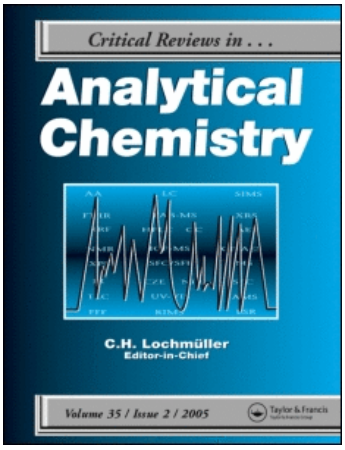


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# Statistical Evaluation of the Effectiveness of Different Extraction Techniques for Determining Robenidine Levels in Poultry Feed

Joanna Wilga[a]; Agata Kot-Wasik[a]; Aleksander Astel[b]; Jacek Namieśnik[a]
[a] Department of Analytical Chemistry, Chemical Faculty, Gdansk University of Technology, Gdansk, Poland [b] Environmental Chemistry Research Unit, Biology and Environmental Protection Institute, Pomeranian Academy, Stupsk, Poland

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# Statistical Evaluation of the Effectiveness of Different Extraction Techniques for Determining Robenidine Levels in Poultry Feed

**Joanna Wilga,[1] Agata Kot-Wasik,[1] Aleksander Astel,[2] and Jacek Namieśnik[1]**

[1]*Department of Analytical Chemistry, Chemical Faculty, Gdansk University of Technology, Gdansk, Poland*
[2]*Environmental Chemistry Research Unit, Biology and Environmental Protection Institute, Pomeranian Academy, Stupsk, Poland*



**The importance of sample preparation techniques cannot be overstated as the accurate sample preparation is essential. Often overlooked, it is the midway point where the analytes from the sample matrix are transformed so they are suitable for analysis. Even the best analytical techniques cannot rectify problems generated by sloppy sample pre-treatment. In the present work effectiveness of six different commonly applied extraction techniques for the determination of robenidine in poultry feed has been compared. The result analysis was performed using non-parametric Kruskal-Wallis (K-W) test and cluster analysis. The extraction performance of shaking, Soxhlet, Soxtec, ultrasonically assisted extraction (UAE), microwave–assisted extraction (MAE) and accelerated solvent extraction (ASE) was investigated. Operation parameters such as extraction pressure, extraction temperature and extraction time were evaluated as the variable parameters under the experimental ranges of 1000–2500 psi, 60–140°C and from 1 minute up to 6 hours, respectively. The effect of operation parameters during extraction on the recovery of robenidine from poultry feed, reproducibility of the methods and solvent consumption were investigated using spiked samples. Every single extract was subjected to clean–up using aluminium oxide column (Pasteur pipette filled with 1 g of aluminium oxide) from which robenidine was eluted with 10 ml of methanol. The eluate from the clean-up column was collected in a volumetric flask and analyzed by HPLC–DAD–MS technique. In general, all extraction techniques allowed the isolation of robenidine from poultry feed, but the recovery obtained by means of modern extraction techniques (ASE, MAE) was higher than that obtained with conventional techniques (Soxhlet, UAE). Also, RSD showed to be lower for novel extraction techniques.**

**Keywords** Poultry feed, sample preparation, robenidine, extraction technique, non-parametric tests, cluster analysis

## INTRODUCTION

Common analytical procedures include several important steps, i.e. sampling, sample preparation, isolation, identification, quantification, statistical evaluation and final decision. Each step is equally important to obtain correct results in order to fulfil the analytical purpose. But from all of them, sample preparation is a key procedure in modern chemical analysis. By some estimates, 60–80% of the work activity and operating cost in analytical laboratories is spent preparing samples for introduction into an analytical device (1). Sample preparation is necessary to isolate the desired components from some sample matrices, since most instruments cannot handle the matrix directly. Sample preparation can also include clean-up procedures for complex dirty samples. In this step, also known as pre-concentration, the analytes have to be concentrated to a suitable level that can be measured by the method chosen for the real analysis. An ideal sample preparation technique should be simple, inexpensive, efficient, selective and compatible with various analytical techniques. It should give as high as possible recovery and the supreme sample clean-up, be environmentally friendly and should reduce

Address correspondence to Aleksander Astel, Enviornmental Chemistry Research Unit, Biology and Environmental Protection Institute, Pomeranian Academy, 22a Arciszewskiego Str., Słupsk 76-200, Poland. E-mail: astel@pap.edu.pl

amount of solvent used. In practice, it is difficult to fulfil all these requirements. Usually, the sample preparation is the most labor-intensive and very often the slowest and the most costly step in the whole analytical procedure, especially if multi-step procedures are used (2, 3).

Multi-step analytical procedure is often required when analyte is extracted from the complex matrix. The determination of coccidiostatic in feeding stuff serves as a good example. Feeding stuff include a lot of substances, which should be already under control on the manufacture's production lines (e.g., hormones, enzymes, dyestuff, aromatic and tested substances, antioxidants, herbs and coccidiostatics). Coccidiostatics are compounds that are widely used to prevent and treat coccidiosis, a contagious amoebic disease affecting livestock and agricultural poultry that is associated with warm and humid conditions. Coccidiosis is caused by protozoa of the phylum Apicomplexa, family Eimeridae. In poultry, most species belong to the genus *Eimeria* and infect various sites in the intestine. The infectious process is rapid (4–7 days) and is characterized by parasite replication in host cells with extensive damage to the intestinal mucosa. Poultry coccidian are strictly host-specific and the different species parasitize specific parts of the intestine. Coccidia are distributed worldwide in poultry and wild birds. Coccidia are almost universally present in poultry raising operations, but clinical disease occurs only after ingestion of relatively large numbers of sporulated oocysts by susceptible birds. Both clinically infected and recovered birds shed oocysts in their droppings, which contaminate feed, dust, water, litter and soil. Oocysts may be transmitted by mechanical carriers, equipment, clothing, insects and other animals (4, 5). European Committee (EC) regulations established minimum and maximum content of coccidiostatics in complete feeding stuffs.

Although the importance of the sample preparation process has long been recognized, the development and implementation of new sample preparation methods are very slow as compared to other parts of the mentioned analytical processes. Even in modern gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) analyses, a very traditional sample preparation process such as shaking or Soxhlet extraction is still performed. The reasons for the lack of progress in this area are many and include low level research activity in this area, largely caused by the complexity of natural matrices, which discourages more fundamental research, even though sample preparation is considered to be the rate determination step of whole analytical separation.

Currently, extraction techniques available for an analyst can be divided into two groups:

- classical ones, like shaking flask extraction, Ultrasonic Assisted Extraction (UAE), Soxhlet extraction and Soxtec extraction and (the extraction efficiency depends mainly on the type of solvent applied for the isolation and extraction time) [6, 7];
- modern ones, like Microwave Assisted Extraction (MAE) and Accelerated Solvent Extraction (ASE) (the extraction efficiency depends not only on the type of solvent applied for the isolation and extraction time, but also on many different parameters characteristic for every technique used) [2, 8].

In this paper, the pros and cons of six different extraction techniques applied to isolate coccidiostatics from feeding stuff have been discussed. To this end, the investigation of numerous sample preparation techniques with special attention paid to the conditions needed for efficient isolation of robenidine (1,3-bis[(4-chlorobenzylidene)amino]quanidine-hydrochloride), one of the most popular coccidiostatics in the world, was carried out. Comparative studies of classical techniques such as Soxhlet extraction, Soxtec extraction and shaking flask extraction and other modern techniques e.g., UAE, MAE and ASE have been performed (Table 1). All extraction procedures were compared in terms of extraction time, extraction yields, solvent consumption and reproducibility. Furthermore, the influence of different parameters and characteristics of every extraction technique on robenidine recovery was evaluated.

## MATERIALS AND METHODS

### Chemicals

Methanol (high performance liquid chromatography (HPLC) grade), molecular sieve 3Å, as well as acetic and formic acids (chemically pure) were purchased from POCH (Gliwice, Poland). Methanol for mobile phase composition (HPLC grade), aluminium oxide and trifluoroacetic acid (TFAA) were purchased from Merck (Warsaw, Poland). Water was purified in a Millipore Super-Plus water purification system (Millipore, Milano, Italy). Quartz sand used as extraction cell filler was collected at the local beach. Quartz sand was thoroughly washed with hot solution of water and nitric acid (1:1v/v), rinsed several times with distilled water until reaching pH 7, and finally dried at 200°C. After drying it was cleaned-up with methanol in Soxhlet, dried and sifted with a $\leq$1 mm sieve. Aluminium oxide (activity grade I) was prepared by drying at 170°C for 10 hours, then cooled and wetted with water (1%).

### Standards and Samples

Due to difficulties with finding a supplier of pure robenidine, the compound was extracted from Robenvit, a commercially available premix used in the manufactured feedstuff, obtained as a kind donation from Cargil (Pruszcz Gdanski, Poland). Robenidine was extracted with chloroform and purified by recrystallization. The identity and the purity of the substance were confirmed by $^{13}$C NMR and HPLC-MS techniques.

Robenidine stock solution (63.67 $\mu$g/ml) used to prepare the calibration curve was obtained by dissolving 63 $\mu$g of robenidine in 98.95 ml of methanol in an ultrasonic bath.

TABLE 1
Classical and modern extraction techniques

| Extraction temperature | Extraction technique | Extraction pressure | Additional parameters set during extraction |
|---|---|---|---|
| Ambient | Shaking | Atmospheric | Intensity of shaking (number of oscillations per time unit) |
| | UAE | | Can be performed at elevated temperature |
| Elevated | Soxhlet | | Can be done in combination with microwave heating |
| Boiling | Soxtec | | |
| Variable in wide range from ambient up to the boiling point | MAE | Variable and depending on the microwave power | Microwave power |
| | ASE | Variable | Number of cycles; amount of solvent used during flushing |



Standard solutions were prepared by diluting the stock solution with methanol to obtain concentrations ranging from 0.01$\mu$g/ml to 4.46 $\mu$g/ml. Since robenidine is light sensitive, all flasks were wrapped with aluminium foil and stored in a dark place. Optimization of the procedure was performed by using commercially available poultry feeds, produced by Koudijs companies, with and without robenidine addition. All samples had been ground prior to analysis and then processed as described in Section 2.3.

## Chromatographic Equipment

The analyses were performed using a Hewlett-Packard HP 1100 LC system equipped with a binary pump, an automatic sampler, a diode-array detector and mass spectrometer. The separation was carried out on a Purospher (Merck, Poland) C18 analytical column (125x3.0 mm, 3 $\mu$m particle size) maintained at 10°C, using isocratic elution mode. The mobile phase was a mixture of 70% methanol and 30% water acidified with 0.1% TFAA (v/v), and the flow rate was 0.7 ml/min. The diode-array UV detector (HP 1100 Series) was set to 317 nm. A single quadrupole mass spectrometer (Hewlett-Packard HP 1100) with electrospray ionisation was used with the following parameters: fragmentor 95 eV, spray voltage 5kV, nebulizer pressure 310 kPa (45 psi), drying gas temperature 350°C, and drying flow 12 l/min. Positive ionization with selected ion monitoring (SIM) (multiplier gain set to 5, dwell times set to 289 ms) was used for all analyses. Pseudomolecular ion $(M+H)+$ $m/z$ 334 and ions at $m/z$ 336 and $m/z$ 337 were monitored.

## Shaking Flask Extraction

An 8 g portion of spiked poultry feed with 8 g of pure quartz were accurately weighed, mixed and shaken with a 60 ml of acidified methanol for 30 and 60 minutes at ambient temperature. During that step a home-made shaker (Department of Analytical Chemistry, Gdansk University of Technology, Poland) was used. Real poultry feed samples, originally containing robenidine (66 mg/kg), were extracted within 30 minutes under optimized conditions. For method development, extractions were repeated eight times under each specific condition.

## Ultrasonic Extraction Method

Extractions were performed with a Sonorex ultrasonic instrument (Bandelin Electronic, Berlin, Deutschland), the power of which was set on 80 W. A 5 g portion of the spiked poultry feed (accurately weighed) mixed with 5 g of pure quartz were extracted with 25 ml of acidified methanol at ambient temperature for 15 and 30 minutes. Real poultry feed samples, originally containing robenidine (66 mg/kg), were extracted within 30 minutes under optimized conditions. For method development, extractions were repeated eight times under each specific condition.

## Soxhlet

A 4 g portion of spiked poultry feed was accurately weighed and extracted with 45 ml of acidified methanol for 6 and 12 hours. Real poultry feed samples, originally containing robenidine (66 mg/kg), were extracted within 6 hours. For method development, extractions were repeated eight times under each specific condition.

### Soxtec

A Soxtec system HT6 (Tecator, Höganä, Sweden) was used. A 2.5 g portion of spiked poultry feed was placed in a 33 mm × 80 mm extraction thimble (supplied by the manufacturer) and extracted with 25 ml acidified methanol in boiling solvent for 15, 30, and 45 minutes. Real poultry feed samples, originally containing robenidine (66 mg/kg), were extracted within 30 minutes. All procedures were repeated eight times under each specific condition.

### Microwave-Assisted Extraction

A CEM MARS 5 Microwave Accelerated Reaction System (CEM Corporation, Matthews, NC, USA) was used. The 0.5 g portion of spiked poultry feed was accurately weighed and loaded into extraction cylinders containing 10 ml acidified methanol. The extraction temperature was 110°C (after ramping to the final temperature in 10 minutes). Microwave power was set at 300 W (100%). After extraction had been completed, ~5 g of molecular sieve was added. MAE parameters (temperature, extraction time and watt power) were optimized in order to maximize the amount of robenidine extracted. Extraction temperature was optimized in the range of 60–120°C, while time extraction was set between 1 and 5 minutes. Each type of sample was repeated eight times.

### Accelerated Solvent Extraction

An ASE 200 system (Dionex, Sunnyvale, CA, USA) was used. The ASE extractor was equipped with an autosampler carousel and a collection tray that allowed up to 24 separate samples to be extracted sequentially. Approximately 8 g of spiked feed sample were thoroughly mixed with ~ 8 g quartz and then placed in the 22-ml extraction cell. Extractions were performed with acidified methanol.

ASE parameters (temperature, pressure, extraction, number of cycles and solvent composition) were optimized in order to maximize the amount of robenidine extracted. Extraction temperature was optimized in the wide range from 60 up to 140°C, while pressure was kept between 1000 and 3000 bars. Extraction time was evaluated between 1 and 5 minutes. For method development, extractions were repeated eight times under each specific condition.

### Sample Clean-Up

After extraction each extract was collected in vials containing ~5 g of molecular sieve (drying agent). The content of the collecting vials was shaken vigorously for 5 minutes. An aliquot (2 ml) of the extract was then subjected to clean-up using a handmade column, which was prepared from Pasteur pipette filled with 1 g of aluminium oxide (activity grade I). Robenidine was eluted from the clean-up column with 10 ml of methanol. The eluate from the clean-up column was collected in a volumetric flask (10 ml). After careful mixing 20 $\mu$l aliquot of the purified extract was analyzed by HPLC-diode array detector (DAD)-MS technique.

## RESULTS AND DISCUSSION

The whole set contained 400 analytical results since six extraction methods were applied to 52 different test samples, and each combination of the sample and the extraction method was performed eight times. All the obtained data were subjected to the statistical analysis in order to establish which of the extraction methods investigated in this study gave significantly higher results than the other methods. The influence of different parameters on the effectiveness of extraction process was also checked.

In the first stage of statistical evaluation of results, the descriptive statistical parameters for specific variants of modified extraction procedures were estimated, in particular the values of Shapiro-Wilk (S-W) test for normality. S-W test tests the null hypothesis $h_0$ that a sample $x_1, \ldots, x_n$ came from a normally distributed population; for p-values $\leq 0.05$ the null hypothesis is rejected and the alternative hypothesis is accepted [sample did not come from a normally distributed population], while for p-values $> 0.05$ there are no grounds to reject $h_o$).

In Table 2 the descriptive statistical parameters for the modified extraction procedures are presented.

Only in 11 cases the p-values calculated for the S-W test for normality allowed us to accept the null hypothesis; therefore, it has been decided that the differences among mean recovery values for the specific modified procedures (modification of time or temperature) would be tested with the non-parametric Kruskal-Wallis's (K-W) test (this test can be used for relatively small samples [in this case n=24] drawn from a population that is not normally distributed). The null hypothesis $h_0$: there are no statistically significant differences among the measured median recovery values for robenidine. It has been emphasized at this point that for all extraction techniques the median/mean recovery value ratio ranges from 0.97 to 1.01. Because of this, median value and mean recovery value are comparable despite slight differences in the data distribution patterns and K-W test results can be understood in way of median as well as in way of mean recovery values.

### Shaking

In the case of shaking, the null hypothesis was not rejected because the calculated p-value equaled 0.8933 ($p > 0.05$). Therefore there was no statistically significant difference between the median recovery values for robenidine, measured in time, which has been shown graphically in Figure 1.

It is noteworthy that the compared mean recovery values, i.e. 64.05 and 63.92%, differed very slightly from the median. Therefore it was concluded that the lack of statistically significant difference between the mean recovery values could be assumed (no difference).

### Ultrasonic Assisted Extraction (UAE)

In the case of UAE with solvent, the null hypothesis about the lack of statistically significant difference between the median recovery values for robenidine, measured in time, was rejected ($p = 0.00000005$).

TABLE 2
Statistical parameters used for the evaluation and comparison of modified extraction procedures

| | N | Mean recovery value | Median | Median/Mean recovery value ratio | Min recovery [%] | Max recovery [%] | S.D. | Mean standard error | Skewness factor | S-W test |
|---|---|---|---|---|---|---|---|---|---|---|
| Shaking_30 | 24 | 64.05 | 65.55 | 1.02 | 54.30 | 73.50 | 6.67 | 1.36 | −0.2 | 0.0086 |
| Shaking_60 | 24 | 63.92 | 63.50 | 0.99 | 54.60 | 74.30 | 6.78 | 1.38 | 0.2 | 0.0037 |
| Ultra_30 | 24 | 74.62 | 75.95 | 1.01 | 65.80 | 83.90 | 6.69 | 1.37 | −0.1 | 0.0031 |
| Ultra_60 | 24 | 91.64 | 89.80 | 0.97 | 80.70 | 108.40 | 9.50 | 1.94 | 0.5 | 0.0138 |
| MAE_2_60 | 24 | 87.08 | 87.30 | 1.002 | 85.60 | 88.00 | 0.79 | 0.16 | −0.4 | 0.0237 |
| MAE _2_80 | 24 | 94.48 | 95.30 | 1.009 | 91.30 | 97.80 | 2.28 | 0.46 | −0.2 | 0.0060 |
| MAE _2_100 | 24 | 95.16 | 94.60 | 0.99 | 92.20 | 99.00 | 2.31 | 0.47 | 0.4 | 0.0129 |
| MAE _2_120 | 24 | 82.91 | 82.05 | 0.99 | 79.80 | 86.80 | 2.35 | 0.48 | 0.4 | 0.0170 |
| MAE _1_100 | 24 | 91.62 | 91.85 | 1.002 | 90.00 | 92.60 | 0.80 | 0.16 | −0.7 | 0.0430 |
| MAE _3_100 | 24 | 90.73 | 90.85 | 1.001 | 87.60 | 93.40 | 1.97 | 0.40 | −0.2 | 0.0390 |
| MAE _4_100 | 24 | 89.48 | 89.55 | 1.00 | 89.00 | 89.90 | 0.29 | 0.06 | −0.2 | 0.0143 |
| Soxhlet_6h | 24 | 73.97 | 76.35 | 1.03 | 62.90 | 83.30 | 7.61 | 1.55 | −0.4 | 0.0034 |
| Soxhlet_12h | 24 | 71.17 | 72.25 | 1.01 | 62.60 | 80.30 | 7.37 | 1.51 | −0.0 | 0.0003 |
| Soxtec_10 | 24 | 90.08 | 89.30 | 0.99 | 83.70 | 97.80 | 4.54 | 0.93 | 0.6 | 0.0048 |
| Soxtec_20 | 24 | 91.64 | 89.80 | 0.98 | 80.70 | 108.40 | 9.50 | 1.94 | 0.5 | 0.0138 |
| Soxtec_30 | 24 | 92.78 | 93.00 | 1.002 | 84.10 | 104.80 | 6.72 | 1.37 | 0.4 | 0.0306 |
| E1_ASE | 24 | 55.01 | 55.05 | 1.00 | 52.70 | 58.20 | 1.48 | 0.30 | 0.7 | 0.0323 |
| E2_ASE | 24 | 53.52 | 53.65 | 1.002 | 49.00 | 57.00 | 2.44 | 0.50 | −0.4 | 0.1689 |
| E3_ASE | 24 | 77.20 | 78.20 | 1.01 | 68.80 | 80.30 | 2.87 | 0.59 | −1.9 | 0.0001 |
| E4_ASE | 24 | 72.78 | 72.70 | 0.99 | 69.00 | 75.80 | 2.07 | 0.42 | −0.3 | 0.1100 |
| E5_ASE | 24 | 76.15 | 76.15 | 1.00 | 73.00 | 78.40 | 1.65 | 0.34 | −0.3 | 0.1274 |
| E6_ASE | 24 | 78.71 | 78.65 | 0.99 | 77.80 | 79.60 | 0.47 | 0.10 | −0.0 | 0.7674 |
| E7_ASE | 24 | 84.50 | 84.60 | 1.00 | 82.00 | 87.40 | 1.45 | 0.30 | 0.1 | 0.6359 |
| E8_ASE | 24 | 68.35 | 67.25 | 0.98 | 66.20 | 72.50 | 2.01 | 0.41 | 1.0 | 0.0014 |
| E9_ASE | 24 | 75.06 | 74.85 | 0.98 | 70.50 | 79.40 | 2.54 | 0.52 | −0.0 | 0.2881 |
| E10_ASE | 24 | 72.00 | 72.10 | 1.001 | 70.80 | 73.40 | 0.70 | 0.14 | 0.2 | 0.1424 |
| E11_ASE | 24 | 62.04 | 61.65 | 0.99 | 58.10 | 67.10 | 3.05 | 0.62 | 0.4 | 0.0224 |
| E12_ASE | 24 | 75.78 | 75.25 | 0.99 | 68.70 | 80.70 | 3.95 | 0.81 | −0.3 | 0.0240 |
| E13_ASE | 24 | 77.91 | 76.90 | 0.98 | 74.20 | 83.00 | 3.37 | 0.69 | 0.5 | 0.0010 |
| E14_ASE | 24 | 81.79 | 81.60 | 0.99 | 77.20 | 86.70 | 2.48 | 0.51 | 0.2 | 0.0134 |
| E15_ASE | 24 | 74.83 | 74.40 | 0.99 | 70.80 | 78.90 | 2.54 | 0.52 | 0.2 | 0.0912 |
| E16_ASE | 24 | 79.52 | 78.90 | 0.99 | 73.80 | 88.40 | 4.57 | 0.93 | 0.6 | 0.0291 |
| E17_ASE | 24 | 86.39 | 86.30 | 0.99 | 84.50 | 89.60 | 1.48 | 0.30 | 0.9 | 0.0139 |
| E18_ASE | 24 | 87.12 | 86.85 | 1.00 | 85.80 | 88.90 | 1.07 | 0.22 | 0.3 | 0.0130 |
| E19_ASE | 24 | 86.78 | 86.50 | 0.99 | 81.00 | 90.80 | 3.22 | 0.66 | −0.3 | 0.0243 |
| E20_ASE | 24 | 89.16 | 89.35 | 1.002 | 86.20 | 92.50 | 1.91 | 0.39 | 0.1 | 0.1177 |
| E21_ASE | 24 | 89.79 | 89.65 | 0.99 | 87.10 | 92.50 | 1.70 | 0.35 | 0.2 | 0.1610 |
| E22_ASE | 24 | 88.60 | 88.90 | 1.01 | 80.70 | 92.90 | 3.11 | 0.64 | −0.5 | 0.1846 |
| E23_ASE | 24 | 82.63 | 82.55 | 0.99 | 80.20 | 85.30 | 1.56 | 0.32 | 0.1 | 0.2613 |
| E24_ASE | 24 | 94.26 | 95.05 | 1.01 | 87.70 | 97.00 | 2.61 | 0.53 | −1.9 | 0.0000 |

*coding of extraction techniques e.g., Shaking_30 means shaking for T = 30min; MAE_1_100 stands for microwave assisted extraction for T = 1 min at Temp = 100°C; etc.

It should be pointed out that the mean recovery values, i.e. 74.62 and 91.64% differed only slightly from the median. Therefore, the lack of statistically significant difference could also be assumed for the mean recovery values. Figure depicts the influence of extraction time on the recovery of robenidine from poultry feed using UAE as a technique for the isolation of coccidiostatic.

As shown in Figure 1, a two-fold increase of extraction time (t) improved the recovery by about 23% in comparison to the recovery values obtained for t = 30 minutes

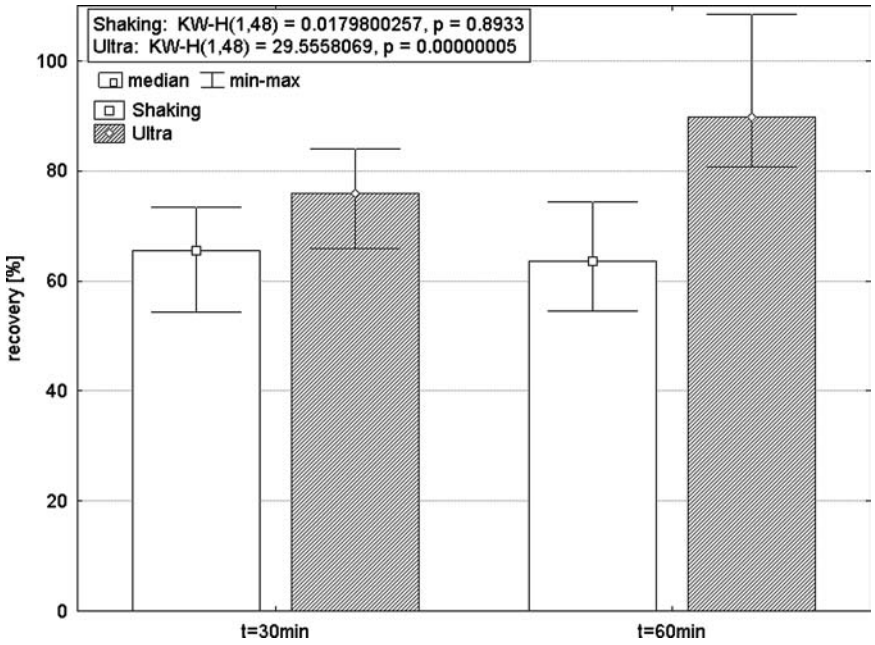


FIG. 1. Recovery of robenidine from poultry feed by shaking and UAE applied at two different extraction times.



(the improvement was calculated from the appropriate mean values).

## Soxhlet Extraction

In the case of Soxhlet extraction, the null hypothesis about the lack of statistically significant difference between the median recovery values for robenidine, measured in time, was not rejected ($p = 0.0777$; $p > 0.05$). Figure 2 presents the obtained results.

Again, it is noteworthy that the respective mean recovery values, i.e. 73.97 and 71.17% differed very slightly from the median, therefore it could be assumed that there was no statistically significant difference between the means.

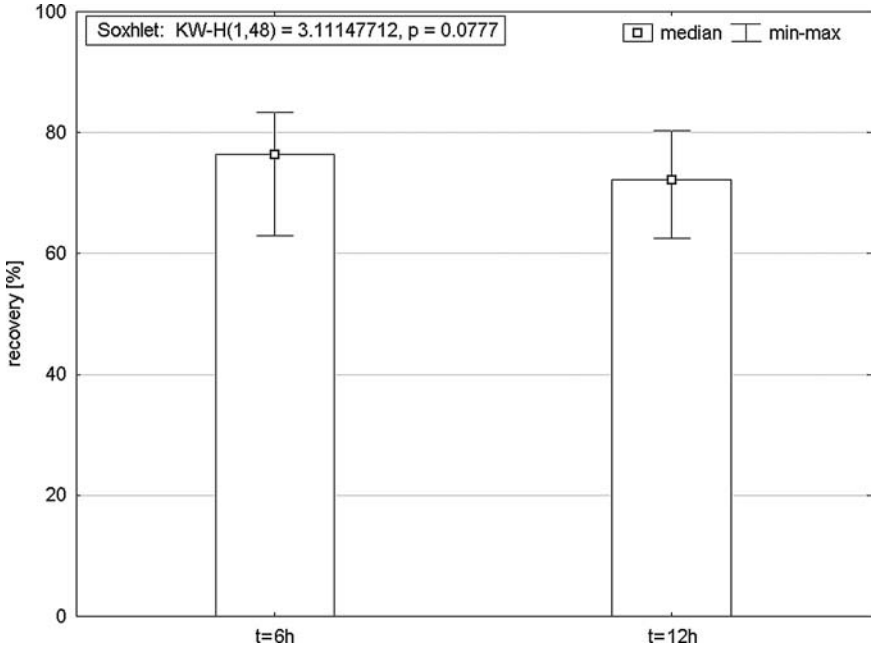


FIG. 2. Recovery of robenidine from poultry feed using Soxhlet extraction applied at two different extraction times.

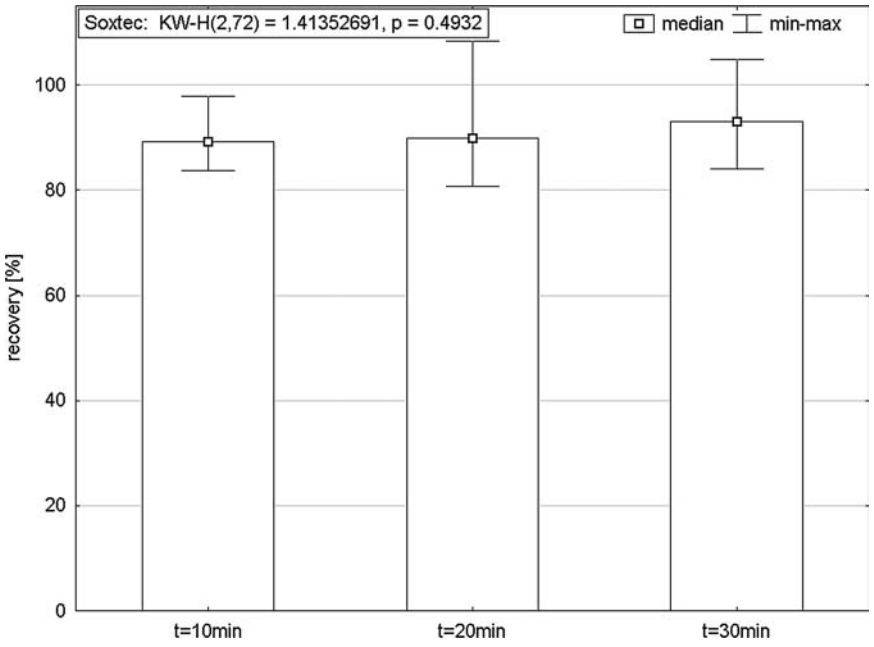


FIG. 3. Recovery of robenidine from poultry feed using Soxtec extraction at three different extraction times.



## Soxtec Extraction

As the obtained p-value for the Soxtec extraction equaled 0.4932 ($p > 0.05$), the null hypothesis about the lack of statistically significant differences among the median values of robenidine recovery, measured in time, was accepted. The obtained results are shown in Figure 3.

At the same time, it was shown that a stepwise change of extraction time by 10-minutes intervals resulted in the linear increase of yield values as described by the following linear regression equation: $y = 1.3479 \times + 88.803$ (coefficient of determination $R^2 = 0.9916$). The results are presented in Figure 4.

According to the equation, a 10-min increase of extraction time results in the increased recovery, estimated at about 1.5%. This linear increment of robenidine recovery from the poultry feed samples implicates that in order to enhance recovery from 90% up to 100% the extraction time should be increased 10

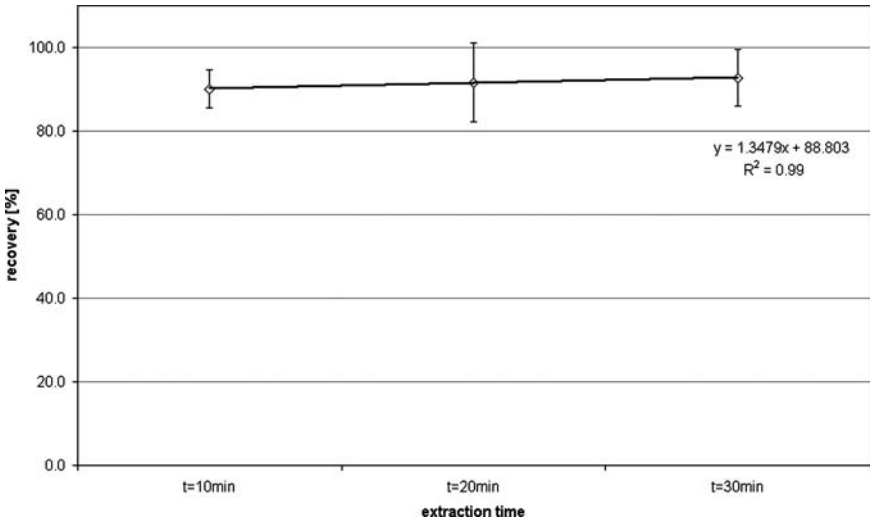


FIG. 4. Linear increase of robenidine recovery from poultry feed using Soxtec extraction at different extraction times.

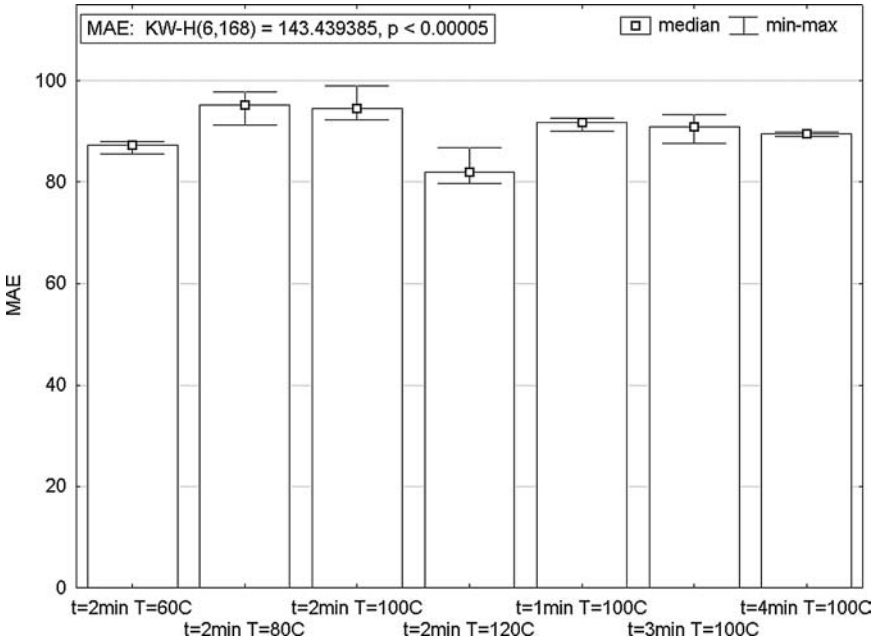


FIG. 5. Recovery of robenidine from poultry feed using MAE applied at different extraction times and temperatures (microwave power).



times. In total, this would lengthen the procedure time by only one and a half hour. However, it has to be remembered that each additional hour incurs costs due to the electricity and water usage.

### Microwave Assisted Extraction (MAE)

In the case of MAE with solvent, the null hypothesis about the lack of statistically significant differences among the median values of robenidine recovery, measured as a function of time and temperature, was rejected ($p < 0.00005$). Figure 5 presents the obtained results.

The highest recovery values were obtained for t = 2 minutes and temperatures (T) ranging from 80 to 100°C (note: no statistically significant differences among those recovery values were found). Based on these results, it can be assumed that t = 2 minutes and T = 80–100°C are the optimal extraction conditions. The lengthening of extraction time at the constant T = 100°C leads to higher yield, which is probably due to thermal degradation of organic compounds; raising the temperature to 120°C results in the highest recovery.

### Accelerated Solvent Extraction (ASE)

In case of a data set for the modified ASE, the p-value was also less than 0.05. Therefore the null hypothesis about the lack of statistically significant differences among the median values of robenidine recovery, measured as a function of chosen extraction parameters, was rejected. The obtained results are shown in Figure 6.

Under all variant extraction conditions no preheating was used (preheat = 0) and purging was always conducted for 60 seconds therefore those two parameters were not considered as differing criteria. Based on the analysis of the obtained mean recovery values, it had been concluded that the type of solvent used was a critical factor. The application of a mix of acetonitrile and $CH_3COOH$/HCOOH (1% v/v) resulted in the lowest mean yields of about 55% (see group 55–64% in cluster analysis). In cases when a MeOH-based solvent was used, the obtained higher mean recovery values were statistically significant ($p = 0.05$). The use of modified solvents i.e., (ACN:MeOH (1:1) + 1% $CH_3COOH$/HCOOH) resulted in the increased mean yields at 77.20% and 72.70%, respectively.

Within the set of modified extraction procedures there are two in which different solvent mixes were used i.e., E5 and E6. The application of 1% of HCOOH instead of $CH_3COOH$ did not increase robenidine recovery in a significant way, as it reached 76.15%; this can be attributed to the similarity of molecules and chemical properties of the acetic and formic acids. Similarly, the increased content of acetic acid in the solvent mix to 10% did not result in a higher yield. Most likely, the pH change observed in the 1 and 10% methanol-based solutions did not influence the extraction process (acidification posed a barrier strong enough for robenidine molecules).

The remaining 17 variants of extraction procedure were conducted with the use of solvent mix of constant composition (MeOH +1% $CH_3COOH$ v/v) therefore solvent was not influencing the effectiveness of extraction in this group, i.e. E7–E10 and E12–E24.

In order to investigate the influence of one parameter at the time on the extraction effectiveness in the ASE group, the modified procedures were sorted into five sub-groups in such a

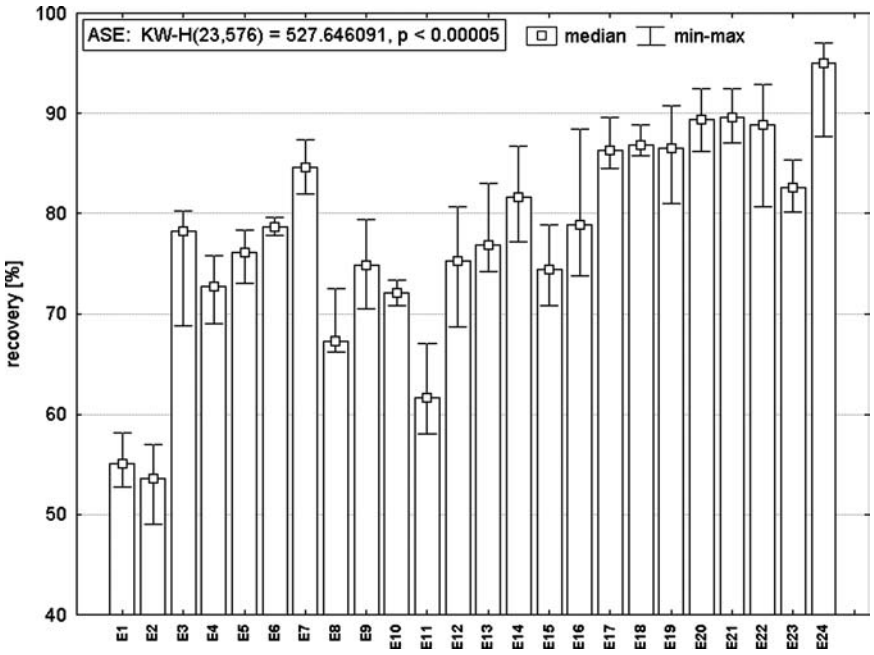


FIG. 6. Recovery of robenidine from poultry feed using ASE at different extraction conditions.



way that only one parameter varied while the others remained constant.

*ASE: Temperature as a Variable.* Temperature during ASE was increased from 60 up to 140°C in 20°C intervals, while other parameters were constant (static time = 5 minutes; pressure = 1500 psi, number of cycles = 1 and flush = 60%).

The maximum yield was reached at 80°**C**, and then the mean recovery was decreasing proportionally with the increasing temperature. The linear regression line $y = -7.0442x+91.01$ ($R^2 = 0.96$) describes the decreasing recovery for the 80–140°C temperature range. In Figure 7 the graphical depiction of the results is presented.

Mean value of robenidine recovery from poultry feed at 80°C by means of ASE was significantly higher than the other values obtained with this particular technique ($p = 0.05$).

*ASE: Pressure as a Variable.* During the extraction procedure pressure was raised from 1000 up to 2500 psi in 500-unit intervals. Other parameters remained constant (static time = 5 minutes; temperature = 80°C, number of cycles = 1 and flush = 60%).

As shown in Figure 8, the maximum mean recovery was obtained for the pressure of 1500 psi.

In the case of ASE with varying pressure, no linear relationship was found between pressure and the mean yield values. However, the outcome of the non-parametric K-W test indicated

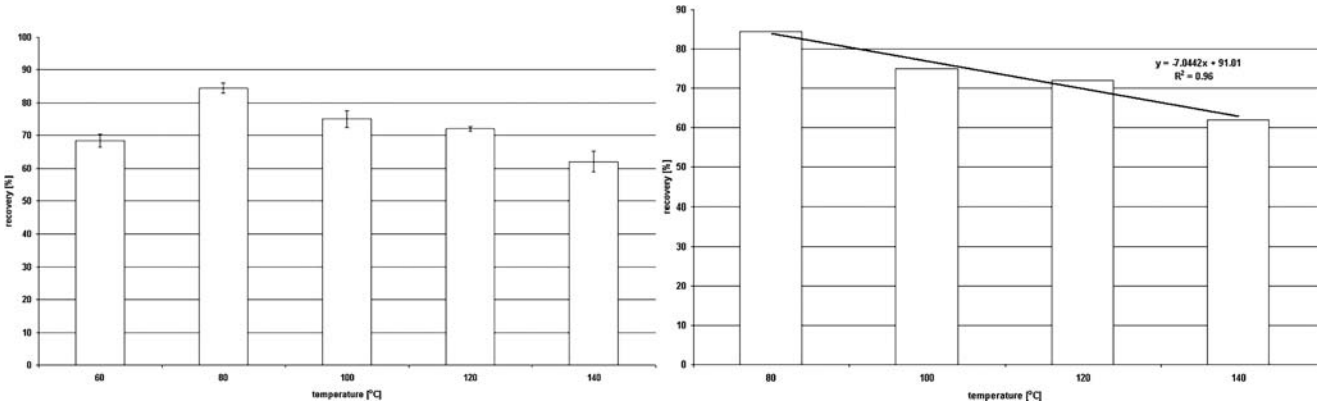


FIG. 7. Recovery of robenidine from poultry feed using ASE at five different extraction temperatures.

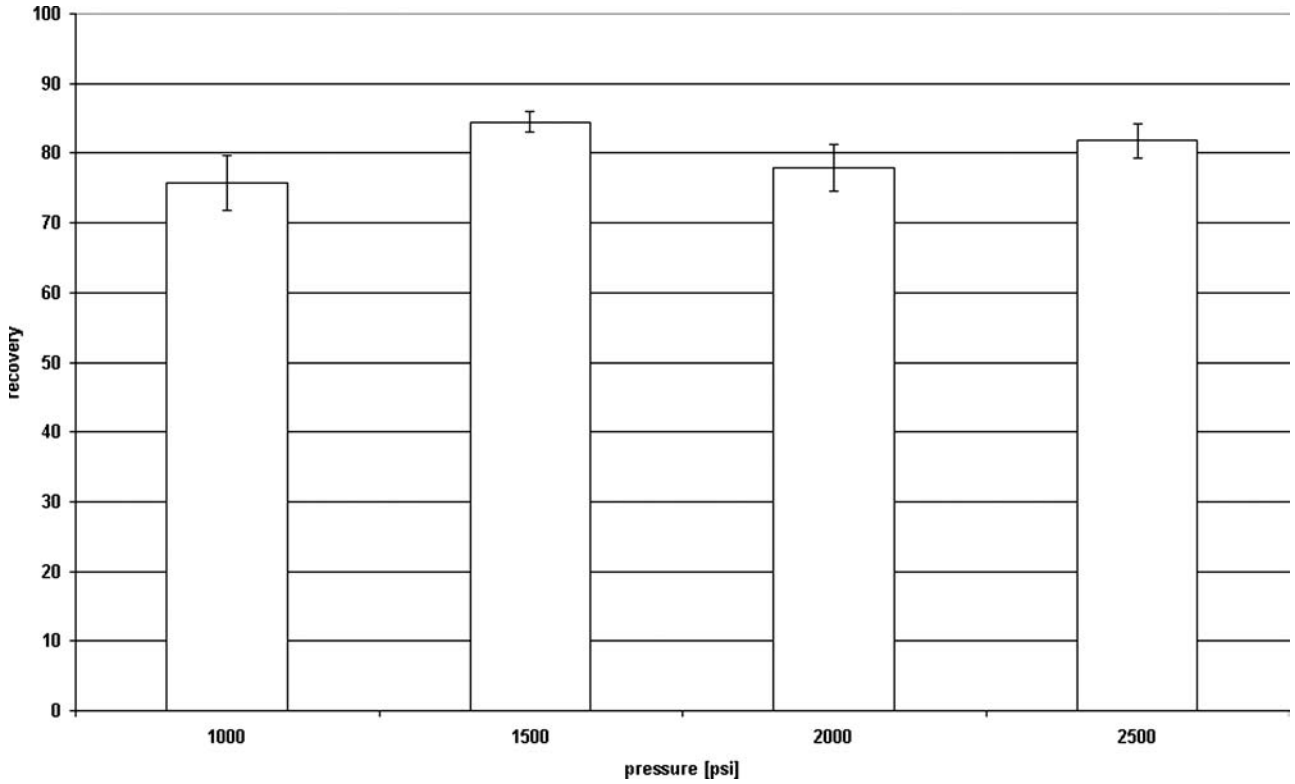


FIG. 8. Recovery of robenidine from poultry feed using ASE at four different extraction pressures.



that the null hypothesis about the lack of differences among the median recovery values should be rejected. Therefore, it has been concluded that the median recovery for the pressure 1500 psi was significantly higher than the median values obtained at 1000 and 1200 psi. As the mean recovery values were quite similar to the respective medians, it can be assumed that the above statistical conclusions hold for the mean concentrations as well.

*ASE: Static Extraction Time as a Variable*

Static extraction time during the procedure ranged from 1 to 5 minutes by 1-min intervals. Other parameters were constant (temperature = 80°C, static time = 5 min; pressure = 1500 psi, number of cycles = 1 and flush = 60%). The maximum yield was reached for the static time of 4 minutes (see Figure 9). Within the time range from 1 to 4 min, the mean recovery was increasing

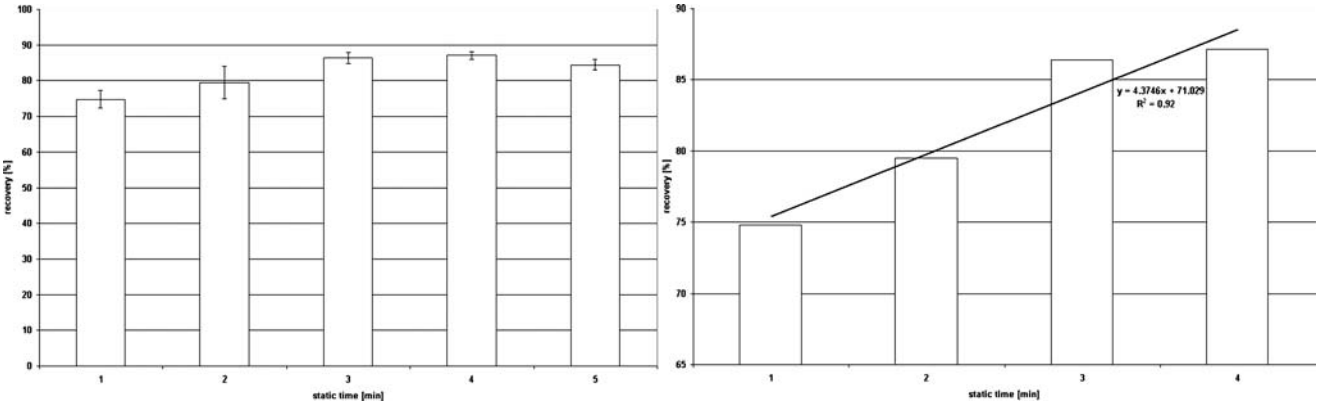


FIG. 9. Recovery of robenidine from poultry feed using ASE at five different static extraction times.

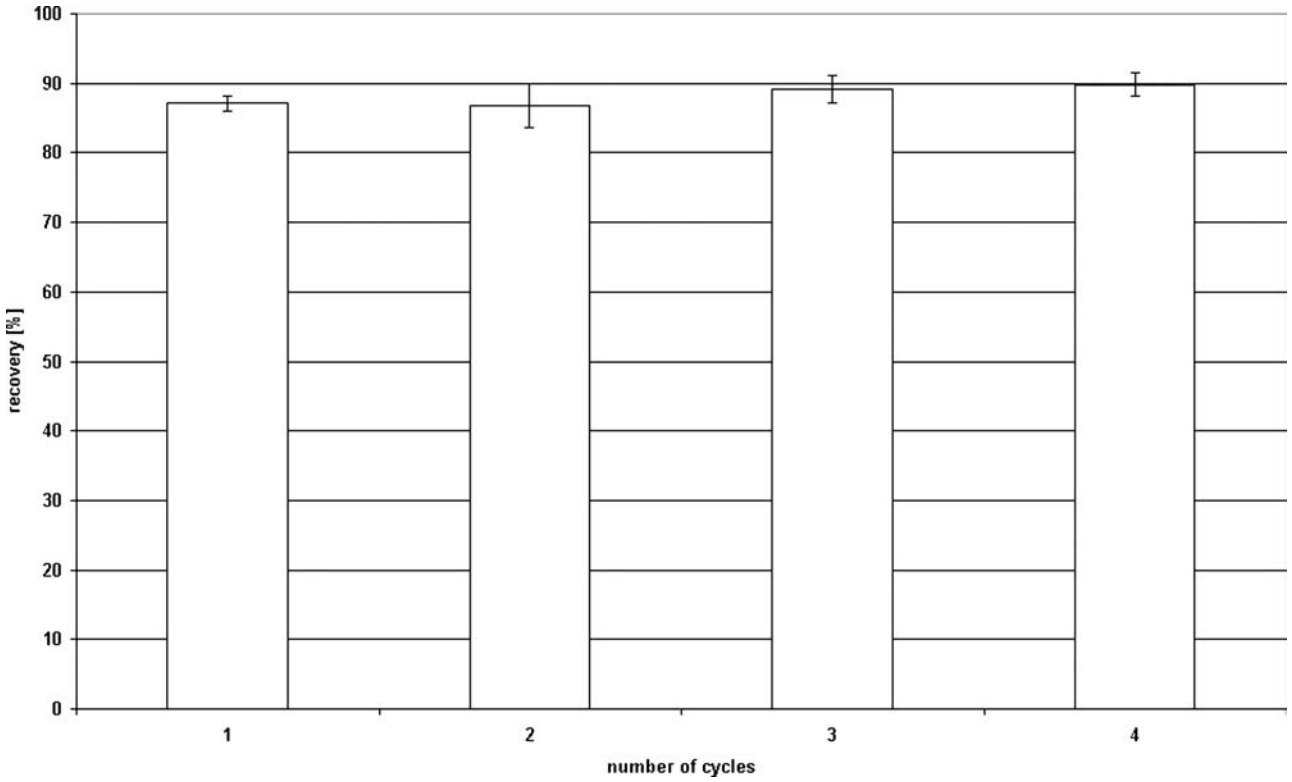


FIG. 10. Recovery of robenidine from poultry feed using ASE at four different numbers of extraction cycles.



with the increasing time; the relationship was described by the following linear regression equation: $y = 4.3746x + 71.029$ ($R^2 = 0.93$).

For the static times = 3 and 4 minutes, the obtained mean yield values were significantly higher than the values for 1, 2 and 5 minutes ($p = 0.05$). Based on the outcome of the K-W test, the null hypothesis about the lack of differences among median values was rejected. Therefore, the conclusion was formulated that the median yields for t = 3, 4 and 5 minutes was significantly higher than those for $t = 1$ or $t = 2$ minutes; no statistically significant differences in median recovery values were found for the time range from 3 to 5 minutes. Similar to the previous point, the statistical conclusions reached for the median values are applicable to the mean concentrations because the respective median and mean recovery values do not differ much.

*Number of Cycles and Flushing Volume in ASE*

The number of cycles performed for one sample and flushing volume was varied during the extraction, so the influence of those parameters on robenidine recovery from poultry feed sample could be checked. The obtained results are shown in Figures 10 and 11.

The maximum yields of 89.16 and 89.79% were reached for three and four extraction cycles, respectively. Because of the low standard deviation values for a series of measurements the statistically significant differences were found between the median recovery values for the extraction with four cycles as compared to that with one cycle. There were no differences between the extractions with one and two cycles, and the procedures with three and four cycles. No linear relationship was found either.

The maximum yield of 94.26% was attained for the extraction with the flush parameter equal to 100%. The statistically significant differences in median recovery values for specific variants of the modified procedures were found.

*Cluster Analysis.* In order to compare all the methods and all the modifications applied to extraction techniques, a cluster analysis was employed (Ward's agglomeration method using Euclidean distance) (Fig. 12).

As the outcome of cluster analysis, all the used extraction variants have been separated into three distinct groups that differ from each other by the mean recovery values, as follows:

1) 55–64%
2) 65–85%
3) 85–95%

The extraction technique based on shaking and ASE procedure (E1 and E2) with the mix of acetonitrile and formic/acetic acid (1% v/v) are the least efficient. Moreover, the modified ASE technique coded as E11, in which the temperature of 140°C was applied, is also in the above mentioned group. It is likely that in this case a partial, thermal degradation of organic compounds

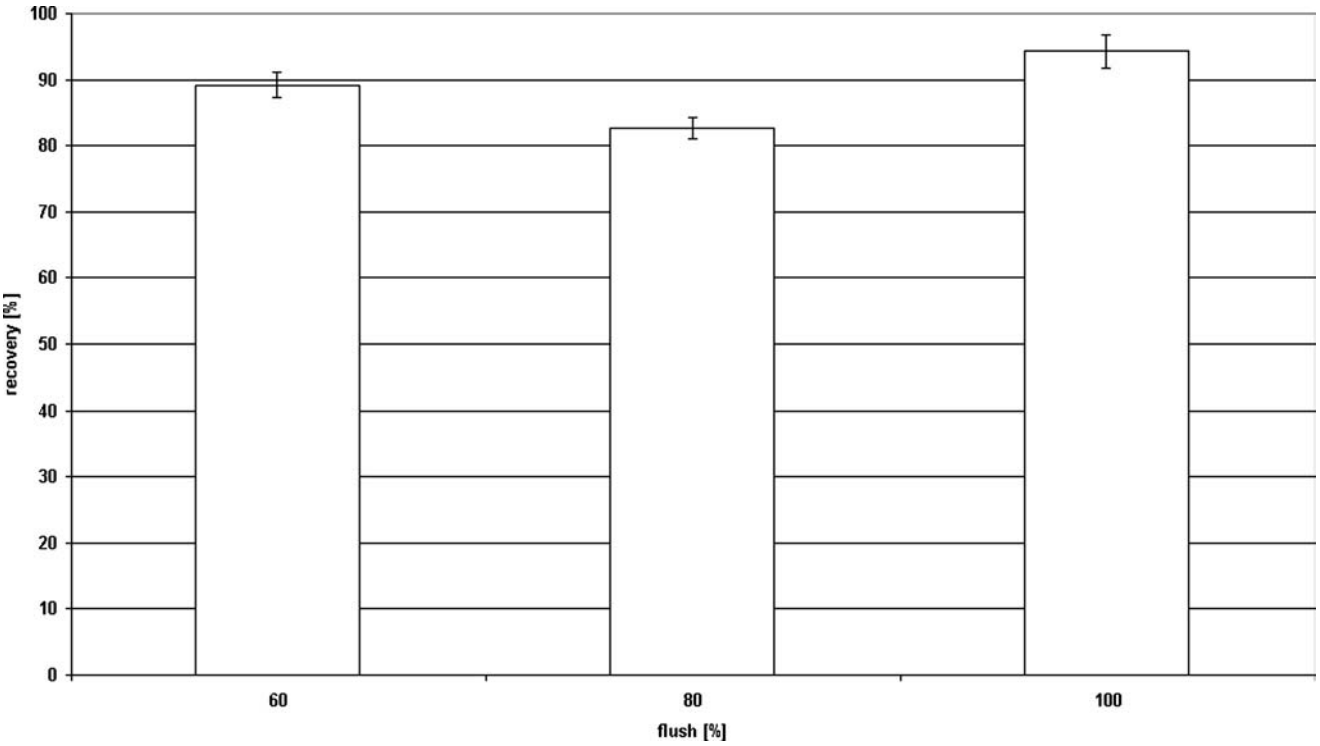


FIG. 11. Recovery of robenidine from poultry feed using ASE with three different flushing volumes.



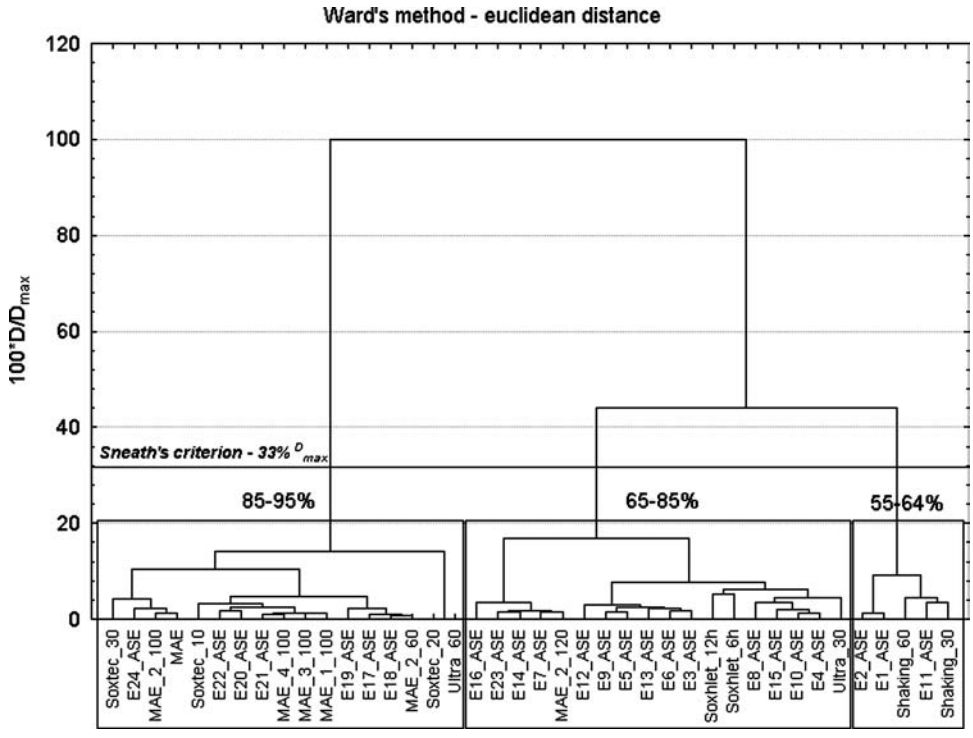


FIG. 12. Dendrogram of the cluster analysis according to Ward: the mean robenidine recovery by six compared extraction techniques.

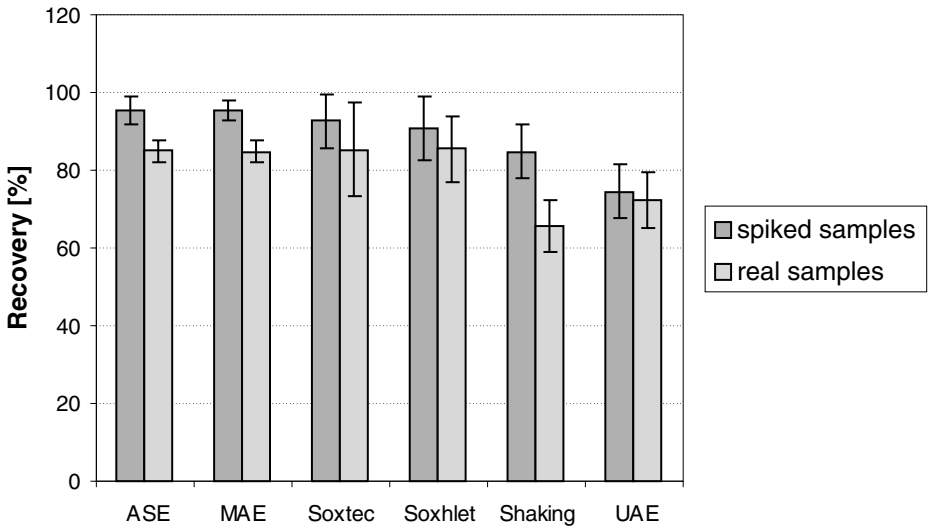


FIG. 13. An overall comparison of robenidine recovery from poultry feed samples obtained with different extraction techniques (Soxhlet, Soxtec, UAE, MAE, ASE and shaking).



took place which caused the decrease of mean recovery; a similar explanation applies to MAE t = 2 minutes and T = 120°C.

The second group of extraction techniques yielded mean recovery values ranging from 65 to 85%. The group consists of Soxhlet extraction, MAE (t = 2 minutes and T = 120°C), ultrasonic extraction (t = 30 minutes) and a number of ASE techniques with modified solvents. It is noteworthy that all the modified ASE techniques agglomerated in that cluster have one common feature, i.e. in 13 cases (93%) the number of extraction cycles is 1. Moreover, in most cases the static time was established to be 5 minutes. The two lowest recovery values were measured at the two extreme temperatures, i.e. 68.35% for T = 60°C and 72.00% for T = 120°C; therefore, it has been concluded that the optimal temperature range for this cluster of procedures was 80–100°C. A decreased mean yield seems to confirm the assumption about thermal degradation due to the raised temperature.

While analyzing the modified ASE techniques it was determined that the yield value of ca. 85% can be achieved for the following parameters: static time from 3 to 5 minutes, pressure = 1500 psi, temperature = 80°C, number of cycles = 1 and flush = 60%. Raising the pressure to 2500 psi, with the other parameters remaining constant, did not result in the statistically significant higher mean yield.

The results of cluster analysis indicated that the third cluster of procedures was mainly characterized by the number of cycles larger than 1 for flush = 60%. From among 24 analyzed variants the highest recovery value (94.26%) was obtained for the modified procedure coded E24.

*Analyses of Real-World Samples*. An overall comparison of robenidine recovery values from poultry feed samples obtained with all different extraction techniques presented in this paper (namely Soxhlet Soxtec, UAE, MAE, ASE and shaking) is shown in Figure 13.

Interestingly, four out of six techniques employing acidified methanol (1% $CH_3COOH$) and sound choices of extraction time and temperature produced nearly identical data. The optimal parameters in terms of efficiency and extraction time are shown in Table 3.

Based on the obtained data, it was concluded that the best extraction efficiency was achieved using MAE and ASE techniques. Moreover, the outcome of the statistical analysis revealed that the relative standard deviations (RSD) for those methods were very comparable, e.g. 2.9% and 3.0% for ASE and MAE, respectively.

The use of microwave energy enables rapid heating of the solvent mixture, accelerating the speed of heating, and consequently reducing the extraction time required. Thus the

TABLE 3
Optimal parameters for the investigated extraction techniques, i.e. ASE, MAE, Soxtec, Soxlet, shaking and UE.

| Extraction technique | Conditions |
|---|---|
| ASE | Temperature 80°C |
| | Time of extraction 4 min |
| | Flush 100% |
| | Pressure 1500 psi |
| | Number of cycles 3 |
| MAE | Temperature 100°C |
| | Time of extraction 2 min |
| | Power 150 W |
| Soxtec | Time of extraction 30 min |
| Soxhlet | Time of extraction 6 h |
| Shaking | Time of extraction 30 min |
| UE | Time of extraction 15 min |

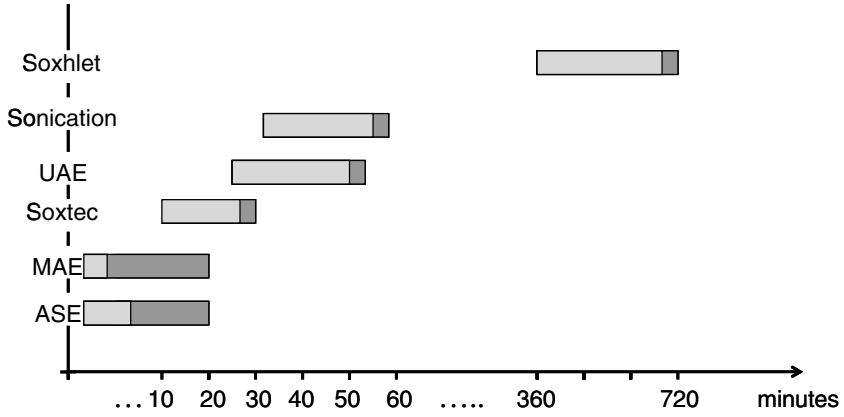


FIG. 14. Comparison of extraction times for Soxhlet, Soxtec, UAE, MAE and ASE obtained under the condition of optimal extraction efficiency. Light grey box represents time needed for the extraction; dark grey box represents time spent to obtain extract ready for further analysis.



extraction time becomes extremely short (2 minutes!), with the advantage of enabling the simultaneous extraction of even 12 samples. But cooling of the extract takes up to 20 minutes, so the final extraction time in case of MAE is usually about 20–25 minutes. Alternately, ASE combining high temperature with high pressure and dynamic extraction leads to slightly better extraction efficiency than conventional techniques. Although this technique requires a little more time for packing the thimbles, it has the great advantage of being fully automatic. Moreover, samples can be easily extracted overnight, extra handling is not needed to separate the matrix from the solvent (as it is the case for all the others techniques), and reduced manpower is required.

The results showed that a very good efficiency was achieved for Soxtec and Soxhlet extractions. In general, data obtained by using Soxtec and Soxhlet were very similar, and in both cases robenidine was isolated from the feeding material with the efficiency reaching 90%. In other words, Soxtec extraction performed for 30 minutes at boiling step and for 15 minutes at rinsing step can be substituted for Soxhlet extraction, which takes 6 hours. However, relatively high RSD values calculated for both methods pose the most important disadvantage.

The lowest recovery value was obtained for shaking and UAE. Nevertheless shaking is the most frequently used extraction technique as the instrumentation required for its application is not expansive and commonly available at all analytical laboratories. UAE is also used often despite the fact that the better extraction efficiency, expected due to mechanistic aspects of ultrasonic energy, was not observed. The calculated RSD values for both methods varied a lot (7–9%).

The most important features of all extraction techniques investigated in this study are summarized in Table 4.

TABLE 4
Characteristic features of the investigated extraction techniques.

| | Shaking | UAE | ASE | MAE | Soxhlet | Soxtec |
|---|---|---|---|---|---|---|
| Full time of extraction per one sample (includes heating, cooling and filtration) | 40 min | 30 min | 20 min | 45 min | 6.5 h | 45 min |
| Volume of solvent used | 60 ml | 25 ml | 30 ml | 10 ml | 45 ml | 25 ml |
| Assisted parameters of extraction | Shaking | Ultrasonic | Temperature pressure | Microwave | Temperature | Temperature |
| Level of difficulty | Low | Low | Medium | Medium | Low | Medium |
| Number of samples which can be extracted simultaneously | One sample or series | One sample or series | One sample or series | One sample or series | one | 6 |
| Level of automation | Low | Low | High | High | Low | Medium |
| Equipment cost | Low | Low | High | High | Low | Medium |

## CONCLUSIONS

Sample preparation is an essential part of every solvent-based extraction procedure. While many types of samples can be efficiently extracted without any pre-treatment, lots of them will require some manipulation to achieve an efficient extraction.

For any efficient extraction, the solvent should be able to solubilize the target analytes while leaving the sample matrix components intact. The polarity of the extraction solvent should closely match polarity of target compounds. For an efficient extraction, the solvent has to create sufficient contact with the analyte, so the higher surface area of the sample, the faster extraction. For that reason all samples in this study were ground before analysis [5, 6]. Based on the obtained results, methanol containing $CH_3COOH$ (1% v/v) was selected as optimal solvent used for the extraction of robenidine from feeding stuff. This solvent composition was characterized by the highest recovery and the lowest relative internal standard.

Temperature plays an important role during any extraction process. As the temperature rises, the viscosity of the matrix and solubilization of the target analytes increases. The added thermal energy also enhances breaking off analytes from the matrix surface. However, in most techniques isolation takes place either at ambient temperature or at the boiling point of a solvent used for the extraction. The only exceptions are ASE and MAE for which extraction temperatures can be evaluated.

Certain sample matrices can retain analytes within their pores or other structures. Increasing the extraction time at elevated temperatures can allow these compounds to diffuse into the extraction solvent. Time is one of the most important parameters during extraction; the other parameters cannot influence analyte recovery from the sample in such a critical way. In this study, time of extraction was evaluated for all types of extraction techniques that had been applied. It turned out that all techniques used for the isolation of robenidine from poultry feed have different optimal extraction times. Shortening the extraction time is crucial since sample preparation step is often obligatory and the most time-consuming part of an analytical procedure. A comparison of the extraction times for all techniques investigated in this study, i.e. Soxhlet, Soxtec, UAE, MAE and ASE is presented in Figure 14.

Hence, the results showed that extraction time is the shortest in case of MAE and ASE, and it is comparable with that used for Soxtec. However, time that an analyst must spend until the extract is ready for further analysis differs among techniques. For ASE, the extract is ready immediately, while in the case of MAE, it is mandatory to allow nearly half an hour for cooling, and up to ten filtration or centrifugation cycles have to be performed. Although the extraction time for Soxtec technique is comparable with the optimised extraction time for ASE, two contrary effects were observed in Soxtec procedure. On one hand, cooling of the extract is necessary (which takes some time), on the other hand, evaporation of the excess solvent can be achieved in the same time. In case of Soxhlet, the optimal extraction time is very long in comparison with those established for all the other techniques. Moreover, filtration and clean-up steps are essential.

In case of ASE technique, many parameters should be optimized. In addition to the extraction time and temperature, parameters such as pressure, number of cycles and volume of extract have to be evaluated. Thus method development can be complicated and time-consuming. Moreover, robenidine recovery from complex matrices can be enhanced by yet another very important factor, which is selectivity.

This study demonstrates that the six different extraction techniques used for determining robenidine levels in feeding stuff are, in principle, interchangeable. Although ASE and MAE have previously been considered as alternative methods, the results clearly indicate that those techniques should be considered as conventional methods with performance similar to the recommended by European Union (EU) extraction by shaking. Agglomerative cluster analysis and statistical comparisons have revealed that ASE and MAE were statistically more efficient than the other conventional methods.

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