# DETERMINATION OF 5-NITROIMIDAZOLE RESIDUES IN MILK BY CAPILLARY ELECTROCHROMATOGRAPHY WITH PACKED $C_{18}$ SILICA BEDS

*Maykel Hernández-Mesa, Francisco J. Lara, Carmen Cruces-Blanco and Ana M. García-Campaña**

*Department of Analytical Chemistry, Faculty of Sciences, University of Granada, Campus Fuentenueva s/n, E-18071 Granada, Spain*

*Correspondence: *Ana M. García-Campaña*

**Phone:** +32958242385; **Fax:** +32958243328

**E-mail:** amgarcia@ugr.es

**Keywords:** Capillary electrochromatography, Packed capillary columns, Slurry pressure packing, 5-Nitroimidazoles, Milk

**Abstract**

This work presents a novel methodology for analysing 5-nitroimidazole residues in milk samples by capillary electrocromatography using lab-made packed columns, produced by carrying out a high pressure packing procedure using acetone as driving solvent and $C_{18}$ silica uncapped particles (5 µm particle size) as packing material. Column frits resulted from sintering the proper stationary phase by heating the packed material for 20 s with a nichrome ribbon (80% Ni - 20% Cr, 28 cm × 2 mm × 0.2 mm, electric resistance 1.3 Ω) connected to a 7 V AC power supply. Lab-made $C_{18}$ silica packed capillaries (40 cm x 50 µm id) were employed for the determination of 5-nitroimidazole drugs. Milk samples were treated by a salting-out assisted liquid-liquid extraction followed by a solid phase extraction with Oasis®HLB cartridges prior to their injection. Samples were hydrodynamically injected into the column for 120 s at 11.5 bar. Afterwards eight 5-nitroimidazole compounds were separated in isocratic mode under an applied voltage of 27 kV and a temperature of 30 °C. The selected mobile phase consisted of a mixture 60:40 acetonitrile:ammonium acetate (2.5 mM, pH = 5). Separation was monitored at 320 nm and it was performed in less than 15 min. The method was characterized in terms of linearity ($R^2 \geq 0.993$) and precision (repeatability, RSD≤ 12.2% and reproducibility, RSD≤ 14.5%), obtaining detection limits lower than 29 µg/L for all compounds under study.

## 1. Introduction

5-Nitroimidazoles (5-NDZs) are constituted by an imidazole ring and a nitro group in fifth position. These antibiotics are used in human medicine against most Gram negative and some Gram positive anaerobic bacteria as well as against anaerobic protozoans. However, resistance bacteria against 5-NDZ based compounds have been reported [1] and other studies have attributed carcinogenic, genotoxic and mutagenic properties to 5-NDZ drugs [2,3]. In this sense, considering that residues of these substances could represent a risk to human health, their use in veterinary medicine is restricted, being banned in food-producing animals. European Union (EU) regulation [4] sets that 5-NDZ residues cannot be found in animal products intended to human consumption, but there are still alerts collected by the Rapid Alert System for Food and Feed (RASFF) about the presence of these compounds in some foods derived from animals [5]. Furthermore, the use of 5-NDZ antibiotics in veterinary medicine has also been forbidden in other countries such as China [6] and United States of America (USA) [7]. Considering the globalisation of food production, any alert about the illegal use of these antibiotics should be taken into account as international food safety concern. Therefore, simple, cheap and green analytical methodologies are required in order to detect 5-NDZ residues in animal derived foodstuffs.

During the last years several contributions have been reported about analytical methods intended to determine 5-NDZ in food. Liquid chromatography (LC) with UV [8,9,10] or mass spectrometry (MS) [11,12,13,14] detection is the most employed technique. Also, gas chromatography (GC) coupled to MS [15,16] or capillary electrophoresis (CE) with UV detection [17,18] have been proposed. The high solvent consumption and waste generation is the main drawback of LC methods, which could be avoided by employing greener techniques such as CE. However, the applicability of CE is limited due to the low sensitivity reached by this methodology as consequence of the injection of low sample volumes. In order to combine the advantages of both methodologies, capillary electrochromatography (CEC) has been proposed as a hybrid separation technique which shows high efficiency and selectivity [19].

In a CEC separation, the mobile phase is propelled through the stationary phase into the capillary by the electroosmotic flow (EOF) generated as a result of applying an electric field instead of a hydraulic pressure. The plug-like profile of EOF involves much higher CEC column efficiency than that reached by a pressure-induced flow at the same linear velocity in the same column, resulting in a peak resolution improvement [20]. In addition, CEC presents a dual separation mechanism based on differences among analyte electrophoretic mobility and analyte-stationary phase interactions. This combined mechanism gives a unique selectivity to

CEC methods [21]. Three different CEC modalities can be considered depending on the stationary phase morphology: open tubular, monolithic and packed CEC. Open tubular capillaries offer poor sensitivity as consequence of the narrow bore size of the capillary and low separation capability due to the low phase ratio caused by the limited surface area of the stationary phase [22]. On the other hand, monolithic capillaries can suffer from a lack of stability because of polymeric monolithic stationary phases tend to swell in organic solvents. Moreover, the preparation of polymeric monoliths usually leads to micropores which results in low efficiency and peak asymmetry. Monolithic capillaries also possess low column capacity attributed to their low specific surface area, providing some limited applications [23]. In contrast to the above mentioned types of columns, packed capillaries offer higher surface area which improves sample loading capacity. The success of these packed capillaries lies in this advantage, becoming the most commonly employed CEC capillaries. They are commercially available but the problems of bubble formation, column fragility and above all, their high price, retard their extensive use [24] so, researchers often prepare their own columns and as a result, several protocols have been proposed for packing capillaries. However, some authors still consider these procedures too arduous, an art or even as "black magic" [25] methodologies because they require specific skills to achieve highly efficient and reproducible capillaries.

Since packed columns in CEC were introduced by Pretorius *et al.* in 1974 [26], different packing techniques have been tested in order to obtain the perfect packed column, even comparisons among them have been reported [27,28,29]. But in the last years, all of them have a common challenge that is to avoid poorly packed capillaries which can lead to low efficiency, poor resolution and asymmetric peaks. Several procedures for capillary packing have been described [25] including electrokinetic packing [30,31], packing with supercritical carbon dioxide [32], with centripetal forces [33] or by gravity [34]. However slurry packing with pressure [35,36,37] is the most established methodology because it is simple to implement and it does not require long recipes for start-up. The main difficulty of column packing resides in the production of the frits because they have to retain the stationary phase in the capillary when a separation voltage is applied and, at the same time, to allow the mobile phase to penetrate freely through them. If frits are too thin, they will not retain the sorbent, and if they are too thick and non-porous, the mobile phase will not pass through them. In these packed columns, EOF is not homogeneous along the capillary, showing different zones (packed part, frit and open part) which produce pressure differences across the frit [38]. As a result, bubbles are formed in the boundary region between the frit and the unpacked part of the capillary,

causing lost of efficiency and current disruptions. In order to solve these problems, the use of different kind of frits, such as monolithic [39], sol-gel [40], magnetic [41], single particle [42] as well as new methods for sintering silica particles [43] have been proposed and compared [44,45]. Moreover, methodologies that attempt to carry out CEC separations in fritless capillaries have been recently reported [46,47]. Therefore, the major challenge of the packing procedure is the satisfactory fabrication of retention frits. Excluding methodologies such as single particle and internal taper approaches, sintering is the prevailing mode in frit fabrication, because it leads to the least frit-related band broadening [24].

The aim of this work is to propose an easy methodology for the fabrication of packed capillaries and their subsequent use for the monitoring of 5-NDZ residues by CEC. At the best of our knowledge, there is just one CEC method published for the determination of 5-NDZs [48]. Nevertheless, this work is focused on monolithic MIP-based capillary development and 5-NDZ standard separation is proposed without any application to real matrixes. In this work, 5-NDZ drugs are analysed for the first time by CEC using packed capillaries and the proposed methodology is evaluated for the analyses of milk samples. It is worth to mention that most of the papers about CEC are focused on testing new sorbents or developing new procedures to make CEC columns. However applications of these developments to solve practical problems with real samples are not usually carried out. In this sense, CEC remains as a technique with a great potential to be explored and this work could contribute to establish CEC as a mature technique that can move from research to routine laboratories.

## 2. Experimental

### 2.1 Materials and reagents

All reagents were analytical reagent grade, unless indicated otherwise, and solvents were HPLC grade. Ammonia (30%), sulphuric acid (98%), sodium chloride (NaCl) and sodium hydroxide (NaOH) were obtained from Panreac-Química (Madrid, Spain). Methanol (MeOH) and acetone were purchased from VWR International (West Chester, PA, USA) while acetonitrile (MeCN) and acetic acid were supplied by Sigma-Aldrich (St. Louis, MO, USA). Formic acid (98–100%), tris(hydroxymethyl)aminomethane (TRIS) and hydrochloric acid (HCl) 37% were acquired from Merck (Darmstadt, Germany). Ultrapure water (Milli-Q plus system, Millipore, Bedford, MA, USA) was used throughout the work.

Analytical standards of dimetridazole (DMZ; 1,2-dimethyl-5-nitroimidazole), ronidazole (RNZ; 1-methyl-2-(carbamoylmethyl)-5-nitroimidazole), carnidazole (CRZ; [2-(2-methyl-5-nitro-

imidazol-1-yl)ethyl]thiocarbamic acid o-methyl ester), ornidazole (ORZ; 1-(3-chloro-2-hydroxypropyl)-2-methyl-5-nitroimidazole), metronidazole (MNZ; 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole) and tinidazole (TNZ; 1-(2-ethylsulfonylethyl)-2-methyl-5-nitro-imidazole) were supplied by Sigma-Aldrich (St. Louis, MO, USA) while ipronidazole (IPZ; 2-isopropyl-1-methyl-5-nitroimidazole), secnidazole (SCZ; α,2-dimethyl-5-nitro-1H-imidazole-1-ethanol hemihydrate) and ternidazole (TRZ; 1-(3-hydroxypropyl)-2-methyl-5-nitroimidazole) hydrochloride were purchased from Witega (Berlin, Germany).

Packed columns consisted of uncoated fused silica capillaries of 50 µm, 75 µm and 100 µm internal diameter (i.d.) which were purchased from Polymicro Technologies (Phoenix, AZ, USA) and LiChrospher RP-C18 non-encapped particles (5 µm particle size) (Agilent Technologies, Waldbronn, Germany) which were recycled from a damage LC column.

Oasis®HLB cartridges (60 mg, 3 cc) (Waters, Milford, MA, USA) were considered for the sample treatment procedure. Clearinert™ 13 mm syringe filters with 0.22 µm nylon membrane (Wilmington, DE, USA) were used for sample filtration prior to sample injection into the CEC system.

**2.2 Standard preparation**

Individual standard solutions of each 5-NDZ were prepared at 1000 mg/L by dissolving each pure compound in MeCN. These solutions were stored in dark bottles at -20°C and equilibrated to room temperature before use. They were stable for at least six months.

Intermediate standard solution containing 100 mg/L of each 5-NDZ except for CRZ (200 mg/L) was obtained by mixing individual standard solutions and subsequent dilution with MeCN. It was stored at 4°C avoiding exposure to direct light. It was stable for at least three months. Working solutions were prepared in water from the intermediate standard solution. Milk samples were also fortified from the intermediate standard solution according to desired concentrations.

**2.3 Instrumentation**

A SP-400 Nanobaume™ column packing unit (Western Fluids Engineering, Wildomar, CA, USA) coupled to a PU-2080 high pressure pump (Jasco, Easton, MD, USA) was employed for capillary packing. Capillary packing process was assisted by a MC-8 magnetic stirrer (Bunsen, Madrid, Spain). Capillary frits were made using a nichrome ribbon (80% Ni-20% Cr, 28 cm × 2 mm × 0.2 mm, electric resistance 1.3 Ω) connected to a 7 V AC power supply which was made by a local technician.

CEC experiments were carried out with an Agilent 7100 CE System (Agilent Technologies, Waldbronn, Germany) equipped with a diode-array detector. Data were acquired using the supplied software with the CE system (HP ChemStation, Version B.02.01).

A Universal 320R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany) and a vortex-2 Genie (Scientific Industries, Bohemia, NY, USA) were also used. Buffer pH was adjusted with a pH meter (Crison model pH 2000, Barcelona, Spain) with a resolution of ±0.01 pH unit.

**2.4 $C_{18}$ silica packed capillaries fabrication procedure**

A fused silica capillary (50 µm i.d. and 20 cm longer than the desired packed capillary length) was rinsed with a NaOH 1 M solution for 10 min at 5 bar. Subsequently it was flushed with deionised water for 5 min at 5 bar, followed by acetone for 5 min at 5 bar. After conditioning, the capillary was mounted in the packing unit and a seven step protocol was carried out for capillary packing and frit formation (Figure 1). The experimental set-up, which mainly consisted of a high pressure pump connected to a Nanobaume™ column packing unit, is shown in Figure 2.

As it can been seen in Figure 1, the driving solvent (acetone) is initially propelled by a high pressure pump to the packing unit where $C_{18}$ silica particles (20 mg) are suspended in 1.5 mL of slurry solvent (acetone) by magnetic agitation (step A). At the same time that the system pressure increases from 0 to 420 bar, acetone carries the particles along the capillary. Particles are initially retained inside the capillary because of a mechanic frit made of a 0.5 µm membrane filter is placed at the end of it. As a consequence, the capillary is packed. Packing velocity is increased and much more homogeneous packing is obtained if a plastic screw is coupled to the mechanic frit and it is removed when the system pressure reaches 200 bar. When the capillary is partially filled with $C_{18}$ silica particles, 12 cm far away from the mechanic frit, the high pressure pump is turned off. As result, system pressure dropped down and capillary packing is interrupted. Afterwards, capillary is rinsed with deionised water for 1 h at 420 bar to ensure a proper packing of this capillary portion and to guarantee acetone is totally drained from the capillary (step B). Then, outlet frit is made at 10 cm far away from the mechanic retainer. To make the frit, the capillary is introduced through a small hole (380 – 400 µm) that was previously made in the center of the 28 cm nichrome ribbon. A 7 V AC power supply is connected to the nichrome ribbon for 20 s in order to heat the $C_{18}$ silica particles so they are sintered. Only particles in contact with the ribbon surface and the closer ones are sintered. Frit formation is done by passing deionised water through the capillary at 420 bar.

Once frit is made, mechanic frit is taken out and the stationary phase is removed from the capillary (steps C and D).

Then, the capillary is totally packed in one single step in order to obtain a compact packing (step E). The beads travel up the capillary using acetone as driving solvent at 420 bar. The packing procedure can be monitored by the observed light/dark transition in the capillary. After filling the capillary, deionised water is used to flush the column for 1 h to consolidate the packed bed (step F). As it was done before, inlet frit is fabricated at the desired length from outlet frit.

Finally, a detection window is made 2 mm from the outlet frit using hot sulphuric acid to remove the capillary coating (step G). In addition, the outlet capillary end is cut 8.5 cm from the detection window and the inlet capillary end is cut 2 mm from the inlet frit.

**2.5 Sample treatment protocol**

Whole cow's milk samples were purchased in a local supermarket. A sample of 3 mL was placed in a conical tube and it was centrifuged for 5 min at 9000 rpm for removing the majority of the fat content. Liquid phase was collected avoiding the upper fat layer. Sample deproteination was carried out by adding 4 mL of MeCN to the liquid sample. Mixture was homogenized by vortex for a few seconds and it was centrifuged for 10 min at 9000 rpm, occurring protein precipitation. Supernatant was collected and 0.8 g of NaCl were dissolved in it by vortex for 2 min. Sample was centrifuged for 5 min at 9000 rpm and two separated phases were obtained based on the salting-out assisted liquid-liquid extraction (SALLE). Highly saline aqueous phase was discarded while 3.3 mL from the upper organic phase were dried under nitrogen current at 25 °C. Sample was reconstituted in 1.5 mL of deionised water.

Afterwards, a sample clean-up was performed following a solid phase extraction (SPE) protocol. An Oasis®HLB cartridge (60 mg, 3 cc) was conditioned with 1 mL of MeOH and 2 mL of deionised water. Then the sample was loaded onto the cartridge at 1 mL/min. Later the cartridge was washed up with 2 mL of deionised water at 1 mL/min and analytes were eluted with 2 mL of MeOH at 1 mL/min by applying vacuum. Sample was dried under a nitrogen current at 25 °C and it was reconstituted in 200 µL of deionised water. Finally, it was filtered through a 0.22 µm nylon filter and it was analysed by the proposed CEC method.

**2.6 Capillary electrochromatography method**

A $C_{18}$ packed capillary (50 µm i.d. x 40 cm packed length) was used for 5-NDZ determination. New packed capillaries were initially rinsed with mobile phase in the CE

instrument by pressure (11.5 bar) for one hour. Then, a voltage of 27 kV was applied between two vials containing mobile phase for 30 min. During voltage application, inlet and outlet vials were pressurized to 5 bar in order to suppress bubbles formation. After capillary conditioning, a stable baseline was observed. At the beginning of the working day, capillary conditioning consisted of discarding the first two runs of the day. Between runs the capillary was flushed with mobile phase for 2 min at 11.5 bar of pressure. After a working day, the capillary was stored with mobile phase and capillary ends were placed in vials containing mobile phase.

Samples were hydrodynamically injected for 120 s at 11.5 bar. After sample injection, a plug of mobile phase was hydrodynamically injected for 20 s at 11.5 bar to ensure sample injection reproducibility. Analyses were performed in isocratic mode using a mixture 60:40 MeCN:ammonium acetate (2.5 mM, pH = 5) as mobile phase. 5-NDZ separation was carried out at 30°C under an applied voltage of 27 kV. A voltage ramp from 0 to 27 kV for 0.5 min was programmed at the beginning of the run, obtaining a separation current of 0.4 µA. UV detection was employed and analytical signals were monitored at 320 nm (244 nm for CRZ detection).

## 3. Results and discussion

### 3.1. Capillary packing optimization

In the fabrication of packed capillaries we selected $C_{18}$ as sorbent because it has been shown that this phase has high selectivity for 5-NDZ separation by LC methods [9,11,13].

Although MeCN is a quite common driving solvent for packing stationary phases and it was our first choice, after several experiences, an alternative solvent was needed. Capillaries that were packed using MeCN presented holes through the stationary phase after some CEC runs, leading to irreproducible analyses in terms of elution times. This effect has been already noticed by Van den Bosch *et al.* [49] when methanol was used as driving solvent as consequence of particle rearrangement when voltage was applied. Finally, acetone was selected as driving solvent instead of MeCN, accomplishing more stable and very efficient capillaries. Packing was carried out at 420 bar that is very close to the pressure limit of our pump (500 bar). Lower pressures are not recommended because they produced poor and inefficient packed capillaries.

While the capillary packing procedure resulted relatively easy to develop, to find an adequate way for making the frits was a harder task. At the beginning, thermal polymerization of silica particles wetted with sodium silicate solution was tested as frit formation

methodology [50]. Although different experimental parameters were changed, it always resulted in inconsistent or nonporous frits. Particle sintering was proposed as alternative to make the frits, considering that the selected $C_{18}$ silica particles are non-endcapped, hence they can sinter. Two parameters are involved on a sintering process: time and temperature. It is difficult to measure with precision the sintering temperature and, although most of the CEC papers refer to this parameter to explain frit fabrication, they do not indicate how to control it. We think frit fabrication can be more reproducible if instead of the temperature value, the parameters leading to that sintering temperature are given, i.e., dimensions and electric resistance of the wire or ribbon and applied voltage to the wire/ribbon for producing the frit, which are easier to measure. In any case, if the sintering temperature is very high, the sintering time has to be very short to obtain permeable frits and not to make the capillary fragile at this zone. Sintering times as short as just a couple of seconds can lead to irreproducible results.

In our case, nichrome ribbon (80% Ni-20% Cr; cross section area of 2 mm × 0.2 mm, electric resistance 1.3 Ω) of different lengths were tested. Finally, a 28 cm metallic strip was chosen because higher sintering times could be applied avoiding the formation of nonporous frits. Sintering times from 5 to 25 s were considered, reaching an optimum at 20 s as a good compromise between permeability and robustness.

Moreover, frit sintering must be carried out in absence of driving solvent. Acetone is rapidly expanded when heat is applied to the capillary and as a result, air bubbles can be formed and ineffective frits can be obtained. So inlet and outlet frits were made under a water flow at high pressure for avoiding bubble formation.

### 3.2. Electrochromatographic separation of 5-NDZs

#### *3.2.1. Effect of mobile phase composition*

Initially a mixture (65:35) of MeCN:ammonium acetate (14 mM, pH = 5) buffer was considered as mobile phase, obtaining a final ammonium acetate concentration in the mobile phase of 5 mM. Other buffers and pH values (14 mM ammonium formate, pH = 3; 71 mM Tris-HCl, pH = 8) were also evaluated. Ammonium formate buffer (pH = 3) resulted in worse peak resolution as well as Tris-HCl buffer (pH = 8) in comparison with the firstly used ammonium acetate buffer (pH = 5). Moreover, Tris-HCl buffer was discharged because of high pH values can damage $C_{18}$ silica particles according to manufacturer's specifications (recommended pH range between 2 and 7.5).

CEC separations are carried out under isocratic conditions; therefore the selection of the proportion between the organic solvent and the buffer in the mobile phase is crucial to achieve satisfactory peak resolutions. Different mixtures of MeCN:ammonium acetate buffer containing percentages of the organic solvent from 55 to 70 % were studied (see Figure caption 3). As it was expected, longer retention times were observed when MeCN percentage in the mixture was decreased due to lower elution strength. In terms of peak resolution, a greater number of compounds were resolved when a mixture MeCN:ammonium acetate buffer (13 mM, pH =5) (60:40) was considered as mobile phase. Therefore, this mixture proportion was set up as optimum mobile phase composition.

The use of MeOH instead of MeCN as organic solvent in the mobile phase was also explored. As it occurs in LC separations, longer retention times were obtained when MeOH was added to the mobile phase due to its lower elution strength. As result, MeCN was selected because 5-NDZ separation with MeOH took more than twice the time required when MeCN was employed.

The pH value of the mobile phase buffer was deeply studied between 4 and 6, but peak resolution improvement was not observed, so pH = 5 was kept. Ammonium acetate and ammonium formate were evaluated as mobile phase buffers. Solutions of each buffer (50 mM) were prepared from their respectively acids and adjusted to pH 6 with ammonium hydroxide solution (1:5 v/v). The required volume of buffer was added to the mobile phase and diluted with water, reaching the desired final ammonium acetate concentration. This assay was carried out setting 12.5 mM as buffer concentration. Better peak resolutions were obtained when ammonium acetate was employed. Finally, ammonium acetate concentration was studied from 2.5 mM to 25 mM. The main observed effect was the decrease of the analysis time when lower buffer concentrations were considered without any effect on the resolution, so a final concentration of 2.5 mM ammonium acetate (pH = 5) was selected.

### *3.2.2. Effect of capillary dimensions*

It is more affordable to study the effect of both, the packed length and the i.d. with lab-made CEC columns than with the commercial ones due to their high price. In this work different packed capillary lengths have been evaluated (8, 25, 32 and 40 cm) using lab-made packed capillaries of 75 µm i.d. As it was expected, higher separation time was obtained when the capillary length was increased. Nevertheless, peak resolution was improved by using longer packed capillaries. Figure 4 shows how peaks 1, 2 and 3 are much better resolved when analyses were carried out in a 40 cm length packed capillary.

Also, packed capillaries (40 cm) with different i.d. (50, 75 and 100 µm) were tested. A packed capillary with higher i.d. provides higher sensitivity because a much greater amount of sample is injected under the same conditions (injection time and pressure). However, lower peak efficiency is reached when higher i.d. columns are used, reducing peak resolution. Diffusion along the axis is reduced when a smaller packing diameter is selected and column efficiency is improved. Peak efficiency in terms of theoretical plates was improved 1.3-1.7 times when i.d. was decreased from 75 µm to 50 µm. Furthermore, it was improved 1.6-2.3 times when 50 µm i.d. packed capillary was employed instead of a 100 µm i.d. one. A 50 µm i.d. column was chosen as optimum for 5-NDZ separation because peak resolution was crucial for the proposed method.

#### *3.2.3. Effect of separation voltage and temperature*

Separation voltage was evaluated between 20 and 30 kV, reducing the analysis time from 20 to 13 min in this interval. However, peak resolutions between RNZ and MNZ and between MNZ and TRZ decreased when higher separation voltages were applied. As a compromise between analysis time and resolution, a separation voltage of 27 kV was established.

Separation temperature was studied in the range of 17 to 35 °C. An increase of the temperature improved CRZ peak efficiency, however, resolution between MNZ and TRZ peaks was drastically reduced. Considering both effects, a separation temperature of 30 °C was selected.

#### *3.2.4. Injection optimization*

Standard solutions in water were hydrodynamically injected in the capillary at 11.5 bar. Injection time was studied from 60 to 180 s. Maximum sensitivity for each 5-NDZ in terms of peak height was accomplished for an injection time of 120 s. Longer injection times resulted in band-broadening and resolution losses, so injection time was set up at 120 s.

### 3.3. Instrumental performance characteristics

In order to check the suitability of the lab-made packed capillaries for 5-NDZ determination, the proposed analytical method was instrumentally evaluated in terms of linearity, limits of detection (LODs), limits of quantification (LOQs), intra-day and inter-day precision. Besides, reproducibility among different lab-made packed capillaries was also studied. Method characterization was finally proposed for eight of the nine firstly considered

5-NDZs, excluding TNZ because it co-eluted with SCZ under the final separation conditions. All peak signals have been monitored at 320 nm, except for CRZ peak (244 nm). An electrochromatogram of a 5-NDZ standard solution showing the separation of the eight compounds under the optimized conditions is included in Figure 5.A.

### *3.3.1. Standard calibration curves*

Standard calibration curves were established with 5-NDZ standard solutions at six concentration levels. Two replicates of standard solutions of each concentration were analysed in duplicate. Peak area was considered as function of analyte concentration. Instrumental LODs and LOQs were calculated as the minimum analyte concentration yielding a signal-to-noise ratio equal to three and ten respectively (Table 1). According to the results, satisfactory instrumental LODs have been reached. All 5-NDZ drugs were detected at low µg/L levels, ranging from 38 to 67 µg/L (except for CRZ, 160 µg/L).

### *3.3.2. Precision studies*

Precision studies were carried out in order to evaluate the repeatability (intra-day precision) and intermediate precision (inter-day precision) of the proposed CEC methodology for 5-NDZs. In the repeatability study, three standard solutions at three concentration levels were analysed in triplicate. Intermediate precision (inter-day) was assayed at three different concentration levels by analysing a standard solution in triplicate per day for five consecutive days. Results expressed as relative standard deviations (RSD, %) of peak areas are shown in Table 1. In all cases RSDs were lower than 11.2 %. In terms of retention times, RSDs ranged from 0.4 to 1.3 % in repeatability studies for all the compounds, while for intermediate precision, RSDs between 4.7 and 10.7 % were obtained.

In addition, four CEC columns were prepared following the same experimental conditions and they were tested in order to verify the reproducibility. Standard solutions at three different concentrations were analysed in triplicate in each capillary. In all cases the RSDs % of peak areas were in general lower than that observed in the intra-day and inter-day precision studies, which means that the change of the capillary during the method application does not involved a higher variability among the data and a low precision of the results. RSDs lower than 7.9 % were achieved for reproducibility assays in four different packed capillaries. High reproducibility was also obtained in terms of elution times (RSD ≤ 8.8 %). This fact supposes a great success considering the reported disadvantages about packing lab-made columns for CEC analyses [51]. Packed capillaries that were properly made showed a half-life

of at least one hundred and fifty runs. After that, the use of a new lab-made packed column is recommended because the charged particles themselves tended to move in the electrical field during the CEC process and the column performance is no longer optimum.

### 3.4. Method characterization in whole milk samples

Contamination of milk samples with 5-NDZs is a relevant problem in the food safety field. Therefore, the proposed analytical method was applied to whole milk samples in order to test its usefulness and potential for complex samples. The analytical method was evaluated in terms of linearity, LODs and LOQs, intra-day and inter-day precision. All peak signals have been monitored at 320 nm although CRZ presents a maximum UV absorption at 244 nm because of the baseline was unstable at 244 nm when milk samples were analysed. Whole milk samples were treated following a two steps extraction procedure (see section 2.5). In the preliminary studies, the samples were treated by applying only the first step of the sample treatment (SALLE with MeCN in presence of NaCl). Sample extracts were reconstituted in water and analysed by the proposed CEC method. Although peak interferences were not found during 5-NDZ separation, peak elution times were not reproducible after several runs. It was attributed to matrix constituents. In order to remove them, a second step was proposed. It was based on the application of SPE using Oasis®HLB cartridges. Treated blank milk samples were analysed and no endogenous interferences were detected at the same 5-NDZ elution times. Moreover, no unknown peaks were observed during all the analyses. The combination of the proposed sample treatment with the developed CEC method shows a high selectivity for 5-NDZ determination.

Matrix-matched calibration curves were established in milk samples fortified at six concentration levels: 25, 50, 100, 200, 350 and 500 µg/L for all considered 5-NDZs, except for CRZ (50, 100, 200, 400, 700 and 1000 µg/L). Two spiked milk samples per level were treated following the SALLE-SPE combined extraction procedure previously described. Afterwards, each sample was analysed in duplicate according to the proposed CEC-UV method. Peak area was considered as a function of analyte concentration on the sample. LODs and LOQs of the method were calculated as the minimum analyte concentration yielding a signal-to-noise ratio equal to three and ten, respectively (Table 2). In spite of the lack of sensitivity attributed to UV detection, LODs lower than 12 µg/L were accomplished for all studied 5-NDZ compounds except for CRZ (29 µg/L). Figure 5.B shows two electrochromatograms obtained from milk samples by applying the proposed SALLE-SPE procedure and analysed with the developed CEC method. An electrochromatogram of a blank sample is also shown.

The precision of the whole method, including the sample treatment, was evaluated by analysing three spiked milk samples at two concentration levels in the same day (intra-day studies) and one spiked milk sample at two concentration levels for four different days (inter-day studies). Each sample was injected in triplicate. Results are shown in Table 2. Satisfactory results were obtained in terms of RSD (%) being lower than 12.2 % and 14.5 % for repeatability and intermediate precision, respectively.

Trueness assays were carried out over whole milk samples spiked at 50 and 350 μg/L with each 5-NDZ drug except for CRZ (100 and 700 μg/L). For each concentration level three samples were analysed in triplicate. Obtained data were compared with those obtained by analysing extracts of blank samples submitted to the sample treatment and spiked with 5-NDZ just before the measurement. Recoveries over 68 % were obtained for all 5-NDZ antibiotics, reaching RSDs lower than 12.2 % for all cases (Table 3). All the presented results show that the developed method is suitable for the analysis of these compounds in milk samples.

In comparison with other previously reported methods for the analysis of 5-NDZs using HPLC or CE with UV detection in food matrixes, the proposed CEC method involves a shorter analysis time for a similar or higher number of analytes, showing also higher selectivity when the advantages of both chromatographic and electrophoretic separation modes are combined, reaching LODs at similar low ppb levels [10,17,18].

**4. Conclusions**

A new method to determine eight 5-NDZs in whole milk samples by CEC using lab-made columns has been proposed and evaluated. A detailed study of the procedure proposed to pack the capillaries and make the frits has also been described. This procedure results in robust and reproducible CEC capillaries considerably cheaper than the commercial ones. The optimized CEC method is able to separate eight 5-NDZs in less than 15 min with good sensitivity and precision. The selectivity of a chromatographic separation was achieved together with the advantage of a lower reagent consumption and waste generation of an electrophoretic separation. A selective sample treatment based on the combination of SALLE and SPE was used to extract 5-NDZ from whole milk samples. The satisfactory results obtained with this complex sample prove that CEC is a separation technique that could be used in routine analysis laboratories.

**Acknowledgements**

The authors gratefully acknowledge the financial support of the Andalusian Government (Junta de Andalucía) for supporting this work (Excellence Project Ref: P12-AGR-1647). Maykel Hernández-Mesa thanks to Plan Propio of University of Granada for a pre-doctoral fellowship.

**Figure captions**

Figure 1.- Capillary packing procedure scheme. A) Capillary is partially filled with $C_{18}$ silica particles. B) Deionised water is passed through packed capillary for 1 hour at 420 bar and outlet frit is made 10 cm far away from the mechanic retainer. $C_{18}$ particles are sintered and consequently frit is made by heating a nichrome ribbon for 20 s. Deionised water is passed through the capillary for 1 hour at 420 bar when frit formation is carried out. C) Mechanic retainer is removed. D) Capillary is emptied. Outlet frit is able to support a pressure of 420 bar. E) Capillary is fully packed at high pressure. F) Deionised water is passed through packed capillary for 1 hour at 420 bar. Afterwards, inlet frit is sintered considering the desired packed capillary length as outlet frit was made. G) The excess of stationary phase at capillary inlet is removed. Detection window is done and capillary ends are cut according to the desired capillary dimensions.

Figure 2.- Packing capillary setup diagram. A) Solvent containers. B) High pressure pump. C) Magnetic stirrer. D) Packing capillary unit. E) Capillary placed through the hole made on nichrome ribbon. Particles from the stationary phase are sintered at this capillary position. F) 7 V AC power supply.

Figure 3.- Influence of MeCN content in the mobile phase on a standard solution containing 2 µg/mL of each compound injected for 30 s at 11.5 bar. Electrochromatograms obtained using different mixtures of MeCN:ammonium acetate buffer pH = 5 (in all cases, final ammonium acetate concentration in the mobile phase, 5 mM): A) MeCN:ammonium acetate buffer (17 mM, pH = 5), 70:30; B) MeCN:ammonium acetate buffer (14 mM, pH = 5), 65:35; C) MeCN:ammonium acetate buffer (13 mM, pH = 5), 60:40; D) MeCN:ammonium acetate buffer (11 mM, pH = 5), 55:45. Separation conditions: 20 kV and 20 °C. Packed capillary dimensions: 25 cm x 75 µm i.d. Signals monitored at 320 nm. Peaks are numbered in elution order.

Figure 4.- Influence of the packed capillary length evaluated in capillaries of 75 µm i.d. on a standard solution containing 2 µg/mL of each compound injected for 60 s at 11.5 bar. Electrochromatograms obtained using different capillary lengths: A) 40 cm, B) 32 cm, C) 25 cm and D) 8 cm. Separation conditions: 20 kV and 20 °C. Mobile phase: MeCN:ammonium acetate buffer (pH = 5, 2.5 mM), 60:40. Signals monitored at 320 nm. Peaks are numbered in elution order.

Figure 5.- Electrochromatograms at optimum conditions of A) a 5-NDZ standard solution containing 1200 µg/L of each compound except for CRZ (2400 µg/L) and B) milk samples treated following the SALLE-SPE procedure and analysed in a packed capillary (40 cm x 50 µm i.d.) according to the proposed CEC method: I) spiked with 350 µg/L of each analyte except for CRZ (700.0 µg/L), II) spiked with 50 µg/L of each analyte except for CRZ (100.0 µg/L), III) blank sample. Separation conditions: 27 kV and 30 °C. Mobile phase: MeCN:ammonium acetate buffer (2.5 mM, pH = 5), 60:40. Sample injection: 120 s at 11.5 bar. Signals were monitored at 320 nm. Peaks: 1, RNZ; 2, MNZ; 3, TRZ; 4, SCZ; 5, ORZ; 6, DMZ; 7, CRZ; 8, IPZ.

Table 1. Statistical and instrumental performance characteristics of the proposed CEC-UV method for 5-NDZ determination.

| Analyte | Linear range (µg/L) | $R^2$ | LOD (µg/L) | LOQ (µg/L) | Repeatability (% RSD, n = 9) ("intra-day") | | | Intermediate precision (% RSD, n = 15 ) ("inter-day") | | | Reproducibility among 4 different capillaries (% RSD, n = 12 ) ("inter-capillary") | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 3 S/N | 10 S/N | 300 µg/L | 1500 µg/L | 3000 µg/L | 300 µg/L | 1500 µg/L | 3000 µg/L | 300 µg/L | 1500 µg/L | 3000 µg/L | Elution time (min); (% RSD, n = 36) |
| Ronidazole | 177 - 5000 | 0.996 | 53 | 177 | 6.9 | 2.6 | 8.2 | 10.5 | 7.1 | 5.3 | 6.4 | 5.3 | 4.7 | 7.7; 8.4 |
| Metronidazole | 128 - 5000 | 0.996 | 38 | 128 | 4.9 | 2.7 | 8.2 | 11.2 | 7.5 | 5.0 | 6.8 | 4.5 | 4.8 | 7.8; 8.4 |
| Ternidazole | 203 - 5000 | 0.996 | 61 | 203 | 8.1 | 4.0 | 7.9 | 8.6 | 7.7 | 5.0 | 7.7 | 5.5 | 5.7 | 8.0; 8.4 |
| Secnidazole | 177 - 5000 | 0.997 | 53 | 177 | 7.6 | 2.6 | 8.5 | 8.2 | 8.4 | 5.8 | 5.8 | 4.4 | 5.1 | 8.3; 8.5 |
| Ornidazole | 222 - 5000 | 0.996 | 67 | 222 | 5.4 | 3.1 | 8.8 | 10.0 | 9.3 | 6.7 | 7.9 | 4.6 | 4.0 | 8.9; 8.7 |
| Dimetridazole | 153 - 5000 | 0.997 | 46 | 153 | 6.7 | 2.8 | 7.4 | 5.7 | 9.3 | 6.7 | 5.7 | 4.2 | 3.6 | 9.5; 8.6 |
| Ipronidazole | 186 - 5000 | 0.996 | 56 | 186 | 6.9 | 3.2 | 7.5 | 7.2 | 9.7 | 9.4 | 7.4 | 4.0 | 3.3 | 11.5; 8.8 |
| | | | | | 600 µg/L | 3000 µg/L | 6000 µg/L | 600 µg/L | 3000 µg/L | 6000 µg/L | 600 µg/L | 3000 µg/L | 6000 µg/L | |
| Carnidazole | 534 - 10000 | 0.996 | 160 | 534 | 6.8 | 3.5 | 7.9 | 8.7 | 6.4 | 8.4 | 4.4 | 3.1 | 3.5 | 9.9; 8.8 |

Table 2. Statistical and performance characteristics of the proposed SALLE-SPE-CEC-UV method for the determination of 5-NDZ residues in whole milk samples.

| Analyte | Linear range (µg/L) | Linear regression equation $y = m \cdot x + a$ | | | | $R^2$ | LOD (µg/L) | LOQ (µg/L) | Repeatability (% RSD, n = 9) ("intra-day") | | Intermediate precision (% RSD, n = 12 ) ("inter-day") | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Slope, $10^{-2}$ | SD for the slope, $10^{-4}$ | Intercept | SD for the intercept | | 3 S/N | 10 S/N | 50 µg/L | 350 µg/L | 50 µg/L | 350 µg/L |
| Ronidazole | 19 – 500 | 1.8 | 2.4 | 0.51 | 0.06 | 0.997 | 6 | 19 | 7.3 | 4.0 | 10.4 | 8.1 |
| Metronidazole | 11 - 500 | 3.3 | 3.8 | 0.84 | 0.10 | 0.997 | 3 | 11 | 6.5 | 6.3 | 11.4 | 9.3 |
| Ternidazole | 17 - 500 | 2.4 | 3.3 | 0.53 | 0.08 | 0.996 | 5 | 17 | 9.0 | 5.3 | 11.4 | 8.2 |
| Secnidazole | 14 - 500 | 3.2 | 5.1 | 0.91 | 0.13 | 0.995 | 4 | 14 | 9.7 | 7.1 | 10.7 | 9.3 |
| Ornidazole | 19 - 500 | 2.3 | 3.7 | 0.65 | 0.09 | 0.995 | 6 | 19 | 8.5 | 4.1 | 10.8 | 7.0 |
| Dimetridazole | 24 - 500 | 1.8 | 4.0 | 0.80 | 0.10 | 0.993 | 7 | 24 | 9.7 | 4.1 | 11.1 | 14.5 |
| Ipronidazole | 38 - 500 | 1.3 | 3.5 | 0.66 | 0.10 | 0.992 | 12 | 38 | 12.2 | 6.3 | 11.4 | 11.4 |
| | | | | | | | | | 100 µg/L | 700 µg/L | 100 µg/L | 700 µg/L |
| Carnidazole | 96 – 1000 | 1.0 | 1.9 | 0.22 | 0.10 | 0.995 | 29 | 96 | 10.8 | 5.8 | 10.1 | 7.3 |

Table 3.- Recovery studies (%) in whole milk samples.

| Analyte | Level 1 (% RSD, n = 9) | Level 2 (% RSD, n = 9) |
|---|---|---|
| | 50 µg/L | 350 µg/L |
| Ronidazole | 107 (7.3) | 81 (4.0) |
| Metronidazole | 94 (6.5) | 85 (6.3) |
| Ternidazole | 100 (9.0) | 85 (5.3) |
| Secnidazole | 107 (9.7) | 93 (7.1) |
| Ornidazole | 104 (8.5) | 91 (4.1) |
| Dimetridazole | 79 (9.7) | 70 (4.1) |
| Ipronidazole | 81 (12.2) | 68 (6.3) |
| | 100 µg/L | 700 µg/L |
| Carnidazole | 102 (10.8) | 75 (5.8) |

% RSD of peak areas is given in parentheses.

**References**


[1] H.S. Leiros, S. Kozielski-Stuhrmann, U. Kapp, L. Terradot, G.A. Leonard, S.M. McSweeney, Structural basis of 5-nitroimidazole antibiotic resistance: The crystal structure of NimA from Deinococcus radiodurans, J. Biol. Chem. 279 (2004) 55840-55849.

[2] G. Rodríguez Ferreiro, L. Cancino Badías, M. Lopez-Nigro, A. Palermo, M. Mudry, P. González Elio, M.A. Carballo, DNA single strand breaks in peripheral blood lymphocytes induced by three nitroimidazole derivatives, Toxicol. Lett. 132 (2002) 109-115.

[3] M.D. Mudry, R.A. Martinez, M. Nieves, M.A. Carballo, Biomarkers of genotoxicity and genomic instability in a non-human primate, Cebus libidinosus (Cebidae, Platyrrhini), exposed to nitroimidazole derivatives, Mutat. Res-Gen. Tox. En. 721 (2011) 108-113.

[4] Commission Regulation (EU) No. 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. Off. J. Eur. Union L15 (2010) 1-72.

[5] Rapid Alert System for Food and Feed (RASFF) portal (2011) Website. https://webgate.ec.europa.eu/rasff-window/portal/. Accessed July 10, 2014.

[6] USDA Foreign Agriculture Service (2011) Website. http://www.usdachina.org/info_details1.asp?id=2772. Accessed July 10, 2014.

[7] Food Animal Residue Avoidance & Depletion Program (2010) Website. http://www.farad.org/eldu/prohibit.asp. Accessed July 19, 2014.

[8] J. Zhou, J. Shen, X. Xue, J. Zhao, Y. Li, J. Zhang, S. Zhang, Simultaneous determination of nitroimidazole residues in honey samples by high-performance liquid chromatography with ultraviolet detection, J. AOAC Int. 90 (2007) 872-878.

[9] X. Huang, J. Lin, D. Yuan, Simple and sensitive determination of nitroimidazole residues in honey using stir bar sorptive extraction with mixed mode monolith followed by liquid chromatography, J. Sep. Sci. 34 (2011) 2138-2144.

[10] H. Sun, F. Wang, L. Ai, Simultaneous determination of seven nitroimidazole residues in meat by using HPLC-UV detection with solid-phase extraction, J Chromatogr B. 857 (2007) 296-300.

[11] M. Cronly, P. Behan, B. Foley, E. Malone, S. Martin, M. Doyle, L. Regan, Rapid multi-class multi-residue method for the confirmation of chloramphenicol and eleven nitroimidazoles in milk and honey by liquid chromatography-tandem mass spectrometry (LC-MS), Food Addit. Contam. 27 (2010) 1233-1246.

[12] R.H.M.M. Granja, A.M.M. Nino, K.V.G. Reche, F.M. Giannotti, A.C. de Lima, A.C.B.A. Wanschel, A.G. Salerno, Determination and confirmation of metronidazole, dimetridazole, ronidazole and their metabolites in bovine muscle by LC-MS/MS, Food Addit. Contam. 30 (2013) 970-976.

[13] A. Toelgyesi, V.K. Sharma, S. Fekete, J. Fekete, A. Simon, S. Farkas, Development of a rapid method for the determination and confirmation of nitroimidazoles in six matrices by fast liquid chromatography-tandem mass spectrometry, J. Pharm. Biomed. Anal. 64-65 (2012) 40-48.

[14] M. Kanda, T. Sasamoto, K. Takeba, H. Hayashi, T. Kusano, Y. Matsushima, T. Nakajima, S. Kanai, I. Takano, Rapid determination of nitroimidazole residues in honey by liquid chromatography/tandem mass spectrometry, J. AOAC Int. 95 (2012) 923-931.

[15] C. Ho, D.W.M. Sin, K.M. Wong, H.P.O. Tang, Determination of dimetridazole and metronidazole in poultry and porcine tissues by gas chromatography–electron capture negative ionization mass spectrometry, Anal. Chim. Acta 530 (2005) 23-31.

[16] J. Polzer, C. Stachel, P. Gowik, Treatment of turkeys with nitroimidazoles: Impact of the selection of target analytes and matrices on an effective residue control, Anal. Chim. Acta 521 (2004) 189-200.

[17] Y. Lin, Y. Su, X. Liao, N. Yang, X. Yang, M.M.F. Choi, Determination of five nitroimidazole residues in artificial porcine muscle tissue samples by capillary electrophoresis, Talanta 88 (2012) 646-652.

[18] M. Hernández-Mesa, A.M. García-Campaña, C. Cruces-Blanco, Novel solid phase extraction method for the analysis of 5-nitroimidazoles and metabolites in milk samples by capillary electrophoresis, Food Chem. 145 (2014) 161-167.

[19] L. Yan, Q. Zhang, J. Zhang, L. Zhang, T. Li, Y. Feng, L. Zhang, W. Zhang, Y. Zhang, Hybrid organic–inorganic monolithic stationary phase for acidic compounds separation by capillary electrochromatography, J. Chromatogr. A. 1046 (2004) 255-261.

[20] P. T. Vallano, V. T. Remcho, Capillary electrochromatography: a powerful tool for the resolution of complex mixtures, J. AOAC Int. 82 (1999) 1604-1612.

[21] J. Simal-Gándara, The place of capillary electrochromatography among separation techniques—A review, Crit. Rev. Anal. Chem. 34 (2004) 85-94.

[22] Z. Liu, K. Otsuka, S. Terabe, Evaluation of extended light path capillary and etched capillary for use in open tubular capillary electrochromatography, J. Chromatogr. A. 961 (2002) 285-291.

[23] H. Zou, X. Huang, M. Ye, Q. Luo, Monolithic stationary phases for liquid chromatography and capillary electrochromatography, J. Chromatogr. A. 954 (2002) 5-32.

[24] W.J. Cheong, Fritting techniques in chromatography, J. Sep. Sci. 37 (2014) 603-617.

[25] L.A. Colón, T.D. Maloney, A.M. Fermier, Packing columns for capillary electrochromatography, J. Chromatogr. A 887 (2000) 43-53.

[26] V. Pretorius, B.J. Hopkins, J.D. Schieke, Electro-osmosis: A new concept for high-speed liquid chromatography, J. Chromatogr. A 99 (1974) 23-30.

[27] S. Roulin, R. Dmoch, R. Carney, K.D. Bartle, P. Myers, M.R. Euerby, C. Johnson, Comparison of different packing methods for capillary electrochromatography columns, J. Chromatogr. A 887 (2000) 307-312.

[28] T.D. Maloney, L.A. Colón, Comparison of column packing techniques for capillary electrochromatography, J. Sep. Sci. 25 (2002) 1215-1225.

[29] S.K. Wiedmer, G. D'Orazio, J. Smått, D. Bourdin, C. Baños-Pérez, M. Sakeye, M. Kivilompolo, M. Kopperi, J. Ruiz-Jiménez, S. Fanali, M. Riekkola, Polyethylenimine-modified metal oxides for fabrication of packed capillary columns for capillary electrochromatography and capillary liquid chromatography, J. Chromatogr. A 1218 (2011) 5020-5029.

[30] R. Stol, M. Mazereeuw, U.R. Tjaden, J. van der Greef, Pseudo-electrokinetic packing of high efficiency columns for capillary electrochromatography, J. Chromatogr. A 873 (2000) 293-298.

[31] Q. Qu, X. Lu, X. Huang, X. Hu, Y. Zhang, C. Yan, Preparation and evaluation of C18-bonded 1-µm silica particles for pressurized capillary electrochromatography, Electrophoresis 27 (2006) 3981-3987.

[32] J.C. Rodrigues, F.M. Lanças, Preparation of packed capillary columns using supercritical carbon dioxide on cyclone-type slurry reservoir, J. Chromatogr. A 1090 (2005) 172-177.

[33] T.D. Maloney, L.A. Colón, A drying step in the protocol to pack capillary columns by centripetal forces for capillary electrochromatography, Electrophoresis 20 (1999) 2360-2365.

[34] K. J. Reynolds, L. A. Colón, Capillary electrochromatography in columns packed by gravity. Preliminary study, Analyst 123 (1998) 1493-1495.

[35] S. Fanali, G. D'Orazio, T. Farkas, B. Chankvetadze, Comparative performance of capillary columns made with totally porous and core–shell particles coated with a polysaccharide-based chiral selector in nano-liquid chromatography and capillary electrochromatography, J. Chromatogr. A 1269 (2012) 136-142.

[36] Z. Aturki, M.G. Schmid, B. Chankvetadze, S. Fanali, Enantiomeric separation of new cathinone derivatives designer drugs by capillary electrochromatography using a chiral stationary phase, based on amylose tris(5-chloro-2-methylphenylcarbamate), Electrophoresis (2014) DOI: 10.1002/elps.201400085.

[37] D. Albals, A. Hendrickx, L. Clincke, B. Chankvetadze, Y.V. Heyden, D. Mangelings, A chiral separation strategy for acidic drugs in capillary electrochromatography using both chlorinated and non-chlorinated polysaccharide-based selectors, Electrophoresis (2014) DOI: 10.1002/elps.201400169.

[38] M. Mayer, E. Rapp, C. Marck, G.J.M. Bruin, Fritless capillary electrochromatography, Electrophoresis 20 (1999) 43-49.

[39] G. D'Orazio, S. Fanali, C18 silica packed capillary columns with monolithic frits prepared with UV light emitting diode: Usefulness in nano-liquid chromatography and capillary electrochromatography, J. Chromatogr. A 1232 (2012) 176-182.

[40] M. Kato, M.T. Dulay, B.D. Bennett, J.P. Quirino, R.N. Zare, Photopolymerized sol–gel frits for packed columns in capillary electrochromatography, J. Chromatogr. A 924 (2001) 187-195.

[41] S. Oguri, C. Oga, H. Takeda, Micro-magnetic particles frit for capillary electrochromatography, J. Chromatogr. A 1157 (2007) 304-308.

[42] B. Zhang, Q. Liu, L. Yang, Q. Wang, Performance of single particle fritted capillary columns in electrochromatography, J. Chromatogr. A 1272 (2013) 136-140.

[43] S. Keunchkarian, P.J. Lebed, B.B. Sliz, C.B. Castells, L.G. Gagliardi, New method for sintering silica frits for capillary microcolumns, Anal. Chim. Acta 820 (2014) 168-175.

[44] S.M. Piraino, J.G. Dorsey, Comparison of frits used in the preparation of packed capillaries for capillary electrochromatography, Anal. Chem. 75 (2003) 4292-4296.

[45] M. Franc, J. Sobotníková, P. Coufal, Z. Bosáková, Comparison of different types of outlet frits in slurry-packed capillary columns, J. Sep. Sci. (2014) DOI: 10.1002/jssc.201400434

[46] J. Zheng, D. Norton, S.A. Shamsi, Fabrication of Internally Tapered Capillaries for Capillary Electrochromatography Electrospray Ionization Mass Spectrometry, Anal. Chem. 78 (2006) 1323-1330.

[47] W. Bragg, S.A. Shamsi, Development of a fritless packed column for capillary electrochromatography–mass spectrometry, J. Chromatogr. A 1218 (2011) 8691-8700.

[48] S. Liao, X. Wang, X. Lin, Z. Xie, Preparation and characterization of a molecularly imprinted monolithic column for pressure-assisted CEC separation of nitroimidazole drugs, Electrophoresis 31 (2010) 2822-2830.

[49] S.E. van den Bosch, S. Heemstra, J.C. Kraak, H. Poppe, Experiences with packed capillary electrochromatography at ambient pressure, J. Chromatogr. A 755 (1996) 165-177.

[50] H. Yamamoto, J. Baumann, F. Erni, Electrokinetic reversed-phase chromatography with packed capillaries, J. Chromatogr. A 593 (1992) 313-319.

[51] Q. Tang, M.L. Lee, Column technology for capillary electrochromatography, TrAC, Trends Anal. Chem. 19 (2000) 648-663.

Figure 1

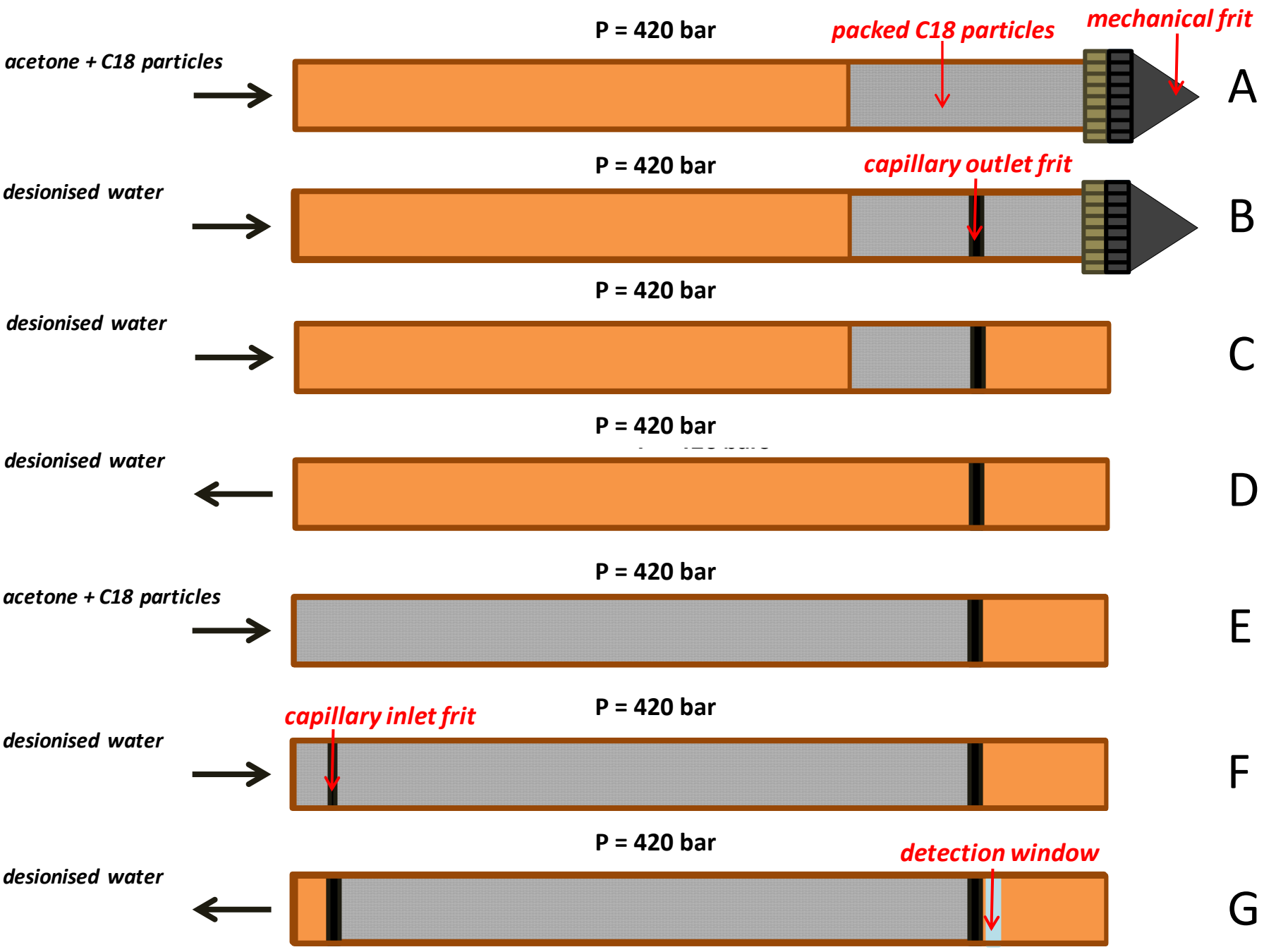

P = 420 bar
packed C18 particles
mechanical frit
acetone + C18 particles
A
P = 420 bar
capillary outlet frit
desionised water
B
P = 420 bar
desionised water
C
P = 420 bar
desionised water
D
P = 420 bar
acetone + C18 particles
E
capillary inlet frit
P = 420 bar
desionised water
F
P = 420 bar
detection window
desionised water
G

**Figure 2**

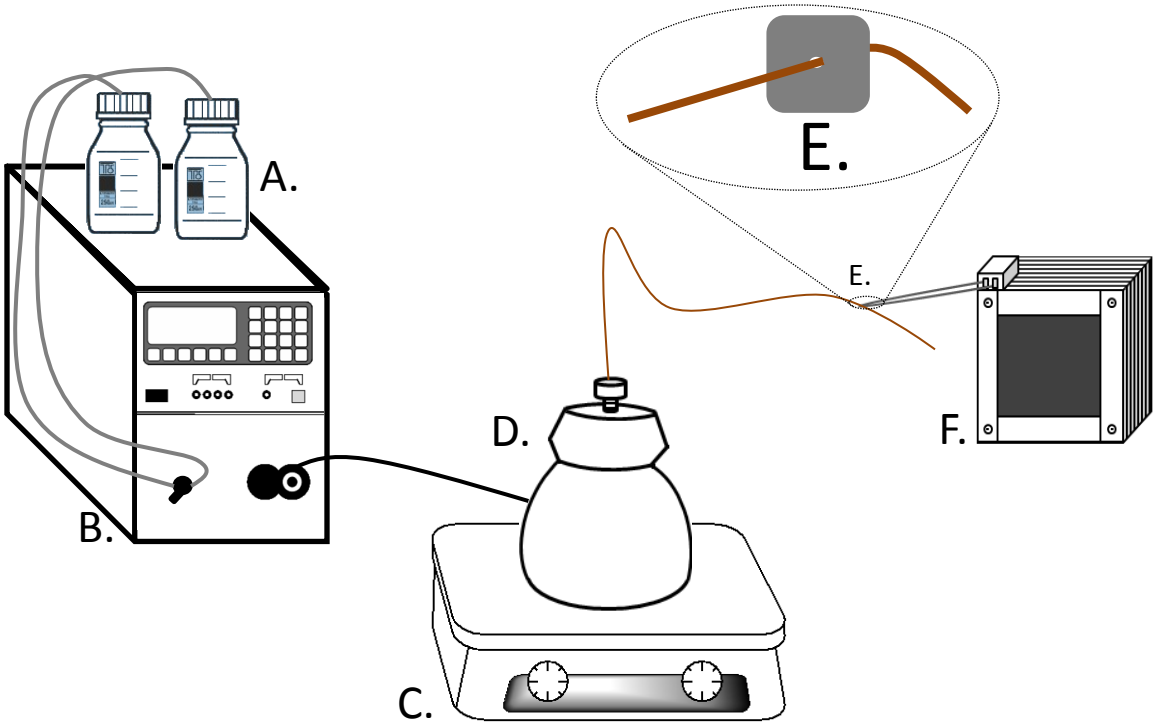


**Figure 3**

Click here to download high resolution image

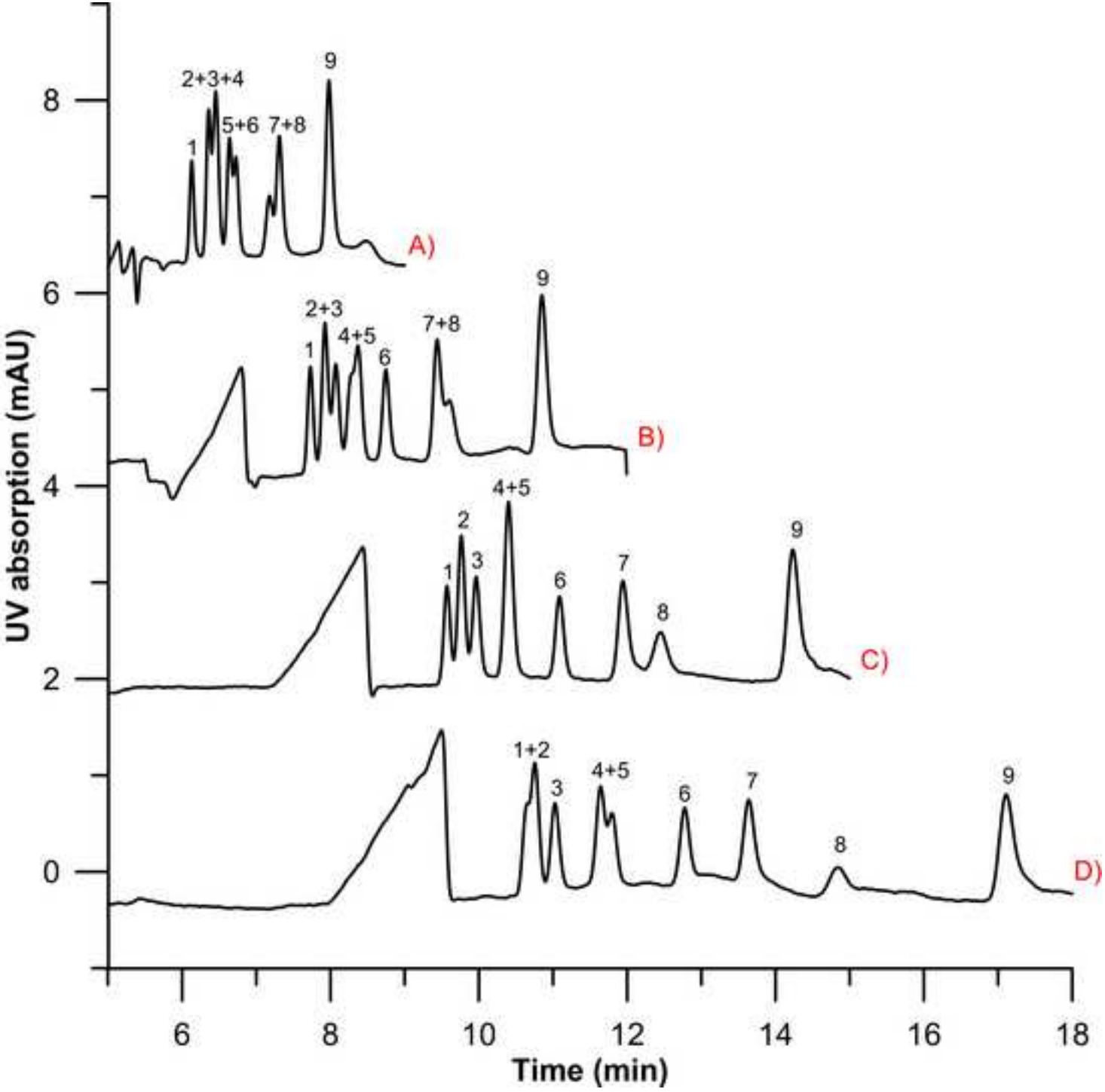


**Figure 4**

Click here to download high resolution image

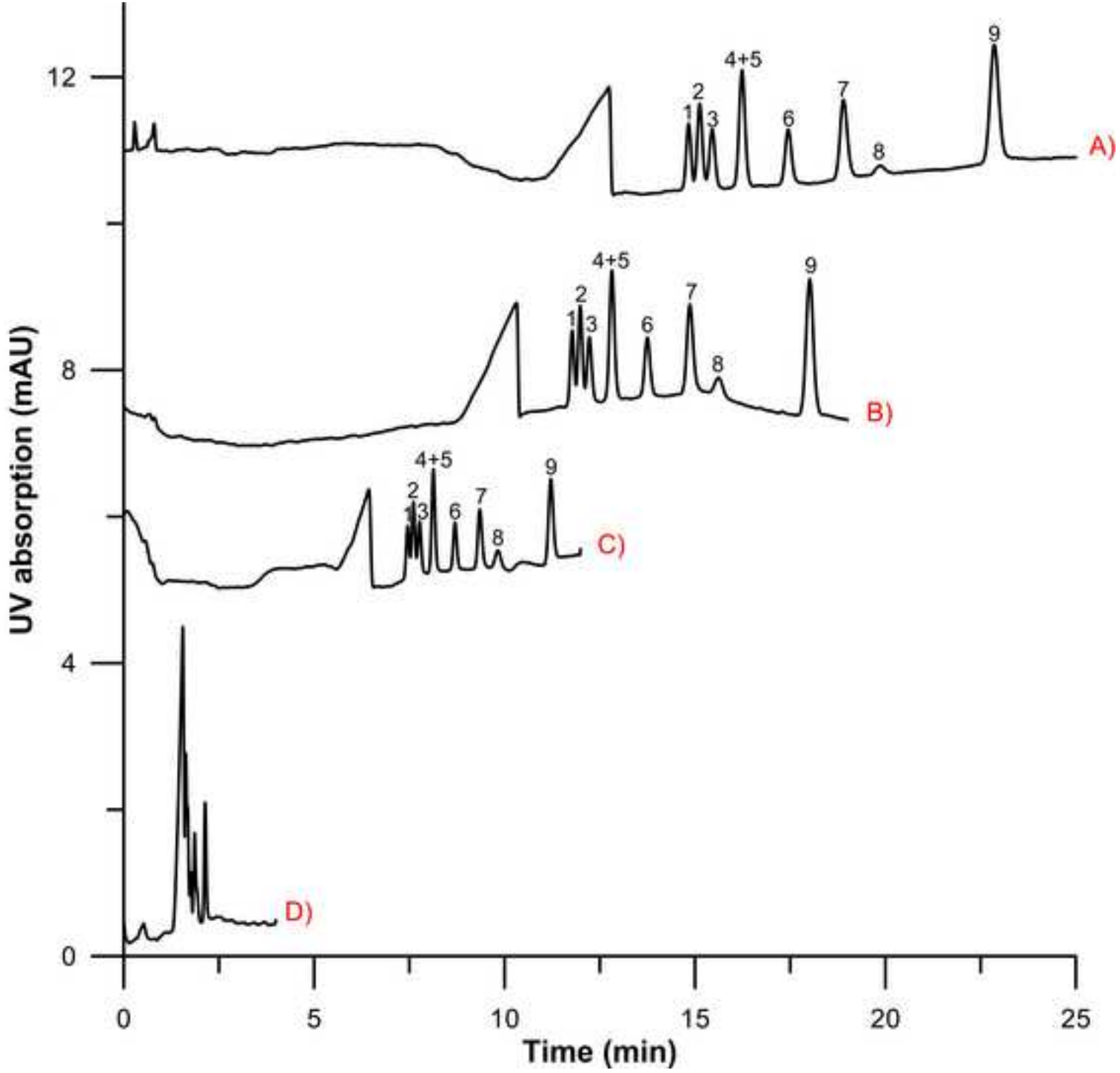
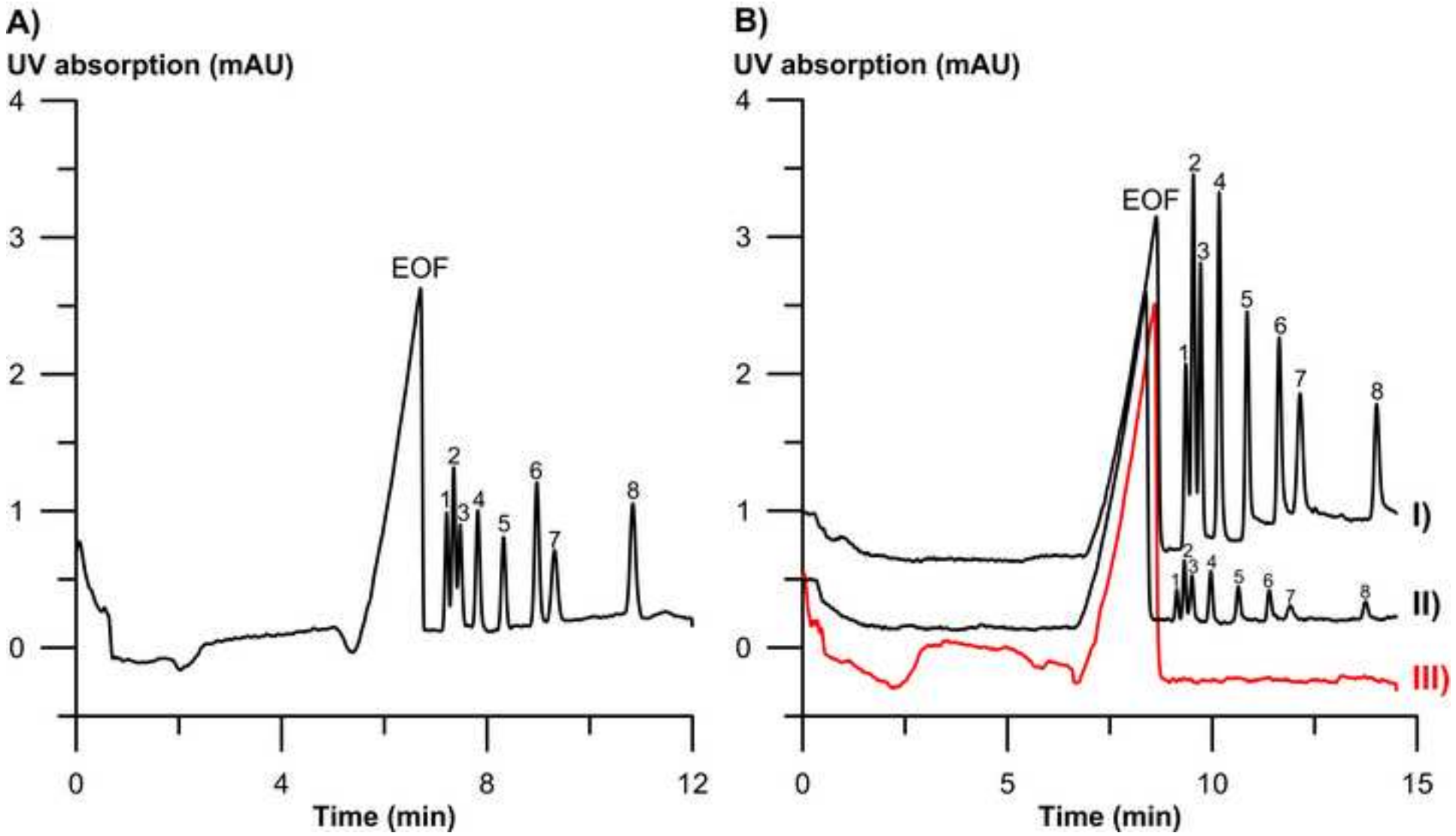


Figure 5

Click here to download high resolution image



*Graphical Abstract (for review)