1. Introduction

Pharmaceuticals are used worldwide to safeguard both human and animal wellbeing. Unfortunately, some of the pharmaceuticals have been increasingly penetrating into our environment either in an unchanged form or as metabolites. The main emission source of the pharmaceuticals generally takes the form of sewage from pharmaceutical plants, hospitals and households. Sewage typically contains excreted but not quite metabolized medications. Furthermore, some unused and/or expired pharmaceuticals from households and hospitals, instead of being safely utilized, are disposed of into wastewater or waste landfills. Agriculture constitutes a major source of pharmaceuticals particularly those used in animal husbandry [1-5]. Pharmaceuticals by design are biologically active substances and in spite of the fact that there is no definitive evidence concerning direct and harmful effects on organisms caused by a large number of medications, some subtle influences of these substances is possible, and if the process continues for generations, it may result in vital changes in our ecosystems [6-9].

Although analytical techniques are being constantly improved, identification and analysis of pharmaceuticals present in the environment causes enormous difficulties to researchers as these chemical compounds occur in low level concentrations in samples of complex matrix compositions. Pharmaceutical residues are not subjected to any norms defining their allowed concentration, and sewage treatment procedures ignore or disregard their possible existence. For this reason these compounds...
together with their metabolites are often found in the treated effluent penetrating into the water environment where they may undergo different physicochemical changes.

This study focuses on the development of analytical method which permits simultaneous determination of five human pharmaceuticals: caffeine, carbamazepine, clomipramine, chlorprothixene and clotrimazole found at low concentrations in wastewater. The first four analytes are psychoactive substances and clotrimazole is popular antimicrobial drug. Caffeine is an alkaloid, a derivative of xanthine found in coffee, tea and cola nuts. Caffeine is classified as a drug with analeptic effects as well as stimulant of the central nervous system. Used along with painkillers it enhances their effectiveness. Carbamazepine is a medication with 3-membered structure. It has antiseizure and psychotropic effects, it also regulates mood and is used in the treatment of trigeminal nerve neuralgia. Clomipramine is a derivative of dibenzoazepine. It is used in the treatment of depression syndrome and in obsessive-compulsive neurosis. Chlorprothixene is a neuroleptic medication, a derivative of tioxantene. It is a drug with strong, anxiety-relieving and antipsychotic impact and weak antiaustistic and antidepressive function. Clotrimazole is a medication from imidazol group with antimycotic, antitrichomonal and weak antibacterial effects. The structures and some properties of the discussed compounds are given in Table 1.

Caffeine, carbamazepine and clotrimazole were found in different environmental samples: river, sea, surface and underground water as well as influent and effluent wastewater [10-22], clomipramine and chlorprothixene have not been discovered in environmental samples so far. The content of caffeine in natural water reaches 160 ng L⁻¹, wastewater contains even 66 μg L⁻¹ of this compound [10-18]. Carbamazepine appears in natural water in concentrations up to 0.9 μg L⁻¹, the concentration of this pharmaceutical in sewage ranges from several dozens of ng L⁻¹ to 2.5 μg L⁻¹ [14-20]. Clotrimazole was discovered in rivers in concentrations from several to several dozens of ng L⁻¹. Similar concentrations of this substance were recorded in sewage [21-23].

In order to determine caffeine, carbamazepine and clotrimazole in environmental samples liquid chromatography (LC), high performance liquid chromatography (HPLC) and gas chromatography (GC) were used. Detection techniques involved mainly the use of mass spectrometry (MS) with electron ionization (EI-MS) as well as ionization in electric field (ESI-MS). With the HPLC technique commonly available spectrophotometer with diode array matrix (DAD) and fluorescent detector (FLD) were also used. To isolate the analyzed compounds from environmental samples solid phase extraction (SPE) was applied, and for caffeine determination liquid-liquid extraction (LLE) was also used. It should be noted that SPE is relatively time consuming as it requires multi-step procedures and it is also expensive because cartridges must be disposed of after each conducted extraction. On the other hand, LLE uses large amounts of toxic organic solvents and as a result it is hazardous to human health, causes environmental contamination and generates additional costs for residue treatment. Compared to the above mentioned techniques of isolation, solid phase microextraction (SPME) developed by Pawliszyn is very advantageous [24]. It is a solvent free isolation technique that is simple, efficient and clean. To conduct SPME isolation a relatively small sample is needed and its preparation is quick. In the headspace mode of SPME (HS-SPME), the fiber is exposed to vapor phase above liquid or solid matrix. In direct immersion SPME (DI-SPME), the fiber is directly placed in the sample. HS-SPME is usually used for volatiles and semi-volatiles while DI-SPME is suitable for isolation of non-volatile compounds [25].

The aim of this study was to develop a new simple and sensitive solvent-free method for simultaneous determination of caffeine, carbamazepine, clomipramine, chlorprothixene and clotrimazole at low concentrations in municipal wastewater. DI-SPME was used for the isolation and GC-MS in the selected ion monitoring (SIM) mode was used for separation and determination of analytes.

2. Experimental procedure

2.1. Chemicals and solutions

Caffeine, carbamazepine, clomipramine, chlorprothixene and clotrimazole were purchased from Sigma-Aldrich (Germany). Methanol (analytical grade), sodium chloride, hydrochloric acid and sodium hydroxide were obtained from POCh Gliwice (Poland). Stock solution of the standards was prepared by dissolving an appropriate amount of each substance in methanol and it was stored at -18°C. Concentrations of compounds in stock solution were as follows: 10 mg mL⁻¹ for caffeine and carbamazepine, 2 mg mL⁻¹ for clomipramine and 1 mg mL⁻¹ for chlorprothixene and clotrimazole. Working standard solution was freshly prepared by dilution of stock solution in ultrapure water (from Milli-Q RG, Millipore, USA purification system) daily, and was stored at 4°C.
2.2. Wastewater samples
Municipal wastewater samples were obtained from the six wastewater treatment plants (WWTPs) located in central and north-eastern Poland. Purification process conducted in these plants consists of two stages: mechanical purification and biological purification. Average daily samples of influent and effluent wastewater were taken between March and May 2010. Samples of wastewater were drawn into glass bottles. Upon arrival the samples were immediately acidified (pH = 2) with the use of hydrochloric acid, filtered through filter paper to obtain clear solution and then stored at -18°C until analyzed.

2.3. Direct immersion solid-phase microextraction
A SPME holder and several different fibres were purchased from Supelco (USA). The fibres used in the
study were 85 μm polyacrylate (PA), 30 μm (PDMS30), 100 μm (PDMS100) polydimethylsiloxane, 65 μm polydimethylsiloxane/divinylbenzene (PDMS/DVB) and carboxen/polydimethylsiloxane (CAR/PDMS). The fibers were conditioned before the usage according to manufacturer’s instructions by inserting them into the GC injector. Fiber blanks were run each day to exclude analyte carryover effect. When real samples were extracted the fiber was rinsed with water prior to analysis.

The working solution together with sodium chloride (enhance SPME adsorption) and sodium hydroxide or hydrochloric acid (to obtain proper pH value) were placed into 18 mL SPME vial. The vial was fully filled and then sealed tightly using screw cap with PTFE silicone-faced septum. The fiber was introduced through the septum and extraction was carried in the direct immersion mode. During extraction, the sample was magnetically stirred at 750 rpm. After extraction the fiber was exposed directly in the GC injector for thermal desorption and analysis. The same fiber was used for about 60-80 extraction cycles.

2.4. Chromatographic analysis
Gas chromatographic analyses were carried out using an HP 6890 gas chromatograph with electronic pressure control connected with mass spectrometric detector MSD 5973 (electron impact source and quadrupole analyzer) from Agilent Technologies, USA. This device was equipped with HP-5MS column (5% phenylmethylsiloxane) size 30 m length × 0.25 mm, i.e., coated with 0.25 μm film thickness and split/splitless injector. The injector worked in splitless mode. Helium of 99.999% purity was used as a carrier gas at a flow rate of 1 mL min⁻¹. The oven temperature was programmed from 120°C (2 minutes hold) increasing at a rate of 10°C min⁻¹ to 280°C and maintained in maximum temperature for 4 minutes. The total run time was 22 minutes. The MS detector worked in Selected Ion Monitoring (SIM) mode. The electron impact source temperature was 230°C with electron energy of 70 eV. The quadrupole temperature was 150°C, and the GC interface temperature was 280°C. The instrument was tuned on perfluorotributylamine. The retention times of analytes and masses chosen for quantification and confirmation are presented in Table 1. Detector calibration was carried out for the mixture of all analytes in methanol solutions. One microliter of solutions with concentrations of 0.1, 0.25, 0.5, 1, 2, 5, 10 and 200 mg L⁻¹ were injected with the autosampler into GC-MS device four times for each concentration solution. Calibration revealed linearity of MS detector functioning in the whole concentration range considered. The determination coefficients equal 0.999 for caffeine, carbamazepine and clomipramine, 0.997 for clotrimazole and 0.996 for chlorprothixene.

3. Results and discussion
3.1. Optimization of extraction and desorption procedure
With regard to low volatility of the analytes, the direct immersion variant of SPME was chosen for realizing isolation. The sensitivity of analytes isolation in this option of extraction depends on the value of the distribution coefficient for the sample and the fiber stationary phase, and thus on the type of fiber coating. The efficiency of isolation of the target compounds was compared by the use of four commercially available stationary phases, differing in polarity and thickness of the coating: PDMS, PA, PDMS/DVB and CAR/PDMS. The experiments were carried out using 18 mL aliquots of water spiked with the compounds at 10 ng mL⁻¹, 0.5 g of sodium chloride at pH=7. Extractions were performed at room temperature for 30 minutes. The normalized detector responses of target compounds obtained with the use of the individual fiber coatings are shown in Fig. 1. The best extraction for carbamazepine, clomipramine and caffeine was achieved with the PA fiber, while for chlorprothixene and clotrimazole the PDMS fiber has the best efficiency. Finally, the PA and PDMS100 fiber coatings were chosen for further investigation.

The effects of extraction temperature on the detector responses of the target compounds were examined at 22, 50, 70 and 90°C. Figs. 2a and 2b show the results of the extraction temperature optimization. The results revealed that for the PA and PDMS fibers the amounts of the absorbed analyte are increasing together with the increase of temperature of sample extraction. Thus, 90°C was selected as the optimum extraction temperature. The effects of the isolation time on the detector responses are illustrated in Figs. 3a and 3b. It is evident from the figures that in the case of the PA fiber that the peak area of each compound increased up to 30 minutes and then decreased or remained constant, depending on the analyte. In the case of the PDMS fiber the optimal time of extraction equals 60 minutes.

The ion strength of the sample solution was studied by adding different amounts of NaCl in the range from 0 to 10% to spiked water samples. Figs. 4a and 4b show the effect of sodium chloride concentration on the responses of the target compounds. For all analytes their responses increased up to 5% of NaCl but the addition of larger quantities of NaCl had a negative effect on the DI-SPME efficiency. The possible reason for this is evident.
Use of direct immersion solid-phase microextraction on polyacrylate and polydimethylsiloxane stationary phases for simultaneous determination of the neutral and basic pharmaceuticals in wastewater was that the high salt content in the water affects the hydration of the ionizable process, causing their increased solubility in the aqueous phase. Therefore, all extractions were conducted with 5% of NaCl. The pH can affect the extraction efficiency by decreasing the solubility of the target compounds when they are mainly present in their neutral form. The effects of the sample solution pH were examined for PDMS fiber at pH 5, 7 and 9 (the maximum pH value for work with this fiber coating equals 10). pH was adjusted by HCl or NaOH. As shown in Fig. 5, the efficiency of DI-SPME isolation of target pharmaceuticals is increasing together with the increasing pH of the solution. pH 9 was selected as the optimum value for extraction with use of the PDMS fiber. The isolation of analytes by fiber with PA coating was done in pH 10.

The desorption temperature and time needed for the desorption of analytes from fibers were investigated. Temperature was varied between 250 and 280°C for PDMS and between 250 and 300°C for PA fiber. 280 and 300°C are the maximum temperatures recommended by the producer for operating the PDMS100 and PA stationary phases, respectively. The signals for all target pharmaceuticals increased up to maximum temperatures, which were selected. The 10 min desorption time was selected as the optimal to achieve the total desorption of all analytes with no memory effect.

3.2. Validation of the method
Analyses of spiked deionized water samples were performed in order to validate the method for linearity, detection and quantification limits, as well as selectivity and precision. The tested concentration range was from 1 to 100 µg L⁻¹ for caffeine and carbamazepine, from 0.1 to 20 µg L⁻¹ for clomipramine, and from 0.05 to 10 µg L⁻¹ for chlorprothixene and clotrimazole. The DI-SPME/GC-MS validation parameters for the determination of the studied pharmaceuticals are shown in Table 2. The analytical curves were obtained by least-squares linear regression analysis of the peak area in relation to the analyte concentration, using five concentration levels in triplicate. The regression
coefficients ($R^2$) for all studied compounds were between 0.9957 and 0.9985. Similar linearity was obtained, when using both types of stationary phases. The limits of detection (LODs), calculated as three times the signal-to-noise ratio, ranged from 10 ng L$^{-1}$ to 90 ng L$^{-1}$ in the case of determination done using PA fiber and from 3 to 82 ng L$^{-1}$ for the PDMS fiber. The limits of quantification (LOQs) were calculated as 10 times the signal-to-noise ratio.

| Compound     | Linearity [µg L$^{-1}$] | $R^2$ | LOD [ng L$^{-1}$] | LOQ [ng L$^{-1}$] | Intra-day precision RSD [%] | Inter-day precision RSD [%] |
|--------------|------------------------|-------|-------------------|-------------------|----------------------------|----------------------------|
| PA Caffeine  | 1 - 100                 | 0.9981| 84                | 277               | 9.3                        | 9.8                        |
| Carbazepine  | 1 - 100                 | 0.9985| 89                | 292               | 9.2                        | 9.7                        |
| Clomipramine | 0.1 - 20                | 0.9957| 8                 | 59                | 8.8                        | 8.9                        |
| Chlorprothixene | 0.05 – 10            | 0.9962| 12                | 40                | 7.3                        | 8.0                        |
| Clotrimazole | 0.05 - 10               | 0.9976| 10                | 34                | 7.1                        | 7.5                        |
| PDMS Caffeine | 1 - 100                 | 0.9968| 75                | 248               | 9.0                        | 9.3                        |
| Carbazepine  | 1 - 100                 | 0.9973| 82                | 269               | 8.9                        | 9.1                        |
| Clomipramine | 0.1 - 20                | 0.9973| 11                | 31                | 8.4                        | 8.5                        |
| Chlorprothixene | 0.05 – 10            | 0.9968| 7                 | 23                | 5.6                        | 6.0                        |
| Clotrimazole | 0.05 - 10               | 0.9980| 3                 | 8                 | 5.0                        | 5.7                        |
Use of direct immersion solid-phase microextraction on polyacrylate and polydimethylsiloxane stationary phases for simultaneous determination of the neutral and basic pharmaceuticals in wastewater.

Repeatability, expressed as the relative standard deviation of the analysis of five samples spiked at 1 µg L⁻¹ was below 9% in the case of the determination done with the PDMS fiber and below 9.3% for the determination with the PA. The reproducibility between days, expressed as the relative standard deviation of five samples, spiked at 1 µg L⁻¹, and analyzed on five different days was below 10% for both stationary phases used for determination. Slightly better repeatability and lower LOD values were obtained with use of the fiber with PDMS coating, and this stationary phase was chosen for further investigations. The selectivity of the method was assessed by the absence of interfering peaks at the elution times of the pharmaceuticals for blank chromatograms of different wastewater samples.

### 3.3. Matrix effect

The analyses of spiked wastewater samples were conducted in order to evaluate the influence of the matrix (influent wastewater and effluent wastewater) on the analytical parameters of the method. The graphs presenting the calibration of standard additions for different wastewater samples, obtained by plotting the concentration (at five levels) against the peak areas and following the linear regression analysis, were compared. The evaluated parameters are shown in Table 3. The slopes for wastewater samples differ greatly from those for the aqueous standards, although a good linearity ($R^2 > 0.987$) was obtained. The values of LOD and LOQ, determined for the wastewater matrix were higher than those obtained from pure water. This is due to complicated matrices, since different substances present in the wastewaters would interfere with the extraction step. The maximum RSD values amounted to 14.8% for raw and 14.2% for purified wastewaters, showing good repeatability for all target pharmaceuticals.

### 3.4. Fiber/water distribution coefficients

The fiber/water distribution coefficient ($K_{fw}$) is an important property for understanding the sorption mechanism of SPME. $K_{fw}$ could be used for quantitative determination of the amounts of extracted analytes, in order to predict the limits of detection and also to facilitate the choice of the best analytical conditions.

The distribution coefficient of a compound between two phases is defined as the concentration ratio of this compound in each of the phases. In the case of partition between the SPME fiber and water, the distribution coefficient is equal to the concentration ratio of the compound dissolved in the stationary phase and in water. In this work the following equation was used for $K_{fw}$ calculation:

$$K_{fw} = \frac{m_{s}}{V_{s} \cdot C_{0}}$$  \hspace{1cm} (1)

where $m_{s}$ is the mass of the compound (ng) absorbed by the fiber, determined on the basis of the detector calibration, $V_{s}$ the volume of film of the fiber stationary phase (µL) and $C_{0}$ is the concentration of the compound in the water phase (ng µL⁻¹). The used volumes of stationary phases of the fibers are as follows:
0.612 μL for PDMS100 and 0.494 μL for PA 85 [28]. The $K_{fw}$ were determined by analyzing the solutions with concentrations of 0.5, 1 and 10 µg L⁻¹ in deionized water. The solutions were prepared in 18 mL SPME vials, the vials were fully filled. The determination was performed at 90°C for 30 minutes with the use of PA fiber and for 60 minutes with the use of the PDMS fibers. Four independent determinations were performed for each concentration and the $K_{fw}$ value was calculated as an arithmetic mean of twelve values.

Table 4 gives the determined values of distribution coefficients in the PDMS/water and PA/water systems, as well as those in the octanol/water system ($K_{ow}$).

### Table 4. PDMS/water and PA/water distribution coefficient ($K_{fs}$) and log $K_{ow}$ values for caffeine, carbamazepine, clomipramine, chlorprothixene and clotrimazole.

| Compound          | log $K_{ow}$* (based on: http://logkow.cisti.nrc.ca/logkow.html (LOGKOW Sangster Research Laboratories), in parentheses recommended value is given) |
|-------------------|---------------------------------------------------------------|
|                   | PDMS                            | PA                  |
|                   | $K_{fs}$ ± SD                      | $K_{fs}$ ± SD        |
| caffeine          | -0.21 ± 0.10 (0.07)               | 41 ± 3              |
|                   | 1.61                             | 57 ± 6              |
|                   | 1.76                             | 1.76                |
| carbamazepine     | 1.54 ± 2.45 (2.30)                | 1112 ± 71           |
|                   | 3.05                             | 1555 ± 121          |
|                   | 3.19                             | 3.19                |
| clomipramine      | 1.75 ± 5.71 (5.19)                | 14215 ± 796         |
|                   | 4.15                             | 16818 ± 992         |
|                   | 4.23                             | 4.23                |
| chlorprothixene   | 2.06 ± 5.18 (5.18)                | 3156 ± 334          |
|                   | 3.50                             | 3843 ± 165          |
|                   | 3.58                             | 3.58                |
| clotrimazole      | 3.50 ± 4.91                      | 7119 ± 327          |
|                   | 3.85                             | 10307 ± 495         |
|                   | 4.01                             | 4.01                |

* Slightly higher $K_{fs}$ values were registered for the PA fiber than for the PDMS fiber. It is connected with the high affinity of the polyacrylate stationary phase for the compounds with moderate polarity. In previous publications a strong correlation between the log $K_{fs}$ and log $K_{ow}$ for different groups of compounds was reported [27,28]. Table 4 shows, however, that little or no correlation exists between log $K_{fs}$ and log $K_{ow}$ for the analyzed compounds. Such a situation was previously reported for pesticides [29,30]. One of the reasons for the lack of correlation between the distribution coefficients in the octanol/water and fiber/water systems could be the large divergence between log $K_{fs}$ values obtained with the use of different methods (see Table 4).

### 3.5. Wastewater samples analysis

The DI-SPME/GC-MS method, developed in this study, was applied in the determination of caffeine, carbamazepine, clomipramine, chlorprothixene, and clotrimazole in influent and effluent wastewater from the municipal wastewater treatment plants located in central and north-eastern Poland. The PDMS100 stationary phase was used for the isolation of analytes. NaCl (5 g) was added to 100 mL of filtered wastewater and the sample was then adjusted to pH 10 with NaOH. An aliquot of 18 mL of the sample solution was then transferred to the SPME vial and submitted to SPME and GC-MS analysis in the conditions described previously.

The concentrations of the target compounds in influent wastewater were determined from calibration curves registered by analysis of spiked influent wastewater samples and the concentrations of the target compounds in effluents were calculated on the basis of the calibration curve registered by the use of effluent wastewater samples. Three pharmaceuticals: caffeine, carbamazepine and clotrimazole were found in the influents and effluents from the WWTPs (Table 5). Caffeine was detected in all analyzed samples at concentrations between 31.9 and 52.3 µg L⁻¹ in influents and between 0.36 and 1.66 µg L⁻¹ in effluents.
Carbamazepine was detected in five samples at a concentration ranging from 90 to 321 ng L\(^{-1}\). Clotrimazole was detected in four samples of influents and three samples of effluents at concentrations lower than 30 ng L\(^{-1}\). The concentrations of the studied compounds are similar with those reported by other authors. The removal rates of caffeine ranged between 94 and 99% and those for clotrimazole were between 82 and 86%. Practically no removal rates were observed for carbamazepine. These results are coincident with those given in previous publications [16,26]. The situation is connected with high persistence of carbamazepine. Some quantities of this pharmaceutical probably could also be retained in activated sludge and subsequently rinsed with new portions of wastewaters. The result of such situations is that sometime higher concentrations of carbamazepine are observed in effluents than in influents. Clomipramine and chlorprothixene were not detected in any wastewater samples in concentrations above LOQ.

### Table 5. Levels of target compounds in municipal wastewater from six Polish wastewater treatment plants (WWTPs).

| WWTP\(^*\) | Concentration of compound (ng L\(^{-1}\)) ± SD |
|-----------|---------------------------------------------|
|           | Caffeine | Carbamazepine | Clotrimazole |
|           | Influent | Effluent | Influent | Effluent | Influent | Effluent |
| WWTP1     | \(Q_d = 70000 m^3 \cdot d^{-1}\) | 5183 ± 916 | 532 ± 69 | 123 ± 11 | 187 ± 11 | 27 ± 9 | 4 ± 1 |
| WWTP2     | \(Q_d = 38000 m^3 \cdot d^{-1}\) | 4129 ± 436 | 763 ± 63 | 269 ± 38 | 321 ± 25 | 14 ± 6 | 3 ± 1 |
| WWTP3     | \(Q_d = 25600 m^3 \cdot d^{-1}\) | 4464 ± 242 | 357 ± 34 | 97 ± 8 | 90 ± 8 | 15 ± 3 | 4 ± 1 |
| WWTP4     | \(Q_d = 20000 m^3 \cdot d^{-1}\) | 3278 ± 371 | 440 ± 69 | 110 ± 9 | 110 ± 7 | <LOD | <LOD |
| WWTP5     | \(Q_d = 9600 m^3 \cdot d^{-1}\) | 3191 ± 959 | 118 ± 33 | <LOD | <LOD | <LOD | <LOD |
| WWTP6     | \(Q_d = 100000 m^3 \cdot d^{-1}\) | 5231 ± 745 | 160 ± 34 | 210 ± 28 | 240 ± 19 | 12 ± 2 | <LOD |

\* \(Q_d\) - average daily capacity

### 4. Conclusions

The new sensitive analytical method, based on the direct immersion SPME as the isolation technique, is proposed for simultaneous determination of five base and neutral pharmaceuticals: caffeine, carbamazepine, clomipramine, chlorprothixene and clotrimazole. The proposed method was successfully applied and determined the studied compounds in influent and effluent municipal wastewater samples. The developed method is very simple and does not contain any clean-up steps. As the procedure omits the use of organic solvents, the possibility of loss of analytes and secondary pollution is considerably reduced and, therefore, the method is environmentally friendly. The time saving character of the proposed method makes it suitable for routine determinations.
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