Capillary electrophoresis-tandem mass spectrometry combined with molecular imprinted solid phase extraction as useful tool for the monitoring of 5-nitroimidazoles and their metabolites in urine samples

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Abstract

A novel capillary electrophoresis-tandem mass spectrometry approach is proposed for the determination of eleven 5-nitroimidazoles in urine samples for therapeutical drug monitoring purposes. A comparison between two separation modes, namely micellar electrokinetic chromatography and capillary zone electrophoresis was carried out, obtaining higher selectivity when 1 M formic acid (pH 1.8) was selected as background electrolyte. 5-Nitroimidazoles were hydrodynamically injected in water for 40 s at 50 mbar and their separation was performed at 28 kV and 25°C. To improve migration time repeatability, a pressure of 50 mbar was applied to the inlet vial during runs without any loss of peak resolution. Electrospray ionization parameters were established as follow: 6 L/min, dry gas flow rate; 51021.2 Pa, nebulization pressure; 160°C, dry gas temperature. Sheath liquid consisted of a mixture propan-2-ol/water/acetic acid (60.0:38.8:0.2% v/v/v) supplied at 3.3 µL/min. MS parameters were optimized for analyte identification through their MS^2 and MS^3 spectra. The method was applied to the determination of 5-nitroimidazoles in urine samples, applying molecular imprinted solid phase extraction for sample clean-up. Recoveries higher than 79.2% demonstrated the suitability of the procedure. Limits of detection ranged from 9.6 to 130.2 µg/L while precision assays resulted in relative standard deviations for peak areas lower than 16.1%.

Keywords: capillary electrophoresis, mass spectrometry, 5-nitroimidazoles, molecular imprinted polymers, urine samples.

1. Introduction

5-nitroimidazole (5-NDZ) drugs are antimicrobial substances which are mainly used for treating anaerobic bacteria infections caused by *Bacteroides* species, fusobacteria and clostridia. They have proved to be effective against *Gardenerella vaginalis* bacteria and they are also employed as antiprotozoal agents against *Giardia lamblia* and *Entamoeba histolytica* protozoans. Furthermore, their application has been extended to the treatment of *Helicobacter pylori* infections by multidrug regimens, *Clostridium difficile* infections and in Crohn disease therapy [1,2]. Their importance has been expressed by the inclusion of the most representative 5-NDZ family compound, metronidazole (MNZ), in the World Health Organization (WHO) Model List of Essential Medicines where it is listed as one of the two essential antiamoebic and antigiardiasis medicines [3]. MNZ undergoes hepatic metabolism, being mainly metabolized to hydroxyl derivative, namely hydroxyl-metronidazole (MNZ-OH), and an acetic acid compound. Metabolites are mostly excreted in the urine, while a small proportion of the unchanged drug is also excreted in the urine (about 10-15%) [4]. Moreover, pharmacokinetic studies carried out over patients treated with 200 mg of MNZ three times daily during 7 days have shown drug levels in urine of 1.6 μg/mL at the twelfth day after the first administration day [5]. Similar metabolic and excretion characteristics have been reported for other 5-NDZ drugs, namely tinidazole (TNZ), ornidazole (ORZ) and secnidazole (SCZ) [6]. Dimetridazole (DMZ) and ronidazole (RNZ) are metabolized to the same hydroxyl metabolite (hydroxyl-dimetridazole, HMMNI), although both compounds follow different metabolism pathways [7]. In this work, ipronidazole (IPZ) metabolite, namely hydroxy-ipronidazole (IPZ-OH) has been also considered.

Traditionally, liquid chromatography coupled to mass spectrometry (LC-MS) has been the most employed technique for 5-NDZ analyses [8-11], although in less extension other methods based on gas chromatography (GC)-MS [12] or capillary electrochromatography (CEC)-MS [13] has been proposed. In spite of capillary electrophoresis (CE) advantages, i.e. low solvent consumption, low sample volume requirement and high separation efficiency, this separation technique coupled to MS has not been explored for 5-NDZ identification and quantification yet. On the contrary, some approaches have been reported for 5-NDZ separation by CE, but always coupled with ultraviolet (UV) absorbance as detection mode. Due to 5-NDZs characteristics, they are neutral molecules in almost the entire pH range, being

cationic species at low pH values because their pKa values range from 1.32 to 2.81 (data obtained from Scifinder database); they can be separated by capillary zone electrophoresis (CZE) at low pH [14] or their separation can be accomplished by micellar electrokinetic chromatography (MEKC) [15]. However, both methods could present drawbacks when the use of MS detection is attempted as alternative to UV detection.

Long analysis times result from CZE separations performed at low pH because the electroosmotic flow (EOF) is minimized. Moreover, a slow EOF can also be problematic in CE-MS analysis, as the transfer rate of analytes from the CE to the mass spectrometer depends on the magnitude of the EOF [16]. This inconvenience can be solved by applying pressure to the inlet vial when the analysis is running and even though peak resolution loss is associated to it, this effect could not be so critical [17]. On the other hand, sodium dodecyl sulfate (SDS) as pseudo-stationary phase is a usual choice when MEKC separations are carried out. Nevertheless, this surfactant is not volatile and therefore micelles in the background electrolyte (BGE) solvent tend to soil the mass spectrometer and as a consequence, lower concentration sensitivity is obtained. In order to avoid this problem, partial filling strategies were firstly explored [18,19], however in the last years the use of volatile surfactants has emerged as a robust alternative, allowing the direct MEKC-MS coupling [20,21]. Some recent applications involve the use of a volatile pseudostationary phase based on ammonium perfluorooctanoate (APFO) [22-24].

Regarding sample treatment, the compatibility between the sample and the features of CE methods has been widely described as an important drawback for their implementation. Sample matrix inherent compounds can disturb CE separations through the action of saline constituents, macromolecules and other substances [25,26]. Moreover, low concentration sensitivity is attributed to these methods due to the small sample volume that is injected in a CE analysis. In order to achieve sample clean-up and an improvement on method concentration sensitivity, solid phase extraction (SPE) procedures have been proposed as suitable sample treatments in CE analysis. Several sorbents have been evaluated for SPE [27-30], being molecular imprinted polymer (MIP) an attractive alternative due to its selectivity. For 5-NDZ extraction, this approach has barely been evaluated and the proposed applications have been focused on food analysis [31,32].

In this work, a comparison between MEKC and CZE separation modes was carried out in order to establish the best strategy for analyzing 5-NDZ drugs by CE-MS. MEKC separation was performed using APFO surfactant as pseudostationary phase while the application of pressure to the inlet separation vial was considered for CZE separation due to the benefits obtained for CZE-MS coupling. The optimization of both methods is widely discussed as well as the evaluation of electrospray ionization (ESI) and MS parameters. At the best of our knowledge, this supposes the first proposal of a CE-MS method for 5-NDZ determination and quantification. Furthermore, the developed method was applied to the analysis of 5-NDZ antibiotics in urine samples for therapeutic drug monitoring. With the aim of avoiding matrix effects, a SPE procedure using MIP cartridges was also assayed.

2. Materials and methods

2.1 Chemicals and materials

All reagents used through this work were analytical reagent grade and solvents were HPLC grade, unless otherwise specified. Ammonium hydroxide solution (30% v/v), sodium hydroxide (NaOH), toluene and heptane were obtained from Panreac-Química (Madrid, Spain). Methanol (MeOH), acetonitrile (MeCN), propan-2-ol and hexane were purchased from VWR International (West Chester, PA, USA) while acetic acid (MS grade) and perfluorooctanoic acid (PFOA, 96% m/m) were supplied by Sigma-Aldrich (St. Louis, MO, USA). Formic acid (98–100% v/v) was acquired from Merck (Darmstadt, Germany). Ultrapure water (Milli-Q plus system, Millipore, Bedford, MA, USA) was used throughout the work. Ammonium acetate, ammonium formate and APFO solutions were prepared from acetic acid, formic acid and PFOA, respectively, adjusting the pH with 5 M ammonium hydroxide solution.

Analytical standards of DMZ (1,2-dimethyl-5-nitroimidazole), RNZ (1-methyl-2-(carbamoylmethyl)-5-nitroimidazole), IPZ-OH (1-methyl-2-(2′-hydroxyisopropyl)-5-nitroimidazole), HMMNI (2-hydroxymethyl-1-methyl-5-nitroimidazole), ORZ (1-(3-chloro-2-hydroxypropyl)-2-methyl-5-nitroimidazole), MNZ (1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole), MNZ-OH (1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole) and TNZ (1-(2-ethylsulfonylethyl)-2-methyl-5-nitro-imidazole) were supplied by Sigma-Aldrich (St. Louis, MO, USA) while IPZ (2-isopropyl-1-methyl-5-nitroimidazole), 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).

Stock standard solutions were obtained by dissolving the appropriate amount of each 5-NDZ drug in MeCN, reaching a final concentration of 1 mg/mL. Stock standard solutions were kept at -20°C avoiding exposure to light. Intermediate standard solution was obtained by mixing the appropriate amount of each stock standard solution and its subsequent dilution with MeCN. The concentration of the analytes in the intermediate standard solution ranged between 50-100 µg/mL. It was stored in dark at 4°C and it was equilibrated to room temperature before its use. Working standard solutions were prepared by dilution of the intermediate standard solution with water to the desired 5-NDZ concentration.

SupelMIP®SPE-Nitroimidazole cartridges (50 mg, 3 mL) (Sigma Aldrich; St. Louis, MO, USA) were considered for the sample treatment procedure. Clearinert^{TM} 13 mm syringe filters (0.22 µm pore size) were supplied by Bonna-Agela Technologies (Wilmington, DE, USA).

2.2 Instrumentation

CE experiments were carried out with an HP^{3D}CE instrument (Agilent Technologies, Waldbronn, Germany). An Agilent 1100 Series LC/MSD SL mass spectrometer equipped with an ion trap (IT) mass analyzer from Agilent Technologies (Waldbronn, Germany) was employed as detector. A coaxial sheath-liquid flow interface (Agilent Technologies, Waldbronn, Germany) was chosen for CE-MS coupling. A KD Scientific 100 series syringe pump (KD Scientific Inc., Holliston, MA, USA) was selected for sheath liquid supplying. MS and MS/MS spectra and ion electropherograms were collected and processed by Esquire software 4.1 from Bruker Daltonics (Bremen, Germany).

Molecular imprinted solid phase extraction (MISPE) treatment was carried out on a VisiprepTM DL vacuum manifold for 12 cartridges from Supelco (Bellefonte, PA, USA). A Universal 320 R centrifuge (HettichZentrifugen, Tuttlingen, Germany), a nitrogen dryer EVA-EC System (VLM GmbH, Bielefeld, Germany), a mechanical shaker (model 384 from Vibromatic, Noblesville, USA) and a vortex-2 Genie (Scientific Industries, Bohemia, NY, USA) were also used. Solution pH was adjusted with a pH meter (Crison model pH 2000, Barcelona, Spain) with a resolution of ±0.01 pH unit.

2.3 Sample treatment procedure

Urine samples were supplied by a healthy volunteer. Aliquots of 5 mL were made from them and they were fortified at the desired 5-NDZ concentration. Prior to sample analyses, a sample clean-up procedure was performed using commercial MISPE cartridges. The extraction protocol recommended by the supplier [33], considering slight modifications, was followed in this work. MISPE cartridges were sequentially conditioned with 1 mL of toluene, 1 mL of MeCN and 1 mL of ammonium acetate buffer (10 mM, pH 5). Afterwards, 2 mL of spiked sample were passed through the extraction cartridge by gravity. Then, the column was washed considering four steps. First, 0.5 mL of deionized water were charged onto the cartridge, followed by loading twice 1 mL of hexane and 1 mL of a mixture heptane:toluene 3:1 (v/v). Between washing steps, the cartridge was vacuum dried (-400 mbar) for 10 s. Finally, sample was eluted in two stages passing through the column 1 mL of MeCN:water 60:40 (% v/v) containing acetic acid 0.5% (v/v) in each stage. Between elution steps, the cartridge was vacuum dried (-400 mbar) for 10 s. Both elution fractions were mixed and the eluted sample was dried under nitrogen current at 40°C. Sample was re-dissolved in 250 µL of deionized water using vortex agitation and it was filtered through a syringe filter to the vial for the CE-MS/MS analysis.

2.4 Capillary electrophoresis separation

CE experiments were carried out in a bare fused silica capillary (110 cm total length, 50 µm inner diameter (i.d.), 375 µm outside diameter) from Polymicro Technologies (Phoenix, AZ, USA). APFO buffer (100 mM, pH 9) was employed as BGE for MEKC analyses and samples were hydrodynamically injected at 50 mbar for 10 s. Separation was performed at 25 kV and 25°C. On the other hand, 1 M formic acid solution (pH 1.8) was considered as BGE for CZE separation. A pressure of 50 mbar was applied to the inlet vial during the separation in order to improve migration time repeatability. A voltage of 28 kV and a temperature of 25°C were applied for 5-NDZ separation. Samples were hydrodynamically injected at 50 mbar for 40 s when CZE analyses were carried out.

Before the first use, capillary was flushed with 1 M NaOH solution for 10 min at 5 bar and 25°C, followed by 10 min with deionized water and 20 min with BGE. At the beginning of each session, capillary was subsequently rinsed at 5 bar and 25°C with 5 M ammonium hydroxide solution for 5 min,

deionized water for 5 min and running buffer for 10 min. Between runs, capillary was conditioned with BGE for 2 min at 4 bar and 25°C. At the end of the working day, capillary was cleaned with deionized water for 5 min at 5 bar and 25°C and, afterwards, it was dried with compressed air for 5 min at 5 bar and 25°C.

2.5 Mass spectrometry and electrospray interface

Sheath liquid consisted of a mixture of propan-2-ol:water:acetic acid 60:38.8:0.2 (% v/v/v) and was supplied with a flow rate of 0.2 mL/h. Compounds ionization was achieved in positive mode under an ESI voltage of -4900 V. Other electrospray characteristics were established as follow: nebulizer pressure, 51021.2 Pa; dry gas flow rate, 6 L/min; and dry gas temperature, 160°C.

For MS experiments, IT parameters were evaluated using the ion charge control mode, setting a target of 100,000 ions, a maximum accumulation time of 300 ms and four averages per experiment. Scan range was established from 125.0 to 250.0 m/z. For MS/MS experiments, a target of 90,000 ions was selected while the maximum accumulation time was set at 100 ms and two averages per experiment were considered. Parent molecular ions $[M+H]^+$ were fragmented by means of collision induced dissociation with the helium present in the trap for 40 ms in multiple reaction monitoring (MRM) mode. Product ions were scanned in the range of 35.0-252.0 m/z. MS/MS parameters are summarized in Table S1 on Supplementary Data.

For method characterization, any electrophoretic parameters such as migration time, signal to noise ratio (S/N), peak height and peak area, were acquired from extracted ion electropherograms of each 5-NDZ.

3. Results and discussion

3.1. Electrophoretic separation

A comparison between MEKC and CZE was carried out in order to establish the best strategy for analyzing 5-NDZ drugs by CE-ESI-MS. MEKC separation was performed using APFO surfactant as pseudostationary phase while formic acid was selected as BGE for CZE separation. Both approaches were evaluated in a bare fused silica capillary (50 µm i.d. x 110 cm), under a separation voltage of 25 kV and a

separation temperature of 25°C. Additionally, samples were hydrodynamically injected for 10 s at 50 mbar.

First, MEKC separation was evaluated in terms of the solution pH. Using a 75 mM APFO solution as BGE, pH value was investigated from 8.5 to 10.0 and better peak resolution was observed when pH 9 was tested. Because 5-NDZs were not baseline resolved under these conditions, APFO concentration was further studied between 75 to 150 mM. Lower APFO concentrations were not considered because peak resolution usually improves when micelle concentration is increased although it tends to increase analysis time. As a compromise between peak resolution and analysis time, a concentration of 100 mM was established as optimum. In order to improve 5-NDZ separation, addition of 5% (v/v) of MeCN, MeOH and propan-2-ol to the separation solution was assayed. However, no improvement in peak resolution was obtained when BGE modifiers were employed, so finally, a BGE consisting of an APFO solution (100 mM, pH 9) was established.

On the other hand, the evaluation of formic acid concentration in the BGE was firstly assessed for the CZE method. Formic acid concentration was studied between 0.1 and 2.0 M, but some 5-NDZ peaks were not baseline resolved under any of the tested conditions. Higher concentrations were not tested because a separation current of 40 µA was observed when 2.0 M formic acid was considered as BGE. Finally 1.0 M formic acid solution was established as BGE. Under this condition co-migrating peaks were not observed, but 5-NDZ separation was only partially achieved. At least two co-migrating peaks were observed under other separation conditions as shown in Figure S1 on Supplementary Data. Additionally, separation pH was evaluated from 1.8 that corresponds to the 1.0 M formic acid solution pH, to 2.5. Solution pH was adjusted by adding 5 M ammonia solution to the 1.0 M formic acid solution. Higher pH values were not assessed because separation current reached to the limit of 50 µA when pH 2.5 was tested. It was observed that an increase of the pH value caused an increase on the running time without achieving any improvement on peak resolution. As a consequence, 1.0 M formic acid solution was finally selected as BGE and no pH adjustment was considered. Because all analytes were not baseline resolved, the addition of an organic modifier to the separation media was investigated. A 10% (v/v) of MeCN or MeOH was added to the BGE (1.0 M formic acid aqueous solution). As can be seen in Figure S2 on Supplementary data, peak resolution between IPZ, MNZ and TRZ was improved when an organic modifier was added to

the BGE but IPZ-OH, TNZ and MNZ-OH co-migrated under these conditions. Moreover, longer analysis times were obtained when an organic solvent was added to the BGE due to the increase of the separation solvent viscosity. Consequently the use of an organic modifier was discarded and 1.0 M formic acid (pH 1.8) was established as BGE for CZE separation.

The application of pressure (50 mbar) to the inlet vial was considered during CZE separation in order to obtain better repeatability in terms of migration times and for overcoming the lack of EOF under the established conditions. When pressure was applied during the separation, it was observed that migration time repeatability, in terms of relative standard deviation (RSD, %), ranged from 0.5 to 1.2% when 54 runs were randomly performed during three days. These values were doubled when separations were performed without applying any pressure to the inlet vial during the run.

Figure 1 shows the electropherograms resulted from the analysis of a 5-NDZ standard solution by MEKC and CZE. Considering peak resolution, higher selectivity was observed when CZE was considered as separation mode, although similar analysis time was achieved in both cases. Therefore, CZE was considered for further experiments.

Once separation mode was chosen, other parameters regarding electrophoretic separation were evaluated. Separation voltage was ranged between 20 and 30 kV, observing shorter analysis times at higher applied voltages, while peak resolution was not affected by this parameter. Because running time was not significantly decreased when separation voltage was increased from 28 to 30 kV, 28 kV was established as optimum. Moreover, capillary temperature was evaluated from 20 to 35°C, choosing 25°C as optimum. Higher analysis times were observed at lower temperatures while higher temperatures did not involve any improvement on analysis time. Furthermore, peak resolution was not significantly affected by capillary temperature.

On the other hand, deionized water and BGE solution were evaluated as injection media considering a hydrodynamic sample injection for 10 s at 50 mbar. Standard solutions of 5 µg/mL of each 5-NDZ were prepared in each injection solvent and analyzed according to the proposed separation method. Similar results were obtained when both media were considered and no significant sensitivity differences, in terms of peak height, were observed. In this case, water was selected as injection medium. Additionally, injection time was assayed from 10 to 50 s. Maximum signal intensity was reached when 40 s was tested,

so it was selected as optimum. Moreover, it should be remarked that higher injection times resulted in wider peaks, involving lower peak efficiencies.

3.2. CE-ESI-MS/MS optimization

Once separation conditions were selected, parameters related to CE-MS and CE-MS/MS were optimized. MS instrument was operated in ESI positive mode as it has been already reported [34,35]. Other acquisition parameters such as scan range, accumulation time and ion accumulation target have been defined in Section 2.5. The selection of sheath-liquid parameters is also very important in CE-ESI-MS/MS methods. In order to achieve optimum signals, sheath-liquid composition and its flow rate, nebulizer pressure and dry gas flow rate and temperature were optimized by analyzing 5-NDZ standard solutions (5 µg/mL for HMMNI, IPZ-OH, ORZ, TNZ, MNZ-OH, RNZ and SCZ; 3 µg/mL for MNZ and TRZ; 2 µg/mL for IPZ and DMZ). Signal-to-noise ratio (S/N) for each studied analyte was selected as response variable.

3.2.1. Sheath liquid composition and flow rate

Initially, sheath liquid consisted of a mixture 70.0:29.9:0.1 (% v/v/v) propan-2-ol:water:formic acid and was supplied at a flow rate of 3.3 µL/min. Both parameters were evaluated considering a dry gas flow rate of 7.0 L/min, a nebulizer pressure of 55158.1 Pa and a dry gas temperature of 200°C. The use of MeCN and MeOH instead of propan-2-ol was tested. Higher signal sensitivity in terms of S/N was obtained when propan-2-ol was employed (Figure 2.A). Furthermore, the replacement of formic acid for acetic acid reported higher sensitivity in terms of S/N (Figure 2.B). Therefore, sheath liquid composition was also evaluated in terms of S/N by ranging propan-2-ol percentage between 40.0 and 80.0% (v/v) and acetic acid percentage from 0.01 to 0.50% (v/v).

Low organic solvent content caused worse desolvation process while high content involved unstable electrospray currents and no analytical signals were obtained. This phenomena has been previously described [36] and it occurs because of the capillary tip gets dry off and the electric circuit is not closed. As can been seen in Figure 2.A, maximum S/N was obtained when 60.0% (v/v) of propan-2-ol was selected. Regarding acetic acid percentage, higher S/N were observed when low quantities of acid were

added to the sheath liquid (Figure 2.B) because as it is known, organic acids increase background noise which deteriorates S/N [37]. However, it was observed that low acetic acid concentrations increased electrospray current instability resulting in higher RSDs for the considered analytical response. As a compromise between both effects, 0.20% (v/v) acetic acid was chosen as optimum. Consequently, selected sheath liquid consisted of a mixture 60.0:39.8:0.2 (% v/v/v) propan-2-ol:water:acetic acid, obtaining maximum signal sensitivity in terms of S/N under these conditions which guarantee separation and electrospray current stability.

Finally, sheath liquid flow rate was assayed between 2.0 and 8.0 µL/min. High flow rates resulted in sample dilution which gave low S/N as it was expected. However, flow rates lower than 3.3 µL/min were discarded because in addition to parent molecules ionization, some of their fragmentation ions were also observed. Therefore 3.3 µL/min was established as optimum flow rate (Figure S3 on Supplementary Data).

3.2.2. Electrospray ionization interface parameters

Dry gas flow rate was assessed from 2 to 10 L/min, resulting in maximum peak sensitivity in terms of S/N as well as in terms of peak height when 6 L/min was selected. Nebulization pressure was studied between 13789.5 and 68947.6 Pa. As S/N was improved when high nebulization pressures were tested, 68947.6 Pa was established as optimum. Higher pressures were not evaluated due to electrospray instability observed at high nebulization pressures. Regarding to dry gas temperature, it was studied from 150 to 310°C. Although differences among analytes behavior were noticed, in general, maximum S/N was reached for almost all 5-NDZs at 190ºC.

These obtained optimum values did not agree with those observed during preliminary assays because initial studies showed higher S/N values at low nebulization pressures and high dry gas temperatures. Therefore, in order to clarify the influence of nebulization pressure and dry gas temperature on the considered analytical response (S/N) as well as their interaction, an experimental design involving both parameters was proposed. Dry gas flow rate was set to 6 L/min (univariate assay optimum) while a central composite blocked cube-star design (12 runs, 4 central points) was performed for the evaluation of nebulization pressure (27579.0 - 55158.1 Pa) and dry gas temperature (180 - 270°C). Lack of fit P-value

for the proposed model was 65% (confidence level of 95%) and determination coefficient (R^2) was 89.7%. Consequently, experimental data satisfactorily fitted to the predicted model. Estimated response surface and main effects plot are shown in Figure S4 on Supplementary Data. Optimal values (51021.2 Pa, nebulization pressure; 160°C, dry gas temperature) were close to those obtained from the univariate assays, selecting these values for further studies.

3.2.3. Mass spectrometer parameters

MS parameters (such as capillary voltage, skimmer, cap exit, Oct 1 DC, Oct 2 DC, Trap drive, Oct RF, Lens 1 and Lens 2) were evaluated by performing direct infusions of 5-NDZ solutions. Each analyte (5 µg/mL) was dissolved in BGE solution (1 M formic acid, pH 1.8) and each standard solution was flushed from the CE instrument to the MS instrument by applying a pressure of 1 bar to the inlet vial. Optimized sheath liquid characteristics (60.0:39.8:0.2 (% v/v/v) propan-2-ol:water:acetic acid; 3.3 µL/min) and optimized nebulization parameters (6 L/min, dry gas flow rate; 51021.2 Pa, nebulization pressure; 160°C, dry gas temperature) were considered. For MS parameter optimization, the enhancement of ion signals was the target, considering that $[M+H]^+$ resulted in the most abundant ions. Because of some 5-NDZs showed similar migration times, MS detection windows were established, so optimum MS parameters were considered as an average of the MS parameters previously estimated for each compound (Table S1 on Supplementary Data). Figure 3 shows the total ion electropherogram, the base peak electropherogram and the extracted ion electropherogram for the separation and detection of eleven 5-NDZs under the optimized conditions.

Hereafter, MS/MS mode optimization was carried out. Considering that some peaks were not well-resolved, MRM mode was chosen. For fragmentation experiments, a cut-off of 27% of the precursor mass was set (i.e., the minimum m/z of the fragment ion able to be trapped by the analyzer). 5-NDZs were fragmented using the SmartFrag™ option that automatically ramps the fragmentation energy from 30 to 200% of the excitation amplitude. Fragmentation amplitude was manually varied and it was optimized considering that the maximum signal should be reached for parent ion and at least two fragmentation ions should be obtained. However, several 5-NDZ parent ions, namely MNZ, TRZ, SCZ, HMMNI, IPZ-OH, MNZ-OH and RNZ, only resulted in one fragmentation ion when MS/MS experiments were performed.

In order to guarantee the unambiguous determination of these compounds, a second fragmentation ion was required. Therefore, MS^3 mode was selected for the detection of previously mentioned analytes. For these compounds, first fragmentation amplitude value was established considering a maximum signal for the fragmentation ion. On the other hand, second fragmentation amplitude value was selected under the consideration of a fragmentation ion ratio equal to 1:10 (second fragmentation ion:first fragmentation ion). Final fragmentation data is appointed on Table 1. Observed fragmentation ions (m/z) were confirmed by previously reported data [35,38].

3.3. Method characterization

Generally, 5-NDZ determination in urine samples has been carried out by LC-MS, however CE-MS is a good alternative because it is a more cost-effective and greener analytical tool. In order to test the usefulness and potential of the proposed method, urine samples were treated by the previously mentioned MISPE procedure (see Section "Sample treatment procedure") and subsequently analyzed by CZE-tandem MS. The optimized method was instrumentally evaluated in terms of linearity, limits of detection (LODs), limits of quantification (LOQs), extraction recoveries (R), matrix effect (ME), overall process efficiency (PE) and peak area repeatability and intermediate precision.

3.3.1. Calibration curves and analytical performance characteristics of the method

Matrix-matched calibration curves were established in urine samples fortified at the following concentration levels: 37.5, 125, 250, 625 and 500 µg/L for DMZ, MNZ, TRZ, SCZ and ORZ; 22.5, 75, 150, 375 and 600 µg/L for IPZ, IPZ-OH, TNZ and MNZ-OH; 60, 195, 400, 1000 and 1600 µg/L for HMMNI and 156.3, 312.5, 781.3 and 1250 µg/L for RNZ. Two spiked urine samples per level were treated following the MISPE procedure. Afterwards, each sample was analyzed in duplicate according to the proposed CZE-tandem MS method. A urine blank sample was analyzed as it has been described and no matrix interferences were found at any analyte migration time. The sum of peak areas from all product ions was considered as function of analyte concentration on the sample. LODs and LOQs of the method were calculated as the minimum analyte concentration yielding a S/N equal to three and ten, respectively (Table 2). In all cases LODs were lower than 39.1 µg/L. Additionally, electropherograms obtained from

the analysis of urine samples spiked at different 5-NDZ concentrations are shown in Figure 4. An electropherogram of a blank sample is also shown.

3.3.2. Recovery studies

Recovery studies were performed over urine samples fortified at three different concentration levels. Three samples per concentration level were treated and analyzed in triplicate. R (%) was estimated by comparing the analytical responses obtained from the application of the full MISPE-CZE-tandem MS to spiked samples with those obtained from the analysis of blank urine samples spiked after the application of the MISPE protocol and prior to the measurement, expressed in percentage (Equation 1). These studies evaluated the efficiency of the extraction process. As can be seen on Table S2 on Supplementary Data, R values higher than 79.2% were obtained for all the compounds, proving the convenience of using MISPE for 5-NDZ determination in urine samples.

$$\boldsymbol{R}(\%) = \frac{\boldsymbol{Signal\ of\ a\ sample\ spiked\ before\ its\ extraction}}{\boldsymbol{Signal\ of\ a\ sample\ spiked\ after\ its\ extraction}} \times \mathbf{100} \quad \textbf{\textit{Equation 1}}$$

3.3.3. Process efficiency and matrix effect

Matrix effect (ME) is the result caused on the analytical response by other components of the sample excepting the target analytes. It can be attributed to many sources affecting the analyte ionization, resulting in ion suppression or signal enhancement when MS is employed as detection tool for quantification and/or identification [39]. In order to evaluate both phenomena, Matuszewski et al. introduced the term of absolute ME which is directly related to R and PE [40]. It can be estimated by comparing the analytical response obtained from a sample spiked post-extraction at any given analyte concentration to the response resulted from a standard solution of the same analyte concentration according to the Equation 2. ME (%) significantly higher than 100 means that matrix components produce a signal enhancement whereas ME (%) significantly lower than 100 can involve ion suppression. Additionally, overall process efficiency (PE) can be defined by Equation 3, in which R and ME parameters are involved as it can be deduced from the combination of Equation 1 and Equation 2.

$$\boldsymbol{ME}(\%) = \frac{\boldsymbol{Signal\ of\ a\ sample\ spiked\ after\ its\ extraction}}{\boldsymbol{Standard\ solution\ signal}} \times \mathbf{100} \quad \textbf{\textit{Equation 2}}$$

$$\boldsymbol{PE}(\%) = \frac{\boldsymbol{Signal\ of\ a\ sample\ spiked\ before\ its\ extraction}}{\boldsymbol{Standard\ solution\ signal}} \times \mathbf{100} \quad \underline{\boldsymbol{Equation\ 3}}$$

ME and PE were calculated according to Equation 2 and 3 respectively, at same concentration levels for which R studies were performed. Average values obtained for each 5-NDZ are shown on Table 3. MEs range from 71.3 to 97.9%, suggesting a slight ion suppression which was more significant for MNZ, TRZ and SCZ. Satisfactory results were obtained for overall PE, ranging between 70.4 and 102.9% that correspond to MNZ and ORZ, respectively. From these results, it can be concluded that the proposed method is suitable for 5-NDZ determination in urine samples because of it results in high Rs and low MEs, which supposes high PE for all studied analytes.

3.3.4. Repeatability and intermediate precision assays

Precision studies were carried out in order to evaluate the repeatability (intra-day precision) and intermediate precision (inter-day precision) of the proposed MISPE-CE-MS/MS method. Repeatability was assayed at three concentration levels by analyzing three spiked samples per level in triplicate. Intermediate precision was assessed at three concentration levels by analyzing one spiked sample in triplicate per level and per day for five consecutive days. Results expressed as RSD of peak areas (%) are shown on Table 4. In all cases RSDs were lower than 11.9% and 16.1% for repeatability and intermediate precision, respectively.

3.4. Comparison of the proposed method with previously reported methods

The developed MISPE-CZE-MS/MS methodology supposes a new contribution for the monitoring of 5-NDZs and has been compared with methods previously proposed for the analysis of these kinds of compounds in urine samples (Table 5). As it can be seen, the separation of a higher number of compounds has been achieved by the proposed method. Moreover, the determination of three 5-NDZ metabolites has been included in this work whereas the analysis of 5-NDZ metabolites in urine samples has only been reported in two previous papers [41,42]. On the other hand, the use of CE as separation technique supposes a great advantage over LC methods [41-43], considering that the use of organic solvents is reduced. In the proposed separation method only the sheath liquid using in the CE-MS interface, containing propan-2-ol and supplied at 3.3 μL/min involves the use of organic solvent, whereas

LC methods require the use of MeOH or MeCN as organic component of the mobile phase, which is supplied at levels of mL/min. Regarding LODs, the developed method allows the determination of 5-NDZs at low μg/L levels, being lower [44] or similar [13,42,43] than other LODs previously reported. Xia et al. [41] have also reported lower levels but the method is applied in porcine urine, being the application focused on food safety, so the presence of 5-NDZ residues in urine involves an illegal use of these compounds, which are forbidden as veterinary drugs. On the contrary, 5-NDZ concentrations at high μg/L or mg/L levels can be expected when the 5-NDZ monitoring is carried out in human urine samples for therapeutically drug monitoring [5]. In terms of repeatability and reproducibility, RSDs lower than 16.1% have been achieved in this work, being in accordance with precision results reported by other methods including whose applying LC (Table 5). Furthermore, the extraction recoveries for the proposed MISPE method are such good as those reported for other traditional SPE methods [13,41,43]. However, MISPE methodology provides higher selectivity than SPE, and as a consequence, lower matrix effects are showed.

4. Conclusions

In this work a comparison between two separation modes, namely MEKC and CZE, was evaluated for 5-NDZ determination by CE-MS. Higher selectivity was achieved under CZE conditions, establishing a novel strategy for the analysis of eleven 5-NDZ drugs, including some of their most relevant metabolites, in urine. On the other hand, high concentration sensitivity has been reached through nebulization and MS parameters optimization, achieving LODs in accordance with those previously reported in human urine samples by LC methods [42,43]. However, further research is required in order to decrease the LODs of the proposed method for its application to the determination of 5-NDZ traces in food matrices. This proposal represents a good alternative to traditional LC methods because it accomplishes with the basis of Green Chemistry, involving low consumption of solvents, reagents and samples. Furthermore, it has been shown the usefulness of MISPE as a selective and efficient sample treatment for 5-NDZ extraction from complex biological samples such as urine, avoiding complicated clean-up procedures. The optimized MISPE-CZE-tandem MS method constitutes a powerful tool for therapeutic drug monitoring of a high number of 5-NDZs in urine samples, being also possible the analysis of some metabolites.

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List of abbreviations

5-nitroimidazole, 5-NDZ; acetonitrile, MeCN; ammonium perfluorooctanoate, APFO; background electrolyte, BGE; dimetridazole, DMZ; extraction recoveries, R; hydroxyl-dimetridazole, HMMNI; hydroxy-ipronidazole, IPZ-OH; hydroxyl-metronidazole, MNZ-OH; ion trap, IT; ipronidazole, IPZ; matrix effect, ME; metronidazole, MNZ; molecular imprinted polymer, MIP; molecular imprinted solid phase extraction, MIPSE; ornidazole, ORZ; perfluorooctanoic acid, PFOA; process efficiency, PE; ronidazole, RNZ; secnidazole, SCZ; sodium dodecyl sulfate, SDS; ternidazole, TRZ; tinidazole, TNZ.

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Figure captions.

Figure 1: Comparison of 5-NDZ drugs separation carried out by (A) MEKC-MS and (B) CZE-MS. BGE: A) 100 mM APFO (pH 9), B) 1 M formic acid (pH 1.8). Both separations were performed in a fused silica capillary: 50 µm i.d., 110 cm effective length. Other experimental conditions: applied voltage, 25 kV; temperature 25°C. Standard solutions (8 µg/mL of each 5-NDZ) were hydrodynamically injected for 10 s at 50 mbar. Peak identification: 1, DMZ; 2, IPZ; 3, MNZ; 4, TRZ; 5, SCZ; 6, ORZ; 7, HHMNI; 8, IPZ-OH; 9, TNZ; 10, MNZ-OH; 11, RNZ.

Figure 2: A) Optimization of the organic solvent nature and percentage (% v/v) added to the sheath liquid. B) Optimization of the acid nature and percentage (% v/v) added to the sheath liquid.

Figure 3: 5-NDZ separation by CZE-ESI-MS. (A) Total ion electropherogram, (B) base peak electropherogram, and (C) extracted ion electropherogram. Separation was performed in a fused silica capillary: 50 µm i.d., 110 cm effective length using 1 M formic acid (pH 1.8) as BGE. Other experimental conditions: applied voltage, 28 kV; temperature 25°C. Standard solutions (2 µg/mL of DMZ and IPZ; 3 µg/mL of MNZ and TRZ; 5 µg/mL of SCZ, ORZ, HMMNI, IPZ-OH, TNZ, MNZ-OH and RNZ) were hydrodynamically injected for 40 s at 50 mbar. Sheath-liquid consisted of a mixture 60.0:39.8:0.2 (% v/v/v) propan-2-ol:water:acetic acid and it was supplied at 3.3 µL/min. ESI and MS parameters are described on Section "Mass spectrometry and electrospray interface". Peak identification: 1, DMZ; 2, IPZ; 3, MNZ; 4, TRZ; 5, SCZ; 6, ORZ; 7, HHMNI; 8, IPZ-OH; 9, TNZ; 10, MNZ-OH; 11, RNZ.

Figure 4: Electropherograms obtained from the analysis of urine samples fortified at different 5-NDZ concentrations. MISPE-CZE-MS/MS conditions are described in Section "Materials and methods". A) 600 µg/L for IPZ, IPZ-OH, TNZ and MNZ-OH; 1000 µg/L for DMZ, MNZ, TRZ, SCZ and ORZ; 1600 µg/L for HMMNI; and 1250 µg/L for RNZ. B) 240 µg/L for IPZ, IPZ-OH, TNZ and MNZ-OH; 400 µg/L for DMZ, MNZ, TRZ, SCZ and ORZ; 640 µg/L for HMMNI; and 500 µg/L for RNZ. C) 30 µg/L for IPZ, IPZ-OH, TNZ and MNZ-OH; 50 µg/L for DMZ, MNZ, TRZ, SCZ and ORZ; 80 µg/L for HMMNI; and 62.5 µg/L for RNZ. D) Blank urine sample.

Table 1. Main parameters of the CZE-MS/MS method.

	Segment time (min)																
	0.0-15.6	15.6-18.2							18.2-20.8			20.8-24.4				24.4-30.0	
	DMZ	IPZ	MNZ		TRZ		SCZ		ORZ	HMMNI		IPZ-OH		TNZ	MNZ-OH	RNZ	
	M^2	M^2	M^2	M^3	M^2	M^3	M^2	M^3	M^2	M^2	M^3	M^2	M^3	M^2	M^2	M^2	M^3
Width (m/z)	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	4.0	2.0	2.0	2.0	2.0	4.0	2.0
Cutoff (m/z)	38.0	46.0	46.0	35.0	50.0	35.0	50.0	35.0	59.0	43.0	38.0	50.0	45.0	67.0	51.0	54.0	38.0
Amplitude (V)	0.23	0.23	0.23	0.20	0.25	0.20	0.25	0.25	0.26	0.24	0.16	0.25	0.18	0.20	0.18	0.28	0.14
Precursor ion $[M+H]^+$	142.0	170.0	172.0	128.0	186.0	128.0	186.0	128.0	220.0	158.0	140.0	186.0	168.0	248.0	188.0	200.9	140.0
Observed fragmentation ions	112.0 96.1	140.0 124.0 109.1	128.0	82.1	128.0	82.1	128.0	82.1	128.0 82.1	140.0	110.0 55.5	168.0	122.0	202.0 121.0 110.0	170.0 123.0	140.0	55.5

Table 2. Matrix-matched calibration curves and statistical and performance characteristics of the proposed MISPE-CE-MS method.

Analyte	Linear range (µg/L)	R^2	Linear regression equation y = m·x + a; (SD, standard deviation)				LOD (µg/L)	LOQ (µg/L)
			Slope × 10^{-3}	SD for the slope × 10^{-2}	Intercept × 10^{-5}	SD for the intercept × 10^{-5}	3 S/N	10 S/N
DMZ	22.8 - 1000	0.9955	3.05	1.18	2.28	0.64	6.9	22.8
IPZ	11.6 – 600	0.9972	9.05	2.78	0.63	0.91	3.5	11.6
MNZ	37.5 – 1000	0.9940	3.57	1.60	1.70	0.87	11.3	37.5
TRZ	15.4 – 1000	0.9949	2.64	1.10	1.53	0.60	4.6	15.4
SCZ	21.0 - 1000	0.9960	3.17	1.16	1.11	0.63	6.3	21.0
ORZ	19.9 - 1000	0.9981	4.11	1.02	0.08	0.55	6.0	19.9
HMMNI	51.3 - 1600	0.9969	4.33	1.40	0.33	1.21	15.4	51.3
IPZ-OH	9.6 - 600	0.9960	9.87	3.61	0.97	1.17	2.9	9.6
TNZ	19.1 - 600	0.9992	6.88	1.10	-0.20	0.36	5.7	19.1
MNZ-OH	21.4 - 600	0.9975	6.56	1.91	1.25	0.62	6.4	21.4
RNZ	130.2 - 1250	0.9983	2.16	0.52	-0.22	0.35	39.1	130.2

Table 3. Estimated process efficiency (%) and matrix effect (%) averages.

Analyte	Matrix effect (%)	Process efficiency (%)
DMZ	84.0	80.1
IPZ	90.1	76.2
MNZ	71.4	70.4
TRZ	74.3	74.7
SCZ	71.3	70.9
ORZ	97.9	102.9
HMMNI	92.6	88.4
IPZ-OH	91.4	84.8
TNZ	85.3	84.4
MNZ-OH	84.5	79.7
RNZ	83.6	82.7

Table 4. Precision studies in terms of relative standard deviations (RSDs, %) for spiked urine samples.

Analyte	Repeatability *(n = 9)*			Intermediate precision *(n = 15)*		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
	50 µg/L	*400 µg/L*	*1000 µg/L*	*50 µg/L*	*400 µg/L*	*1000 µg/L*
DMZ	11.7	3.2	3.3	13.1	8.9	8.4
MNZ	11.9	5.5	3.0	12.7	7.9	9.1
TRZ	11.6	3.3	5.1	16.1	6.9	8.5
SCZ	10.2	4.2	3.8	13.9	6.9	14.9
ORZ	7.6	3.2	9.2	7.0	15.1	13.7
	30 µg/L	*240 µg/L*	*600 µg/L*	*30 µg/L*	*240 µg/L*	*600 µg/L*
IPZ	5.8	3.1	4.2	12.2	8.9	8.4
IPZ-OH	8.3	4.3	2.8	11.7	7.5	8.3
TNZ	8.5	4.4	3.0	11.6	5.0	10.2
MNZ-OH	8.5	4.0	3.9	13.0	6.3	15.8
	80 µg/L	*640 µg/L*	*1600 µg/L*	*80 µg/L*	*640 µg/L*	*1600 µg/L*
HMMNI	4.3	4.6	4.8	10.9	10.1	14.4
	500 µg/L	*1250 µg/L*		*500 µg/L*	*1250 µg/L*	
RNZ	5.4	6.6		13.1	12.9	

Table 5. Comparison of methods proposed for 5-NDZ determination in urine samples.

Analytes	Sample	Sample treatment	Technique	LODs (μg/L)	Extraction recoveries	Precision (RSDs)	Reference
MNZ, MNZ-OH, acetic acid-MNZ metabolite, 2-carboxy-MNZ metabolite, and their glucoronide conjugates	Human urine	Dilution	LC-UV	2 – 37	NA	< 7.7%	[42]
RNZ, MNZ, DMZ, HMMNI	Porcine urine	Extraction with ethyl acetate + SPE (Oasis® MCX cartridges)	LC-ESI-MS/MS	0.03 – 0.05	83 – 107%	< 16% (RNZ, 24.5%)	[41]
MNZ, ORZ, TNZ	Human urine	Dilution	CE-UV	500 – 1800	NA	< 14.5%	[44]
MNZ, DMZ, RNZ, IPZ, TRZ, TNZ, SCZ, ORZ, CRZ	Human urine	SPE (Oasis® MCX cartridges)	CEC-ESI-MS	30 – 130*	67 – 103%	< 16.1%*	[13]
ORZ, TNZ, ofloxacin, norfloxacin	Human urine	SPE (Strata-X™ cartridges)	LC-UV	60 – 160	94 – 102%	< 7.4%	[43]
MNZ, MNZ-OH, DMZ, HMMNI, RNZ, IPZ, IPZ-OH, TRZ, TNZ, SCZ, ORZ	Human urine	MISPE	CE-ESI-MS/MS	2.9 – 39.1	79 – 105%	< 16.1%	This work

*Experiments were carried out using standard solutions.
NA, Not applicable

Figure 1

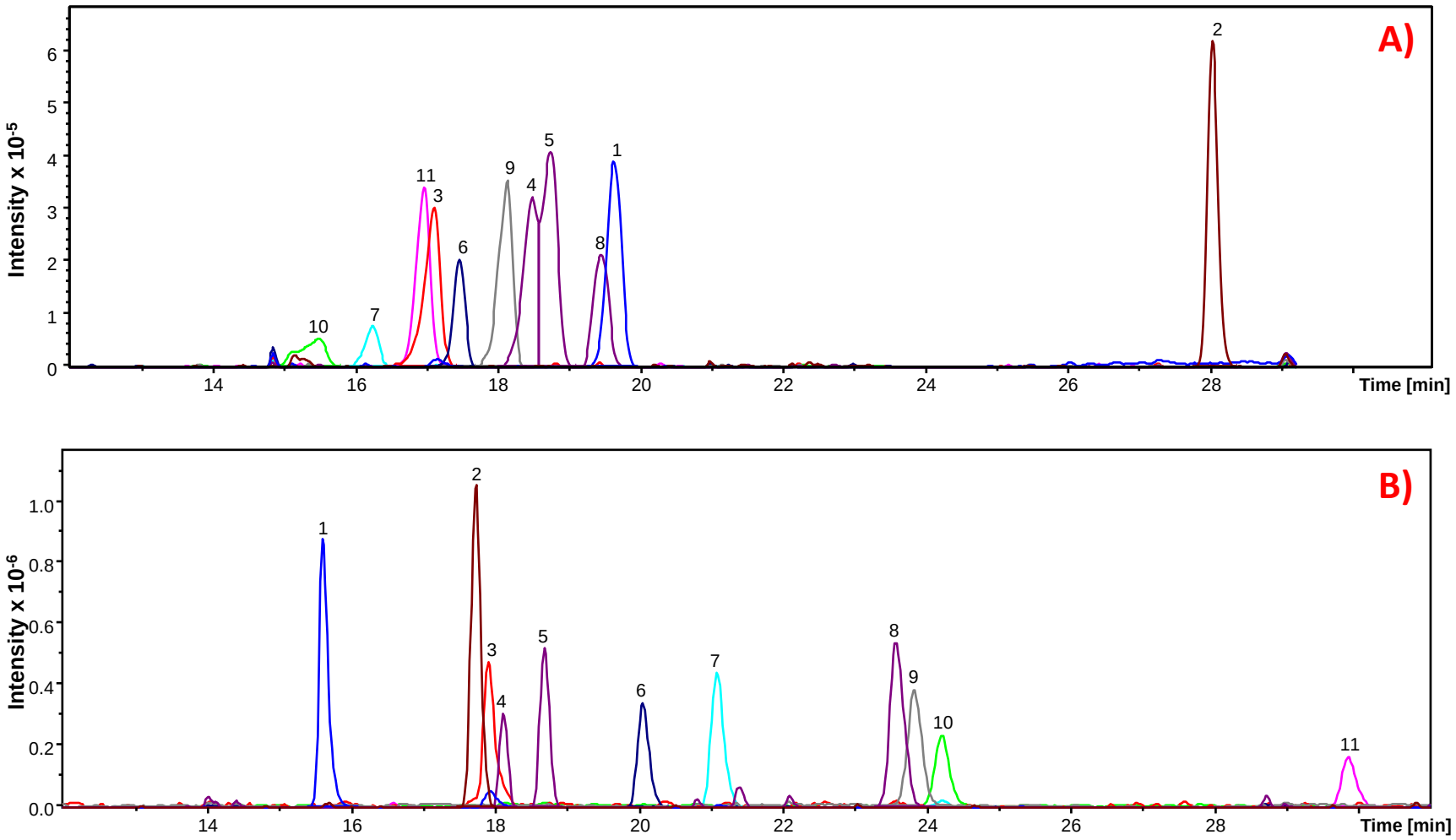


Figure 2

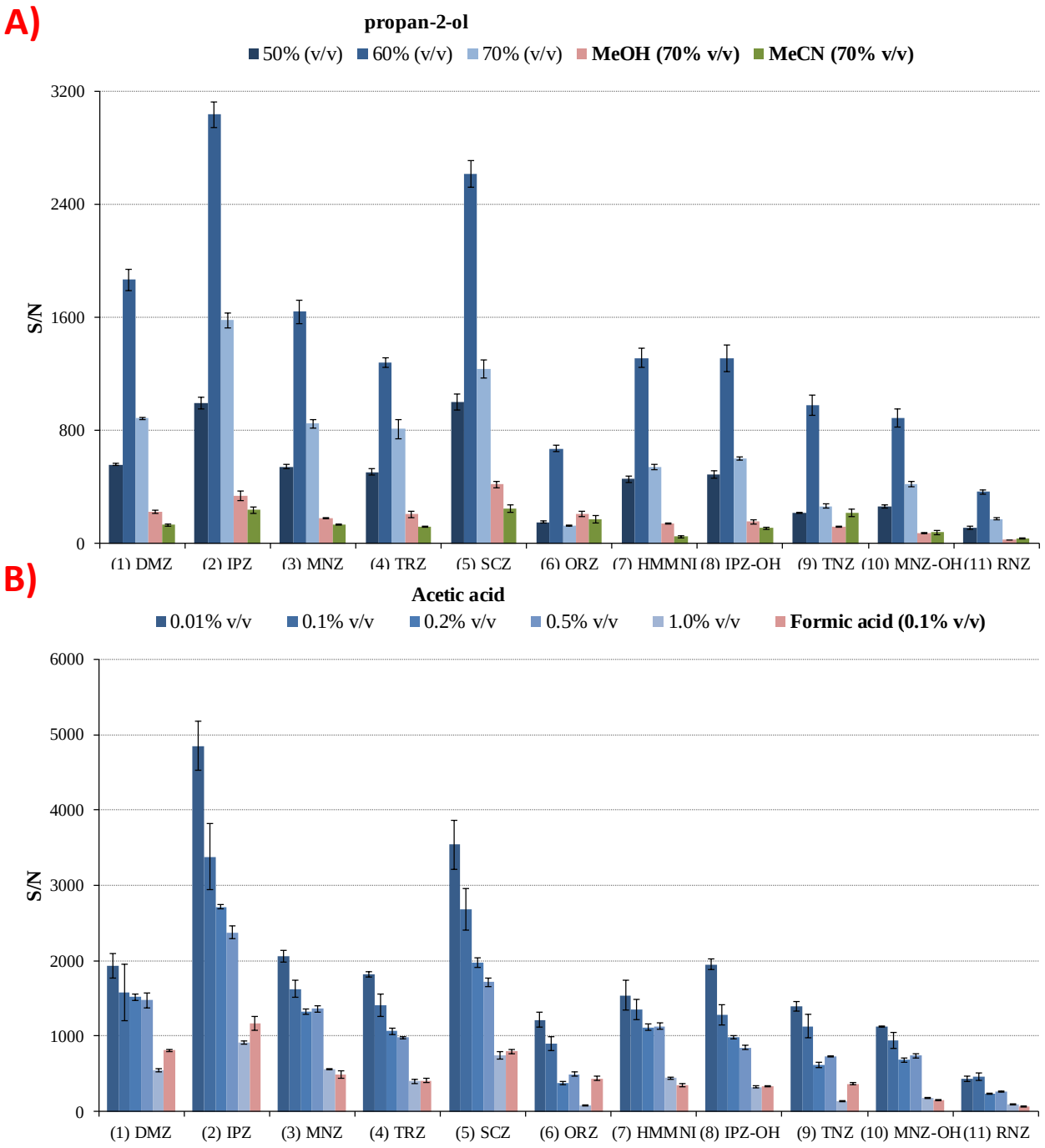

A)
propan-2-ol
50% (v/v)
60% (v/v)
70% (v/v)
MeOH (70% v/v)
MeCN (70% v/v)
S/N
3200
2400
1600
800
0
(1) DMZ
(2) IPZ
(3) MNZ
(4) TRZ
(5) SCZ
(6) ORZ
(7) HMMNI
(8) IPZ-OH
(9) TNZ
(10) MNZ-OH
(11) RNZ
B)
Acetic acid
0.01% v/v
0.1% v/v
0.2% v/v
0.5% v/v
1.0% v/v
Formic acid (0.1% v/v)
S/N
6000
5000
4000
3000
2000
1000
0
(1) DMZ
(2) IPZ
(3) MNZ
(4) TRZ
(5) SCZ
(6) ORZ
(7) HMMNI
(8) IPZ-OH
(9) TNZ
(10) MNZ-OH
(11) RNZ

Figure 3

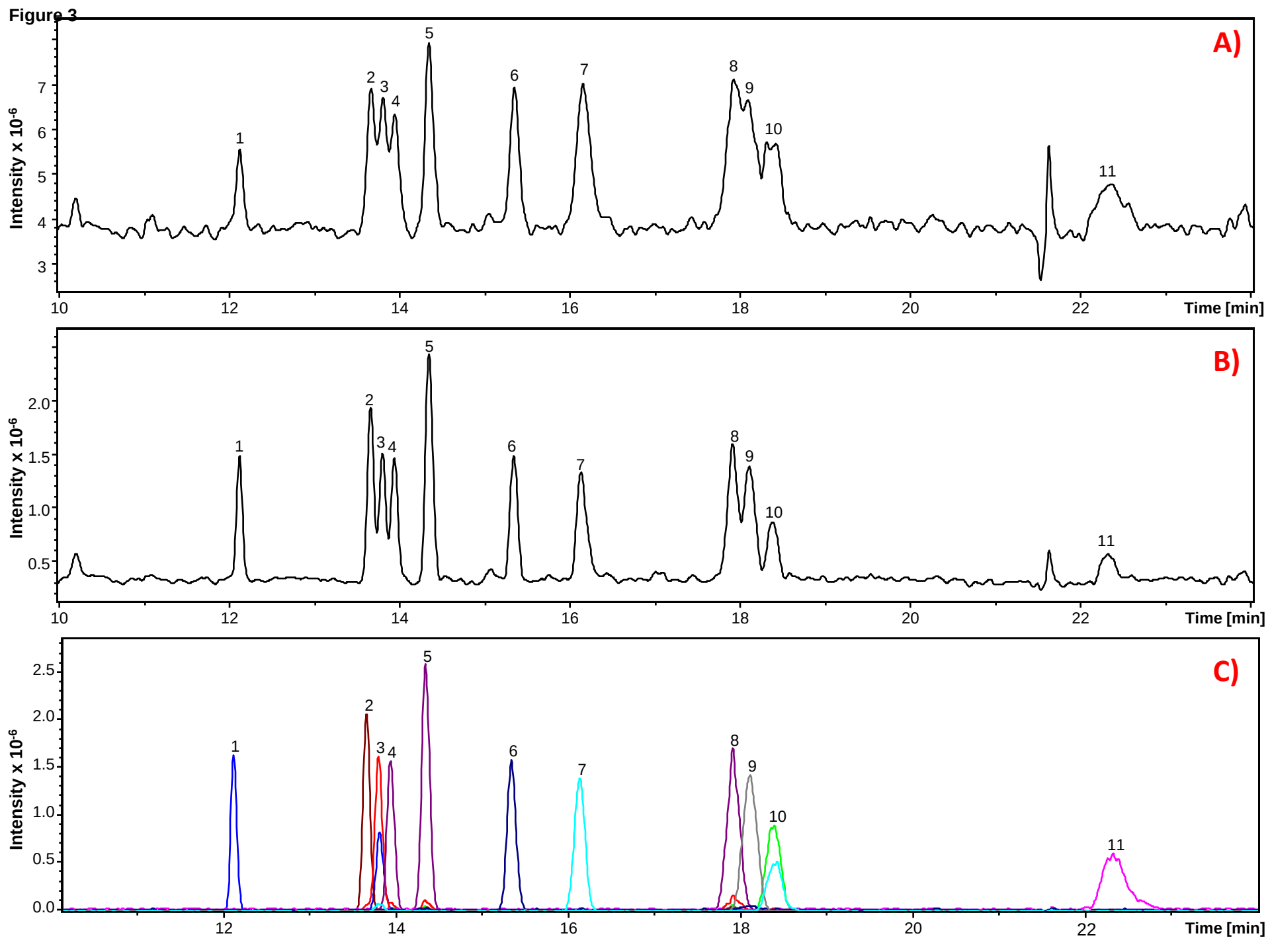
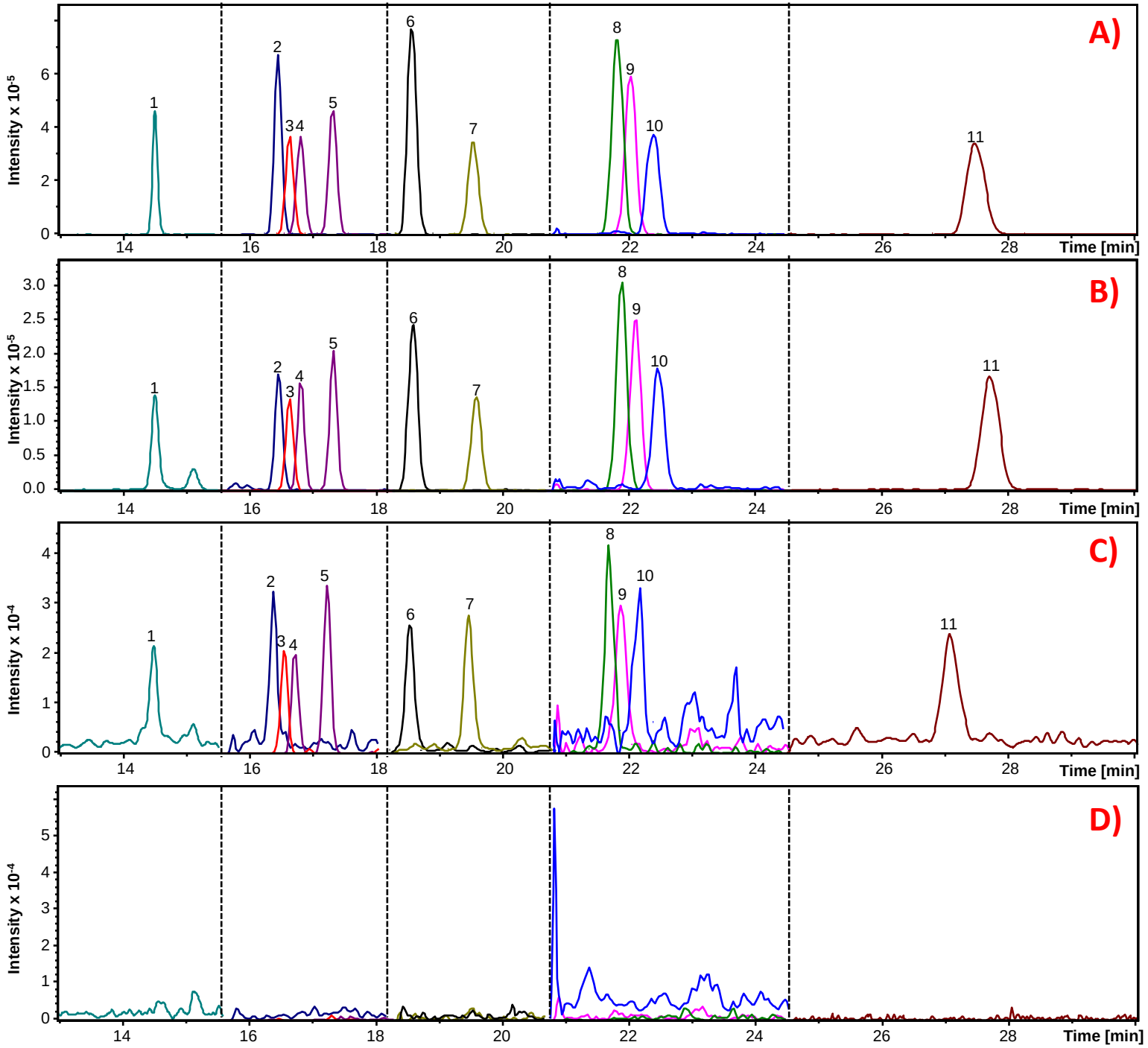


Figure 4


A)
B)
C)
D)
Intensity x 10⁻⁵
Intensity x 10⁻⁴
Time [min]
1
2
3
4
5
6
7
8
9
10
11