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ABSTRACT

A separation of zinc bacitracin, polymyxin B, oxytetracycline and sulfacetamide in animal feedstuffs by micellar electrokinetic capillary chromatography (MEKC) was developed. The running buffer was 20 mmol L^{-1} borate 20 mmol L^{-1} phosphate, pH 8.4, containing 20 mmol L^{-1} sodium dodecylsulphate and 10 % (v/v) methanol. MEKC was performed at 25°C, the applied voltage was 25 kV and running pressure of 10 mbar was applied. Simultaneous UV detection for all analytes was at 215 nm. The method was validated for specificity, accuracy, linearity, precision and robustness. It was shown to be specific, accurate (recoveries were 99.7 ± 0.3, 99.9 ± 0.9, 99.8 ± 1.0 and 99.5 ± 0.4, respectively for oxytetracycline, sulfacetamide, polymyxin B and zinc bacitracin spiked samples of feed for cow, pigs, chicken and cattle), linear over the tested range (correlation coefficients ≥ 0.9987), and precise (RSDs below 1.8 % for each analyte). The method was applied to determine zinc bacitracin, polymyxin B, oxytetracycline and sulfacetamide as additives in animal feed.

KEYWORDS: Animal feed additives, Zn bacitracin, oxytetracycline, polymyxin B, sulfacetamide, micellar electrokinetic capillary chromatography (MECK)

INTRODUCTION

Feed additives are intended to improve feed quality, nutritional aspects, animal health and animal performance. According to Regulation (EC) No. 1831/2003 (European Union, 2003) there are a wide range of substances considered as feed additives and classified as technological, organoleptic, nutritional and zootechnical (i.e. increasing animal production or performance).

Antibiotics have been used in the rearing of food-producing animals in livestock farm industries as active disease treatment agents for prophylactics and in cases of pulmonary, urinary and digestive infections as feed additives. The use of these drugs to control and treat animal disease and to promote fast, more efficient growth of livestock is a common practice. Improper use, as well as the systematic administration of such additives at sub-therapeutic doses can lead to the development of antibiotic-resistant bacteria that can be transferred to humans (Witte, 1998; Smith and Coast, 2002). Since 1999, the European Union has forbidden the use of those antibiotics as additive in animal feed (Molterer, 1998), but they are still in use in non EU countries.

Recently, Serratosa *et al.*, (Serratosa *et al.*, 2006) published an overview of the presence of residues from veterinary medicinal products, growth-promoting agents and performance enhancers in food-producing animals, as a result of legally or illegally administration of these substances, as well as the current

situation in EU and practical challenges for uncovering illegal uses and prevention public health risks.

In order to carry out an efficient official controls of the possible illegal use of these drugs in feeding stuffs, reliable methods of analysis need to be available with efficient separation and high sensitivity. Oka *et al*., (2000) reviewed the literature for the assay of tetracycline antibiotics in food, among which oxytetracycline, tetracycline, chlortetracycline and doxycycline are commonly applied to food-producing animals. They have summarized the application of thin-layer chromatography, capillary electrophoresis, and HPLC for the separation and detection, as well as extraction and clean up procedures for real samples.

Capillary electrophoresis (CE) has become a very useful method for pharmaceutical analysis and in comparison with HPLC has many advantages such as: high resolution, speed, the exceptionally small sample volume required and low content of organic solvents in running buffer and short run time for the separation. Oka *et al*., (2000) indicated that CE had less applications in the analysis of drugs in food due to low sample injection volume, and on the other hand authors pointed out that due to properties of tetracycline's these bind with silanol groups in the stationary phase interfere with the establishment of a simple analytical method.

Recently, Kowalski *et al.,* (2003) proposed a CE method with UV detection for eight of the most frequently used antibiotics (among those was doxycycline) with detection limit of drug residue in tissues at level below 20 μg/kg. One of the commonly used CE modes is micellar electrokinetic capillary chromatography (MEKC), which is efficient for the separation of both ionic and neutral analytes. Success of the separation is based mainly on appropriate selection of the surfactant. The results of MECK application for Zn Bacitracin and nystatin separation and assay in animal feed, recently developed in our laboratories (Injac *et al*., 2006) demonstrated that this technique could be the alternative to HPLC method, but also the simple extraction procedure was proposed. For zinc bacitracin in animal feed Capitán-Vallvey *et al.* (2001 and 2002) proposed HPLC methods with UV and fluorescence detection, but with difficult extraction procedures using LLE as well as SPE techniques.

In the continuation of our research studies we selected, beside Zn Bacitracin (Zn BAC), oxytetracycline (OXY), polymyxin B (POL B) and sulfacetamide (SUL) to investigate the separation applying MECK with the similar extraction procedure described in previously published paper (Injac *et al*., 2006). OXY, SUL, Zn BAC and POL B have been determined in pharmaceutical formulations by CZE and MEKC (Gallego and Arroyo, 2002; Gallego and Arroyo, 2003a; Gallego and Arroyo, 2003b; Gallego and Arroyo, 2003c; Gallego and Arroyo, 2003d; Mamani *et al*., 2006; Nozal *et al*., 2004). Kang *et al.* (Kang *et al*., 2001) have used MEKC to separate bacitracin components, with Brij 35 and PAPS as surfactants.

Counter-current chromatography and high-performance liquid chromatography were used for the separation of Zn-bacitracin (Oka *et al.*, 1998; Capitan-Vallvey *et al.*, 2002; Sin *et al.*, 2005). Tetracyclines and sulfonamides were analysed by liquid chromatography-electrospray ionization tandem mass spectrometry, as well as with HPLC-UV, LC/MS/MS in wastewaters, pharmaceuticals and food (Yang *et al.*, 2005; Zhao *et al.*, 2004; Mandens *et al.*, 2004; Rao *et al.*, 1999).

Zn BAC interferes with bacterial cell wall synthesis by bloking the function of the lipid carrier molecule that transfers cell wall subunits across the cell membrane. It is active against many Gram-positive bacteria. Acquried bacterial resistance to bacitracin rarely occurs (Martindale, 2005). Oxytetracycline is bacteriostatic antibiotic with a wide spectrum of activity and has been used in the treatment of a large number of infections caused by susceptible organisms. Polymyxin B and the other polymyxin antibacterials act primarily by binding to membrane phospholipids and disrupting the bacterial cytoplasmic membrane. The use of sulfacetamide and other sulfonamides has been limited by the increasing incidence of resistant organisms (Wang *et al.*, 2006). Their main use has been in the treatment of acute, uncomplicated urinary-tract infections, particularly those caused by Escherichia coli (Martindale, 2005).

Selected analytes, OXY, SUL, Zn-BAC and POL used as additives in animal feed at levels 100-200 mg kg^{-1}, 100 mg kg^{-1}, 50-250 mg kg^{-1} and 50-150 mg kg^{-1},

respectively (Swine antibiotics and feed additives, 2006; Frost & Sullivan, 2005; National Department for Veterinary Science, 2006).

The aim of this paper was to establish the optimal conditions for MECK separation and assay using the model mixture of OXY, Zn BAC and POL B in spiked feedstuff samples, antibiotics belonging to different cases of antibiotics, in combination with SUL as these can be found as feed additives. The model mixture is also of interest since, polymyxins have been reported to demonstrate antimicrobial synergy with a variety of other drugs, including chloramphenicol, tetracycline, and the sulfonamides and trimethoprim (Martindale, 2005), and as such the prophylactics in food could be covered with these representative drugs.

MATERIALS AND METHODS

Apparatus

The HP^{3D} Capillary Electrophoresis system (Hewlett Packard, Waldbronn, Germany) with a diode-array detector, controlled by HP ChemStation software, was used to perform MEKC. Compounds were separated on a 48 cm (40 cm to the detector) x 50 μm i.d. fused silica capillary (with bubble cell, 150 μm) (Agilent, Waldbronn, Germany).

A Crison MicropH 2002 pH meter (Barcelona, Spain) was used for pH measurement.

Reagents and solutions

All solvents and reagents were of analytical grade unless indicated otherwise. Samples solutions were prepared with deionized water (Milli-Q-quality). Sulfacetamide sodium was obtained from Vetprom (Belgrade, Serbia) and Zn-bacitracin from Sigma (Deisenhofen, Germany). The quality of the both complied with BP requirements. Oxytetracycline hydrochloride and polymyxin B sulphate were obtained from Sigma (Deisenhofen, Germany) and Fluka (Buchs, Switzerland), respectively (USP quality).

Animal diet and feedstuff mixtures for cow K-19 (protein min 19 %, crude fat max 15 %, ash max 15 %, crude fiber max 10 %, calcium 0.8 %, phosphorus 0.45 %, sodium 0.25 %, moisture max 13.5 %, vitamins A, D_3, E, zinc, manganese, selenium, cobalt, copper, iron and iodine), young cattle TL-TIP (protein min 14 %, ash max 10 %, crude fiber max 12 %, moisture max 13.5 %, vitamins A, D_3, E, zinc, manganese, selenium, cobalt, copper and magnesium), pigs from 25 to 60 kg BEK-1 (protein 17 %, ash max 8 %, crude fiber max 7 %, moisture max 13.5 %, vitamins A, D_3, E, B-complex, zinc, selenium, manganese, iodine, cobalt, copper, iron and antioxidants Sanox), chicken feed NSK-1 (protein min 15 %, moisture max 13.5 %, crude fiber max 8 %, ash max 12 %, vitamins A, D_3, E, K_3, B-complex, zinc, selenium, manganese, iodine, cobalt, copper, iron, carophyll red and antioxidants Sanox), and minerals and vitamins for cattle BOVISAL (calcium 16 %, phosphorus 11 %, sodium 10 %, magnesium 0.6 %, moisture max 7 %, vitamins A, D_3, E, zinc, manganese, selenium, iodine, cobalt and copper), were manufactured by JATA EMONA (Ljubljana, Slovenia).

Buffer solutions were prepared by dissolving the appropriate amount of NaH_2PO_4 (20 mmol L^{-1}) and $Na_2B_4O_7$ (20 mmol L^{-1}) in deionized water and the pH was adjusted to 8.4 with HCl. $Na_2B_4O_7$ x $10H_2O$ was p.a. from Kemika (Zagreb, Croatia) and NaH_2PO_4 x H_2O was p.a. from J.T. Baker Inc. (Phillipsburg, USA). Sodium dodecylsulphate (SDS) was from Riedel Line AG (Seelze,Germany).

The background electrolyte (BGE) was 20 mmol L^{-1} borate + 20 mmol L^{-1} phosphate buffer, pH 8.4, containing 20 mmol L^{-1} SDS and 10 % (v/v) methanol.

Preparation of standard stock solutions

Stock solutions of antibiotics were prepared by weighing 50 mg of the drugs and dissolving in 50 mL methanol - deionized water (1:1; v/v). The stock solutions were diluted with running buffer to obtain the concentration ranges required (linearity was check in range of 0.1 – 100 mg kg^{-1} for each drug).

Sample preparation and extraction

Spiked animal feed were prepared by grinding 500 g of feedstuff and adding 50 mg of each of the antibacterial. Blank feed or spiked feed with added antibiotics, were weighed (100 g) and extracted with methanol (5 x 20 mL), first by shaking, then in an ultrasonic bath for 15 min. The extracts were combined, filtered (0.22 µm), transferred to 100 mL volumetric flask, and made up with methanol. Different known volumes were placed in 1.5 mL calibrated vials and diluted to volume with running buffer.

Operating conditions

The capillary was conditioned prior to its first use by flushing with 0.1 mol L^{-1} NaOH for 20 min and then with water for 10 min. The capillary was conditioned, in the optimized method, at the beginning of each day with methanol under high pressure for 3 min, than rinsed for 2 min with 0.1 mol L^{-1} NaOH and 3 min with background electrolyte. This was followed by hydrodynamic sample injection at 600 mbars. Separations were performed at 25 kV and 25°C (under applied pressure of 10 mbar) in 12 min; under these conditions the current was 97-98 μA. UV detection was at 215 nm.

RESULTS AND DISCUSSION

Preliminary studies

Preliminary investigations of Zn-BAC, SUL, OXY and POL B were carried out, to optimize the separation. MEKC methods were published for the determination of hydrocortisone and its most important associated compounds (including Zn-BAC, POL B and OXY) in topical pharmaceutical preparations (Gallego and Arroyo, 2002; Gallego and Arroyo, 2003c) SUL were determined in pharmaceuticals by MEKC with others associated compounds (Gallego and Arroyo, 2003a). Some modifications of the SDS concentration, organic modifier and applied pressure were assessed to obtain a shorter run time and better resolutions in separation of of all analytes from animal feed.

The published literature (Gallego and Arroyo, 2002; Gallego and Arroyo, 2003a) pointed out the effect of pH values (6 - 11) of phosphate-borate (1:1) buffer, buffer component concentrations (10 to 60 mmol L^{-1}) and effect of organic modifier (methanol, acetonitrile; 3 – 12 %). In our research the presence of 10 % methanol in the electrolyte resulted, in well resolved peaks and the shoulders disappeared. The results demonstrated the separation is better when the pH is between 8 and 9. A 40 mmol L^{-1} (20 mmol L^{-1} borate/20 mmol L^{-1} phosphate buffer) concentration was considered suitable for its good resolution and peak shape. Higher concentration of buffer resulted in peak broadening.

Running voltages effects in the range 5-30 kV were tested using a background electrolyte of 20 mmol L^{-1} borate/20 mmol L^{-1} phosphate buffer, pH 8.4, containing 10-80 mmol L^{-1} sodium dodecylsulphate (SDS) and 10% methanol, without running pressure, at 25℃. The best results were at 25 kV, and an acceptable level of baseline noise was achieved by performing experiments at 25℃.

The influence of SDS in the electrolyte on the migration time (without running pressure) is very significant. The results show that the SDS concentration dramatically affects the migration time of the Zn-BAC and POL B, but not that of SUL and OXY. A concentration of 70 mmol L^{-1} was selected for the experiment as to give the best resolution, but without a short analysis time (20 min) and without good symmetry and widths of Zn-BAC and POL B peaks.

Running pressure was tested in the range 5-30 mbar, using the above experimental conditions. Migration times were decreased with increasing running pressure, and the value of 10 mbar was selected as optimum. It gives the best resolution and symmetric peaks in all cases, and also the analysis time is shorter. Under such experimental conditions the concentration of 20 mmol L^{-1} SDS was selected as the most advantageous. It gave the best separation without broadened and deformed peaks, and acceptable resolution for Zn-BAC and POL B (Figure 1).

The best results were with applying pressure of 10 mbar at 25 kV. UV detection was at 215 and 254 nm for Zn-BAC, SUL and OXY, 205 and at 215 nm for POL B, for simultaneous detection 215 nm was selected. The electropherograms obtained in the separation under selected conditions are presented in Figure 2. It is remarkable that all peaks have good resolutions in a run time of 12 min (OXY 3.1; SUL 3.7; POL B 9.3; Zn-BAC 10.6). The conditions of MECK with SDS was exceptionally suitable for OXY, since it is eluted as the first peak without any interferences, confirming that this technique could be more efficient in comparison to HPLC, since Oka et al. (2000) pointed out that EDTA and oxalic acid should be used during each analytical step (extraction, clean up and HPLC separation). Our recently published paper (Injac *et al.* 2007) denoted sufficient difference in migration time for two tetracycline antibiotic OXY and doxycycline.

Validation of the method

The characteristics and the procedures used for validation were those described in USP 24 (USP 24, 2000), the International Conference of Harmonization (ICH) Guidelines (ICH Q2A, 1995; ICH Q2B, 1997) and other literature (Heyden et al., 1999; Shabin, 2003; Brown et al., 2001; Toro et al., 2004; Altria and Chanter, 1993; Injac et al., 2007).

Selectivity

The selectivity of the method was investigated by observing interfering peaks from matrix present in the feedstuffs. Five different feedstuff mixtures were tested for the interferences. There was no interference in MEKC results by the feed's ingredients in any of the tested mixtures, which indicates that the method is selective.

Linearity

The linearity of the assay was determined by analysis of a series of standards at five different concentrations in range of 0.1 to 100 mg kg^{-1} for each compound.

The linearity of calibration curves (peak area vs. concentration) for OXY, SUL, POL B and Zn-BAC over the concentration ranges of 6.5 – 77.2 mg kg^{-1} for OXY, 0.3 – 84.5 mg kg^{-1} for SUL, 18.5 – 85.0 mg kg^{-1} for POL B and 12.1 – 62.4 mg kg^{-1} for Zn-BAC gave correlation coefficients of 0.9994, 0.9997, 0.9987 and 0.9991, respectively.

Limit of detection (LOD) and limit of quantification (LOQ)

LODs and LOQs were estimated by the baseline noise method. Baseline noise was evaluated by recording the detector response over a period of ten times the peak width. LOD and LOQ, respectively, were defined as the analyte concentrations resulting in peaks of height three and ten times the baseline noise level ((ICH Q2A, 1995; ICH Q2B, 1997; Heyden et al., 2001). Thus, LODs/LOQs are 1.6/6.2, 0.1/0.3, 6.0/17.5 and 3.7/12.3 mg kg^{-1} for OXY, SUL, POL B and Zn-BAC, respectively.

Accuracy

The accuracy of the method was determined by analyzing a solution of known concentration (working standard solution – spiked feed samples with standards) and comparing the measured and known values. Fifteen determinations were carried out for five concentration levels. The mean recovery was 99.7 ± 0.3, 99.9 ± 0.9, 99.81 ± 1.0 and 99.5 ± 0.4 for OXY, SUL, POL B and Zn-BAC, respectively, proving a good accuracy of the method.

Precision

Precision can be measured as repeatability, reproducibility, and intermediate precision. In this work only repeatability and intermediate precision were studied.

Repeatability

A repeatability test was performed to determine intra-day variation in corrected areas and migration times. Working solutions of concentrations 20, 40 and 60 mg L^{-1} (relative to 20, 40 and 60 mg kg^{-1}) were analyzed (n = 6). The RSD values for

migration times (0.29 % for OXY, 0.13 % for SUL, 0.94 % for POL B and 0.82 % for Zn-BAC) and for peak areas (0.92-1.76 % OXY, 0.57-0.77 % SUL, 1.12-1.53 % POL B and 0.85-1.72 % Zn-BAC) indicate that the repeatability of the method is acceptable.

Intermediate Precision

Intermediate precision was evaluated over three days by performing working solutions of concentrations 20-50 mg L^{-1} (relative to 20-50 mg kg^{-1}). These were injected on each of three days under the same conditions and the results were used for the repeatability study. When stored in the dark refrigerated, the recovery ranged from 100.2 to 99.1 % over three days. The RSD values (0.12-0.34 % for OXY, 0.09-0.25 % for SUL, 0.39-0.83 for POL B and 0.42-0.81 % for Zn-BC) indicate that the intermediate precision is acceptable. When they were stored at room temperature in sunlight, decreasing recovery values from 100.15 to 94.81 % for Zn-BAC, from 100.40 to 95.03 % for POL B and from 100.01 to 92.98 % for OXY were observed for the standards in BGE.

Robustness

The optimum MEKC conditions set for this method have been slightly modified in order to evaluate the robustness. The effects of different concentrations of SDS (20 ± 1 mmol L^{-1}) and organic modifier (10 ± 0.5 % methanol) in the mobile phase, as well as the effects of buffer pH (8.4 ± 0.06), capillary temperature (25 ± 5℃), applied pressure (10 ± 1 mbar), running voltage (25 ± 1 kV) and detection wavelength (± 3 nm), and were determined. The design applied was the

fractional factorial design (Heyden et al., 2001). No significant variations in accuracy, specificity and precision were found over the tested ranges, which indicated that the method conditions are robust.

Stability of antibiotics

The stability of OXY, SUL, POL B and Zn-BAC in acidic (pH < 5) and basic (pH > 10) solutions was checked in test samples at room temperature for 24 and 48 h and the recoveries were 98.2 ± 2.3 % at 24 h and 95.8 ± 3.8 % at 48 h. The stability in the BGE was also checked at 24 h. Recoveries of each compounds were ≥ 99.3 %. It was indicating good stability.

Application

The present method was tested by determining OXY, SUL, POL B and Zn-BAC in animal feedstuffs. When analyzing spiked commercial products, the amounts and recoveries obtained were determined by comparing the results with a standard solution containing the same concentration as expected in the spiked commercial products. The results presented in Table I show good agreement between the claimed and found values. Recovery values also confirmed that, due to the sufficient solubility of investigated compounds, extraction procedure was efficient.

According to LOQ values for each analyte, the minimum quantifiable amounts with the proposed method are 6.2, 0.3, 17.5 and 12.3 mg kg^{-1} (ppm) of OXY,

SUL, POL B and Zn-BAC, respectively in feed. The sensitivity of the proposed method is acceptable in relation to the efficiency levels of OXY, SUL, POL B and Zn-BAC as additives and can be utilized for routine control of animal feedstuffs. The obtained results are in correlation that CE is already an official method in USP will also be valuable method as the alternative to HPLC (S. Görög, 2007) and the precision of MECK (Injac *et al.*, 2007) confirmed the applicability in routine quality control.

The advantage of proposed method over the HPLC and TLC methods described in literature for analysis OXY, SUL, POL B and Zn-BAC in different samples is its lower running costs and higher environmental friendliness. In the proposed method, 20-30 analyses with MEKC require 2 mL of phosphate/borate buffer containing SDS and 10 % (v/v) methanol, while 20 analyses by HPLC require 300 to 1000 mL of mobile phase with different proportion of most commonly used organic solvent (methanol, acetonitrile, acetone, THF). The advantage of LC (MS; MS/MS) methods over MECK is evident for the analysis of residues in samples of different tissues (Bogialli *et al.*, 2003).

CONCLUSION

The MEKC method presented here is a useful technique for rapid separation (within 12 min) of zinc bacitracin, polymixin B, oxytetracycline and sulfacetamide at low concentration of surfactant (SDS 20 mmol L^{-1}) and pH 8.4 (phosphate-borate buffer). A pressure of 10 mbar was applied and it gives the best resolution

and symmetric peaks in all cases, special for POL B and Zn-BAC. This system was also applied successfully to their identification and assay in animal feedstuff, with different matrixes, spiked with zinc bacitracin, polymixin B, oxytetracycline and sulfacetamide.

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Figure captions (R. Injac et al.)

Figure 1.

Effect of SDS concentration on resolution with a applied pressure of 10 mbar. The electrolyte solution was 20 mmol L^{-1} phosphate + 20 mmol L^{-1} borate buffer, pH 8.4, containing 10% methanol, and the temperature and voltage were 25°C and 25 kV, respectively.

Figure 2.

Electropherograms obtained **A**) 100 mg kg^{-1} of Zn-BAC, POL B, SUL and OXY standards, and **B**) 50 mg kg^{-1} of Zn-BAC, POL B, SUL and OXY from spiked animal feedstuff; under the optimized conditions, at 215 nm. The BGE was 20 mmol L^{-1} phosphate + 20 mmol L^{-1} borate buffer, pH 8.4, containing 20 mmol L^{-1} SDS and 10% methanol, the temperature and voltage were 25°C and 25 kV, respectively with applied pressure of 10 mbar.

Table I.

Application results for spiked feedstuff samples.

Table I R. Injac et al.

Tested Sample (*n = 10*)	Amount expected (mg)	OXY Amount found (mg)	OXY Recovery (%)	SUL Amount found (mg)	SUL Recovery (%)	POL B Amount found (mg)	POL B Recovery (%)	Zn-BAC Amount found (mg)	Zn-BAC Recovery (%)
K-19	50	49.9±0.1	99.8	48.9±0.5	97.8	48.9±0.4	97.8	49.3±0.3	98.6
TL-TIP	50	49.9±0.2	99.8	49.8±0.3	99.6	48.8±0.3	97.6	49.2±0.4	98.4
BEK-1	50	50.0±0.1	100.0	49.9±0.1	99.8	49.3±0.8	98.6	50.2±0.3	100.4
NSK-1	50	49.8±0.1	99.6	49.7±0.4	99.4	49.5±0.5	99.0	50.3±0.1	100.6
BOVISAL	50	50.1±0.3	100.2	49.8±0.2	99.6	49.1±0.6	98.2	49.7±0.7	99.4

Figure 1 R. Injac et al.

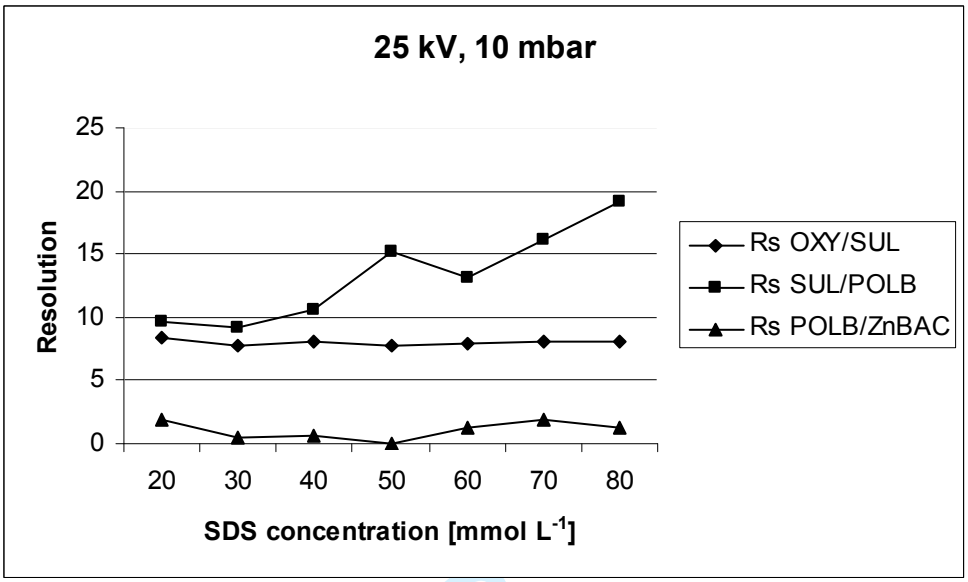

Figure 2A R. Injac et al.

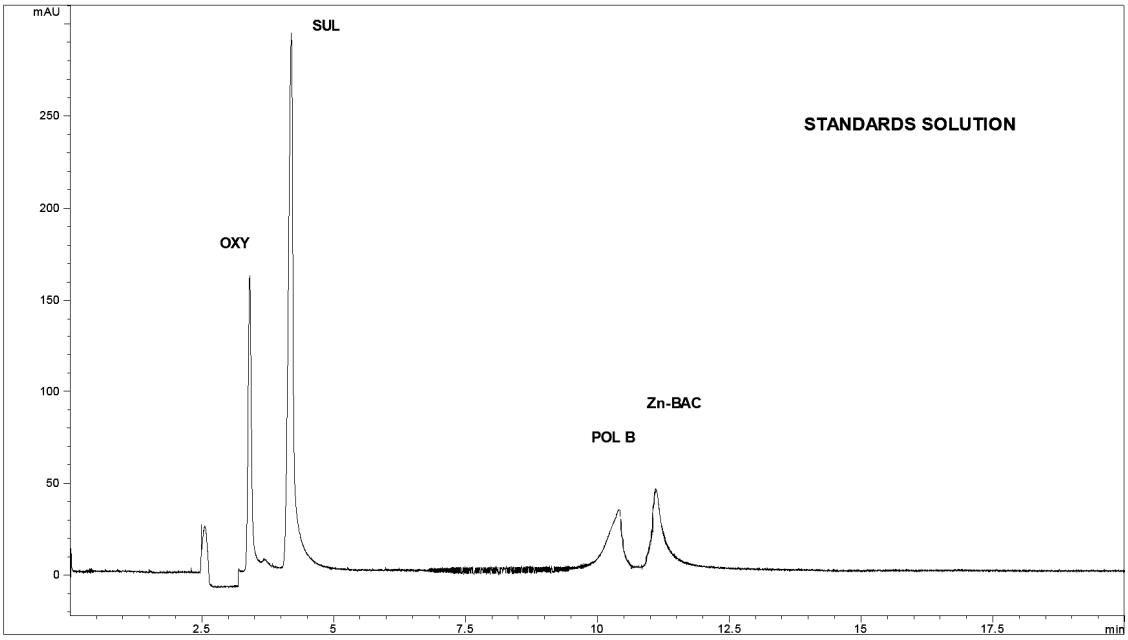

Figure 2B R. Injac et al.