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Simultaneous determination of sulfaquinoxaline, sulfamethazine and pyrimethamine by liquid chromatography

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Abstract

A liquid chromatography method is described to determine sulfaquinoxaline (SQX), sulfamethazine (SMT), and pyrimethamine (PMT), by using a Kromasil C_{18} column and a 40 m*M* NaH_2PO_4 buffer solution, containing 10 m*M* $NaClO_4$ (pH 3.0)–acetonitrile (65:35) as mobile phase. The mobile phase flow-rate and sample volume injected were 1.5 ml/min and 20 μl, respectively and the samples were dissolved in the mobile phase. The limits of quantification were found to be about 180 μg/l (3.6 ng) for each compound. The method was applied in veterinary commercial formulations. Analyses were made by means of the standard addition method, whose results were compared with those obtained by preparing ''tests'' (from the stock solutions) and with those obtained by a capillary electrophoresis method. Both methods showed similar results, and then it was proved that some commercial claimed levels were not in agreement with the obtained results by using our analytical method, as they were in other cases.

Keywords: Sulfaquinoxaline; Sulfamethazine; Pyrimethamine

1. Introduction

Sulfonamides are anti-bacterial, anti-infective agents used widely in medicine and in the normal veterinary use. These drugs are often used in conjunction with other compounds to increase their activities (potentiators).

Sulfonamides have been analysed successfully by thin-layer chromatography (TLC) for decades [1]. Although, thanks to their strong absorption bands with a maximum between 250 and 280 nm, the liquid chromatography (LC) with ultra-violet detection methods have been investigated extensively for the last years. These studies have been applied to biological samples [2–13] as well as in pharmaceutical assays [14,15].

Obviously, there are many possibilities of making combinations among sulfonamides and potentiators. The choice of the combination depends on the illness to treat.

Coccidiosis, consisting in renal stone formation, is one of the best known illnesses in animals. It can be treated with Sulfaquinoxaline (SQX) and/or Sulfamethazine (SMT). For this purpose, Pyrimethamine (PMT) is used as a potenciator (see Fig. 1 for chemical structures). Nevertheless, there is just one reference found for resolving the ternary mixture of SQX, SMT and PMT applied in commercials [16], where the authors propose a method on the basis of the derivative ratio spectrum zero-crossing method, without separation step.

The aim of this work is to point out a more

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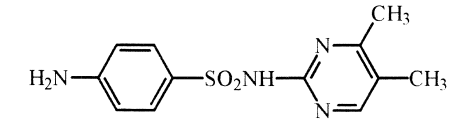
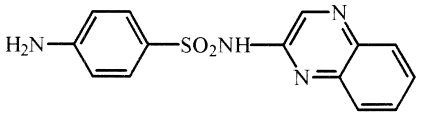
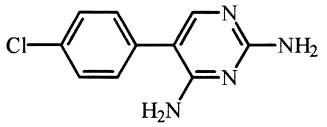


SMT



SQX



PMT

Fig. 1. Chemical structure of the mixture compounds.

selective and sensitive method for the identification and quantification of the sulfonamides (sulfaquinoxaline and sulfamethazine) in association with other compounds, such as pyrimethamine in veterinary formulations, by using LC as analytical technique. Besides, the use of the standard addition method to confirm the obtained results is suggested in order to avoid matrix effect.

2. Experimental

2.1. Apparatus

A Shimadzu L.C. model LC-10A with diode-array detector model SPD-M10A, provided with a double pump system, that allows working in gradient mode, was used. It also comes with a Rheodyne Model 7725 injector with a 20 μl sample loop, and a Silicon 486/33 computer fitted with CLASS-LC 10 software was used for all the measurements and treatment of the data.

The analytical column was a Kromasil C_{18} (150 mm H 4.6 mm's ID, particle size 5 μm).

2.2. Reagents

All solvents and reagents were of analytical grade unless indicated otherwise.

Solutions were made with deionized water (Milli-Q quality).

Sodium salts of Sulfamethazine (SMT) and Sulfaquinoxaline (SQX), as well as Pyrimethamine (PMT) were from Sigma Chemical Co. (Germany and Switzerland). The stock solutions (100 mg/l) of PMT were prepared by dissolving in ethanol, SQX in 25:75 ethanol:water (v:v) and SMT, in deionized water.

NaH_2PO_4, HCl and acetonitrile (LC grade) were from Panreac (Barcelona, Spain).

$NaClO_4$ was from Merck (Darmstadt, Germany).

3. Results and discussion

3.1. Optimization of separation conditions

3.1.1. Preliminary investigations.

In this work, we have used the reverse phase liquid chromatography to study the separation of SQX, SMT and PMT on a Kromasil C_{18} column.

A solution containing 2 mg/l of each one, which shall be called ''Z'', was prepared by diluting the stock solutions of the three compounds. Methanol and acetonitrile as organic solvents in the mobile phase were studied in order to find better selectivity and resolution, using phosphate as a buffer (pH 3.0) in the mobile-phase. The injections resulted in peak broadening when methanol was used, whereas in acetonitrile did not, so acetonitrile was chosen as organic solvent in the composition of the mobile-phase. Before running a separation process, the mobile phase was always filtered by using Millipore filter kit. Dissolved gases were removed with a 10 min helium purge.

At first, a phosphate buffer pH 3 was taken because an acid medium is usually employed in the separation of sulfonamides [16] and because the column works properly from pH 2.7 up to 7. At pH 3, SQX (pK_a=6.7) and SMT (pK_a=8.6) are not charged, but PMT is (pK_a=6.7). This is troublesome for PMT peak accuracy because there might be some interactions with the matrix of stationary phase.

Several experiences about this subject showed that interactions exist indeed: lack of accuracy was observed in PMT peak. Thus, we introduced a bulky anion, perchlorate, to get the ionic pair $PMT^+ClO_4^-$, and avoid PMT interaction with any other chemicals.

Thus, the Kromasil C_{18} column, a 100 m*M* phosphate buffer (pH 3), containing 10 m*M* ClO_4^-: acetonitrile (70:30) as mobile phase and a default selected flow-rate of 1.5 ml/min resulted to be the initial conditions for this chromatographic study.

3.1.2. Effect of the pH and perchlorate concentration

The influence of the pH of the mobile phase on the retention properties was studied. For this purpose, four 0.1 *M* phosphate buffer solutions, adjusted to pH 3, 4, 5 and 6, respectively, were prepared. Chromatograms of the ''Z'' solution were performed in accordance with the above conditions.

Negligible variations on the retention times were reported in these experiments, so a pH 3 was selected for further ones, because at this pH, the sodium phosphate shows a good buffer capacity, SQX and SMT are as neutral molecules and because at lower pH values, the stability of the separation column could be affected.

Nevertheless, it was necessary to adjust the perchlorate concentration in the buffer. For this purpose, some experiments were carried out by changing the $NaClO_4$ concentration between 10 and 50 m*M*. As ClO_4^- concentration was increased, the PMT retention time also increases, whereas the other drug retention times were unaffected. This behaviour confirms that, at pH 3, PMT is as a cation and it forms the ionic pair $PMT^+ClO_4^-$. According to resolution and run time, 10 m*M* was selected as the suitable ClO_4^- concentration (Fig. 2). For a concentration lower than 10 m*M*, SMT and PMT showed the same retention time.

3.1.3. Influence of the phosphate buffer concentration

The phosphate buffer (pH 3.0) concentration was varied from 20 to 100 m*M* and its influence was studied using the experimental conditions mentioned above.

As ionic strength increases, the PMT retention time decreases, whereas no important effect on the others was reported. A 40 m*M* phosphate buffer concentration was considered as suitable because it is sure to keep a pH 3.0 and also a retention time for PMT that provides the best resolutions among the three drugs.

3.1.4. Effect of the organic solvent in the mobile phase and flow-rate on the retention

In these experiences, the ''Z'' stock solution was injected into the column and chromatograms were obtained with mobile phases containing different percentages of acetonitrile (20 up to 40%). Obviously, as acetonitrile concentration increased the analysis time decreased, as expected. A 35% acetonitrile was chosen as suitable, according to the peak resolution and run time as well (Fig. 3).

Then, the influence of the flow-rate of the mobile phase was studied. The retention times and chromatographic resolutions between peaks decreased when the flow-rate of mobile phase increased. A flow-rate of 1.5 ml/min was chosen as a compromise between resolution and analysis time.

Finally, using the conditions above selected, satisfactory chromatographic peak resolutions were obtained in a short analysis time.

3.2. Chromatographic procedure selected

From the studies carried out before, we propose the chromatographic procedure, summarized in Table 1. In Fig. 4, the chromatogram obtained in the separation of SQX, SMT and PMT under the selected conditions is presented. It can be seen that all peaks have good resolution in a run time as short as 4.5 min.

3.3. Quantitative aspects

3.3.1. Limit of detection and limit of quantification

Limits of detection and quantification (LOD and LOQ) were estimated in accordance to the base line noise. The base line noise was evaluated by recording the detector response over a period about 10 times the peak width. The LOD was obtained as the sample concentration that caused a peak with a height three-fold the base line noise level and the

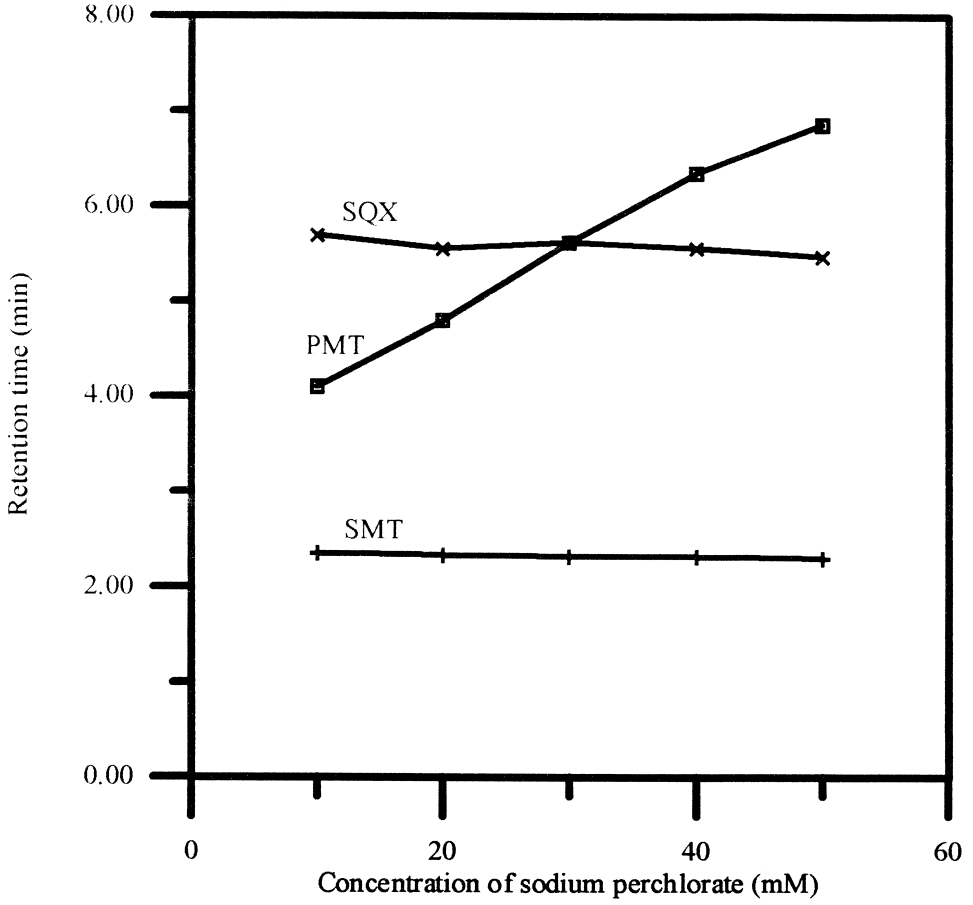


Fig. 2. Influence of the sodium perchlorate concentration on the retention times.

LOQ was calculated as ten-fold the base-line noise level.

Using the parameters above mentioned, LOD and LOQ were estimated to be 60 μg/l and 180 μg/l, respectively.

3.3.2. Linearity range and calibration curves

The linearity of the assay was checked by injecting a set of standards through the chromatographic procedure above described.

The calibration curves were obtained for each component by plotting the peak area and peak height, measured at the maximum absorption wavelengths, 263, 210 and 248 nm for SMT, PMT and SQX, respectively, versus concentration. Since the LOQ was estimated around 180 μg/ml, the calibration curves started in those concentration levels, but it must be considered the relative composition of the commercials to analyse, with regards to determine all the components in just one injection.

The results showed that, for PMT, peak height provided better repeatability than peak area, whereas for SQX and SMT, no significant differences were reported. Then, peak height was selected for the quantification in all cases.

As it can be noted, in Fig. 5, a good linear relationship was obtained between concentration and height for each component. In Table 2, equations, determination coefficients and the linear response ranges for the calibration curves are presented. In all cases, the intercepts were estimated as negligible by using the Student's test "t" (a=0.05).

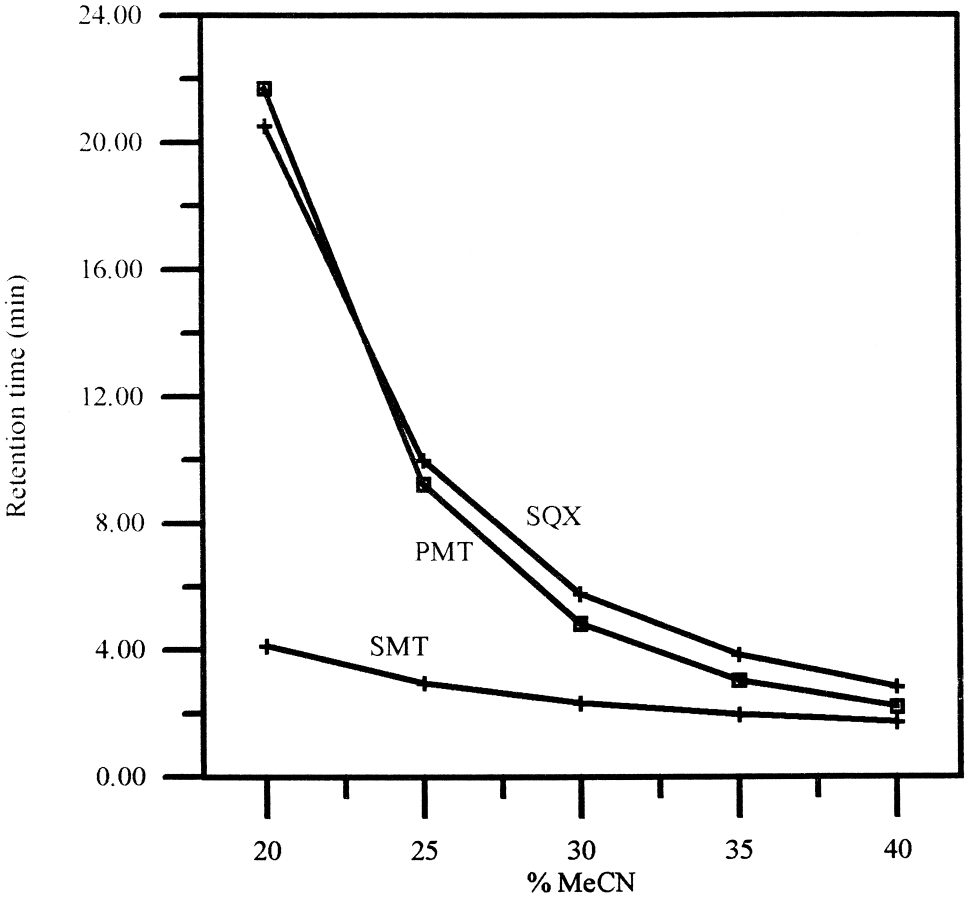


Fig. 3. Influence of the acetonitrile percentage on the retention time.

3.3.3. Repeatability and reproducibility

Repeatability was assessed under the chromatographic conditions previously selected by means of thirteen replicate injections of a ''Z'' solution. Reproducibility was evaluated over two days by performing thirteen injections each day.

The results showed that the repeatability for every component on each day is satisfactory because the relative standard deviation was found to be about 3% for all components. In terms of reproducibility, the comparison of averages with the Snedecor test did not provide any significant difference between both day series, for a signification level of 0.05 ($n=13$).

Thus, it can be assumed that our quantitative

Table 1
Chromatographic conditions selected

Column	Kromasil C_{18}
Mobile phase	40 m*M* phosphate buffer (pH 3.0), 10 m*M* ClO_4^-: Acetonitrile (65:35)
Flow rate	1.5 ml/min
Injection volume	20 μl
Detection	Diode-array

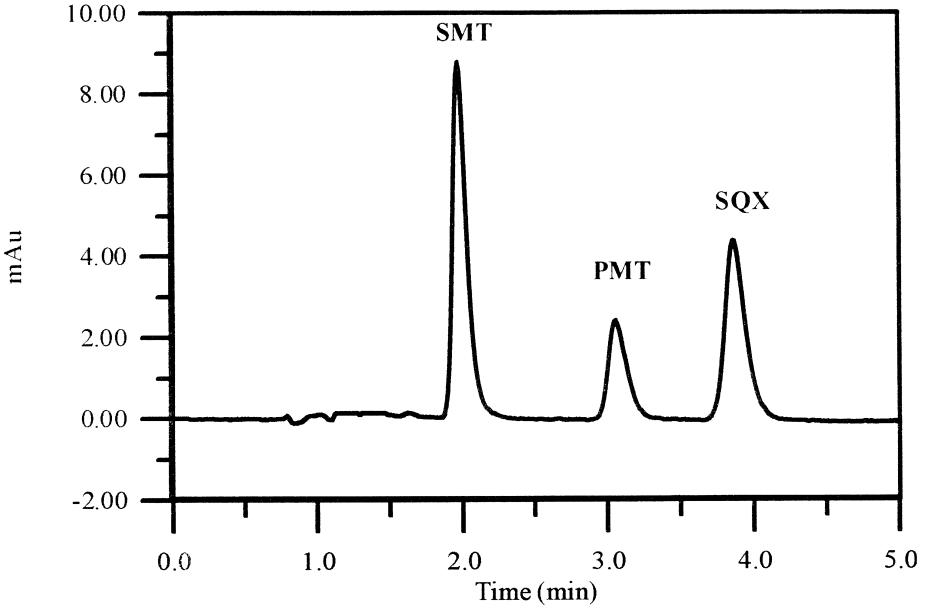


Fig. 4. Chromatogram of a "Z" sample in the optimised conditions. λ=270 nm (see Table 2).

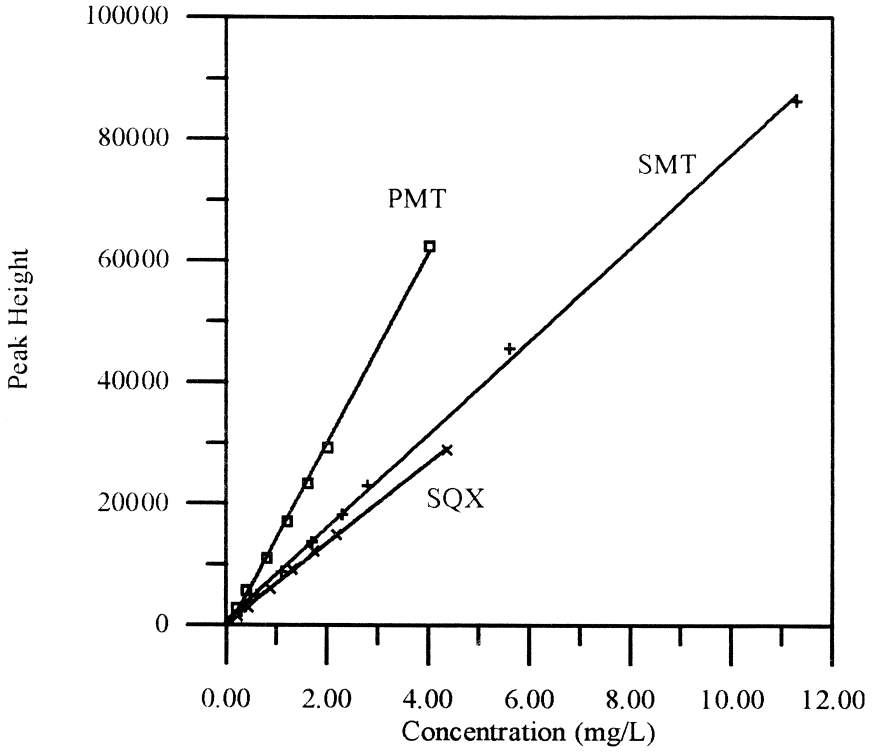


Fig. 5. Calibration curves of SMT, PMT and SQX (α=0.05) under optimised conditions (see Table 2).

Table 2
Linear regression calibration curves

Component	Linear regression curve	r^2	Linearity range (mg/l)
SMT	$H=0.83\ (\pm\ 0.37)\times10^3+7.656\ (\pm\ 0.078)\times10^3$ conc (mg/l)	0.9991	0.2–11
PMT	$H=-1.24\ (\pm\ 0.49)\times10^3+15.63\ (\pm\ 0.26)\times10^3$ conc (mg/l)	0.9985	0.2–4
SQX	$H=0.32\ (\pm\ 0.16)\times10^3+6.616\ (\pm\ 0.079)\times10^3$ conc (mg/l)	0.9992	0.2–4

results are affected by a relative standard deviation of 3%

3.4. Application

Due to the high viscosity of the commercial products, it is not possible to take an exact volume by using a pipette. Thus, the best way to take an exact volume of the commercial products was to use a volumetric flask. So, in all cases, a 10 ml volumetric flask was filled to the mark by using a syringe. Later, the solution was transferred to a 250 ml volumetric flask, washing several times the 10 ml volumetric flask with ethanol. Then, the 250 ml volumetric flask was filled with ethanol to the mark. After some manual shaking, 1 ml was taken and diluted with deionized water to 100 ml (''X'' solution). Five aliquots of 2 ml each were taken from ''X'' solution and carried into a 25 ml volumetric flask each. Then, different concentrations of each component (SMT, SQX and PMT) were added to them.

In the analysis of the commercial products, the found amounts and recoveries were achieved by comparing with test solutions containing the same concentrations than expected for commercials according to their claimed levels. The test solutions were prepared from the stock solutions after convenient dilution.

The results are presented in Table 3, nevertheless here are some comments about it:

(1) For *Cocciamin* and *Cocichemical*, the low recoveries are just due to their own SQX and PMT contents. This was confirmed by means of a micellar electrokinetic capillary chromatography method, pointed out by the same authors.

(2) In *Cocichemical*, no peak was reported for PMT, as marked in the labels.

(3) SMT was found in *Coccilina*, while it is not shown in claimed levels. The presence of SMT in *Coccilina* was confirmed by overlapping the sample and the standard spectra and by means of the standard addition method as well.

Similar recoveries were found when the above mentioned MEKC method was applied to the same samples, so comments 1, 2 and 3 should be made in this case, as well.

4. Conclusion

The presented HPLC method to determine SQX, SMT and PMT showed to be easy to apply in commercials because there are no previous sample treatments, only the dissolution of the commercials in ethanol.

This new method provides better sensitivity and a wide range of application, accurate and exact than the derivative spectrophotometric one proposed in bibliography for this mixture. In this way, by derivative ratio spectrum zero-crossing method, the measurement is performed at the specific wavelength previously selected. However, the presence in the formulations of some unexpected drugs in a derivative form or the presence of different excipients, might cause a big incidence on the measured signal. The proposed method, due to the high separation power of HPLC, provides an useful tool for removing the contribution of these interferences, as well as for their detection.

Similar results were found by both, calibration curve and standard addition quantification and when

Table 3
Application results (g/l)[a]: Recoveries (%)

	Claimed	Found	Test	Addition	MEKC
Cocciamin	SQX 50	Conc.	25.50±0.91	26.71±0.89	28.7±0.31
	PMT 15		9.11±0.34	9.16±0.39	8.32±0.20
	SQX	Rec.	51.1	53.4	57.5
	PMT		60.9	60.8	55.3
Cocichemical	SQX 50	Conc.	28.42±0.91	28.78±0.91	29.62±0.84
	PMT 15		0	0	0
	SQX	Rec.	56.8	57.8	59.1
	PMT		0	0	–
Coccilina	SQX 3.9	Conc.	3.01±0.12	3.12±0.15	3.11±0.10
	PMT 1.3		1.021±0.052	0.897±0.055	0.913±0.043
	SMT 0		0.7013±0.0061	0.7105±0.0062	0.8018±0.0057
	SQX	Rec.	77.4	80.0	80.3
	PMT		73.8	72.5	68.2
	SMT		–	–	–
Coccirex	SQX 44	Conc.	42.9±1.6	44.19±0.96	42.0±0.86
	PMT 12		11.32±0.43	11.06±0.41	10.2±0.36
	SQX	Rec.	97.6	100.2	95.4
	PMT		94.6	91.4	85.2
Coccivet	SQX 36	Conc.	31.32±0.75	30.14±0.68	32.32±0.64
	PMT 9		8.122±0.089	7.53±0.11	8.31±0.12
	MEN 50		–	–	–
	SQX	Rec.	87.0	85.7	89.8
	PMT		89.3	83.5	92.1
	MEN		–	–	–
Quinoxipra-p	SQX 50	Conc.	47.9±1.1	46.63±0.98	48.71±0.89
	PMT 15		14.12±0.34	14.15±0.42	14.20±0.42
	SQX	Rec.	95.9	93.3	97.4
	PMT		94.0	93.6	94.6

[a] The concentration units in *Coccilina* are g/100 g.

a capillary electrophoresis method was applied [17]. So it can be concluded that the presented method is convenient for the determination of the studied compounds with appropriate exactness.

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