Determination of sulphonamides in pork meat extracts by capillary zone electrophoresis

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ABSTRACT

For sixteen sulphonamides the effective mobility was measured as a function of pH and from the effective mobilities determined in two different electrolyte systems the pK value and mobility at infinite dilution were calculated. A pH of 7.0 was found to be the optimum pH for the separation for both standard mixtures and mixtures of sulphonamides dissolved in pork meat extracts. For the determination of the sulphonamides in pork meat only a very simple sample pretreatment consisting of extraction with acetonitrile and centrifugation is suitable, as the matrix effects at pH 7.0 do not affect the separation. Calibration graphs for five sulphonamides were constructed, and regression coefficients of at least 0.999 were obtained. The limit of detection for the method varies from 2 to 9 ppm for a pressure injection time of 10 s (injection volume *ca.* 18 nl) using a Polymicro Technology capillary of length 116.45 cm, distance between injection and detection 109.75 cm and I.D. 50 μm.

INTRODUCTION

Many veterinary drugs in use for food-producing animals are available as injectable preparations. Depending on the formulation, the use of these preparations may give rise to residual amounts of the drug at the site of injection [1,2]. Moreover, the use of these preparations may lead to considerable local irritation of the tissue, leading to visible injection sites when treated animals are slaughtered. According to the law, visible injection sites must be removed from the carcass in Netherlands. For regulatory purposes it is often of interest to know which drug has been used, *e.g.*, when the use of an illegal drug is suspected. In order to identify the drugs present at injection sites of slaughtered animals, a selective and routinely applicable analytical method is required. As often fairly high concentrations of drugs will be found at injection sites, owing to incomplete [3] or slow [4] adsorption of the drugs,

there are no special requirements regarding the sensitivity of the method. Most recently developed methods allow the selective detection of a single component, or a selected group of components, at very low concentrations [5–7], albeit that multiscreening methods have been reported [8]. Owing to this limited applicability and/or the necessary extensive purification of the samples, and sometimes derivatization, these methods are not considered suitable for our purposes.

In this paper the applicability of capillary zone electrophoresis (CZE) for the determination of sulphonamides in pork meat extracts is described. The group of sulphonamides was chosen because its members are present in injectable formulations. Moreover, this group of drugs consists of a relatively large number chemically related compounds and studies of this group will yield information concerning the applicability and the selectivity of the technique when applied to meat extracts. In the

determination of sulphonamides in pork meat extracts using CZE, the most important questions to be answered are: (1) can the sulphonamides be separated from the matrix; (2) how can the sulphonamides be identified; and (3) are linear calibration graphs obtained and what is the limit of detection?

EXPERIMENTAL

Chemicals and reagents

The structural formulae of the compounds studied are shown in Fig. 1. Trimethoprim, sulphamethoxazole, sulphanilamide, sulphadimidine-Na, sulphathiazole, sulphamerazine, sulphadiazine, sulphadoxine, sulphamethoxypyridazine, sulphadimethoxine, sulphatroxazole, sulphaquinoxaline,

sulphamethoxydiazine, sulphaguanidine, sulphachloropyrazine-Na and sulphachloropyridazine were supplied by the Netherlands State Institute for Quality Control for Agricultural Products (RIKILT). Stock solutions standard (1 mg/ml) were prepared by weighing exactly 10.0 mg of the component and dissolving it in 10.0 ml of solvent. These solutions were diluted to the desired concentrations with distilled water to give working standard solutions.

All other chemicals were of analytical-reagent grade.

Instrumentation

All CZE experiments were performed using a P/ACE System 2000 HPCE instrument (Beckman Instruments, Palo Alto, CA, USA). The working temperature was 25°C. Two capillaries were used, *viz.*, an original Beckman standard capillary of length 57 cm, distance between injection and detection 50 cm, I.D. 75 μm, where a 1-s pressure injection equals an injection volume of *ca.* 19 nl, and a capillary from Polymicro Technologies (Phoenix, AZ, USA) of length 116.45 cm, distance between injection and detection 109.75 cm, I.D. 50 μm, where a 1-s pressure injection equals an injection volume of *ca.* 1.8 nl. The sample was introduced by means of pressure injection and in all experiments a constant voltage was applied. The wavelength of the UV detector was 254 nm in all experiments.

Sample pretreatment

For the pork meat extracts, 10 g of pork meat were homogenized in a household food processor and extracted in a stomacher apparatus for 5 min with 100 ml of acetonitrile. The sample was centrifuged for 10 min at 4000 g and filtered through a 0.45-μm filter. If the sample had to be spiked, this was done between the centrifugation and the filtration steps. The filtrate was used for injection.

RESULTS AND DISCUSSION

Determination of effective mobilities

The effective mobility is an important parameter in CZE because differences in effective mobilities determine whether components can be separated or not, and because separated components can be identified by their effective mobilities. As described
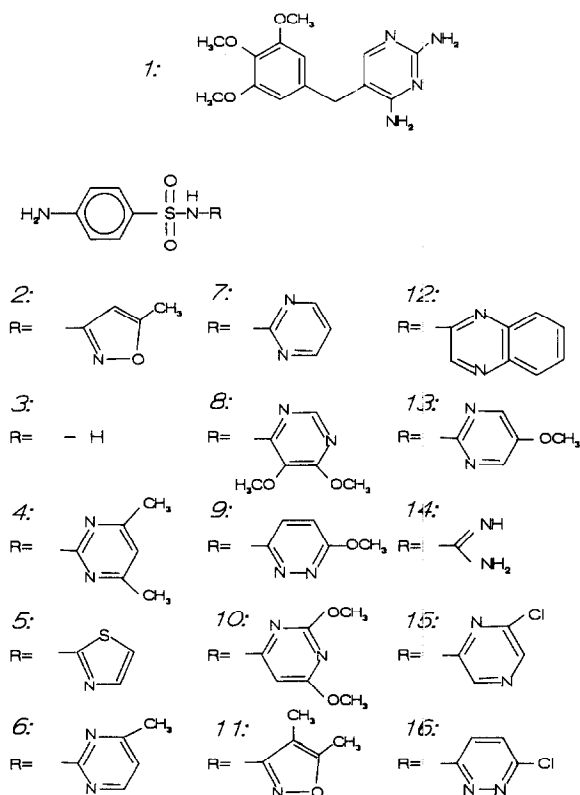

Fig. 1. Structural formulae of (1) trimethoprim, (2) sulphamethoxazole, (3) sulphanilamide, (4) sulphadimidine, (5) sulphathiazole, (6) sulphamerazine, (7) sulphadiazine, (8) sulphadoxine, (9) sulphamethoxypyridazine, (10) sulphadimethoxine, (11) sulphatroxazole, (12) sulphaquinoxaline, (13) sulphamethoxydiazine, (14) sulphaguanidine, (15) sulphachloropyrazine and (16) sulphachloropyridazine.

previously [9], the effective mobility m_{eff} can be calculated from the mobility of the electroosmotic flow (EOF), m_{EOF}, and the apparent mobility, m_{app}, of the component according to

$$m_{eff} = m_{app} - m_{EOF} = \frac{l_d l_c}{V}\left(\frac{1}{t_s} - \frac{1}{t_{EOF}}\right) (cm^2/V \cdot s) \quad (1)$$

where l_c and l_d are the length of the capillary and the length between injection and detection (cm), V is the applied voltage (V) and t_s and t_{EOF} are the migration times of the component and the EOF (s). As the velocity of the EOF, v_{EOF}, can vary with time, the migration time or apparent mobility of a component cannot be used for identification.

For the determination of the effective mobilities, the background electrolytes and separation conditions given in Table I were used. The effective mobilities were determined with both the Polymicro Technologies and the Beckman standard capillaries. Table II gives the effective mobilities for the sixteen sulphonamides for both capillaries at four pH values. The voltage applied in the experiments with capillary II was lower in order to avoid a large difference between the electric currents. At pH 3.2 the effective mobilities in the long Polymicro Technologies capillary could not be determined because the velocity of the EOF was so low that the analysis time became about 80 min, causing very broad peaks so that precise migration times could not be ob-

tained. From Table II it can be concluded that the effective mobilities determined with both capillaries agree. The migration times of components not completely separated from the EOF marker are often difficult to determine, resulting in imprecise effective mobilities. In Fig. 2 the effective mobilities as a function of the pH of the background electrolyte for the Beckman standard capillary are given.

As can be seen in Fig. 2, the differences between the effective mobilities are optimum at pH 7. At this pH most of the sulphonamides can be separated from each other. Nevertheless, two pairs of sulphonamides cannot be separated, viz., sulphanilamide and sulphaguanidine, which are both uncharged at this pH ($m_{eff} = 0$) and can be used as EOF markers, and sulphathiazole and sulphamethoxypyridazine, which have very similar m_{eff} values (average -6.42×10^{-5} and -6.71×10^{-5} cm^2/V · s, respectively). Sulphadiazine, sulphadoxine and sulphadimethoxine are not completely separated, but three tops of peaks can be determined if all three are present.

As expected, the components are better separated in the Polymicro Technology capillary, which has a longer separation length. Fig. 3 shows the electropherograms obtained using the Beckman standard capillary for a mixture of twelve sulphonamides dissolved in water–acetonitrile (90:10) using a background electrolyte of (A) 0.02 M imidazole–acetate

TABLE I

BACKGROUND ELECTROLYTES AND SEPARATION CONDITIONS FOR THE DETERMINATION OF THE EFFECTIVE MOBILITIES OF THE SULPHONAMIDES

I, Polymicro Technologies capillary, length 116.45 cm, distance between injection and detection 109.75 cm, I.D. 50 μm. II, Beckman standard capillary, length 57 cm, distance between injection and detection 50 cm, I.D. 75 μm. The background electrolytes were prepared by adding the acid to the cationic component until the desired pH was reached, except for the phosphate–borate buffer, where KOH was added to the mixture of the acids until the desired pH was reached.

Background electrolyte[a]	pH	Applied voltage (kV) with current (μA) in parentheses		Injection time (s)	
		I	II	I	II
0.02 M Tris–acetate	8.2	30 (3.7)	15 (8.5)	5	2
0.01 M imidazole–acetate	7.0	30 (4.9)	10 (8.4)	5	2
0.02 M phosphate–0.02 M borate	7.0	30 (16.7)	10 (26.8)	5	2
0.01 M Tris–MES	6.5	30 (2.9)	10 (4.6)	5	2
0.01 M Tris–acetate	5.0	30 (3.7)	10 (5.7)	5	2
0.01 M Tris–formate	3.2	30 (5.2)	10 (8.1)	5	2

[a] Tris = tris(hydroxymethyl)aminomethane; MES = 2-(N-morpholino)ethanesulphonic acid.

TABLE II

EFFECTIVE MOBILITIES × 10⁵ (cm²/V · s) FOR THE SIXTEEN SULPHONAMIDES AT DIFFERENT pH VALUES OF THE BACKGROUND ELECTROLYTES

Component	Capillary[a]	Effective mobility			
		pH 8.2	pH 7.0[b]	pH 5.0	pH 3.2
Trimethoprim	I	1.50	11.25	18.76	
	II	1.87	11.73	19.08	20.26
Sulphamethoxazole	I	−21.55	−20.48	−4.02	
	II	−21.96	−21.15	−4.30	0.00
Sulphanilamide	I	0.00	0.00	0.00	
	II	0.00	0.00	0.00	1.30
Sulphadimidine	I	−13.91	−2.92	0.00	
	II	−14.26	−2.91	0.00	1.65
Sulphathiazole	I	−19.41	−6.47	0.00	
	II	−19.67	−6.36	0.00	1.21
Sulphamerazine	I	−18.83	−9.57	−1.63	
	II	−18.98	−9.49	0.00	1.41
Sulphadiazine	I	−21.01	−16.03	−1.41	
	II	−21.29	−16.39	−0.96	0.89
Sulphadoxine	I	−18.54	−16.81	−2.43	
	II	−18.65	−17.24	−2.59	0.31
Sulphamethoxypyridazine	I	−17.77	−6.61	−0.23	
	II	−17.83	−6.81	0.00	1.05
Sulphadimethoxine	I	−19.16	−17.15	−2.19	
	II	−19.33	−17.37	−2.31	0.47
Sulphatroxazole	I	−20.31	−19.22	−3.61	
	II	−20.49	−19.65	−3.69	0.00
Sulphaquinoxaline	I	−19.49	−18.20	−2.38	
	II	−19.60	−18.59	−3.84	0.45
Sulphamethoxydiazine	I	−19.38	−12.49	−0.58	
	II	−19.35	−12.83	−0.76	0.49
Sulphaguanidine	I	0.00	0.00	0.00	
	II	0.00	0.00	0.00	2.21
Sulphachloropyrazine	I	−21.18	−21.21	−10.46	
	II	−21.36	−21.73	−9.50	0.32
Sulphachloropyridazine	I	−20.85	−20.26	−5.65	
	II	−20.89	−20.64	−4.96	0.41

[a] See Table I.
[b] For pH 7.0 the imidazole–acetate system is used.

and (B) 0.02 *M* phosphate–0.02 *M* borate and of a mixture of twelve sulphonamides dissolved in the pork meat matrix using a background electrolyte of (C) 0.02 *M* imidazole–acetate and (D) 0.02 *M* phosphate–0.02 *M* borate. As can be seen in Fig. 3A and B, the separation of the standard mixture is good in both background electrolytes. Nevertheless, the effective mobilities for some components are different in both background electrolytes (possibly owing to a slight difference in the pH and the nature of the two electrolyte solutions) but, as further experiments showed, the effective mobilities are reproducible and constant in each background electrolyte. As can be seen in Fig. 3C and D, the separation of the mixture in the matrix is much better in the phosphate–borate buffer than in the imidazole buffer, showing sharper peaks. For quantitative analysis, therefore, the 0.02 *M* phosphate–0.02 *M* borate buffer is used.
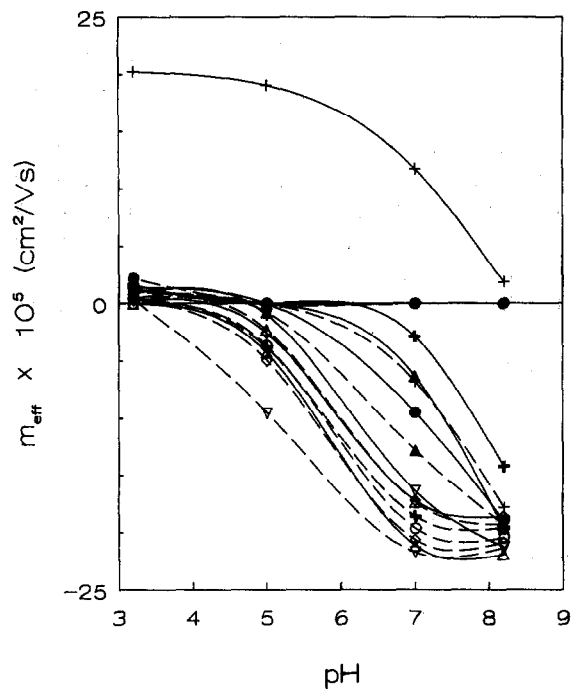
Fig. 2. Effective mobility as a function of the pH of (solid lines) (+) 1; (△) 2; (○) 3; (+) 4; (▲) 5; (●) 6; (▽) 7; (◇) 8; and (dotted lines) (+) 9; (△) 10; (○) 11; (+) 12; (▲) 13; (●) 14; (▽) 15; (◇) 16. For the names of the components, see Fig. 1.
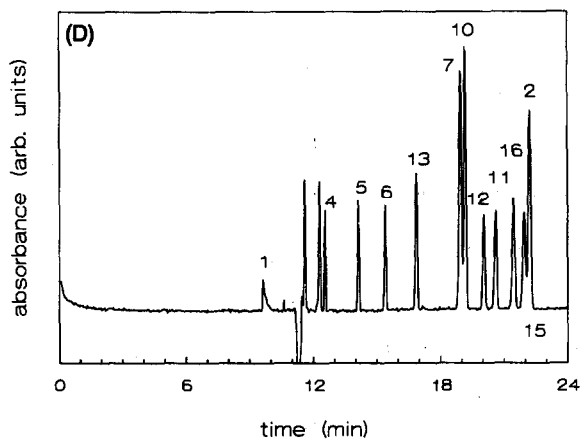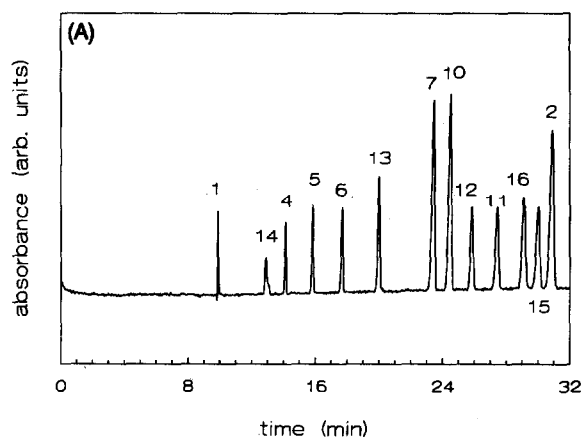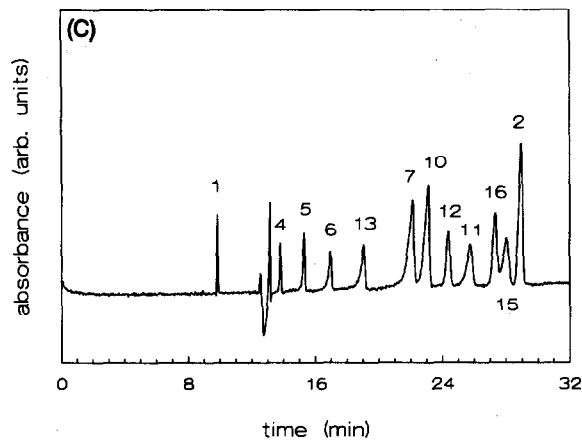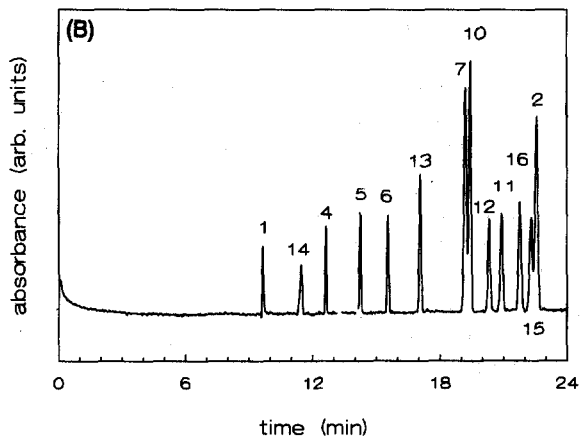
Fig. 3. Electropherograms of the separation of twelve sulphonamides (0.01 mg/ml) in the Beckman standard capillary (length between injection and detection 50 cm, pressure injection time 2 s ≈ 39 nl, applied voltage 10 kV) of standard mixture in the background electrolyte (A) 0.02 M imidazole–acetate at pH 7 and (B) 0.02 M phosphate–0.02 M borate at pH 7 and the standard mixture dissolved in the pork meat matrix in the background electrolyte (C) 0.02 M imidazole–acetate at pH 7 and (D) 0.02 M phosphate–0.02 M borate at pH 7. For the names of the components, see Fig. 1.

Calculation of pK values and mobilities at infinite dilution

If the effective mobility of a component is known for two different electrolyte systems at different pH values, at which the component shows a different degree of dissociation, both its pK value and its mobility at infinite dilution can be calculated [9]. In the calculations the best values are obtained if the pK value of the component lies between the pH values of the background electrolytes and if the effective mobilities are not too small. For components with pK values of about 7–8, background

electrolytes at pH 8.2 and 7 are chosen. For components with pK values of about 5–7, both background electrolytes with pH 7 and 5 and pH 8.2 and 5 are chosen.

The pK values of sulphadiazine and sulphamethoxydiazine were calculated from the background electrolytes at pH 8.2 and 6.5. Table III gives the pK values and mobilities at infinite dilution for fourteen sulphonamides calculated from effective mobilities in the different background electrolytes, and the pK values as found in the literature [10,11]. Sulphanilamide and sulphaguanidine are not mentioned in

TABLE III

CALCULATED pK VALUES AND MOBILITIES AT INFINITE DILUTION, $m_0 \times 10^5$ (cm^2/V·s), FOR THE SULPHON-AMIDES USING THE EFFECTIVE MOBILITIES OF THE COMPONENT IN BACKGROUND ELECTROLYTES WITH THE INDICATED pH VALUES, AND LITERATURE VALUES FOR pK VALUES

Component	Capillary[a]	pK (lit.)	pH 8.2–7.0[b]		pH 8.2–5.0		pH 7.0[b]–5.0	
			pK	m_0	pK	m_0	pK	m_0
Trimethoprim	I	6.6	7.11	23.59	7.13	22.53		
	II		7.26	21.75	7.23	22.83		
Sulphamethoxazole	I				5.73	−25.40	5.72	−25.05
	II				5.70	−25.82	5.70	−25.73
Sulphadimidine	I	7.4	7.82	−22.36				
	II		7.83	−23.09				
Sulphathiazole	I	7.2	7.48	−26.25				
	II		7.50	−26.73				
Sulphamerazine	I	7.0	7.13	−23.90				
	II		7.15	−24.12				
Sulphadiazine[c]	I	6.4			6.55	−25.18		
	II				6.52	−25.45		
Sulphadoxine	I	6.1			5.91	−22.34	5.89	−21.49
	II				5.88	−22.45	5.87	−21.90
Sulphamethoxypyridazine	I	6.7	7.37	−23.81				
	II		7.39	−23.77				
Sulphadimethoxine	I	6.2			5.98	−22.99	5.95	−22.04
	II				5.96	−23.16	5.93	−22.22
Sulphatroxazole	I	5.8			5.75	−24.13	5.74	−23.76
	II				5.75	−24.32	5.74	−24.12
Sulphaquinoxaline	I	5.5			5.94	−23.32	5.94	−23.15
	II				5.70	−23.40	5.68	−22.98
Sulphamethoxydiazine[c]	I				6.75	−23.70		
	II				6.73	−23.76		
Sulphachloropyrazine	I	5.1			5.10	−24.97	5.10	−25.12
	II				5.18	−25.16	5.20	−25.72
Sulphachloropyridazine	I				5.52	−24.66	5.51	−24.47
	II				5.59	−24.71	5.60	−25.00

[a] See Table I

[b] For pH 7.0 the imidazole–acetate system is used.

[c] Calculated with background electrolyte systems at pH 8.2 and 6.5.

Table III because they have very small effective mobilities in all electrolyte systems. From Table III it can be concluded that the calculated pK values and mobilities at infinite dilution obtained from the effective mobilities of both capillaries with the same background electrolyte systems agree.

Matrix effects

Because for a routine method the pretreatment should be very simple, we studied the matrix effects for a pork meat extract where the sample pretreatment consisted only of extraction of the homogenized meat with acetonitrile and centrifugation. This acetonitrile sample was directly used for injection. In Fig. 4 the electropherograms of (a) a mixture of thirteen sulphonamides dissolved in water and (b) the pure matrix in the background electrolytes (A) 0.02 M Tris–acetate at pH 8.2, (B) 0.02 M phos-

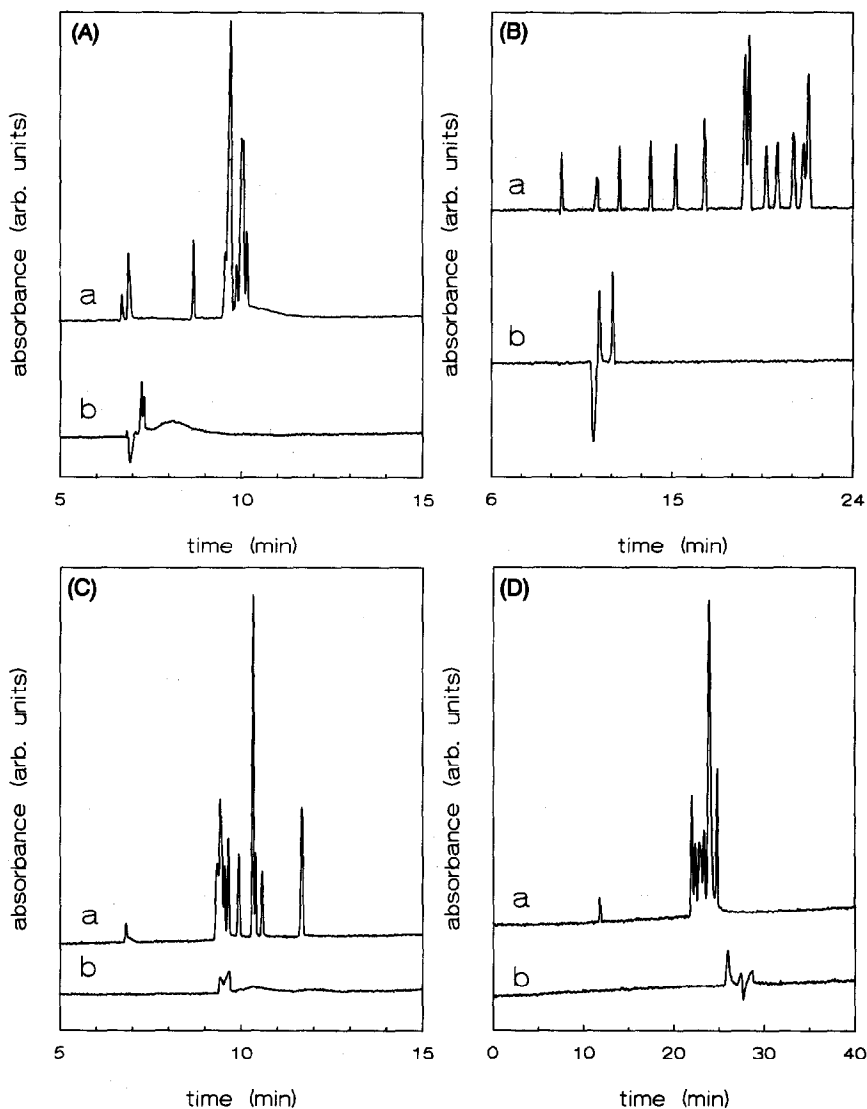Fig. 4. Electropherograms of (a) a mixture of thirteen sulphonamides (0.01 mg/ml) and (b) the pure matrix in the background electrolytes (A) 0.02 M Tris–acetate (pH 8.2), (B) 0.02 M phosphate–0.02 M borate (pH 7.0), (C) 0.01 M Tris–acetate (pH 5.0) and (D) 0.01 M Tris–formate (pH 3.2). Beckman standard capillary, length 57 cm, distance between injection and detection 50 cm, I.D. 75 μm; applied voltage, 10 kV; pressure injection time, 2 s $\approx$ 39 nl.

TABLE IV

EFFECTIVE MOBILITIES, $m_{eff} \times 10^5$ (cm^2/V · s) FOR TWELVE SULPHONAMIDES DETERMINED SEPARATELY, IN THE MIXTURE, AND IN THE MATRIX SPIKED WITH THE MIXTURE

Background electrolyte, 0.02 M imidazole–acetate at pH 7.0; Beckman standard capillary, length 57 cm, distance between injection and detection 50 cm, I.D. 75 μm; applied voltage 10 kV.

Component	Separately	Mixture	Matrix with mixture
Trimethoprim	11.49	11.70	11.19
Sulphadimidine	−2.92	−3.01	−3.40
Sulphathiazole	−6.42	−6.35	−6.52
Sulphamerazine	−9.53	−9.38	−9.44
Sulphamethoxydiazine	−12.66	−12.45	−12.53
Sulphadiazine	−16.21	−15.88	−15.89
Sulphadimethoxine	−17.26	−16.92	−17.02
Sulphaquinoxaline	−18.40	−18.12	−18.28
Sulphatroxazole	−19.43	−19.08	−19.22
Sulphachloropyridazine	−20.45	−20.10	−20.35
Sulphamethoxazole	−20.82	−20.54	−20.70
Sulphachloropyrazine	−21.47	−21.40	−21.28

phate–0.02 M borate at pH 7.0, (C) 0.01 M Tris–acetate at pH 5.0 and (D) 0.01 M Tris–formate at pH 3.2 are given. It can be clearly seen that not only for the separation, but also for the matrix effects the background electrolyte at pH 7.0 gives the best results. The velocity of the EOF can vary from experiment to experiment and from this it seems that the components in Fig. 4D migrate in front of the matrix components. Further experiments showed, however, that this is only due to the change in the velocity of the EOF. The determination of sulphaguanidine and sulphanilamide in the matrix is problematic because these components are uncharged at pH 7.0 and migrate at the position of the EOF in the acetonitrile plug.

Table IV gives the effective mobilities for twelve sulphonamides determined separately, in the mixture and in the matrix spiked with the mixture, applying a background electrolyte at pH 7. For each component the effective mobilities determined in the mixture and in the matrix spiked with the mixture are comparable, and hence the effective mobility can be used for peak recognition.

Quantification

In order to evaluate the quantitative abilities of the method, calibration graphs for five sulphonamides in acetonitrile and in the matrix were constructed using the 0.02 M phosphate–0.02 M borate

buffer (pH 7.0) and the Polymicro Technologies capillary. For the determination of the calibration graphs 10.0 mg of sulphadimidine-Na, sulphamerazine, sulphadoxine, sulphatroxazole and sulphamethoxazole were weighed accurately and dissolved in 10.0 ml of acetonitrile or matrix. From these solutions dilutions were made at concentrations of 0.1, 0.01 and 0.001 mg/ml. Each of the solutions was used for injection with pressure injection times of 3, 5, 10, 15, 20, 25 and 30 s (a 1-s pressure injection equals an injection volume of *ca.* 1.8 nl; sometimes the dimension μg · s/ml is used, which means the product of the pressure injection time and the concentration; 1 μg · s/ml = 1.8 pg). Peak areas were determined using the laboratory-written data analysis program CAESAR. From the calibration graphs, the limit of detection (LOD) for the method can be evaluated. For the calculation of the LOD the value $y_B + 3s_B$ is used, whereby the calculated intercept of the regression line is used as an estimate of y_B and s_B is the standard deviation in the y-direction of the regression line [12]. Table V gives the regression coefficient and LOD (μg · s/ml) for the calibration graphs for the five sulphonamides. The regression coefficients were calculated with the data of the three concentration decades and for the determination of the LOD the calibration graph a sample concentration of 0.001 mg/ml was used. As can be concluded from Table V, the regression

TABLE V

REGRESSION COEFFICIENTS AND LIMITS OF DETECTION FOR THE CALIBRATION GRAPHS OF SULPHADIMIDINE, SULPHAMERAZINE, SULPHADOXINE, SULPHATROXAZOLE AND SULPHAMETOXAZOLE DISSOLVED IN (A) ACETONITRILE AND (B) THE PORK MEAT MATRIX

For the determination of the LOD the calibration graph in the lowest concentration decade was used. The regression coefficient was calculated using the data over the three concentration decades.

System	Component	Regression coefficient	LOD ($\mu g \cdot s/ml$)
A	Sulphadimidine	0.9996	4.8
	Sulphamerazine	0.9995	1.9
	Sulphadoxine	0.9994	8.8
	Sulphatroxazole	0.9993	6.3
	Sulphamethoxazole	0.9994	3.7
B	Sulphadimidine	0.9997	5.5
	Sulphamerazine	0.9994	4.4
	Sulphadoxine	0.9991	3.9
	Sulphatroxazole	0.9991	6.8
	Sulphamethoxazole	0.9991	2.8

coefficients are fairly good (>0.999) and the limit of detection for the method lies between 2 and 9 $\mu g \cdot s/ml$. For the given sample pretreatment and an injection of time of 10 s, the value of the LOD in $\mu g \cdot s/ml$ in the extract equals the value of the LOD in ppm in the meat sample, assuming 100% recovery.

CONCLUSIONS

Effective mobilities can be used for the identification of components, and they were measured for sixteen sulphonamide drugs in two different capillaries as a function of pH. From these experiments it can be concluded that a pH of the background electrolyte of 7 is the optimum pH for separation. At this pH, fourteen of the drugs can be determined and twelve of them can be identified using the effective mobilities. For some drugs, however, a further confirmation such as UV absorbance ratios or diode-array detection should be used. From the effective mobilities the pK values and the mobilities at infinite dilution for the sulphonamides were calculated.

For the determination of sulphonamides in pork meat, a very simple pretreatment consisting of an extraction with acetonitrile and centrifugation can be applied. For five sulphonamides the calibration graphs were set up both in acetonitrile and in the matrix. Linear calibration graphs were obtained with regression coefficients of at least 0.999 and limits of detection in the range 2–9 ppm in the sample for a pressure injection time of 10 s (*ca.* 18 nl) using a Polymicro Technology capillary of length 116.45 cm, distance between injection and detection 109.75 cm and I.D. 50 μm.

The applicability of this method for the determination of a variety of veterinary drugs in meat extracts and the possibilities of CZE with diode-aray detection for the peak identification are under investigation.

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