 1.4 – 8.5 %) was observed
236 (see Table 1), so this fact allowed us to inject higher sample volumes without any loss in peak
237 resolution. In this sense, sample injection time was reevaluated between 15 s (injection time
238 defined in the previous method for water samples) and 35 s. For achieving a sweeping effect,
239 standard solutions prepared in a buffer similar to the running buffer but without micelles were
240 hydrodynamically injected at 50 mbar. When injection times higher than 25 s were applied, poor
241 resolution between HMMNI and MNZ peaks was observed. Besides, sensitivity increase was not
242 significant for injection times higher than 25 s in the case of some analytes, so an injection time of
243 25 s was selected as optimum. Due to the on-line concentration in the sweeping process,

sensitivity was increased in a range between 1.5 times for MNZ and 4.0 times for IPZ compared to a normal injection during 15 s of a sample solution containing micelles.

3.2. Proposed milk sample treatment

3.2.1. Fat removing and protein precipitation

Numerous methods have been proposed for fat removing and protein precipitation in milk samples [(Vera-Candioti, Olivieri, & Goicoechea, 2010), (Lal, Paliwal, Grover & Gupta, 1994), (Tefera, Ehling & Ho, 2007) and (Galeano, Guiberteau, Acedo, Correa & Salinas, 1997). In this work we have proposed to carry out fat removing and protein precipitation simultaneously by centrifugation of the sample after adding a protein precipitation agent. MeCN, TCA and acetic acid were checked as protein precipitation agent, being TCA the most adequate because less suspended matter was observed in the supernatant and it allows to decrease the sample pH and to charge the analytes. Solid TCA has been employed in order to avoid sample dilution. The concentration of the added TCA into the milk samples was evaluated between 0.10 and 0.18 g/mL. No improvement on the analyte recoveries was observed when a higher concentration than 0.10 g/mL was used, so it was chosen as optimum. Once the solid acid was placed in test tubes containing milk samples, they were shaken by vortex a few seconds for homogenizing and then, they were centrifuged at 9000 rpm for 10 min. Protein precipitation can be observed in the bottom of the tube while a layer of fat remains on the top of the acidic supernatant.

The loss of mass from the matrix after defatting and protein precipitation was evaluated for three samples of each studied type of milk (whole pasteurized cow milk, semi-skimmed goat milk, goat raw milk and ewe raw milk). A loss of 25 % in mass was observed for raw goat milk samples, which gives an idea about the complexity of this matrix. For whole pasteurized cow milk, weight difference was about 19 %, while the loss was lower for raw ewe milk and semi-skimmed goat

267 milk (around 7 %). As it will be shown later, the complexity of raw goat milk samples entails low
268 analytes recoveries.

269 *3.2.2. Solid phase extraction*

270 SPE procedure has been proposed as sample treatment for concentration and clean-up. Due to
271 TCA addition to milk sample, supernatant from fat removing and protein precipitation step
272 presents an acidic pH, around 0.6, which is lower than the pk_a of the analytes (Figure 1). At these
273 conditions 5-NDZ drugs are positively charged and for this reason a cation exchange cartridge
274 was chosen for SPE. Previous assays were carried out employing a 60 mg Oasis MCX cartridge
275 (30 µm particle diameter). The proposed protocol consisted on a cartridge preconditioning with 1
276 mL of methanol and 1 mL of 0.1 g/mL TCA aqueous solution. Then milk supernatant was passed
277 through 0.2 µm nylon filters to the SPE cartridge; except for goat raw milk for which 0.45 µm
278 polyethersulfone filter was used. The filtrate was charged at 1 mL/min into the cartridge without
279 vacuum. Afterwards a washing step consisting on 1 mL of 2% formic acid solution (for removing
280 salts and to block alkaline analytes) and 1 mL of methanol (for removing neutral and acidic
281 interferences) was carried out. Both solutions were passed through the cartridge at 1 mL/min.
282 Finally, analytes were eluted with 1.5 mL of methanol containing 2% ammonium hydroxide.
283 Recoveries around 90% were obtained for MNZ-OH, HMMNI, MNZ, RNZ, DMZ, TRZ, IPZ-OH
284 and ORZ. It was shown that methanol in alkaline medium is needed for 5-NDZ elution and
285 therefore no analyte losses occur when pure methanol is used as washing agent. Strong
286 retention of positive charged 5-NDZs is achieved in the Oasis MCX cartridges because of the low
287 working pH. Although satisfactory recoveries were achieved, these results were obtained when 1
288 mL of milk was treated. It was an inconvenience because the obtained concentration level was
289 not enough considering the target value of 3 µg/L proposed by CRLs as the minimum level to be
290 detected for methods focused on 5-NDZ residues. In this sense, volumes between 1 mL and 5
291 mL were tested, however, with these higher volumes, MNZ-OH and RNZ analytical signals were

drastically reduced and an important interference peaks on electropherograms were observed. To avoid these problems, an extra washing step and the use of cartridges with higher sorbent amount were proposed. In this sense, considering 2 mL of samples, volumes of 1 mL of alkaline solutions containing low concentrations of methanol were tested for removing alkaline polar interferences in order to complete the cartridge washing step. So, aqueous solutions containing 0 to 15 % methanol in presence of 2 % ammonium hydroxide or 2 % TEA were tested. In terms of peak area, better results were obtained when ammonium hydroxide was employed as alkaline medium instead of TEA. The introduction of this washing step did not involve significant losses of any analyte (TRZ, ORZ, DMZ, IPZ-OH and IPZ). However for MNZ-OH, RNZ, HMMNI, MNZ a decrease in the analytical signal was observed when the concentration of methanol in the alkaline solution was higher than 5% (see Figure 2 (A)), so this value was selected as optimum. As it can be seen in Figure 2 (B), very clean extract was obtained with this extra washing step.

On other hand, Oasis MCX cartridges (150 mg, 30 µm particle diameter) were tested with the aim of increasing the sample volume, following the SPE previously described. With this purpose, milk samples of 2, 3 and 4 mL spiked with 100 µg/L of each compound were analyzed. The recovery for MNZ-OH decreases drastically (around 20%) when 4 mL samples were treated. For the rest of analytes, recoveries did not suffer so important variation at the studied sample volume range. Finally 3.5 mL was chosen as milk sample volume as a compromise to get good recoveries for MNZ-OH and to achieve satisfactory preconcentration factors. In conclusion, Oasis MCX cartridges with 150 mg of sorbent and 3.5 mL of milk samples were defined as the best conditions for sample treatment in order to reach low detection limits fulfilling the CRL requirements for 5-NDZ determination methods.

Because of the increase in cartridge sorbent mass, washing and elution solution volumes were re-optimized. The washing step consisted on 1 mL of 2 % formic acid solution, 1 mL of pure methanol and 1.5 mL of solution containing 2 % ammonium hydroxide and 5 % methanol. The

effect of the volume for this last solution was investigated between 1 mL and 2.5 mL. In this range, no analyte losses were observed, and extracts were clean enough when 1.5 mL of solution was applied. On other hand, the elution volume was increased from 1.5 mL to 2 mL, which was enough to elute all the 5-NDZs. Because of the highest cartridge volume, it was necessary to apply vacuum between washing and elution step in order to remove the washing solution from the cartridge before elution.

Sample eluate was collected, dried under gentle N_2 current and reconstituted in 200 µL of a 20 mM sodium phosphate aqueous solution (pH 6.5). An off-line concentration of 18 times was achieved.

A summary of the final sample treatment is included in section 2.5 (Preparation of milk samples).

4. Validation of the method

4.1. Calibration curves and analytical performance characteristics

The calibration curves were established for the studied analytes in milk samples treated according to the optimized SPE procedure described above. To validate and characterize the present method, whole pasteurized cow milk was selected.

Matrix-matched calibration curves were established with samples that were spiked with different concentrations (5, 10, 25, 50 and 100 µg L^{-1}) of the nine studied 5-NDZs. Two samples per each concentration level were processed following the SPE method and injected by triplicate. Peak area was considered as a function of analyte concentration on the sample. A blank sample was also processed, and none of the 5-NDZs was detected. Figure 3 shows the electropherograms of a blank and a spiked sample at 10 µg L^{-1} for each compound of whole pasteurized cow milk sample.

339 Statistical parameters, calculated by least-square regression and the performance characteristics
340 of the SPE-MECK-UV method for milk samples, are shown in Table 1. Limits of detection (LODs)
341 and quantification (LOQs) were calculated as 3×S/N and 10×S/N, respectively. As it can be seen,
342 for all the studied compounds, the LODs calculated by applying both off-line and on-line
343 procedures are at the low μg L^{-1} levels, being lower than 3 μg L^{-1} (according to CRL
344 recommendations).

345 ### *4.2. Precision study*

346 The precision of the method was evaluated in terms of repeatability (intra-day precision) and
347 intermediate precision (inter-day precision) by application of the proposed SPE-MEKC-UV
348 method to whole pasteurized cow milk spiked at three different concentration levels of 5-NDZs
349 (10, 50 and 100 μg L^{-1}). Repeatability was evaluated over three samples (experimental
350 replicates) prepared and injected by triplicate on the same day, under the same conditions.
351 Intermediate precision was assessed for five consecutive days with a similar procedure as for
352 repeatability studies. The results, expressed as RSD (%) of the peak areas, are summarized in
353 Table 2.

354 ### *4.3. Trueness assessment*

355 In order to check the trueness of the proposed methodology for the analysis of real samples,
356 recovery experiments were carried out in different types of milk samples spiked at three different
357 concentration levels of 5-NDZs (10, 50 and 100 μg L^{-1}) (see Table 3). Commercial milks as whole
358 pasteurized cow milk and semi-skimmed goat milk, and raw milks, such as goat's and ewe's milk
359 were selected as representative kinds of milks destined to human consumption. Each level was
360 prepared by triplicate and injected three times. In all cases, a blank sample was analysed to
361 check the presence of 5-NDZs. An unknown peak was detected at MNZ migration time in both
362 goat milk samples. This peak was attributed to an interference belonging to goat milk samples

because it appears in samples with different origin. However, analyses by MS should be required to discard a positive result for MNZ in these samples. In spite of this interference, MNZ can be detected in real samples indirectly because the presence of its metabolite can be monitored. For other cases no compounds were co-migrating at the same time of the studied analytes. Figure 4 shows an electropherogram of a blank of each milk sample.

In general, recoveries over 60 % were obtained for all analytes in milk samples. Recoveries ranged between 50 and 70 % for all analytes in raw goat milk samples, except for DMZ and IPZ, being around 50 % or lower.

5. Conclusions

In this work a novel sample treatment combined with MEKC as analytical separation technique has been proposed as quick, simple and low solvent consumption strategy for the simultaneous determination of nine 5-NDZ residues in different milk samples. Sample treatment consisted on two steps in which fat removing and protein precipitation occur simultaneously, following by SPE with Oasis MCX cartridges for cleaning-up and off-line preconcentration. On other hand, it is the first time that these group of analytes have been determined in such a complex matrix by CE-UV, which constitutes a new green alternative for the simultaneous monitoring of a high number of 5-NDZs and their metabolites in foods, with very low detection limits, even lower that the recommended by CRLs.

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Figure captions

Figure 1. Chemical structures of the studied 5-NDZs.

Figure 2. A) Influence on the peak areas of the methanol (MeOH) percentage in the washing step. B) Electropherograms of whole pasteurized cow milk subjected to the proposed SPE method including a washing step consisted on: I) 1 mL of 2% formic acid and 1 mL of methanol; II) 1 mL of 2% formic acid, 1 mL of methanol and 1 mL of 2% ammonium hydroxide solution with 5 % of methanol. Peaks (1) MNZ-OH, (2) HMMNI, (3) MNZ, (4) RNZ, (5) DMZ, (6) TRZ, (7) IPZ-OH, (8) ORZ, (9) IPZ.

Figure 3. Electropherograms of whole pasteurized cow milk sample applying the proposed SPE-MEKC-UV method: (I) blank sample; (II) sample spiked with 10 μg L^{-1} for each 5-NDZ; Peaks (1) MNZ-OH, (2) HMMNI, (3) MNZ, (4) RNZ, (5) DMZ, (6) TRZ, (7) IPZ-OH, (8) ORZ, (9) IPZ and (U) Unknown peaks.

532 **Table 1.** Statistical and performance characteristics of the SPE-MEKC-UV method for the analysis of nine 5-NDZs in whole pasteurized cow milk samples

533

Analyte	Linear dynamic range (μg L^{-1})	Slope	Intercept	R^2 (%)	LOD (μg L^{-1})	LOQ (μg L^{-1})	Migration time (min)*
MNZ-OH	1.80-100	0.062	0.606	99.6	1.80	6.00	6.7
HMMNI	1.57-100	0.099	0.281	99.4	1.57	5.23	7.4
MNZ	1.10-100	0.111	0.799	99.5	1.10	3.66	7.7
RNZ	1.71-100	0.079	0.631	99.5	1.71	5.70	8.3
DMZ	0.95-100	0.137	0.980	99.4	0.95	3.17	9.4
TRZ	1.18-100	0.119	0.710	99.6	1.18	3.95	9.7
IPZ-OH	0.94-100	0.139	1.196	99.5	0.94	3.13	10.7
ORZ	1.12-100	0.141	1.032	99.3	1.12	3.72	11.7
IPZ	1.29-100	0.219	1.716	99.0	1.29	4.29	17.3

534

535 *For the determination of migration time, the 45 samples analysed for intermediate precision studies have been considered.

536

537

538

Table 2. Precision studies of the SPE-MEKC-UV method for the analysis of nine 5-NDZs in whole pasteurized cow milk samples

	Intraday RSD (%) (n=9)			Intermediate precision RSD (%) (n=15)		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
MNZ-OH	10.3	7.5	2.8	11.8	11.3	9.2
HMMNI	4.2	8.0	2.3	15.2	9.7	9.4
MNZ	5.7	4.4	2.7	12.1	9.0	9.3
RNZ	8.4	3.0	3.5	14.4	9.0	10.6
DMZ	8.2	3.8	4.2	13.8	11.1	13.2
TRZ	7.2	6.4	2.4	13.9	10.7	11.5
IPZ-OH	5.0	3.4	3.5	11.0	10.8	13.6
ORZ	5.7	4.1	3.8	16.1	11.8	13.1
IPZ	14.3	4.5	5.1	19.3	18.3	20.1

Level 1: 10 μg L^{-1}

Level 2: 50 μg L^{-1}

Level 3: 100 μg L^{-1}

547 **Table 3.** Recovery studies for each 5-NDZ at different spiked levels in different milk samples (n=9)

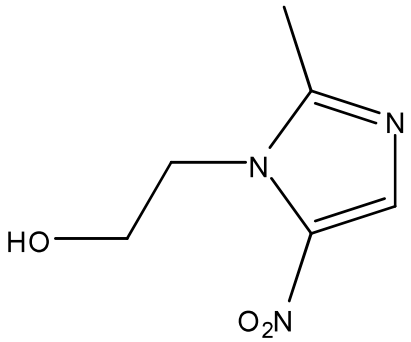
	Whole pasteurized cow milk sample						Semi-skimmed goat milk sample						Raw ewe milk sample					
	Level 1		Level 2		Level 3		Level 1		Level 2		Level 3		Level 1		Level 2		Level 3	
	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)
MNZ-OH	76.3	10.3	65.8	7.5	65.4	2.8	77.5	8.4	65.3	1.7	64.4	2.4	66.8	8.3	68.1	3.4	69.1	4.8
HMMNI	66.7	4.2	73.3	8.0	76.1	2.3	74.9	7.5	85.5	4.1	84.9	2.6	72.5	6.3	83.1	3.9	83.4	2.5
MNZ	76.2	5.7	77.4	4.4	76.8	2.7	No applicable						97.7	3.5	84.1	3.7	84.6	3.0
RNZ	73.1	8.4	70.7	3.0	68.8	3.5	62.6	7.3	72.5	3.0	74.2	1.1	83.9	7.1	76.8	3.5	77.9	3.2
DMZ	63.5	8.2	65.6	3.8	68.4	4.2	79.3	8.4	78.1	3.1	78.1	2.6	79.4	8.1	71.8	3.9	70.2	4.2
TRZ	64.5	7.2	78.4	6.4	78.5	2.4	81.7	9.7	83.8	4.0	84.0	2.5	79.8	4.3	82.3	2.9	82.2	4.1
IPZ-OH	64.6	5.0	70.1	3.4	74.8	3.5	79.3	4.8	80.4	4.7	81.9	3.7	88.1	7.3	78.3	3.7	78.4	4.2
ORZ	66.7	5.7	70.0	4.1	70.4	3.8	85.7	6.2	73.9	3.8	76.4	3.2	83.0	5.4	73.0	4.8	73.4	4.6
IPZ	57.0	14.3	53.0	4.5	58.5	5.1	76.7	12.3	68.5	3.1	68.5	3.6	71.0	5.3	57.5	1.8	59.4	6.2

548 **Level 1:** 10 µg L^{-1}

549 **Level 2:** 50 µg L^{-1}

550 **Level 3:** 100 µg L^{-1}

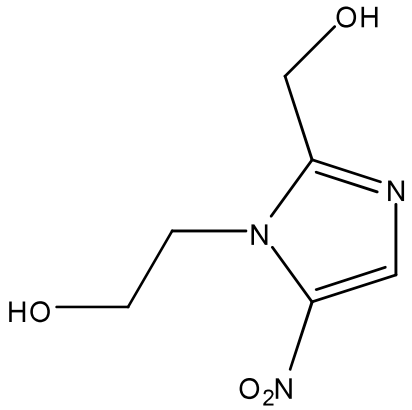
Figure 1



MNZ

$pk_1 = 2.58 \pm 0.34$

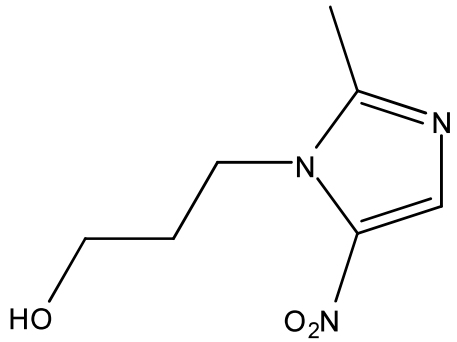
$pk_2 = 14.44 \pm 0.10$



MNZ-OH

$pk_1 = 1.98 \pm 0.34$

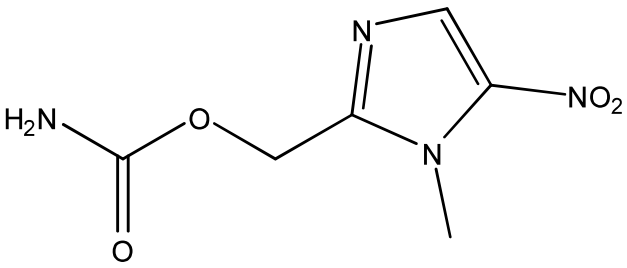
$pk_2 = 13.28 \pm 0.10$



TRZ

$pk_1 = 2.72 \pm 0.34$

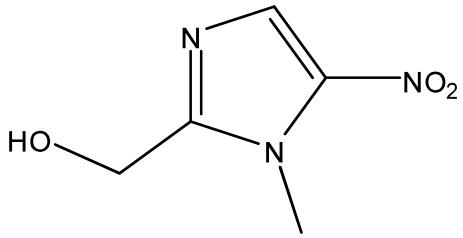
$pk_2 = 14.90 \pm 0.10$



RNZ

$pk_1 = 1.32 \pm 0.25$

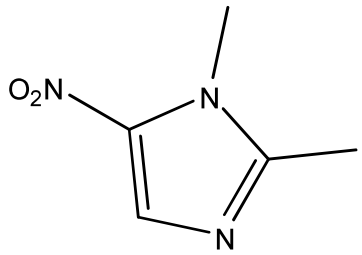
$pk_2 = 12.99 \pm 0.50$



HMMNI

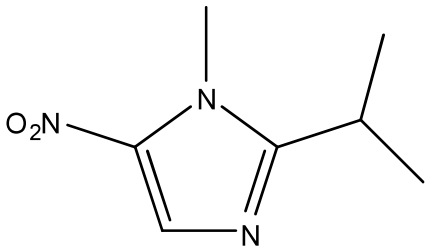
$pk_1 = 2.21 \pm 0.25$

$pk_2 = 13.31 \pm 0.10$



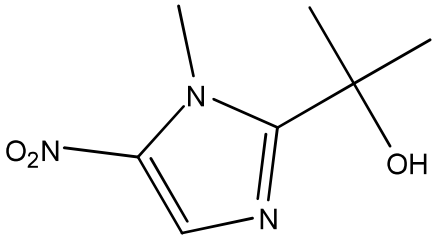
DMZ

$pk_1 = 2.81 \pm 0.25$



IPZ

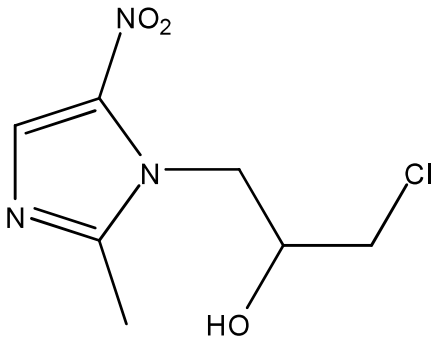
$pk_1 = 2.55 \pm 0.25$



IPZ-OH

$pk_1 = 2.21 \pm 0.25$

$pk_2 = 13.44 \pm 0.29$



ORZ

$pk_1 = 2.72 \pm 0.35$

$pk_2 = 13.29 \pm 0.20$

Figure 2

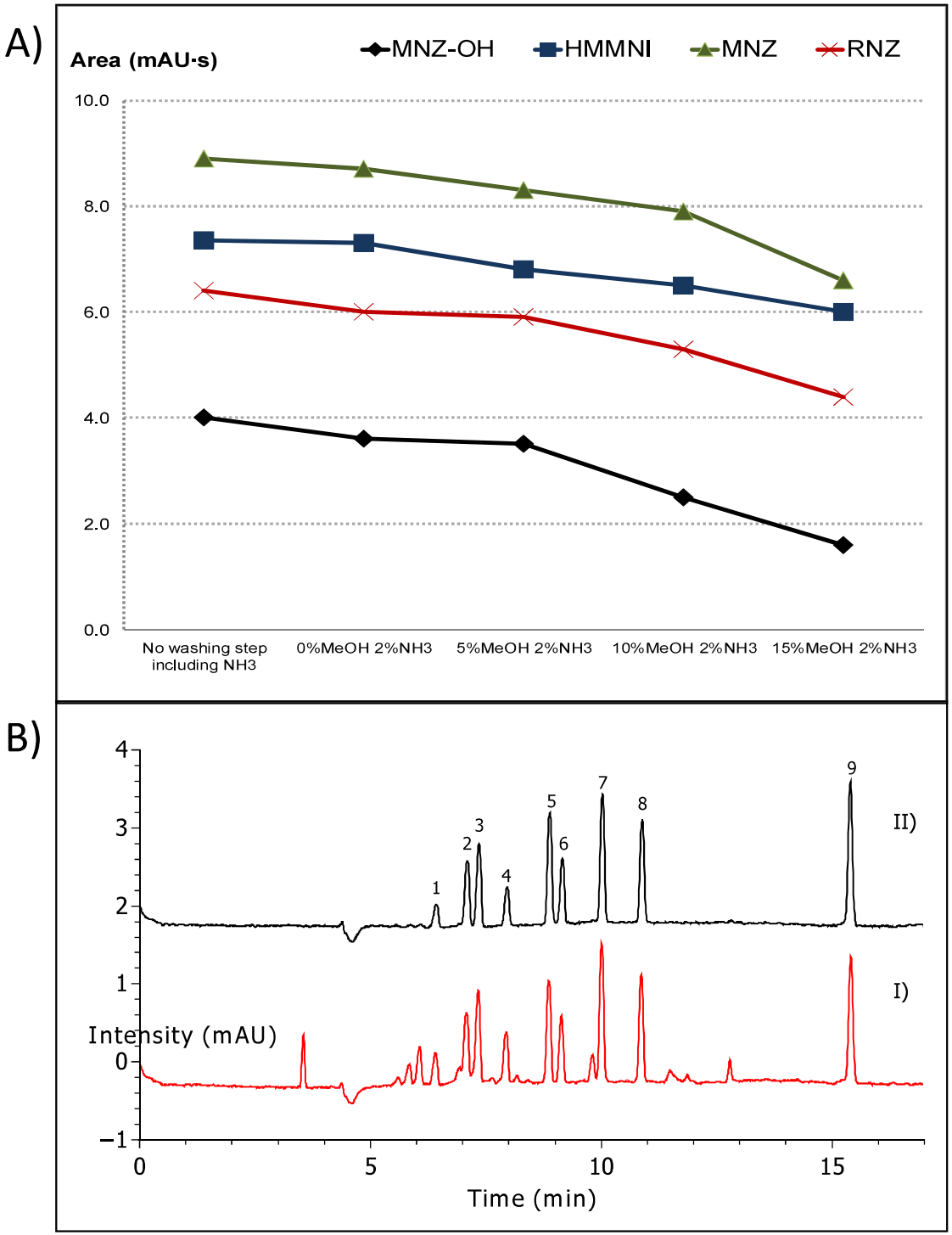

A)
Area (mAU·s)
MNZ-OH
HMMNI
MNZ
RNZ
10.0
8.0
6.0
4.0
2.0
0.0
No washing step including NH3
0%MeOH 2%NH3
5%MeOH 2%NH3
10%MeOH 2%NH3
15%MeOH 2%NH3
B)
Intensity (mAU)
Time (min)
II)
I)
1
2
3
4
5
6
7
8
9

Figure 3