Field amplified sample injection – capillary zone electrophoresis (FASI-CZE) for the analysis of amprolium in eggs

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Abbreviations: MRL Maximum residue limit

FASI Field amplified sample injection

AT Activation time

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Abstract

Veterinary medicines are widely administered to farm animals since they keep animals healthy at overcrowded conditions. Nevertheless the continuous administration of medicines to farm animals can frequently lead to the presence of residues of veterinary drugs in consumption products. Amprolium is a quaternary ammonium compound used in the treatment of coccidiosis. In this paper a method based on capillary zone electrophoresis (CZE) to analyze residues of amprolium in eggs was developed and validated for the first time. Parameters such as electrolyte type, concentration and pH were optimized. In order to improve sensitivity, field amplified sample injection (FASI) was used for in-line preconcentration after a quick and simple sample treatment based on SPE (Envi-Carb). During method validation studies using egg samples a matrix interference was found at the migration time of amprolium. This compound was identified as thiamine and confirmed by MS^n experiments using capillary electrophoresis coupled to mass spectrometry (CE-MS) with an ion-trap mass analyzer. CZE conditions were re-optimized to separate thiamine from amprolium allowing the quantification of amprolium in eggs at concentrations down to 75 μg Kg^{-1}, which are far below the MRL legislated values.

1. Introduction

On modern farms, animals are raised under confined feeding conditions at high temperature and humidity, which can easily lead to the proliferation of microorganisms causing many diseases. Veterinary medicine is in charge of the prevention and treatment of these illnesses via the administration of a varied number of veterinary drugs. One of these diseases that must be controlled is coccidiosis, an infectious disease caused by protozoa of the genus *Eimeria* that affects the intestinal tract provoking weight loss and even death to the infected animal. It affects all farm animals in general but especially poultry, so routine prophylactic treatments are performed adding to water or feeds veterinary medicines in order to prevent the apparition of the illness. Amprolium is a quaternary ammonium compound used since the 1950s for the treatment of coccidiosis. However, although it has been employed successfully against the disease, its use has some drawbacks. On one hand it can produce thiamine deficiency as it is a thiamine analog and an abusive intake of the drug can lead to a poor absorption of this vitamin [1]. On the other hand, after long periods of administration it can become ineffective because of the appearance of resistant strains. Moreover, residues of the drug can remain in eggs and tissues; hence, the drug can arrive to consumers if withdrawal periods are not fully accomplished. Due to the growing concern about the amount of veterinary drugs reaching the food, European governments have restricted the use of veterinary medicines establishing maximum residue limits (MRLs) in different tissues, however for amprolium no MRLs have been set [2,3]. Meanwhile, in non European countries where this drug is still authorized, MRLs have been established at values ranging from 0.03 to 1 mg Kg^{-1} in tissues and from 0.1 to 8 mg Kg^{-1} in eggs (http://www.mrldatabase.com/default.cfm?selectvetdrug=1 (18/07/12)), while the concentration added to feeds usually ranges from 80 to 130 mg Kg^{-1}.

Very few analytical methodologies are available in the literature for the analysis of this compound in matrices such as feed, tissues and plasma are mainly based on ion-pair liquid chromatography [4-6]. Earlier studies used direct ultraviolet (UV) detection [5,6] or fluorescence detection after post-column derivatization [4]. However nowadays there is a trend towards the use

of mass spectrometry [7,8], since it is a more reliable detection method and can provide lower detection limits. Nevertheless the purchase of a mass spectrometer is not always an affordable expense for some laboratories, so alternative sensitive methods must be available. Additionally, moving towards green chemistry, it is always important the reduction of solvents such as methanol or acetonitrile, which are commonly used as mobile phases in liquid chromatography. In this context, it is well known that capillary electrophoresis can represent an alternative to the current methods since it is an affordable technique that requires a minimum amount of solvents besides the high separation efficiency that provides among other advantages. Despite its high efficiency, CE presents relatively low sensitivity because of the small volume of injected sample (2-10 nL) and the short optical path length (25-100 μm). This problem can be overcome by in-column pre-concentration techniques such as field amplified sample injection (FASI), stacking and sweeping [9-11]. These techniques combined with off-line pre-concentration procedures provide limits of detection capable of fulfilling current legislation.

To the best of our knowledge, for the analysis of amprolium only an electrophoretic method based on isotacophoresis has been reported in the literature [12]. The purpose of the work presented here was to develop and validate an easy and cheap CZE method for the determination of amprolium. The present paper reports the optimization of the experimental conditions. The influence of different parameters such as the concentration and pH of the aqueous buffer on the analysis was studied and the applicability of field amplified sample injection (FASI) was evaluated. Additionally the challenge that represents dealing with difficult matrices is hereafter commented since an interfering compound was identified as thiamine and separated. Finally the FASI-CZE method was validated for the analysis of amprolium in egg samples.

2. Materials and methods

2.1. Chemicals and consumables

All the reagents used in this work were of analytical grade. Amprolium hydrochloride was provided by Riedel-de-Haën (Seelze, Germany). Methanol and acetonitrile were supplied by Sigma-Aldrich (Steinheim, Germany). Formic acid (98-100%), acetic acid (100%), ammonium acetate, hydrochloric acid (25%), and sodium hydroxide were obtained from Merck (Darmstadt, Germany), and ammonium formate from Fluka (Buchs SG, Switzerland). A stock solution of amprolium (1 mg mL^{-1}) was prepared in Milli-Q water and was stored at 4ºC for a maximum of 4 weeks.

Solid phase extraction (SPE) cartridges ENVI-Carb (500 mg, 3mL) were provided by Supelco (Bellefonte, PA, USA). Nylon syringe filters 0.45 µm were obtained from Tecknokroma (Barcelona, Spain). Water was purified using an Elix 3 coupled to a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Instrumentation

CZE experiments were performed on a Beckman P/ACE MDQ capillary electrophoresis instrument (Fullerton, CA, USA) equipped with a diode array detection system. Uncoated fused-silica capillaries (Beckman) with a total length of 57 cm (effective length 50 cm) x 50 µm I.D. and a 150 mM acetic acid-ammonium acetate buffer (pH 4.5): Methanol (60:40 *v/v*) solution as background electrolyte (BGE) was used. Capillary temperature was held at 25 ^{o}C. The buffer was filtered through a 0.45 µm membrane filter, and degassed by sonication before use. Samples were loaded by using electrokinetic injection (+10 kV, 35 s), and field amplified sample injection (FASI) as in-line pre-concentration was used. FASI was performed as follows: the capillary was filled with the BGE and a water plug (40 s, 3.5 kPa) was hydrodinamically introduced, then the samples were electrokinetically injected (+10 kV during 50 s) into the capillary. The electrophoretic separation was performed by applying +30 kV through the capillary. Direct UV-detection was carried out at 234 nm. The CE instrument was controlled using a Beckman P/ACE station software version 1.2.

New capillaries were pre-treated using 0.1 M hydrochloric acid for 30 min, water for 30 min, 1 M sodium hydroxide for 30 min, and finally were washed with water for 30 min. At the beginning of each session, the capillary was rinsed with sodium hydroxide, water, and with the carrier electrolyte during 30 min each. The capillary was rinsed with carrier electrolyte for 5 min between runs and stored after rinsing with water at the end of each session.

For MS experiments, the CE instrument was coupled to a LCQ Classic mass spectrometer (Finnigan, San Jose, CA, USA) equipped with a tricoaxial pneumatically assisted electrospray ionization (ESI) source and with an ion-trap mass analyzer. A standard solution of 10 µg mL^{-1} of amprolium was used to optimize CE-MS coupling parameters. This solution was infused into the ESI source applying simultaneously an electrophoretic voltage of +20 kV and an over imposed pressure of 5 kPa on the CE inlet vial and +4 kV as electrospray needle voltage. A solution of methanol:BGE (80:20 *v/v*) at a flow rate of 10 µL min^{-1} was used as sheath liquid. The ESI was pneumatically assisted using nitrogen as sheath gas at a flow-rate of 8 arbitrary units (a.u.). The heated capillary temperature was held at 25 ^{0}C. The CE capillary protrudes from the electrospray needle 0.1mm, and the distance to the heated capillary was 1.5 cm.

CE-MS data acquisition was carried out in positive full scan mode from *m/z* 50 to 300 in centroid mode using a maximum injection time of 50 ms and performing 1 µscan. For the product ion scan experiments, an isolation width of *m/z* 1.5 was applied and the trapping ratio frequency voltage (AQ) was set at 0.300 while the activation time (AT) was 30 ms. Mass spectrometry data were processed with an Xcalibur 2.1 software version.

2.3. Sample preparation and clean-up procedure

Whole egg samples were homogenized using an Ultraturrax T25 basic (IKA-Werke, Staufen, Germany) and stored at -18ºC until analysis. Sub-samples of 0.5 g were weighted into 2 mL eppendorfs and 1.5 mL of acetonitrile was added. The samples were first vortex mixed for 1 min and then they were set in a Sonorex RK100 ultrasonic bath (Bandelin Electronic GmbH & Co.,

Berlin, Germany) for 20 min; finally they were vortex mixed again for 1 min. After that, they were centrifuged at 4000 rpm for 5 min using a Selecta Centronic centrifuge (Selecta, Barcelona, Spain). A 1 mL portion of the supernatant liquid was transferred into a 15 mL Falcon tube and 9 mL of water were added. For clean-up, ENVI-Carb cartridges were conditioned with 3 mL of methanol followed by 3 mL of water:acetonitrile (90:10) using a Supelco Visiprep vacuum manifold (Supelco, Gland, Switzerland). Then the extract was passed through the sorbent material, washed with 3 mL of water and finally eluted with 1 mL of methanol. The methanol solution was evaporated to dryness and finally reconstituted in 500 µL of water. Each extract was filtered through 0.22 µm pore size Ultrafree-MC Centrifugal Filters (Millipore, Bedford, USA) before injection into the CE system using the FASI procedure.

3. Results and discussion

3.1. Electrophoretic conditions

To determine the optimum conditions for the analysis of amprolium by CZE, two acidic buffers were tested as background electrolyte (BGE). Formic acid-ammonium formate ($3.0 \leq pH \leq 4.5$) and acetic acid-ammonium acetate ($4 \leq pH \leq 5.5$) were selected and the effect of their concentration from 25 to 200 mM as well as the pH was studied. For these tests hydrodynamic injection (15 s, 3.5 kPa) of a 20 µg mL^{-1} standard solution prepared in water was used and a capillary voltage of +20 kV was applied to perform the electrophoretic run. This systematic optimization provided very similar results for both electrolytes showing the worst sensitivity at low pHs and concentrations and the bests results at higher values of both studied parameters. As an example of the effect of the buffer concentration on the amprolium response Figure 1A shows the electropherograms obtained when using the acetic acid-ammonium acetate buffer at pH=5.0 at increasing concentrations. As can be seen, the amprolium signal improved considerably with the buffer concentration despite an increase in the analysis time. The increase in the analysis time is easily explained by the raise of the ionic strength which lowers the effective electric field and hence

the ion velocity, while the improvement of the amprolium signal is due to a higher stacking effect at this buffer concentration since the injected sample is prepared in pure water giving as a result a narrowing of the peak. Since signal improvement at concentrations higher than 150 mM was not noteworthy, this value was chosen as the optimum in order to avoid high capillary currents. The effect of pH was then studied from 3.0 to 5.5 with formic /formate and/or acetic/acetate buffers at a concentration of 150 mM. The electropherograms obtained at each tested pH condition for the acetic acid-ammonium acetate buffer are shown in Figure 1B where it can be observed an important increase in the amprolium signal and also a slight increase in migration time when pH raised up to 5.0. Since a pH value over 5 did not significantly improve the response of amprolium and yielded to high capillary current values pH 5.0 was chosen as the optimum. In general, slightly better peak shapes, signal intensity and lower current values were obtained when using acetic acid-ammonium acetate, so, this buffer, (150 mM pH 5.0) was selected as optimum BGE for the CZE determination of amprolium. Finally, injection time for electrokinetic injection was evaluated and as expected, an increase in the injection time yielded to higher responses up to a certain value from which peak broadening was observed. The optimum value was then established at 35 s for electrokinetic injection mode.

To increase sensitivity, the use of an in-line pre-concentration method, FASI, was investigated. This technique takes advantage of the difference in mobility and conductivity between the sample matrix and the BGE to pre-concentrate the analyte. The effect of two high-resistivity solvents, water and methanol was studied. To perform these tests, a 100 ng mL^{-1} amprolium standard solution prepared in water was used. Although a significant increase in the response of amprolium was observed for both solvents, methanol caused the electrophoretic voltage to frequently fail, probably due to the formation of bubbles in the capillary. For this reason, a plug of water was used. Injection times for both the plug of water (hydrodynamic mode) and the sample (electrokinetic mode) were simultaneously optimized. Hydrodynamic injection (3.5 kPa) of a water plug from 5 s to 40 s, and electrokinetic sample injection (10 kV) from 5 s to 60 s were tested. The

best results were obtained with a water plug hydrodynamic injection time of 40 s and a sample electrokinetic injection time of 50 s. Under these conditions, an instrumental sensitive enhancement of 1600-fold with respect to hydrodynamic injection was achieved. Obviously, when increasing injection time an enhancement of the response was observed; however, peak broadening occurred at sample injection times higher than the selected value.

3.2. Instrumental quality parameters

Instrumental quality parameters using electrokinetic injection and FASI under optimal conditions were calculated and the figures of merit are given in Table 1. The limit of detection (LOD) and the limit of quantification (LOQ), based on a signal-to-noise ratio of 3:1 and 10:1, respectively, were calculated using standard solutions at low concentration levels. The use of CZE with electrokinetic injection provided a LOD of 1 μg L^{-1}. When FASI-CZE was applied, a 4-fold enhancement was achieved obtaining a LOD of 0.25 μg L^{-1}. The low LODs achieved with both electrokinetic injection and FASI would allow the analysis of amprolium at the levels required by the current legislation.

Run-to-run and day-to-day precisions for amprolium quantification were calculated at two concentration levels, a low level (LOQ) and a medium level (55 μg L^{-1} for electrokinetic injection, and 27 μg L^{-1} for FASI). In order to obtain the run-to-run precision, six replicate determinations for each concentration level were carried out using the two injection modes under optimal conditions. On the other hand, day-to-day precision was calculated by performing 18 replicate determinations of each concentration level on 3 non-consecutive days (six replicates each day). The relative standard deviations (RSDs) obtained for run-to-run and day-to-day precisions with CZE using injection were 7.6% and 14.9%, respectively, at the medium concentration level. The values increased to 12.8% and 18.8% when the low concentration level was evaluated, as expected, being RSDs values quite acceptable for CZE methodologies. For both, electrokinetic and FASI injection modes, the RSDs obtained for run-to-run and day-to-day precisions were quite similar. This is

explained by the great dependence of the electrokinetic injection mode on the electroosmotic flow (EOF) and the electrophoretic mobility of the solute [13]. In terms of migration times good run-to-run and day-to-day precisions were obtained in all cases (Table 1) with RSDs values from 0.4 % to 3 %.

Calibration curves based on amprolium peak area at the working ranges indicated in Table 1 were obtained showing good linearity, with correlation coefficients (r^2) higher than 0.999.

3.3. Sample treatment and clean-up procedure

In a previous study carried out in our research group, amprolium was extracted from egg samples using 10 mL of acetonitrile and the extracts were directly analyzed by LC-MS/MS [7]. The same procedure was tested for the FASI-CZE method, however the direct injection of the acetonitrile-based extract did not provide good results since current leakage occurred when electrokinetic injection was being performed. Another approach, evaporation of the extract to dryness and subsequent reconstitution in water was neither effective, probably due to the presence of other cationic species such as sodium or calcium in the final extract that prevented the appropriate preconcentration of the analyte under FASI conditions. These facts highlighted the need of a proper clean-up procedure in order to obtain extracts clean enough to be submitted to the FASI-CZE method. For this purpose solid phase extraction (SPE) was used, however, most of the sorbent materials require the loading of a water-based solvent to retain the analytes, which means that the solvent extract must be changed before the loading step. This was performed by the addition of water (9 mL) up to a 90% in the final extract. Two different cartridges were tested; a graphitized carbon material, ENVI-Carb (Supelco) which presents quite important retention for aromatic compounds due to π-π interactions, and a polymeric column, Oasis MCX (Waters) (mixed mode cation exchange). In a first stage of the optimization, standard solutions prepared in ACN:H_2O (10:90) were used. For the elution of amprolium from the ENVI-Carb cartridge just 1 mL of methanol was necessary to obtain a recovery over 90%. However, Oasis MCX could not be used for

the analysis of amprolium since ammonium hydroxide must be used to release this compound from the cationic exchanger, remaining in the final extract and preventing the entrance of amprolium into the capillary by electrokinetic injection. So, the Envi-Carb cartridges were selected for the off-line pre-concentration of egg samples.

3.4. Validation

Blank egg samples previously analyzed by LC-MS/MS in our laboratory [7] were used in order to evaluate the applicability of the FASI-CZE method for the determination of amprolium. Sample treatment was performed following the procedure described in Section 2.4. However, in the electropherograms corresponding to the non-spiked blank samples a peak with a very similar UV spectrum appeared at the migration time of amprolium. That indicated the presence of a matrix interference with characteristics similar to amprolium that must be separated from our analyte in order to avoid false positives and errors in the quantification. In the literature it is described that amprolium is actually a thiamine (Vitamin B1) analogue that blocks the transport of this vitamin preventing the carbohydrate synthesis in the coccidia. Concentration values of thiamine in eggs around 0,1mg/100g product have been reported (http://nutritiondata.self.com/facts/dairy-and-egg-products/111/2, 18/07/12), so, in a first attempt to identify the observed interference, thiamine was considered as a candidate. A standard solution of this product as well as a mixture of thiamine and amprolium were prepared and injected in the electrophoretic system. In both cases only one peak was observed at the migration time of amprolium and identical UV spectra were obtained, which was consistent with the formulated hypothesis. However, in order to conclude unequivocally that the interference of the egg matrix was thiamine some more evidence of identity was necessary. For this purpose the electrophoresis was coupled to a mass spectrometer provided with an ion trap mass analyzer. ESI–MS instrumental parameters such as sheath liquid and sheath gas flow rates, sheath liquid composition and electrospray voltage were optimized in order to obtain the highest response for thiamine and are indicated in the experimental section. To check the optimum operation of the

CE-MS system, a standard solution of thiamine was injected and data were acquired in both, full scan and product ion scan modes. The full scan spectrum showed as base peak the ion at *m/z* 265 which corresponds to the thiamine cation and in the product ion scan spectrum two fragment ions at *m/z* 144 and 122 (Figure 2) corresponding to each of the moieties resulting from the cleavage of the N-C bond were present [14,15]. Under these conditions, a blank egg sample extract was also injected and both full scan and product ion scan spectra were acquired. Although the signal intensity of the interference was quite low, its full scan spectrum showed as base peak an ion at *m/z* 265 while the product ion scan spectrum of this ion provided the same two fragments observed before for the thiamine standard (*m/z* 144 and 122). Considering the cationic nature of the compounds analyzed by CZE, the data provided by the mass spectrometric analysis and the information available in the literature this interference was identified as thiamine (Vitamin B1).

Once the interference was identified, CZE conditions were reoptimized to achieve the separation of both amprolium and thiamine. For this purpose, the addition of an organic solvent into the BGE was evaluated to achieve the complete separation of amprolium and thiamine. Methanol was added to the BGE from 10% to 40%. The addition of methanol had direct consequences on the separation, on one hand it allowed the baseline separation of amprolium from thiamine, but on the other, a substantial increase in the analysis time was observed. In order to reduce analysis time the separation was then performed at a higher voltage (+30 kV) without losing the separation previously achieved while the sensitivity was improved thanks to the narrowing of the amprolium peak (Figure 3A).

In order to evaluate the method quality parameters, blank egg samples were spiked at different known concentrations of amprolium, they were homogenized and left to stand for an hour before they were submitted to the sample treatment. Following this procedure, a method limit of quantitation (MLOQ), based on a S/N ratio of 10, of 75 µg Kg^{-1} was obtained. This MLOQ would make this method suitable for the determination of amprolium in egg samples in all the countries with established MRLs considering that these values are well over the obtained MLOQ. Figure 3B

shows an electropherogram of a blank egg sample spiked at 100 µg Kg^{-1}. To assess the recovery of the method as well, blank extracts were spiked at the same concentrations as before and an overall recovery value of 85 % was obtained. Since amprolium was not detected in any of the commercial egg samples analyzed, in order to check the precision and accuracy of the proposed method a blank egg sample was spiked at two concentration levels, low and medium (75 µg Kg^{-1} and 1000 µg Kg^{-1}), and quantified using matrix matched calibration. This quantitation method was preferred since the slopes of external calibration and matrix matched calibration curves were significantly different mainly due to the variability of the amount of sample injected when using electrokinetic injection. Considerably low relative errors were obtained for the quantification of both concentration levels, 13.1% for the low level and 4.3% for the medium, while for run-to-run precision, relative standard deviations of 14.2% and 9.5% were achieved respectively. From these results we can conclude that the FASI-CZE method showed good accuracy and precision, and it can be proposed as a suitable methodology for the screening and determination of amprolium in egg samples bellow the levels established by the different countries that have set an MRL for this compound in hen eggs.

4. Concluding remarks

In this study it is demonstrated that FASI-CZE can be used for the determination of amprolium in egg samples. A quick and efficient sample treatment based on SPE using ENVI-Carb cartridges is proposed providing extracts suitable for the analysis of eggs by capillary electrophoresis. However, an interference from the matrix eluting at the same migration time as amprolium prevented quantification. This compound was identified as thiamine (Vitamin B1) by coupling CZE to mass spectrometry. The addition of MeOH to the BGE up to a 40% allowed the separation of the two compounds and to propose the method as a simple and cheap procedure for the determination of amprolium in egg samples. Under these new conditions we were able to quantify amprolium in egg samples at concentrations above 75 µg kg^{-1}, which would fulfill the

current legislation of non-European countries. Good results in terms of run-to-run precision (%RSD< 14) and accuracy (<13%) were also obtained.

5. Acknowledgements

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Figure captions:

Figure 1: A) Buffer concentration optimization (ammonium acetate/acetic acid pH=5.0) B) Buffer pH optimization (150 mM ammonium acetate/acetic acid)

Figure 2: Electropherogram of thiamine and its MS and MS/MS spectra

Figure 3: A)Separation of Amprolium and Thiamine with 150 mM acetic acid-ammonium acetate pH 5.0: 40% MeOH buffer at 30 kV separation voltage. B) Blank egg sample spiked at 100 µg Kg^{-1} overlayed to a blank egg sample

381 Table 1: Instrumental quality parameters

	CZE Electrokinetic injection	FASI-CZE
LOD (µg L^{-1})	1	0.25
Sensitive Enhancement	-	4
LOQ (µg L^{-1})	3	0.75
Run-to-run precision (%RSD, n=6)		
Migration time	0.4	0.5
Concentration		
Low level (LOQ)	12	11
Medium level	7[a]	7[b]
Day-to-day precision (%RSD, n=3x6)		
Migration time	1.6	3.0
Concentration		
Low level (LOQ)	18	13
Medium level	14[a]	10[b]
Working range (µg L^{-1})	3.2-480	0.9-470
Correlation coefficient (r^2)	≥0.999	≥0.999

382 [a]55 µg L^{-1}; [b]27 µg L^{-1}

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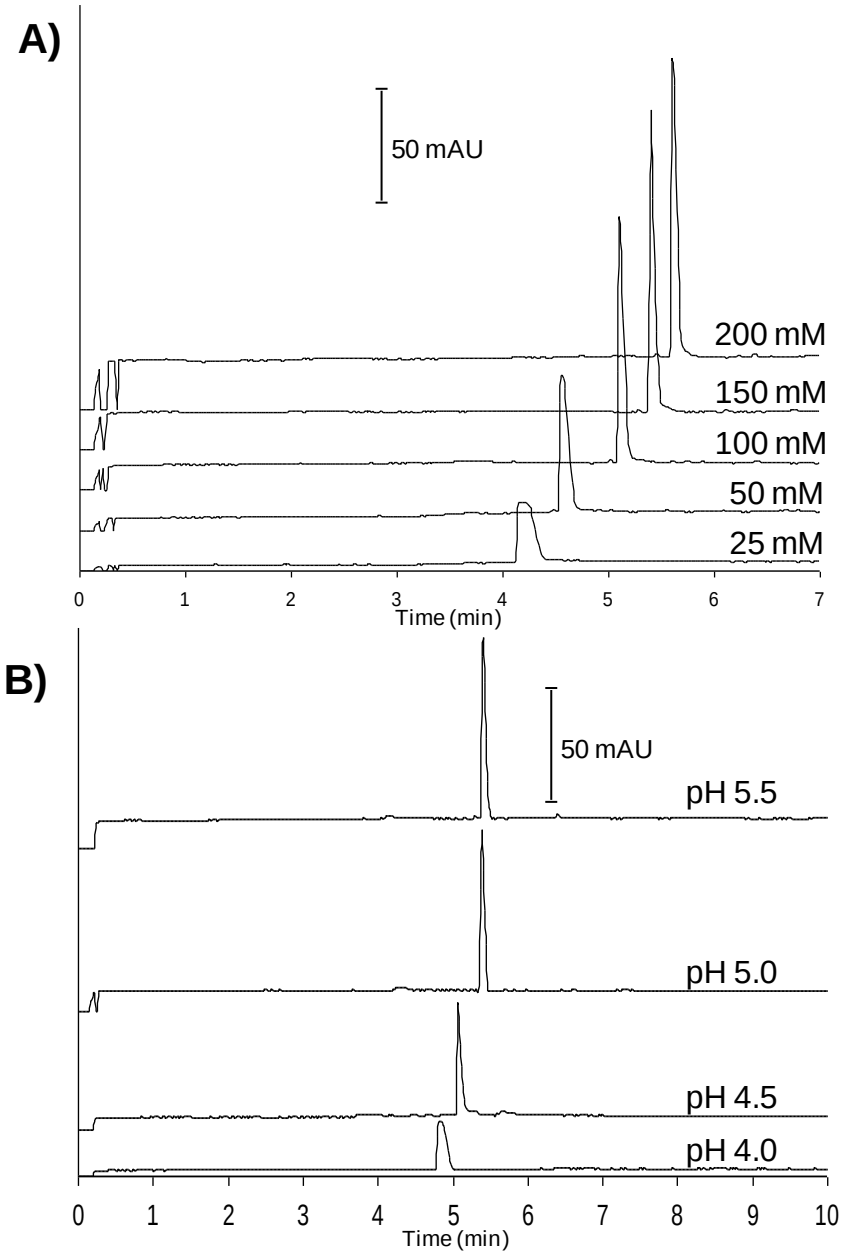

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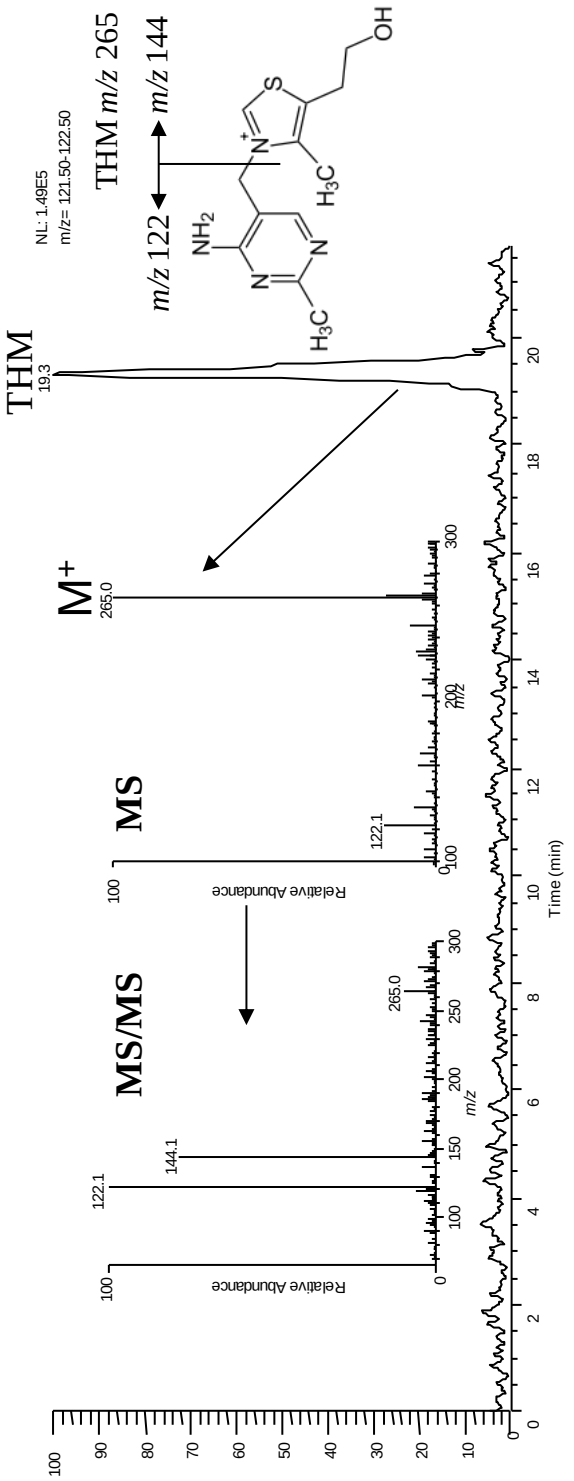

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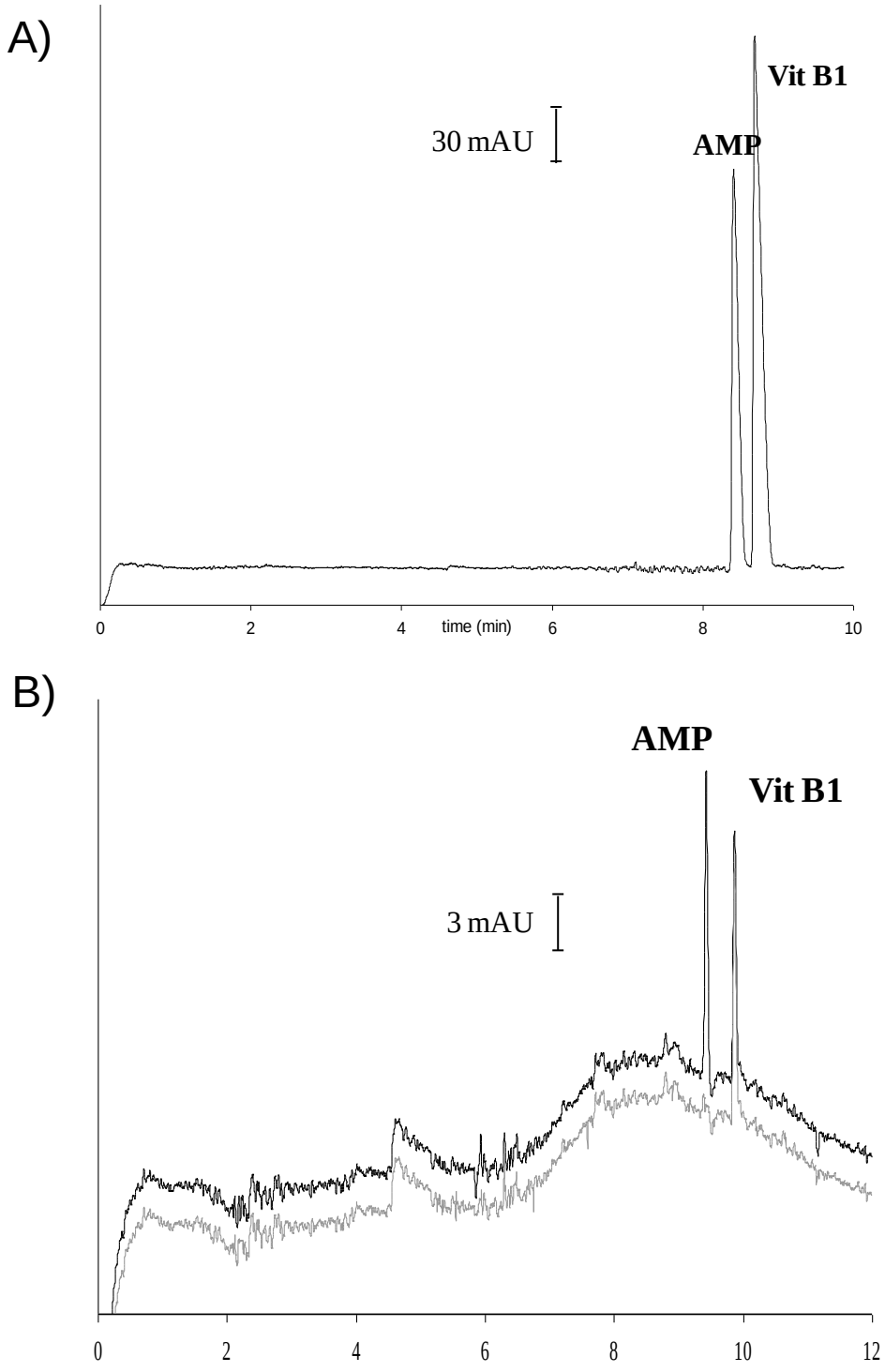

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