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Extraction Efficiency of a Commercial Espresso Machine Compared to a Stainless-Steel Column Pressurized Hot Water Extraction (PHWE) System for the Determination of 23 Pharmaceuticals, Antibiotics and Hormones in Sewage Sludge

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Abstract: Two green chemistry extraction systems, an in-house stainless-steel column Pressurized Hot Water Extraction system (PHWE) and a commercially available Espresso machine were applied for analysing 23 active pharmaceutical ingredients (APIs) in sewage sludge. Final analysis was performed on UPLC-MS/MS using two different chromatographic methods: acid and basic. When analysing all 23 APIs in sewage sludge both extraction methods showed good repeatability. The PHWE method allowed for a more complete extraction of APIs that were more tightly bound to the matrix, as exemplified by much higher concentrations of e.g., ketoconazole, citalopram and ciprofloxacin. In total, 19 out of 23 investigated APIs were quantified in sewage sludge, and with a few exceptions the PHWE method was more exhaustive. Mean absolute recoveries of 7 spiked labelled APIs were lower for the PHWE method than the Espresso method. Under acid chromatographic conditions mean recoveries were 16% and 24%, respectively, but increased to 24% and 37% under basic conditions. The difference between the PHWE method and the Espresso method might be interpreted as the Espresso method giving higher extraction efficiency; however, TIC scans of extracts revealed a much higher matrix co-extraction for the PHWE method. Attempts were made to correlate occurrence of compounds in sewage sludge with chemical properties of the 23 APIs and there are strong indications that both the number of aromatic rings and the presence of a positive charge is important for the sorption processes to sewage sludge.

Keywords: espresso coffee machine extraction; pressurized hot water extraction; pharmaceuticals; antibiotics; hormones; sewage sludge; ion suppression; UPLC MS/MS; basic buffer

1. Introduction

More than 1000 different active pharmaceuticals ingredients (APIs) are today used in Sweden [1]. The release of APIs into the water environment has been a subject of research for more than 30 years [2], and their ubiquitous occurrence at varying concentration levels have been shown in wastewater, surface water, sediment, groundwater and drinking water [3–9]. Large research resources have been spent worldwide on investigating the occurrence of pharmaceutical residues in the water phase, a significantly smaller proportion resources of these compounds' presence in sewage sludge [10]. The production of sewage treatment plant (STP) sludge in Sweden is estimated at approximately 207,500 tons of dry matter, finalized at over 400 STPs. The sludge contains, besides carbon, about 3% phosphorus and 3.5% nitrogen. This means that around 6000 tons of phosphorus and 7000 tons of

nitrogen can be recycled in Sweden and returned to the soil via sludge each year [11]. Sludge spread on farmland is the largest single use category, since it is the most economical outlet for sludge and offers the opportunity to recycle plant nutrients and organic matter to soil for crop production. Agricultural use is estimated at approximately 50,000 tons, which corresponds to 25% of the total Swedish net production [12]. Sludge spreading in Sweden is regarded as an environmentally hazardous activity, but is still not subject to authorization or reporting, though authorities may impose stricter requirements in individual cases. From a European perspective the European Council Directive 86/278/EEC on the protection of soil, regulates sewage sludge use in agriculture in the EU. In several EU countries Directive 86/278/EEC is complemented by national legislation on soil protection; however, such legislation does not include regulation of pharmaceutical residues, even though there is increasing societal awareness and debate on the fate of APIs during sludge management. The fact that the chemical load in the form of pharmaceuticals and most other emerging contaminants (ECs), is unregulated both in wastewater effluents and in produced sewage sludge at the STPs, may in part be due to the fact that sludge chemically is a very complex matrix, which severely complicates the chemical analysis [13,14]. Nevertheless, sewage sludge is a sink for many of these substances [15] and given the spreading of sludge on farmland makes it important to seek valid results on its content of APIs. Traditionally, a variety of techniques have been used for extracting organic pollutants, including APIs, from sewage sludge such as Soxhlet, ultrasound assisted extraction, microwave-assisted extraction, mechanical shaking, supercritical fluid extraction and pressurized liquid extraction [16–20]. Since APIs hold a variety of physicochemical properties it is difficult to find a single method capable of analysing such a large number of differing chemical substances. Furthermore, emerging in recent years, there is a growing environmental concern in chemistry reshaping this field towards "green chemistry" [21], which is defined as the design of chemical products and processes that reduce or eliminate the use or generation of hazardous substances [22]. One possible technique possessing properties with the potential of meeting both these criteria is pressurized hot water extraction (PHWE) [23].

From a scientific perspective, experimental work on PHWE dates back to at least 1994 when Hawthorne et al. [24], interested in finding environmentally friendly extraction methods, suggested water as a clean solvent for the extraction of non-polar analytes from environmental samples. In their pioneering work, performed on their in-house constructed equipment using stainless-steel columns, they found an increase in solubility of non-polar organics such as polyaromatic hydrocarbons (PAHs) in water with increasing temperature. The basic experimental set-up presented by Hawthorne and co-workers [24] has previously been used by us for the extraction of APIs from sediment [9], using a stainless-steel column Pressurized Hot Water Extraction system (PHWE method). This system and methodology was applied in this work as a reference methodology using a temperature of 150 °C as it has previously been shown that temperatures exceeding 100 °C has a very positive effect on extraction efficiency for pharmaceuticals in solid matrices [25]. Yet, too high temperatures (>200 °C) may cause thermal degradation of some compounds [26] and increased ion suppression as a consequence of severe co-extraction of unwanted components. A temperature of 150 °C is in our experience a suitable balance between good extraction efficiency combined with relatively moderate co-extraction of undesired matrix components.

One of the world's most common commercial pressurized hot water extraction apparatuses has been in use since the early 20th century. After the Second World War and in the early 1960s this extraction technique, the espresso machine, underwent technical improvement with the sole purpose of delivering great coffee by the extraction of flavours from grinded coffee beans mounted in the porta filter. Typically, modern espresso machines reach temperatures around 100 °C and operates at pressures of 15–20 bars. Substantial research has been done to better understand what parameters govern the extraction process of coffee with the aim of achieving a balanced coffee aroma [27–30], but commercial espresso machines have also been used to determine different contaminants such as polyaromatic hydrocarbons in soil [31], airborne pesticides in particulate matter trapped in filters [32] and polychlorinated biphenyls in soil [33]. In this work, a commercially available espresso machine

was used for the extraction of APIs holding a variety of physicochemical properties from dewatered stabilized sewage sludge. The efficiency and reproducibility of the espresso machine extraction (Espresso method) was evaluated and results were compared to results from our Pressurized Hot Water Extraction (PHWE) as well as literature data obtained with traditional extraction techniques and methods. Post-extraction analysis with UPLC-MS/MS were conducted in both acid and basic chromatographic environments, as this previously has been shown to have an effect on the level of matrix influence in the chromatographic run [34]. The key advantages of a successful espresso machine extraction (Espresso method) would be very simple packing and maintenance procedures and a very fast extraction process.

2. Materials and Methods

2.1. Chemicals and Reagents

Ultra-pure water (18.2 MΩ) was obtained from an OPTIMA water purification system (Elga Ltd., High Wycombe, Buckinghamshire, UK). Reference standards were purchased from Sigma-Aldrich Sweden AB (Stockholm, Sweden). A list of compound details is found in Table S1, while chemical formulas, structures and molar masses are easily available on the Internet at PubChem, Drugbank, and ChemSpider. Acetonitrile (ACN) and methanol (MeOH), both OPTIMA grade, used for the chromatographic mobile phase were purchased from Fisher Scientific (Gothenburg, Sweden). Formic acid (FoA), ammonium hydroxide solution (25% sol.), ammonium hydrogen carbonate, disodium ethylenediaminetetraacetate (Na_2EDTA), ascorbic acid, and ammonium hydroxide and silicon carbide were purchased from Sigma-Aldrich. Stabilised dewatered sewage sludge was obtained from Kristianstad STP (Scania, Sweden) and lyophilized in-house.

2.2. PHWE

The in-house constructed PHWE system is schematically shown in Figure 1.

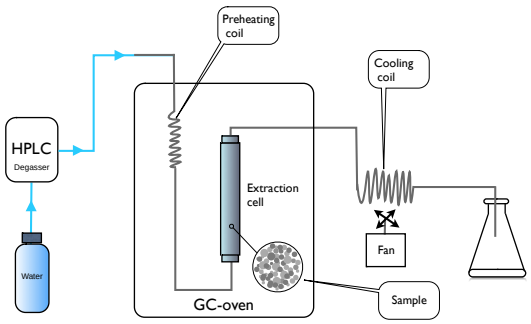

Figure 1. Schematic picture of the in-house stainless-steel column pressurized hot water extraction (PHWE) system.

The PHWE system consisted of a Waters Alliance 2690 HPLC system (Waters, Milford, MA, USA). In all experiments, the HPLC pump supplied water at pH 7, via stainless-steel tubing (1/16 in. o.d. and 0.040 in. i.d.), to the pressurized stainless-steel column (length 7.0 cm × i.d. 1.0 cm), in which the extraction took place. The extraction cell was placed inside a Varian 3400 GC oven (Walnut Creek, CA, USA) heated to 150 °C. A portion of 0.2 g lyophilized sludge was homogenized and mixed with 8.0 g of silicon carbide. A regular coffee filter (Melitta® 102, Melitta Europa GmbH & Co. KG, Minden, Germany) shaped into a cylinder covered the inside of the column and a circular shaped glass microfiber filter (GF/C 47 mm, Whatman, GE Healthcare UK Limited, Buckinghamshire, UK) was positioned at the outlet nut of the extraction column. The column was filled with the mixture of sludge and silicon carbide. The mixture was spiked at the top with 30 µL of an internal standard mixture containing 7 labelled standards (Table S1) and left to soak for 10 min. The content of the column was

then packed with a stomp and the coffee filter edges folded and the column closed with an inlet nut. The column was mounted inside the oven and connected to the stainless-steel tubing. The degasser was activated and the HPLC pump flow set to 1 mL/min. When the eluate emerged at the collection point the oven temperature was set to 150 °C and flow adjusted to 1.8 mL/min. A single run was completed in 13 min and gave 23 mL of extract. Pressure was monitored to between 11 and 12 bars. Additionally, the eluate was centrifuged at 5000 rpm for 10 min on an SIGMA 4-16KS Centrifuge (SIGMA Laboratoriezentrifugen, Osterode am Harz, Germany). Then a SPE protocol followed, as described below. The column was thereafter disconnected and the system was cleaned with a by-pass procedure at 90 °C for 5 min with the flow 1 mL/min. The column was emptied and reinstalled in the system and thereafter washed at 150 °C for 10 min at 2 mL/min. All experiments were conducted in triplicate.

2.3. Espresso Machine Extraction (Espresso Method)

A Rancilio Miss Silvia E Espresso Machine (Rancilia Group S.p.A., Villastanza di Parabiago, Italy) adopted for pods was used to conduct the espresso extractions. A portion of 0.2 g lyophilized sludge was homogenized and mixed with 8.0 g of silicon carbide. The mixture was spiked with 30 µL of internal standard mixture containing 7 labelled standards (Table S1) and left to soak for 10 min. A regular coffee filter cut in half enclosed the mixture and served as an extraction pod. At the bottom of the espresso porta filter a 30 mm 0.45 µm GF/C glass fibre filter was placed. The espresso machine was started when the ready lamp was lit and a total of 60 mL eluate was collected in a beaker. Previous Espresso extraction methods for environmental applications have used extraction volumes of 50 mL for sample sizes up to 5.0 g [31,32]. Our chosen volume of 60 mL was at the high-end of this scale and was also the maximum volume suitable for the SPE protocol, as described below. The cleaning procedure recommended by the supplier (placing 0.5 g of Espresso detergent in a blind filter and flushing for 10 s repeatedly) was performed 10 times in a row after each sample extraction was completed. The espresso experiments were conducted in triplicate.

2.4. Solid-phase Extraction (SPE Method)

A SPE robot RapidTrace+ (Biotage, Uppsala, Sweden) was used for *conditioning* and *eluting samples*, while a SPE manifold (Agilent, Santa Clara, CA, USA) was used for *loading/extracting* the samples. Samples were concentrated by an automatic evaporator TurboVap LV (Biotage, Uppsala, Sweden) using clean air produced by an Atlas Copco SF2 Oil-free air system (Atlas Copco Airpower n.v. B, Wilrijk, Belgium), delivering certified 100% oil-free air, complying with ISO 8573-1 CLASS 0 certification. Both the PHWE extracts and the Espresso extracts were concentrated and purified using 200 mg Oasis HLB SPE cartridges (Waters). The HLB SPE cartridges were conditioned with 5 mL of MeOH followed by 5 mL of reagent water prior to extraction. HLB SPE cartridges were mounted in the sample manifold together with a 70 mL plastic syringe container on top. Thereafter one volume of PHWE-or Espresso extract was added, together with 50 µL FoA (10%) and 50 µL saturated EDTA solution. Samples were then passed through the SPE cartridges at a rate of ca. 5 mL/min. Afterwards, the cartridges were air-dried under a positive pressure for 15 min, a step which proved crucial for both recovery results and evaporation time reproducibility, since drying the cartridges on the manifold was insufficient. SPE cartridges were mounted in the Rapid Trace+ SPE robot and analytes were eluted with 6 mL MeOH into disposable borosilicate glass tubes (PYREX®, 16 × 100 mm, Corning Incorporated, Corning, NY, USA). The extracts were evaporated to complete dryness (22 min) at 40 °C. The dry extracts were reconstituted to a total volume of 1 mL by adding 100 µL MeOH to the dry borosilicate glass tube, followed by a rapid swirling (vortexing) for 15 s. Thereafter 885 µL water was added followed by 15 s of rapid swirling, and transferred to a vial. Finally, 10 µL of *instrumental standard solution* (Thiacloprid-d4, 250 pg/µL, Table S1) was added along with 5 µL saturated EDTA solution. A volume of 1 µL of the final sample was injected into the UPLC-ESI-MS/MS system. The same SPE procedure was used for both extraction techniques.

2.5. Analytical Separation and Detection (UPLC-MS/MS Method)

The liquid chromatographic system, the mass spectrometer and the analytical methodology have all previously been described in detail [34,35]. In short, the UPLC-MS/MS system used was a Waters Acquity UPLC H-Class connected to a Xevo TQ-STM triple quadrupole mass spectrometer (Waters Micromass, Manchester, UK), equipped with a Z-spray electrospray interface. The UPLC H-Class consisted of a Quaternary Solvent Manager (QSM), a Sample Manager with Flow-Through Needle (SM-FTN) and a Column Manager (CM) enabling fast column switching between two different columns, running at two different pH (Waters, Milford, MA). Two Acquity UPLC BEH C18 columns (2.1 mm i.d. × 50mm, 1.8 mm) in parallel, maintained at 40 °C, were installed in the column manager. Nitrogen was used as both drying gas and nebulizer gas delivered by an Infinity Nitrogen Generator (Peak Scientific Instruments Ltd., Inchinnan, UK). For operation in MS/MS mode, the collision gas was argon 99.995% (AGA Gas AB, Malmö, Sweden). Waters UNIFI software (Version 1.7) controlled the UPLC-MS/MS system.

2.6. Calculations

2.6.1. Absolute Recoveries of Isotopic Labelled Compounds

We calculated the absolute recovery expressed as a percentage of the recovery of each isotopic labelled standard. The absolute recovery was then calculated according to Equation (1):

$$\text{Absolute Recovery} = 100 \cdot (A_n)/(A_l) \quad (1)$$

A_n = the area of the daughter m/z for the labelled compound in spiked sample from either the *PHWE* extracts or the *Espresso* extracts. A_l = the area of the daughter m/z for the labelled compound in standard solution.

2.6.2. Quantification of APIs in Sludge

The approach presented in this paper to quantify APIs in sludge is based on the comprehensive analytical methodology described by the United States Environmental Protection Agency; EPA Method1694 (U.S. Environmental Protection Agency 2007). Isotope dilution for calibration of each native compound was used when a labelled analogue was available, and calibration by internal standard was used to determine the concentration of the native compounds when no labelled compound was available. The two approaches have common mathematical operations. For the compounds determined by isotope dilution, the relative response (*RR*) (labelled to native) vs. concentration in the calibration solutions was computed over the calibration range according to Equation (2):

$$RR = (A_n C_l)/(A_l C_n) \quad (2)$$

A_n = the area of the daughter m/z for the native compound. A_l = the area of the daughter m/z for the labelled compound. C_l = the amount of the labelled compound in the calibration standard (pg). C_n = the amount of the native compound in the calibration standard (pg).

Response factors (*RF*) were calculated in a similar way; replacing A_l with A_{is} (area of the daughter m/z for the internal standard), C_l with C_{is} (amount of the internal standard, pg) and A_l with A_{is} (area of the daughter m/z for the internal standard).

The concentration of a native compound was calculated according to Equation (3):

$$C_n = (A_n C_l)/(A_l RR) \quad (3)$$

The calculated API concentration, C_n, was then used to express the content of an API in sludge as µg/kg dry weight sludge.

2.6.3. Statistical Analysis

The standard deviation was calculated for both the recovery of the 7 isotope labelled compounds in the internal standard mixture and the recovery for all 23 compounds investigated. ANOVA at a 95% confidence level were used to evaluate and compare the extraction efficiency of the *Espresso method* vs. the *PHWE method*.

3. Results and Discussion

3.1. Absolute Recoveries of Spiked Isotope Labelled Compounds and Ion Suppression

The eight isotope labelled compounds used in this study include positive, negative and neutral APIs, Table 1. Seven of these were spiked at the top of the extraction cell (internal standard mixture, Table S1), containing the sewage sludge, and underwent the entire extraction process, while the internal standard thiacloprid-D4 was spiked just prior to the analysis. By calculating the absolute recovery of the 7 labelled compounds the extraction efficiency of the Espresso method compared to *PHWE method* could be revealed. The obtained extraction efficiency results also contain a component of ion suppression, which might depend on the applied extraction methodology. To get basic information about possible differences in the magnitude of ion suppression caused by the two individual extraction methods the results for thiacloprid-D4 was used.

Table 1. Absolute recovery and relative standard deviation (RSD %, $n = 3$) for the instrumental standard *thiacloprid-d4* spiked just prior to the analysis and seven labelled standards spiked at the top of the two types of extraction cells, Espresso and PHWE, containing the sewage sludge. Absolute recovery was calculated using Equation (1). Four of the compounds were analysed at both basic and acid conditions and are marked in *italic*.

Compound	UPLC Condition	Espresso ($n = 3$) (%)	RSD (%)	PHWE ($n = 3$) (%)	RSD (%)
Thiacloprid-d4[a]	*Basic*	*70*	*17*	*34*	*17*
Carbamazepine-D10	*Basic*	*44*	*18*	*28*	*4*
Diclofenac-13C6	*Basic*	*30*	*18*	*14*	*24*
Sulfamethoxazole-13C6	*Basic*	*53*	*7*	*26*	*19*
Atenolol-d7	Basic	56	5	45	10
Methiocarb-d3	Basic	20	12	17	5
Metoprolol-d7	Basic	36	15	34	9
Estrone-d4	Basic	21	29	17	2
Average recovery 7 compounds	Basic	37		24	
Thiacloprid-d4[a]	*Acid*	*37*	*22*	*14*	*18*
Carbamazepine-d10	*Acid*	*19*	*31*	*10*	*7*
Diclofenac-13C6	*Acid*	*20*	*11*	*14*	*12*
Sulfamethoxazole-13C6	*Acid*	*34*	*18*	*20*	*13*
Average recovery 3 compounds	Acid	24		16	

The absolute recoveries obtained for all 8 compounds are shown in Table 1. It should be noted that four of these compounds were analysed in both the *acid* and the *basic chromatographic UPLC method*, giving information on possible differences in reduction of ion suppression by better separation of compounds from co-eluting matrix components during the two different UPLC runs. To provide additional knowledge of differences in terms of ion suppression, TIC spectra for both chromatographic methods and both extraction methods were also collected as shown in Figure 2.

Starting with the standard, thiacloprid-D4, added post extraction, it was shown that the absolute recovery for this compound was 70% in the Espresso method and 34% in the PHWE method with basic chromatographic conditions. This difference can be explained by a heavier matrix suppression expressed in the PHWE method, which is further supported by the higher TIC numbers for PHWE compared to Espresso, Figure 2a,b. Interestingly, there are also differences in absolute recovery comparing the thiacloprid-D4 results from the basic and acid chromatographic method separately. Recovery results dropped from the above-mentioned 70% in the basic method to 37% in the acid

method, for samples run in the Espresso method. In the acid method, the recovery dropped from 34 to 14%. One plausible explanation is less ion suppression in the basic method. TIC spectra, generated by the PHWE method and the Espresso method, in the acid method is higher throughout the whole spectrum, reaching a maximum at 1.70 min and 1.6×10^{11} counts, compared to the basic method where the maximum is 1.2×10^{11} counts at 1.75 min, Figure 2a,b.

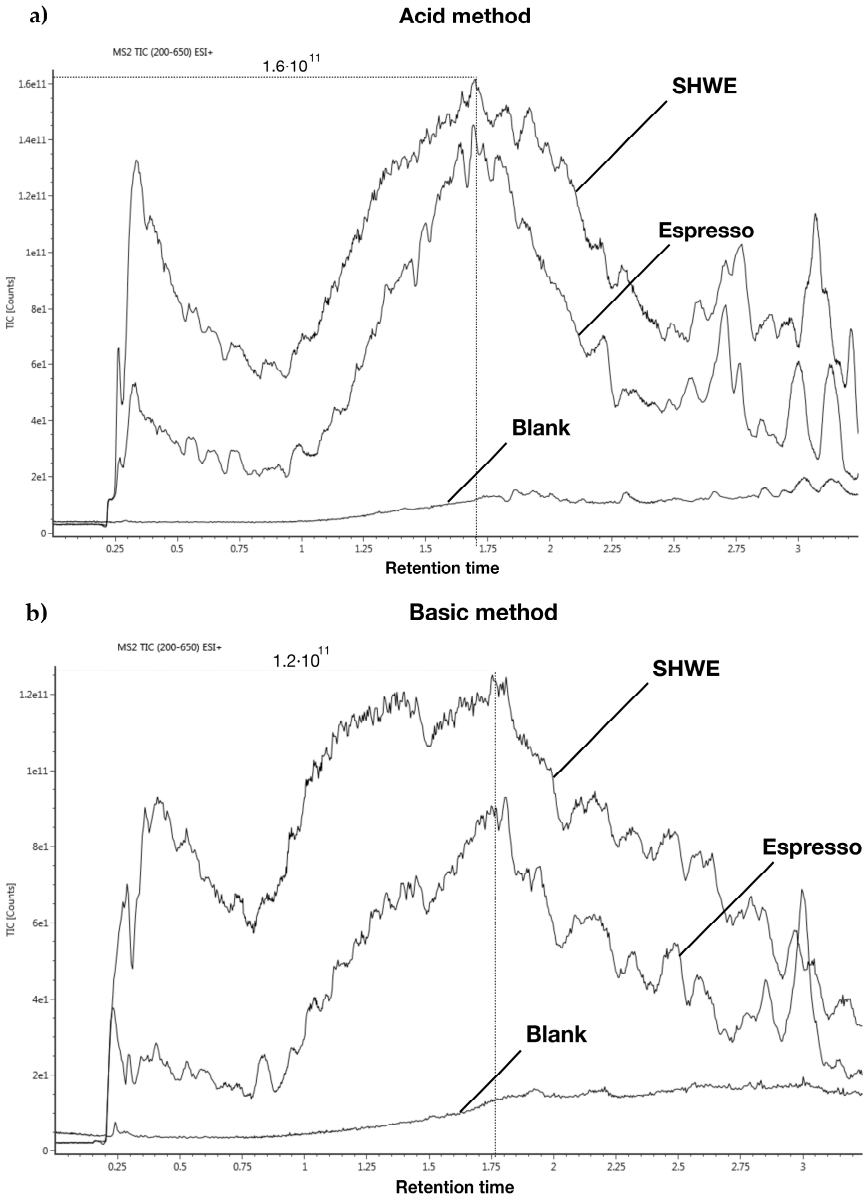

Figure 2. TIC spectra obtained using Waters RADAR^{TM} function of full scan (MS). Panel (**a**) shows TICs obtained for a blank sample as well as sewage sludge extracts applying the *Espresso method* and the *PHWE method* at acid chromatographic conditions. Panel (**b**) shows TICs for the same samples types but analyzed at basic chromatographic conditions.

From Figure 2a,b it is clear that the PHWE method extracts more sample components. Both above mentioned techniques rely upon the same shift in chemical and physical properties that water undergoes as the temperature is elevated. An increase in water temperature changes the relative permittivity from nearly $\varepsilon = 80$ at room temperature to $\varepsilon = 27$ at 250 °C. The organic solvents methanol and ethanol has a permittivity of $\varepsilon = 33$ and $\varepsilon = 24$ at 25 °C [36]. A declining degree of hydrogen bonding and decreased polarizablity can then also be observed. Thus, by increasing the water temperature

the normal behaviour of water as a solvent will shift from polar to less-polar. Kondo and Yang compared the retention properties of superheated water and organic–water eluents and came to the conclusion that approximately 3.5 °C rise in water temperature was equivalent to 1% MeOH increase in MeOH–water mixtures [37]. This may be one reason for the higher co-extraction of matrix components using PHWE, since a 50 °C higher water temperature in PHWE would be equivalent to a 14% increase in MeOH content.

Turning to the three isotope labelled standards carbamazepine-D10, diclofenac-13C6 and sulfamethoxazole-13C6, which all were run in both the acid and the basic chromatographic methods, the effects of both the chromatographic conditions and the applied extraction method became even further accentuated, as seen in Table 1 and Figure 3a.

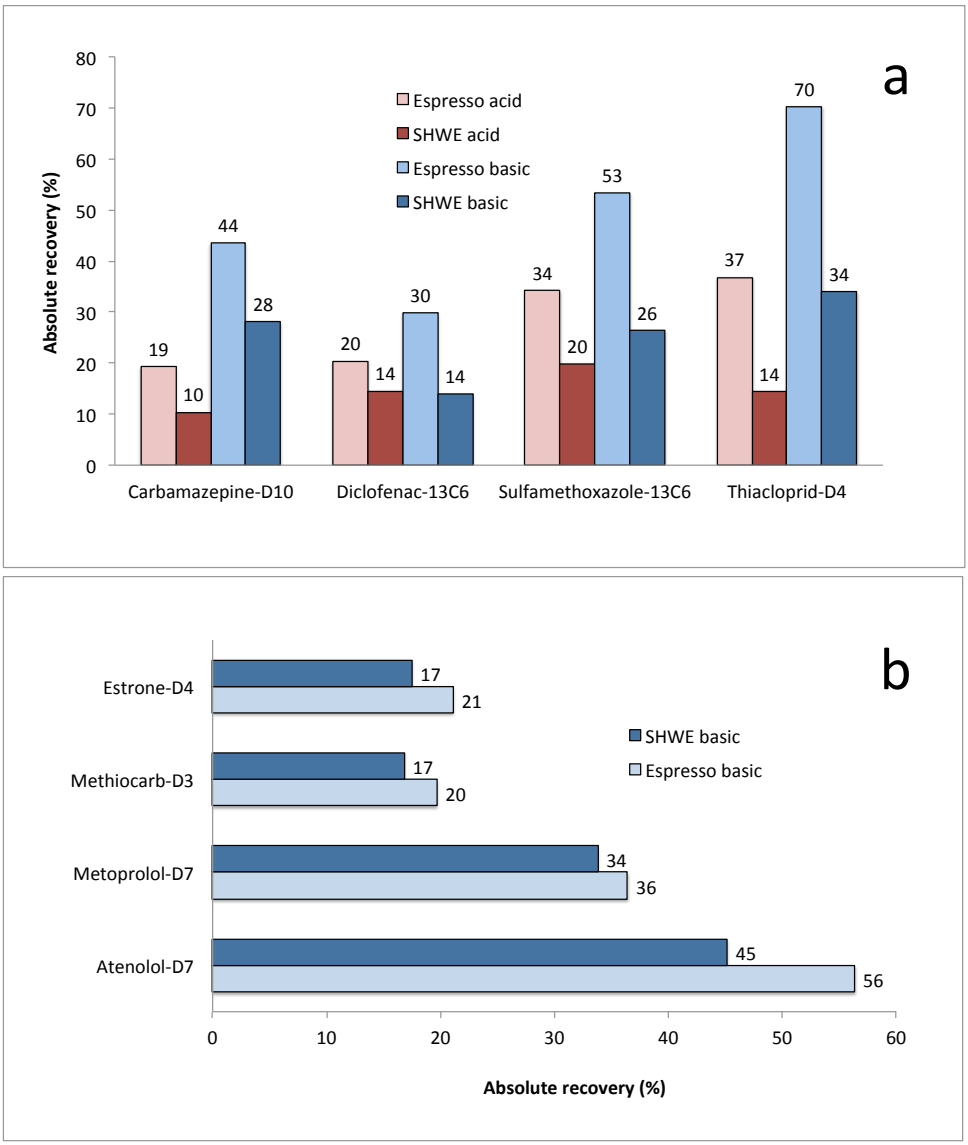

Figure 3. Absolute recovery for eight labelled standards spiked at the top of the extraction cell, containing the sewage sludge, and the instrumental standard thiacloprid-d4 spiked just prior to the analysis. The absolute recovery was calculated using Equation (1). Four analytes were analysed using two different chromatographic methods at acid and basic conditions (**a**), while four of the compounds could only be analysed at basic conditions (**b**).

Mean absolute recovery results for these labelled compounds in the basic method was 37% for the Espresso method and 24% for the PHWE method, while corresponding figures using the acid

method were 24% and 16%, respectively. A first interpretation of the calculated mean values points in the direction of greater extraction efficiency using the Espresso method, but as discussed above, the results also reminds us of heavier ion suppression in the PHWE method. Göbel et al. [17] came to the conclusion that a temperature of 100 °C was optimal when optimizing pressurized liquid extraction (PLE) for the determination of sulfonamide, and macrolide antimicrobials and trimethoprim in sewage sludge. Increasing the temperature above 100 °C led to decreased recoveries. For sulfamethoxazole, they noticed a reduction by 95%, and for the macrolides the reduction was 60–90%. They concluded that this was due to thermal degradation of the analytes; however they also noted increasingly darker extracts at higher extraction temperatures, indicating a larger extraction of soluble organic matter. This notion of lower recoveries might not necessarily be a consequence of thermal degradation as we have shown in a temperature stability study [26], but rather a result of ion suppression, and which may partly be avoided as shown here by basic chromatography methods.

A second conclusion from Figure 3a would be that running the analysis under acid conditions always give lower absolute recoveries independent of compound and extraction method. This further strengthens the above statement made for thiacloprid-D4 that there may be less ion suppression in basic methods as is evident from TIC numbers shown in Figure 2. These results for isotope labelled standards clearly show the impact a chromatographic method might have when developing analytical methods for samples that express heavy ion suppression in mass spectrometry. Yet, a literature survey of methodologies published in high-impact analytical chemistry journals between 2006 and 2016 revealed that a vast majority of the identified methods rely on acidic mobile phases as discussed in a previous paper and thesis from our research group [35].

Among the four remaining isotope labelled compounds, atenolol-D7, methiocarb-D3, metoprolol-D7 and estrone-D4, there was a tendency that the PHWE method gave lower absolute recoveries as shown in Figure 3b, but not as pronounced as for the other compounds (Figure 3a). Atenolol-D7 showed the highest absolute recovery for both extraction methods, but also the largest difference between the two methods, with the Espresso method being most successful (56% recovery). Estrone-D4, methiocarb-D3 and metoprolol-D7 showed minor difference in recovery for the two methods.

3.2. Pharmaceuticals Extracted from Sludge

In a previous work dealing with surface water containing matrix components, we have determined which isotopic labelled standard that best compensates for losses caused by ion suppression and sample preparation losses for a specific API [34,35]. The pairing of labelled standard and API can be found in Table S2. The quantification in µg/kg sludge dry matter of a single API in the different sludge samples was calculated as described in Section 2.6.2. In total, 23 APIs were analysed. They have a molecular range from 236 to 734 D and contain 1-3 aromatic rings. A negative charge might be expressed from oxygenated substituents (e.g., diclofenac) and/or positive charge from nitrogen substituents (e.g., metoprolol). The results from the chemical analysis are presented in Table 2 together with various physicochemical parameters. Additionally, wastewater influent and effluent samples were collected at Kristianstad STP and the concentrations of all APIs were analysed and included in Table 2. Finally, results from a previous National Swedish Screening of API contents in sludge from the three Swedish cities Skövde, Stockholm and Umeå [38] are given for comparison at the end of Table 2 as are values from scientific articles on sludge published the last decade.

All 23 investigated compounds, except sulfamethoxazole, could be detected and quantified in sludge—with both extraction methods. In total, the content of APIs was 1042 µg/kg using the Espresso method and 5027 µg/kg using the PHWE method, i.e., nearly 5 times higher drug levels were estimated using PHWE. Average standard deviations were 22% for the Espresso method and 17% for the PHWE method. An ANOVA comparison of the two methods showed a significant difference ($F_{critical}$ = 7.7) for 15 of the 23 investigated compounds and is marked in bold text in Table 2. One of the compounds, fluconazole, showed higher concentration with the Espresso method, while the remaining 14 compounds all were higher in the PHWE method. These differences can be explained, despite

higher ion suppression as discussed earlier, by a more efficient extraction process using PHWE as it operates at a much higher temperature compared to Espresso. Nieto et al. 2010 [39] describes this in a clear way writing that: "Higher temperatures decrease the viscosity of liquid solvents, thus allowing better penetration of the matrix particles and enhancing extraction. In addition to reducing viscosity, high temperatures also decrease the surface tension of the solvent, the solutes and the matrix, allowing the solvent to "wet" the sample matrix more thoroughly". It has also been concluded that the time of operation have a strong influence on extraction efficiency, as stated by Nieto et al.: "The long exposure to the solvent allows the matrix to swell, thus improving penetration of solvent into the sample interstices and contact between solvent and analyte" [39]. In our study, the PHWE method operates under a longer time period, 13 min, as compared to the Espresso method extracting only for 10 s. As a consequence, more API molecules are likely liberated from the sewage sludge.

Turning to individual compounds, *five* compounds stands out; ciprofloxacin, citalopram, ketoconazole, metoprolol and venlafaxine, which all were detected in concentrations higher than 180 µg/kg using the *PHWE method*, all other APIs being below 70 µg/kg (Table 2, Figure 4). We would here like to make the reader aware of the logarithmic scale applied in Figure 4 due to the concentration range of APIs spanning three orders of magnitude

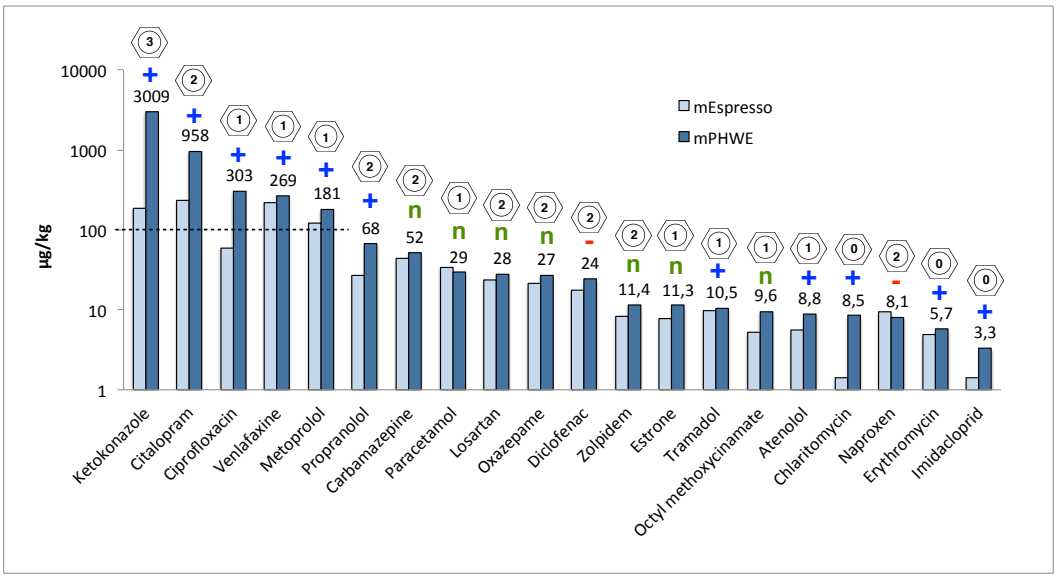

Figure 4. Concentrations in µg/kg of 23 APIs in lyophilized sewage sludge from Kristianstad STP determined using the *Espresso method* (light blue) and the *PHWE method* (dark blue). The APIs are listed in descending order of concentration (*PHWE method*) together with the charge and the number of aromatic ring structures of the compound in question.

The concentration found of ketokonazole in sludge using the *PHWE method* was 3009 µg/kg, which was 15 times higher as compared to the results from the *Espresso method*. Corresponding relative comparison between the methods for ciprofloxacin, citalopram, metoprolol and venlafaxine showed 5.1, 4.1, 1.5 and 1.2 times higher for the four compounds, respectively. In a previous investigation we showed that the matrix content of organic matter measured as total organic carbon content in sediments and sludge dictated drug sorption together with the presence of charged sites [40]. All of these top five compounds carry a positive charge at pH 7 (Table 2), and one of the explanations to these large amounts is probably the electrostatic interaction by the compounds positively charged groups and the negatively charged surfaces of the sludge matrix. Another part of the explanation is likely due to the existence of plural aromatic structures. Ketoconazole, which is by far the most abundant API found in the investigated sewage sludge samples contains three aromatic rings, citalopram, the second most abundant API contains two aromatic rings.

Table 2. Physicochemical properties, and determined concentrations in µg/kg of 23 APIs in lyophilized sewage sludge from Kristianstad STP using an *Espresso method* and a *PHWE method* (RSD %, *n* = 3). The methods were compared by ANOVA, and bold figures indicate statistically higher concentrations for that method. Inlet at outlet concentrations of the 23 APIs from Kristianstad STP are shown in the table along with previously determined API concentrations from 3 cities (Skövde, Stockholm and Umeå) in a Swedish National Screening Programme 2010. Literature data from scientific international studies are also presented at the end of the table.

Substance	pKa	Charge (pH 7)	Log D (pH 7)	log Kow	Espresso (*n* = 3) µg/kg	RSD (%)	PHWE (*n* = 3) µg/kg	RSD (%)	Water Inlet ng/L	Water Outlet ng/L	Skövde Sludge µg/kg	Stockholm Sludge µg/kg	Umeå Sludge µg/kg	Scientific Paper µg/kg	[REF]
Atenolol	9.67	+	−2.14	0.16	5.6	*15*	**8.8**	*3*	1348	215	13	9	12	6;22	[14]
Carbamazepine	-	n	2.28	2.45	43	*26*	52	*10*	1031	547	190	200	120	18;32	[41]
Clarithromycin	8.99	+	1.84	3.16	1.4	*34*	**8.5**	*22*	131	22	13	1.4	4.5	24	[17]
Ciprofloxacin	6.38	(+:–)	−0.81	0.28	60	*38*	**303**	*40*	58	46	450	250	170	2420	[42]
Citalopram	9.78	+	1.27	2.51	233	*31*	**958**	*8*	155	32	760	570	630	725	[43]
Diclofenac	4.00	–	1.37	4.06	18	*16*	**24**	*11*	713	577	59	31	10	192	[18]
Erythromycin	8.80	+	1.2	2.50	4.8	*30*	5.7	*8*	385	267	1000	150	120	62	[18]
Estrone	10.30	n	4.31	3.13	7.9	*6*	**11**	*9*	49	3	33	36	2	23;28	[44]
Fluconazole	2.56	n	0.56	0.25	**0.5**	*15*	0.4	*12*	51	105	3.5	13	47	<LOQ, n.d	[45]
Imidacloprid	5.28	pH6 +	1.09	0.57	1.4	*38*	**3.3**	*24*	8	14	n.a	n.a	n.a		
Ketokonazole	3.96, 6.75	+	4.06	4.30	186	*36*	**3009**	*12*	69	0	510	1200	1100	910	[45]
Losartan	4.12	n	4.94	4.01	23	*7*	28	*22*	326	205	n.a	n.a	n.a		
Metoprolol	9.60	+	−0.81	1.88	123	*21*	**181**	*7*	999	533	180	410	210	29:92	[46]
Naproxen	4.19	–	0.45	3.18	9.3	*25*	8.1	*13*	2421	290	<LOQ	<LOQ	<LOQ	4	[47]
Octylmethoxycinamate	-	n	5.38	6.10	5.2	*5*	**10**	*31*	64	31	n.a	n.a	n.a		
Oxazepame	-	n	2.06	2.31	22	*33*	27	*14*	374	403	43	18	12		
Paracetamol	9.46	n	0.74	1.08	34	*29*	29	*33*	22528	0	73	11	<LOQ		
Propranolol	9.42	+	1.15	3.48	27	*22*	**68**	*9*	47	16	n.a	n.a	n.a	<1.26	[48]
Sulfamethoxazole	6.16	–	0.14	0.89	n.d		n.d		476	118				n.d	[49]
Tramadol	9.23	+	0.24	2.51	10	*12*	11	*10*	168	153	<LOQ	68	<LOQ	43	[49]
Trimethoprim	7.20	+	0.92	0.91	0.2	*33*	**1.2**	*19*	77	17	27	2.2	2.5	5:13	[49]
Venlafaxine	8.91	+	0.84	3.28	219	*7*	**269**	*20*	174	88	86	310	150	318	[43]
Zolpidem	5.65	n	3	3.85	8.4	*9*	**11**	*26*	12	4	7.7	8.3	3.2	38	[49]
Sum concentration					**1042**		**5027**		**31664**	**3686**	**3448**	**3288**	**2593**		

From Table 2 it can be seen that of the 23 compounds analysed, 20 showed a concentration in sludge exceeding 1 µg/kg sludge. These are all shown visually in descending order of concentration in Figure 4, and reveals that 18 of these compounds are all in favour of the *PHWE method*. Figure 4 also shows the clear pattern of positively charged molecules being at the highest concentrations range in the sludge as discussed above, followed by neutrals, and some additional positively and negatively charged compounds. The reason for finding some of the positively charged compounds on the far-right side could likely be attributed to two circumstances. The most obvious is that some of these compounds simply do not occur at high enough concentrations in the incoming water to reach high concentrations in the sludge despite a high degree of affinity to this negatively charged matrix. This is further discussed in the next section presenting APIs in the incoming wastewater. The other option is, as already touched upon, is that there are other chemical interactions at play. One of these is the aromatic ring structure. As mentioned, the two top compounds, ketoconazole and citalopram, have three and two aromatic rings. To reveal any possible relation to aromaticity, Figure 4 also presents the number of aromatic rings present on each of the 20 APIs. From this it becomes evident that those neutral compounds that reach fairly high concentrations in most cases have 2 aromatic rings, the one exception being paracetamol. Yet, finding paracetamol at such high concentrations in sludge might not come as too much of a surprise considering the large amounts of this compound being sold over the counter in Sweden, and being found in enormous concentrations in incoming water (see below). Additionally, on the very far right end there are 4 positively charged APIs. Three of these have no aromatic ring structure at all. In conclusion, there are strong indications that both the number of aromatic rings and the presence of a positive charge are important for the sorption processes of APIs to sewage sludge.

3.3. Comparison of Extraction Results with Effluent and Influent Data

In Table 2 we have included wastewater influent and effluent analysis results for the investigated APIs from Kristianstad STP, performed by us according to our previously published methodology [34,35]. The first observation was that all 23 APIs were present in the *influent* water. Likewise, all investigated APIs except sulfamethoxazole were present in the sewage sludge samples. In the effluent samples, all APIs except ketoconazole and paracetamol could be detected. Based on results from Figure 4 and Table 2 it is likely that the efficient reduction of ketoconazole in the STP primarily is caused by sludge adsorption. Paracetamol on the other hand show much lower sludge concentrations, and the huge loss of paracetamol, with the highest influent values, must be explained by either abiotic or biotic degradation/transformation, or a combination thereof.

Evidently the sludge concentrations measured in PHWE and presented together with two physicochemical properties in Figure 4, and inlet and outlet concentrations cannot fully explain the observed pattern. Yet, a few more chemical remarks can be made in favour of using charge and aromaticity as valuable additional tools in describing sludge sorption apart from primarily looking at more well-established classical parameters such as log *K*ow and log *D*.

For example, in Table 2 high log *K*ow and log *D* values can be found for ketoconazole, but not for ciprofloxacin and citalopram. Here charge may aid in better explaining the high concentration of the two latter on sludge. Metoprolol and carbamazepine have approximately the same influent and effluent concentrations, yet metoprolol is 3.5 times more abundant in sludge compared to carbamazepine, despite that log Kow and log *D* is higher for carbamazepine than metoprolol. This again shows that the positive charge might have a strong impact on sorption. These results show that neither log *K*ow nor log *D* alone are valid tools too fully describing APIs adsorption to sludge.

3.4. Comparison of Extraction Results with Literature Data

In Table 2 we have also included analysis results from the Swedish National Screening Programme 2010 [38] together with a selection of literature data describing concentrations of APIs in digested sludge. The top five compounds in the Swedish National Screening Programme were ketoconazole,

citalopram, ciprofloxacin, metoprolol, and venlafaxine, the same top five compounds that we found in our extracted sludge. With a few exceptions, the absolute values are roughly the same, only the antibiotic compound erythromycin showed a lower concentration in our study compared to the National Screening Programme, representing the only truly deviating value.

In the Swedish report, we couldn't find what extraction technique and method used. If not as exhaustive as our PHWE method this may be one reason for not achieving such high sum concentrations of all investigated compounds. In fact, the two compounds contributing most to the difference is ketoconazole and citalopram, both adsorbing strongly to the sludge. In our work ketoconazole had a concentration of 3009 µg/kg, while the other three sludges (Skövde, Stockholm and Umeå) were in the range 510–1200 µg/kg. Likewise, citalopram had a concentration in our study of 958 µg/kg in our study but was in the range 570–760 µg/kg in the Swedish report.

4. Conclusions

Two green chemistry extraction systems based on either an in-house stainless-steel column pressurized hot water extraction system (PHWE method) or a commercially available Espresso machine (Espresso method) were applied for analysing 23 APIs in sewage sludge. Both methods showed god repeatability. The PHWE method allowed a more complete extraction of APIs that were more tightly bound to the matrix, as exemplified by the results from ketoconazole, citalopram and ciprofloxacin. In fact, 19 out of 23 investigated APIs were quantified in sludge, and with few exceptions they showed higher concentrations using the PHWE method. The Espresso method might still be useful if strong electrostatic forces aren't to be overcome as it gives cleaner extracts (lower ion suppression) and is much faster to operate compared to the PHWE method, allowing higher sample throughput. An Espresso extraction takes 10 s, while the PHWE extraction takes several minutes. The results produced with the PHWE method are in line with results reported from a Swedish National screening program and international literature data. A weakness with both methods, which was more pronounced for the PHWE method, were the low absolute recoveries, which most likely were caused by matrix ion suppression. Further investigations will focus on reducing these effects by investigating fractionating of the extracts and the possibility of matrix precipitation using various techniques. Additionally, UPLC-MS/MS, applying basic conditions, is surprisingly unexplored in environmental analysis, despite its positive effects in reducing ion suppression, and should be further investigated.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/9/7/1509/s1, Table S1: Compounds analysed, Table S2: Labelled standard used for each API.

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