Superheated water extraction (SWE) coupled on-line with superheated water chromatography (SWC)

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SUPERHEATED WATER EXTRACTION (SWE) 
COUPLED ON-LINE WITH 
SUPERHEATED WATER CHROMATOGRAPHY (SWC) 

By 

RUZIYATI TAJUDDIN 

A Doctoral Thesis 

Submitted in partial fulfilment of the requirements 
for the award of 

Ph.D. of Loughborough University
ACKNOWLEDGEMENTS

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Thesis Title: Superheated Water Extraction (SWE) Coupled On-line with Superheated Water Chromatography (SWC)

Ruziyati Tajuddin

Thesis Abstract

Since the inception of analytical superheated water extraction (SWE) by Hawthorne and co-workers in 1994, this technique has been widely employed in the extractions of organic pollutants, pesticides, natural products as well as inorganic compounds from a variety of sample matrices. The rapid development of SWE has led to the direct combination of this technique with conventional chromatographic methods. However, these coupling methods still required a considerable amount of organic solvent. In this study, SWE has been directly coupled to superheated water chromatography (SWC) by using simple switching valves and a solid-phase trap as the interface between the extractor and the chromatograph. The trap column was used to collect the extracted analytes and pre-concentrate them prior to chromatographic analysis. It could also be used as a clean-up step for the removal of the matrix interferences from the extract. Because superheated water was used as the extractant, as the mobile phase, as well as the washing solvent, the use of organic solvent has been avoided in all stages of this on-line SWE-SWC method, and therefore it is compatible with 'green chemistry'.

The on-line SWE-SWC was first examined as a method for the extraction of pharmaceutical compounds from an unretentive matrix, such as sand. This on-line method was then further developed by the inclusion of clean-up steps for the extraction of triazine herbicides from a complicated matrix, such as compost. Two compost samples representing high organic content (100% peat) i.e., ericaceous compost; and lower organic content (up to 60% peat + sand) i.e., seed compost, were selected as the sample matrices to carry out the SWE of the triazines and directly assayed by SWC. The validation of the on-line coupled system method was assessed from the evaluation of the recoveries of triazine herbicides extracted from the spiked compost samples, whilst the relative standard deviation (RSDs) of the results were used to assess the repeatability of the method.

In quantitative analysis of the triazines, the effects of sample amount and analyte concentration, sample matrix, and temperature, were studied with respect to the recoveries. Method comparisons were then made with the off-line SWE followed by SWC or HPLC, and solvent extraction method followed by SWC or HPLC.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>SWE</td>
<td>Superheated Water Extraction</td>
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<tr>
<td>SWC</td>
<td>Superheated Water Chromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>RP-LC</td>
<td>Reversed Phase Liquid Chromatography</td>
</tr>
<tr>
<td>WRP-LC</td>
<td>Water-only Reversed Phase Liquid Chromatography</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas Chromatography – Mass Spectrometry</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>FT-IR</td>
<td>Fourier Transform Infra Red</td>
</tr>
<tr>
<td>UV/Vis</td>
<td>Ultra violet / visible</td>
</tr>
<tr>
<td>FID</td>
<td>Flame Ionisation Detector</td>
</tr>
<tr>
<td>NPD</td>
<td>Nitrogen Phosphorus Detector</td>
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<tr>
<td>PLE</td>
<td>Pressurised Liquid Extraction</td>
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<tr>
<td>ASE</td>
<td>Accelerated Solvent Extraction</td>
</tr>
<tr>
<td>SFE</td>
<td>Supercritical Fluid Extraction</td>
</tr>
<tr>
<td>MAE</td>
<td>Microwave Assisted Extraction</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid-Phase Extraction</td>
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<tr>
<td>SPME</td>
<td>Solid-Phase Microextraction</td>
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<tr>
<td>SBSE</td>
<td>Stir-bar Sorptive Extraction</td>
</tr>
<tr>
<td>MMLLE</td>
<td>Microporous Membrane Liquid-liquid Extraction</td>
</tr>
<tr>
<td>AAS</td>
<td>Atomic Absorption Spectrometry</td>
</tr>
<tr>
<td>ICP</td>
<td>Inductively Coupled Plasma</td>
</tr>
<tr>
<td>ODS</td>
<td>Octadecyl bonded silica</td>
</tr>
<tr>
<td>PGC</td>
<td>Porous graphitic carbon</td>
</tr>
<tr>
<td>PS-DVB</td>
<td>Polystyrene divinylbenzene</td>
</tr>
<tr>
<td>PAHs</td>
<td>Polyaromatic hydrocarbons</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of Detection</td>
</tr>
<tr>
<td>RSD</td>
<td>Relative Standard Deviation</td>
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</tbody>
</table>
# Glossary of Trade Names (HPLC Columns)

<table>
<thead>
<tr>
<th>Trade name</th>
<th>Common name</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Discovery RP-Amide C16</td>
<td>Amide C16 polar embedded phase</td>
<td>Supelco</td>
</tr>
<tr>
<td>2. PRP-1</td>
<td>Polystyrene-divinylbenzene (PS-DVB)</td>
<td>Hamilton</td>
</tr>
<tr>
<td>3. PLRP-S</td>
<td>PS-DVB</td>
<td>Polymer Laboratories</td>
</tr>
<tr>
<td>4. Hypersil® ODS</td>
<td>Octadecyl bonded silica (ODS)</td>
<td>ThermoHypersil-Keystone</td>
</tr>
<tr>
<td>5. Hypersil® BDS C18</td>
<td>BDS C18</td>
<td>ThermoHypersil-Keystone</td>
</tr>
<tr>
<td>6. Hypercarb</td>
<td>Porous graphitic carbon (PGC)</td>
<td>ThermoHypersil-Keystone</td>
</tr>
<tr>
<td>7. Nova Pak C18</td>
<td>C18</td>
<td>Waters</td>
</tr>
<tr>
<td>8. X-Terra RP18</td>
<td>C18</td>
<td>Waters</td>
</tr>
<tr>
<td>9. OASIS HLB™</td>
<td>Poly (divinylbenzene-co-N-vinylpyrrolidone (DVB/NP)</td>
<td>Waters</td>
</tr>
<tr>
<td>10. ZirChrom PDB</td>
<td>Polybutadiene-zirconia</td>
<td>ZirChrom Separations</td>
</tr>
<tr>
<td>11. ZirChrom CARB</td>
<td>Graphitized carbon-zirconia</td>
<td>ZirChrom Separations</td>
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CHAPTER ONE
Green chemistry, which was introduced in the early 1990s, is defined as the design of chemical products and processes that reduce or eliminate the use and generation of hazardous substances [1]. Water as a cheap and readily 'green' source can be used as an alternative solvent in analytical processes, and is therefore compatible with this green ideology.

A few researchers have looked at the applicability of using pure water as a mobile phase in reversed phase-liquid chromatography (RP-HPLC) performed at ambient temperature [2-6]. Unfortunately, little work has been performed since water is the weakest solvent and analyte retentions were extremely high, even for small molecules. Hence, specially modified stationary phases which can operate in 100% water mobile phase were required. However, in the last few years, the use of higher operating temperatures with pure water has been demonstrated to be viable and useful for both extraction [7-83] and chromatographic [84-108] applications. Hot, but subcritical, also known as superheated water [109] is an interesting medium for extraction and chromatography. The changes that occur in the physico-chemical properties of water at high temperatures and pressures are exploited in the extraction and separation processes. The selectivity to compounds of different polarity can be adjusted through changes in temperature and pressure.

Superheated water techniques were originally distinguished from conventional techniques by the use of temperatures above 100 °C and below the critical temperature, 374 °C but with enough applied pressures to maintain the liquid state. With two additional components: an oven and a back pressure regulator, a conventional HPLC system can be readily modified to a
superheated water separation (SWC) or extraction (SWE) system. However, there is still no specific instrument commercially available for SWC or SWE even in large-market areas, including the United States or United Kingdom. The nearest system has been the pressurised liquid extraction (PLE), which marketed under the trademark ASE (accelerated solvent extraction) by Dionex. Unfortunately, ASE is limited due to the limited range of elevated temperatures that can be employed with it.

1.1 Superheated Water Extraction (SWE)

An ideal extraction method should be:

- rapid,
- simple and inexpensive to perform,
- yield quantitative recovery of target analytes without loss or degradation,
- yield a sample that is immediately ready for analysis without additional concentration or class fractionation steps,
- generate no additional laboratory waste and be environmentally friendly.

Extraction methods for solid samples can be divided into conventional methods and new methods. Conventional methods, such as Soxhlet extraction [110], shake-flask [111, 112] and sonication [111] methods, frequently fail to meet the requirements of an ideal extraction method. They often require a long time to perform, are labour intensive, result in a dilute extract (which must be concentrated when trace analysis is desired), and may not result in quantitative recovery of target analytes. Recent concern about the hazardous nature of many commonly used solvents, the costs and environmental dangers of waste solvent disposal, and the emission of hazardous solvents into the atmosphere during sample concentration, further support the development of alternative sample extraction methods. These newer methods, which include supercritical fluid extraction (SFE) [113], microwave assisted extraction (MAE) [114] and pressurised liquid extraction
(PLE) [115, 116], are reported to meet most of the requirements of an ideal extraction method.

However, the main limitation of these methods is that they involve organic solvent consumption, either in extraction or sample concentration. This fueled interest in the development of superheated water as an alternative solvent. Hawthorne and co-workers [9] were the first to fully exploit the altered physico-chemical properties of superheated water in the extraction of phenols, n-alkanes and PAHs from environmental samples. Water could also be used as supercritical solvent, but it is very aggressive in practice and oxidises or decomposes many substances, and thus, supercritical water has been studied as a method to decompose toxic wastes [117]. However, extractions are sufficiently effective with using hot water without the need to go to the supercritical state.

The properties of superheated water are unique. One of the key parameters governing the solute/solvent interactions in SWE, is the dielectric constant or relative permittivity, \( \varepsilon \). The high dielectric constant of water at room temperature, \( \varepsilon = 80 \) [118], favours the solubility of ionic and polar compounds. However, when heated to a higher temperature and under enough pressure to maintain the liquid state, superheated water can have a dielectric constant very similar to typical organic solvents and it can dissolve a wide range of medium and low polarity analytes [9]. For example, at 220 °C, the dielectric constant of water is 30 which is almost equal to the dielectric constant of methanol at room temperature, \( \varepsilon = 33 \) [119]. The large change in the dielectric constant value of water can be explained by the large temperature dependence of hydrogen bonding [27]. A decrease in hydrogen-bonding at elevated temperatures leads to a decrease in the intermolecular forces between water molecules and brings the attractive forces of the water and low polarity solute molecules closer to each other, thus enhancing the dissolution process.
Higher temperatures also decrease the viscosity of superheated water [81], thus enhancing solute mass transfer and allowing a better penetration of matrix particles, therefore, enhancing extraction. Increased temperatures also decrease the surface tension of the superheated water [81] together with solutes and matrix. This will allow the water to better ‘wet’ the sample matrix. Both changes will facilitate better contact of the analytes with superheated water and enhance extraction.

1.1.1 Analyte Collection

Collection methods for the extracted analytes in SWE can generally be classified into two types: collection into organic solvents (e.g., methylene chloride, ethyl acetate etc.) in a beaker or vial i.e., solvent trapping, or collection onto a solid surface or sorbent media i.e. sorbent trapping. Solvent trapping [9, 10, 23, 30, 41, 43, 44, 58, 59] is the most straightforward method but suffers from a few drawbacks. It needs longer analysis time and results in a relatively dilute aqueous extract. Evaporative loss of volatile analytes can also occur during solvent removal, and the sensitivity is limited as only a fraction of the final extract can be injected into the chromatographic apparatus. In an attempt to minimise the dilution of the analyte in the liquid extract as well as to minimise the consumption of organic solvent, solvent trapping was therefore replaced by sorbent trapping, using solid phase extraction (SPE) cartridges [11, 14, 19, 20, 46, 47] or discs [12, 13, 20, 48, 49] or solid phase micro-extraction (SPME) fibres [15, 16, 25, 29, 46, 50, 53, 54]. Recently, microporous membrane liquid-liquid extraction (MMLLE) [120, 121] has also been used in the trapping of analytes, using a hydrophobic membrane placed between two immiscible liquid phases, aqueous and organic.
1.1.2 Applications of Superheated Water Extraction (SWE)

Because the solvent strength of water can be varied simply by changing temperature, this factor leads to variety of applications of SWE for organic pollutants, pesticides, natural products as well as inorganic compounds, mainly in environmental samples [9-51, 72-83], plant materials [57-71] and also foods [52-56, 82].

1.1.2.1 Organic Pollutants in Environmental Samples

The largest application for SWE has been in the field of environmental analysis. There are many areas where harmful organic pollutants persist in the environment, particularly in soil, sediment and sludges. Miller et al. [122, 123] examined the solubilities of a number of PAHs and found that they increased significantly as the temperature increased. However, a large increase in pressure (40 to 400 bars) lowered the solubilities, but had a much smaller effect than temperature and caused only a very small change in PAH solubilities.

Hawthorne et al. [9] demonstrated the class-selective extraction of phenols, n-alkanes and PAHs from environmental samples, such as soils, sludges, sediments and air particulates, using temperature programming. They reported that water can be used to sequentially extract polar, moderately polar, and non-polar organics from environmental solids by increasing the extraction temperature from 50 °C (for polar organics e.g., chlorophenols) to 400 °C (for all non-polar organics e.g., > C-20 alkanes). Low-polarity organics, such as PAHs, can be extracted at relatively mild conditions (e.g., 250 °C and 50 bar) without extracting the bulk of n-alkanes (> C-22) that interfere with GC determinations of the PAHs. Yang et al. [10] also reported the class-selective extraction of polar analytes, PAHs and n-alkanes from sludge samples at different temperatures and pressures. While polar analytes
Chapter 1

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like phenols were already extracted at 100 °C and 50 bar, non-polar PAHs and \( n \)-alkanes remained in the sludge sample. When the extraction temperature was raised to 250 °C at 50 bar, the PAHs were removed from the matrix, but the \( n \)-alkanes (>\( n \)=20) were not extracted until superheated steam conditions (250 °C and 5 bar) were reached. It was suggested that the low dielectric constant of steam was compatible with the polarity of the alkanes. This was confirmed by Hartonen et al. [11] who used superheated water and steam to extract \( n \)-alkanes and PAHs from spiked sea sand and sediment samples, by employing Tenax as the trapping material for the analytes’ collection. The results were compared with those obtained from SFE using neat \( \mathrm{CO}_2 \) and modified \( \mathrm{CO}_2 \). They found that steam at 250 °C and 300 °C gave similar recoveries to those obtained by SFE with the use of a modifier. In addition, extraction with steam also gave higher recovery of the \( n \)-alkanes compared to the recovery with SWE.

In order to avoid any possibility of the PAHs being deposited back onto the sample matrix or into the connecting tubing, Hawthorne et al. [12] analysed PAHs in soils, sediments, and air particulate matter by utilising a styrene-divinylbenzene (SDB-XC) extraction disc as a sorbent using static SWE at 250 °C. The sorbent disc was placed in the extraction cell and acted as an \textit{in-situ} trapping agent whereby the extracted PAHs partitioned into the disc as the extraction solution cooled. After SWE, the sorbent disc can be stored in an autosampler vial without loss of PAHs, thus providing a convenient method for shipment from the fieldwork to the analytical laboratory. Dean and McGowin [13] adapted this method to the analysis of PAHs in sewage sludge. Several other researchers also used the similar technique to trap pesticides from aqueous extraction solutions (see later in section 1.1.23).

Andersson et al. [14] designed a laboratory-made extraction vessel and used it in the SWE of PAHs from sediment, followed by trapping with solid-phase Tenax and thermal desorption with nitrogen prior to GC analysis. They
obtained similar recoveries to those obtained with a commercial extraction vessel at 300 °C with steam.

In most of the extraction studies followed by GC, organic solvents were used for the sample collection either by solvent trapping [9, 10] or sorbent trapping via SPE [11-14]. However, no organic solvents were required if the pollutants were recovered using solid phase micro-extraction (SPME). Hageman et al. [15] coupled static superheated water extraction (SWE) with SPME to determine the concentration of aromatic amines and PAHs in soil samples. With SPME, a polymer-coated silica fiber was exposed to the water sample where equilibrium between analytes in the water and the sorptive polymer phase was established. The analytes were then thermally desorbed from the fibre in a GC injection port for analysis. Daimon and Pawliszyn [16] described two different SPME approaches; dynamic and static, coupled to SWE for the determination of PAHs in solid matrices followed by GC analysis.

Because a solvent-free aqueous extract is obtained, SWE is directly compatible with immunoassay determinations. Kipp et al. [17] introduced the coupling of an enzyme immunoassay (EIA) with SWE for the determination of PAHs in native surface soil and sediment samples. The application of sensitive and relatively inexpensive assays made the coupling an attractive technique. Jimenez-Carmona and Luque de Castro [18] proposed a dynamic approach for the SWE of fluorescent organic compounds, such as pyrene, from solid matrices. They coupled a superheated water extractor to a continuous flow-manifold which incorporates a fluorimetric flow-cell packed with C18 solid support.

In a few cases where the extraction power of the superheated water was limited, organic solvents were added to the water to enhance the SWE. Fernandez-Perez and Luque de Castro [19] reported a new method for the quantitative extraction of PAHs from a soil sample based on the integration of three steps: continuous SWE, solid-phase clean-up/pre-concentration, and
HPLC separation with post-column fluorimetric determination. They added sodium dodecyl sulfate (SDS) as a micellar agent to the extraction water to enhance the extractability of PAHs and thus, the extraction temperature and time needed were reduced. The SDS was separated from the PAHs during the solid-phase clean-up/pre-concentration before the assay. In another study, Field and Reed [20] demonstrated the use of modified superheated water with ethanol to extract nonylphenol polyethoxy carboxylates (NPECs) from industrial and municipal sludges. NPECs were recovered from the water/ethanol sludge extract using strong anion exchange Empore discs. The acid analytes were simultaneously eluted from the disc and derivatised to their methyl esters for analysis by Cl-GC/MS. Analysis of PAHs in municipal solid waste compost was first reported by McGowin et al. [21] using static SWE with solid-phase extraction (SPE). The optimum extraction was achieved at 150 °C for 20 min and the recovery increased when C18 resin was added to the sample to provide an alternate surface for re-adsorption during sample cooling.

Hawthorne et al. [22] compared SWE with pressurised liquid extraction (PLE) for the extraction of PAHs from contaminated soil. One of the advantages of SWE over PLE method, is that substantially cleaner extracts can be obtained (with loss organic matrix), thus avoiding lengthy purification procedures of crude-soil extracts. For PLE extracts, class-fractionation was necessary in order to remove the matrix organic components from the extracts prior to analysis. Unfractionated extracts from PLE yielded more artifact peaks in the GC chromatograms compared to superheated water extracts. In term of selectivity, SWE has a higher potential compared to PLE. Superheated water starts as a very polar solvent at lower temperatures and becomes less polar as the temperature is increased until its polarity becomes similar to methanol or acetonitrile at ~200 °C. Thus, most polar analytes extract most readily in water at lower temperature while less polar analytes require less polar water at higher temperatures up to 300 °C.
Low polarity compounds, such as polychlorinated biphenyls (PCBs), can also be extracted from soil and sediment samples at water temperatures $\geq 250$ °C. Under superheated water optimised conditions of 300 °C and 50 atm, followed by solvent trapping, quantitative extraction of PCBs was achieved by Yang et al. [23] in only 5 min. Pross et al. [24] compared the extraction of spiked samples of 8 different PCB congeners from soil by superheated water with supercritical carbon dioxide (CO$_2$) and sulfur hexafluoride (SF$_6$). They found that the extraction from water and CO$_2$ gave quantitative results with recoveries greater than 95% for all PCBs. SF$_6$ appeared to be most successful particularly for the extraction of low polar PCB. Hawthorne et al. [25] coupled static SWE with SPME for the rapid determination of PCBs from contaminated soils and sediments. They demonstrated the higher selectivity of SWE-SPME, even after storing the SWE water for 24 hours, compared to Soxhlet extraction. Solid-phase trapping with Tenax was introduced by Hartonen et al. [26] to collect the PCBs after SWE, and higher recoveries were achieved relative to Soxhlet extraction.

In other studies, van Bavel et al. [27] demonstrated the extraction of polychlorinated naphthalenes (PCNs) and polychlorinated dibenzofurans (PCDFs) from aged industrial soils, whilst Hyotylainen et al. [28] developed a SWE method for brominated flame-retardants (BFRs) in sediments. Both Bavel and Hyotylainen showed that the extraction efficiency was substantially better than that obtained by traditional Soxhlet methods. It was also faster and provided cleaner extracts, therefore, no further clean-up was necessary with SWE.

Wennrich et al. [29] determined chlorophenols in soil samples by the SWE method, using a commercially available accelerated solvent extractor combined with SPME followed by GC/MS, and compared the results with classical ASE procedure using organic solvents (acetone/n-hexane, 1:1, v/v). They found that superheated water at 125 °C extracted the chlorophenols more effectively from the soil matrix than the organic solvents at 100 °C.
Superheated water has also been used to extract dioxins from contaminated soils, which was more efficient than a common extraction procedure with hexane-methylene chloride (1:1) [30].

Other than solid samples, superheated water is also capable of extracting a broad range of polar to low polarity organic compounds from air particulate matter. Two types of carbonaceous aerosols, diesel exhaust particulate (a relatively non-polar matrix) and wood smoke particulate (a polar matrix), were sequentially extracted by Kubatova et al. [31] using superheated water ranging from 25 °C to 300 °C. Each of the aqueous fraction extracts could be directly used for toxicological testing. The polar fractions which are not expected to be extracted by organic solvents were found to have high toxicity.

1.1.2.2 Transition Metal Ions and Toxic Metals in Environmental Samples

Most superheated water extractions that have been used for sample pretreatment are devoted to organic analytes, such as PAHs, PCBs, or semivolatile organics. However, superheated water has also been used as the extractant for inorganic sample, such as transition metals and toxic metals, for subsequent ICP or AAS analysis.

Rico Varade and Fernandez-Perez [32] reported the use of superheated water for the continuous extraction of different selenium forms, both inorganic selenium (as Se⁴⁺/Se⁶⁺) and organo-selenium compounds, from solid samples. The extraction was completed in 15 min at 250 °C. Continuous derivatisation (hydride formation) and detection by atomic fluorescence was used for the assay.

Acidified superheated water has been used widely to extract metal elements in several reported studies. Fernandez-Perez et al. [33] utilised water
modified with 4% v/v nitric acid as the extractant for the extraction (in a static-dynamic mode) of selenium (Se), arsenic (As) and mercury (Hg), from coal prior to continuous derivatisation and detection by atomic fluorescence. A similar technique was later extended by Jimenez-Carmona et al. [34] for the extraction of major-ash forming elements (aluminium, iron, calcium, magnesium, sodium and potassium) from coal prior to determination by atomic spectroscopy. Again, acidified superheated water was proposed as an extractant for the development of a liquid-liquid extraction method for the demetalisation of used industrial oils [35]. The two immiscible liquid phases (the used oil and water modified with 4% (v/v) HNO$_3$ + 0.1 M KCl) entered into contact in a stainless steel extraction coil located into an electrically heated oven. The metal ions (Cu, V, Pb, Ni, Cd and Cr) extracted into aqueous phase were determined by graphite furnace AAS. Priego-Lopez and Luque de Castro [36] also applied a similar approach for the extraction of metals, such as lead, copper, cadmium, arsenic, selenium and mercury, from contaminated soil samples. Morales-Munoz et al. [37] then employed dynamic extraction with water modified with 1% (v/v) HNO$_3$ for the continuous leaching of cadmium and lead from plant materials prior to electro-thermal AAS.

Boron was also extracted by SWE from soil and was compared with conventional boiling hot water extraction method [38]. SWE was found to be faster and produced a higher value of measured boron. Beichert et al. [39] combined SWE with SPME for the extraction and determination of alkylmercury compounds in solid matrices. The identification and quantification of the extracted alkylmercury compounds was performed by GC-MS after thermal desorption.
Chapter 1

Introduction

1.1.2.3 Pesticide Residues in Environmental Samples

As a consequence of the widespread use of pesticides, the presence of their residues in the different environmental matrices has become an important issue in analytical science. It has been observed that by increasing the water temperature, the solubilities of the pesticides increased. Curren and King [40] measured the solubilities of triazine pesticides atrazine, cyanazine and simazine in pure and modified water at temperatures ranging from 50 to 125 °C and at a pressure of 50 atm. Ethanol and urea were used as the modifiers. Both of these co-solvents are non-toxic and can be safely disposed of with the rest of the aqueous media. They found that cyanazine exhibited higher solubilities in superheated water than either atrazine or simazine which probably due to its more polar character. At 100 °C, the solubility of atrazine was doubled when the water was modified with urea and was increased more when ethanol was used as modifier. This indicated that extractions can be performed at lower temperatures if co-solvents are used in conjunction with temperature to reduce the hydrogen-bond density of water. This approach is preferred for solutes that are thermally unstable.

Recently, Richter et al. [41] evaluated 16 pesticides (with a broad spectrum of polarities) from soil, using SWE followed by solvent trapping with dichloromethane and determination by GC-MS. They found that under the optimised conditions, most of the analytes were extracted quantitatively with recoveries comparable to Soxhlet extraction method and even faster. However, the precision of the SWE method was lower than that of the Soxhlet method. Kramer et al. [42] investigated four extraction techniques: Soxhlet extraction, microwave-assisted extraction (MAE), aqueous microwave extraction (ME), and superheated water extraction (SWE), for the determination of pesticides, such as diazinon, malathion, chlorpyrifos, and chlordane. They discovered that the recoveries of the methods involving organic solvents (Soxhlet and MAE) were substantially higher than those
involving aqueous solvents (ME and SWE), and SWE at 200 °C was more efficient compared to ME with water.

Krieger et al. [43] showed that SWE was an effective technique for the rapid and quantitative extraction of tricyclazole from freshly spiked soils and sediments, but even higher extraction temperatures were required for quantitative recovery of tricyclazole from aged samples (200 days). A triazolopyrimidine sulfoanilide herbicide called chloransulam-methyl was also extracted with SWE from soil [44]. Water was as effective as strong organic solvents for this extraction, however, chloransulam-methyl hydrolysed when extracted at 150 °C. Thus, it is important to verify the stability of analytes under SWE conditions.

Jimenez-Carmona et al. [45] extracted TCP (3,5,6-trichloro-2-pyridinol, a metabolite of chlorpyrifos) from soil and compared the extraction of TCP with CO₂ and H₂O. 95% extraction was achieved in 30 min when supercritical CO₂ at 40 °C and 383 bar was used as the extractant with the presence of a co-solvent (methanol) and an ion-pair reagent. Whereas a complete extraction was achieved within 15 min when superheated water at 250 °C and 200 bar was used as the extractant without additives.

In several of the SWE methods, SPE cartridges [46,47] and discs [48, 49], or SPME fibres [50] were used to collect and trap the extracted pesticides. Crescenzi et al. [46] introduced the extraction of 18 multiresidue herbicides, including phenoxy acid herbicides, from soil at 90 °C, using a Carbograph 4 SPE cartridge as the trap, which was set on-line with the SWE system. The analytes were then recovered by stepwise elution to separate non-acidic from acidic herbicides and both were analysed by LC/MS with an electrospray ion source. They found that at higher extraction temperatures, less polar herbicides were removed. A similar system was used by Di Corcia et al. [47] but using phosphate-buffered superheated water. They determined terbutylazine (CBET) and its degradation products in aged and incubated soil.
via SWE followed by collection with a carbon cartridge and assay with LC/MS. They observed that phosphate-buffered water heated at 100 °C extracted much larger amounts of terbutylazine and its metabolites from a naturally aged soil than water alone at the same temperature. In another work, Field et al. [48] demonstrated the use of a strong anion-exchange (SAX) disc, placed in the extraction cell to trap acid metabolites of Dacthal from the aqueous SWE solution. The acid metabolites were then combined with Dacthal (extracted from soil by supercritical CO₂) by placing the disc into the GC autosampler vial containing the SFE extract. This method of coupling supercritical CO₂ and superheated water proved to be rapid and allowed for the class-selective fractionation of the relatively non-polar herbicide Dacthal from its polar metabolites in soil. The potential of combined SWE/SAX disc extraction was further investigated by Lou et al. [49] with the objective of developing an easy, robust, and field-portable method for the analysis of chlorinated acid herbicides from contaminated solids. They demonstrated that this technique allowed for the simultaneous extraction and trapping of acid herbicides and phenoxy acid esters from soil samples. The SAX disc was stable at water temperatures up to 150 °C and the trapped solutes can be derivatised directly from the disc. In another study, superheated water up to 200 °C was used by Krappe et al. [50] to hydrolyse and extract pyrethrins (insecticides) from soil samples. The SWE was coupled to SPME and GC/MS. They examined three SPME fibres: polydimethylsiloxane (PDMS), polyacrylate (PA), and carbowax/divinylbenzene (CW), and found that the PDMS fibre was the most reproducible and most stable.

Lagadec et al. [51] employed superheated water to remove pesticides along with PAHs, from highly contaminated soils. They used laboratory-scale (8 g of soil) experiments to determine conditions for the pilot-scale (8 kg of soil) extractions. SWE at 250 °C of 8 kg of soil contaminated with 70-400 mg/kg levels each of trifluralin, atrazine, cyanazine, pendimethalin, alachlor, and metochlor removed all the pesticides to below detection limits.
1.1.2.4 Pesticide Residues in Plant Material and Food

The presence of pesticides in food products continues to be of increasing concern and therefore the determination of the levels of pesticides in food products has prompted the development of solvent-free methods, such as SFE and SWE. Superheated water was used to extract two fungicides: thiabendazole (TBZ) and carbendazim (MBC) from food or agricultural commodities, including banana pulp, whole lemons, orange pulp, mushrooms and rice, at a temperature of 75 °C and pressure of 50 atm [52]. The temperature, which has a great impact on extraction efficiency, was studied. Wennrich et al. [53] determined organochlorine pesticides from strawberries by using SWE followed by solid-phase microextraction (SPME) or stir-bar sorptive extraction (SBSE, a stir bar coated with a sorptive polymer), and subsequent thermal desorption-GC-MS. Both SPME and SBSE are solvent-free techniques and require neither clean-up nor concentration steps. SBSE enables more effective extraction of volatile and semivolatile organic compounds from aqueous samples compared to SPME because it provides a more extractive surface than a coated fibre. The same technique was then used to determine organochlorine pesticides and chlorobenzenes in fruit and vegetables [54].

Curren and King [55] utilised ethanol modified superheated water in combination with SPME for the removal of atrazine from beef kidney. SWE was performed with a pressurised liquid extraction unit at 100 °C and 50 atm. In-situ sample clean-up was carried out using matrix solid-phase dispersion utilising the acrylic polymer XAD-7 HP. It was determined that 30% ethanol in water (v/v) was adequate for the complete extraction of atrazine. Triethylammonium phosphate (TEAP) buffer was also added to the extraction solvent to overcome the removal of lipids and proteins with ethanol. With the similar approach, they later extracted avoparcin (an antibiotic) from swine kidney at 75 °C and 50 atm [82]. In another study, Lawrence et al. [56] also
utilised ethanol as a co-solvent during the SWE of fumonisins (mycotoxins) from contaminated corn products.

1.1.2.5 Natural Products (essential oils, fragrances, and botanical drugs & antioxidants) in Plant Material

Other than environmental analysis, researchers have taken advantage of the abilities of superheated water in the extraction and isolation of various natural products. Attention was drawn to the use of new and clean alternative methods for the isolation of essential oils and aroma compounds from plants. Moreover, with the growing needs for the validation of botanicals and herbal preparations, methods that required little or no organic solvent are attractive options.

The use of SWE as a promising alternative to conventional extraction methods for the isolation of aroma compounds was proposed by Basile et al. [57]. They reported that SWE of aroma compounds from rosemary plants gave higher yields than steam distillation. By using water at temperatures between 125 and 175 °C, rapid extraction of oxygenated flavour and fragrance compounds could be achieved, leaving behind monoterpenes, high hydrocarbons and lipids. Since then, the SWE technique has shown its applicability in the field of essences and fragrances. Recently, antioxidant compounds have also been extracted from rosemary leaves by SWE at several temperatures ranging from 25 to 200 °C [71]. The results indicated high selectivity of the superheated water towards the most active compounds of rosemary. The antioxidant activity of the superheated water fractions was very high and comparable to those achieved by SFE.

SWE followed by analysis with GC/FID and identification by GC/MS has been developed by Jimenez-Carmona and Luque de Castro [58] for the isolation of eucalyptus essential oil. Essential oil was extracted from fresh eucalyptus
leaves with water at 150 °C and 50 bar. They compared the SWE method with hydrodistillation and found that SWE was clearly quicker and more efficient. In another work, essential oil from laurel leaves was extracted by Fernandez-Perez et al. [59], using static-dynamic SWE. They also compared this method with others based on the use of both conventional (hydrodistillation and liquid-liquid extraction) and recent (dichloromethane subcritical extraction) techniques. They discovered that SWE has more advantages in terms of rapidity, efficiency, and cleanliness. Jimenez-Carmona et al. [60] and Ayala et al. [61] extracted essential oils from marjoram and oregano leaves respectively, by continuous SWE and compared the results with the hydrodistillation method. They reported that continuous SWE combined with GC-FID was quicker than hydrodistillation coupled to GC-FID. It provided a more valuable essential oil with high precision, saved energy and cost.

The comparisons between continuous SWE and conventional techniques were further examined by Gamiz-Gracia and Luque de Castro [62]. They employed SWE to isolate the essential oil of fennel (a medicinal plant) and compared it with both hydrodistillation and dichloromethane manual extractions. Again, better results were obtained with the continuous SWE in terms of rapidity, efficiency, and cleanliness. In addition, SWE benefits from the possibility of manipulating the composition of the extract by changing the parameters of the extraction, such as temperature, flow rate and static extraction time.

The yields of oxygenated and non-oxygenated flavour and fragrance compounds from savory and peppermint using SWE were then compared to SFE-CO$_2$ and hydrodistillation by Kubatova et al. [63]. Superheated water preferentially extracted more polar (oxygenated) flavour compounds, in contrast with SFE, which easily extracted non-polar compounds and its extracts included plant alkane waxes as well as the same flavoured compounds recovered by hydrodistillation. They also studied the
mechanisms that control the extraction rates achieved with superheated water and supercritical CO₂, using sample extracts of essential oil from savory [64].

SWE was used by Rovio et al. [65] to extract eugenol and eugenyl acetate from clove at various temperatures and pressures. The analytes were collected by using a C18 solid-phase trap. They found that the extraction kinetics with superheated water were very fast at high temperatures (250 °C and 300 °C) compared to extraction at 125 °C. Clifford et al. [66] also extracted eugenol and eugenyl acetate from clove buds by SWE and compared this method with supercritical CO₂ extraction (SC-CO₂) and conventional extraction: steam distillation and Soxhlet extraction. The yields that were obtained by all these methods were found to be similar.

SWE of kava lactones (oxygenated substituted α-pyrone and dihydro-α-pyrone) from kava (Piper methysticum) root was compared to a Soxhlet extraction with water, to boiling in water, and to a sonication in acetone by Kubatova et al. [67]. Higher recoveries of kava lactones were obtained by SWE at 175 °C in 20 min. Sonication with acetone also yielded high recoveries, however, it required 18 hours to complete the extraction. Both conventional methods that employed water under atmospheric pressure exhibited ~50% lower recoveries than superheated water.

Luque de Castro et al. [68] reported a critical overview of conventional methods (based on either organic solvent extraction or distillation) and new alternative methods, including microwave-assisted extraction (MAE) as well as SC-CO₂ and SWE. Special emphasis was given to the use of continuous SWE which emerged as clearly advantageous over conventional methods by avoiding the use of organic solvents and considerably shortening the extraction time, as well as increasing the efficiency.

Superheated water was also applied for the extraction of thermally labile and reasonably polar marker compounds, such as berberine, baicalein, and
glycyrrhizine, in several medicinal plants [69]. The dynamic SWE was carried out at temperatures between 95 and 140 °C and an applied pressure of 10 – 20 bar. The efficiencies of SWE were comparable to Soxhlet extraction for baicalein, and sonication for berberine and glycyrrhizine. Since berberine and glycyrrhizine showed signs of degradation above 120 °C, ethanol was added into the water to increase the efficiencies at lower temperatures. In another study, Suomi et al. [70] reported several methods (PLE, hot water extraction and SWE) for the extraction of two iridoid glycosides, catalpol and aucubin, from plant materials. PLE performed at 100 °C (103 atm) and SWE at 150 °C (145 atm) gave lower recoveries compared to SWE at 100 °C (145 atm) and hot water extraction at 100 °C (1 atm).

1.1.3 Other Applications of SWE

The fact that most non-polar environmental pollutants can be extracted using water is especially interesting in the development of remediation techniques for contaminated soil. SWE at moderate temperatures cleans the soil and makes the target compounds available for further chemical or biological treatment. Yak et al. [72] described preliminary studies of a method for remediation of PCB-contaminated soil and sediments by using zero-valent iron as the dechlorination agent and SWE as the transporting medium. With SWE conditions of 250 °C and 10 MPa, the PCBs were completely degraded. Weber et al. [73] reported that >99% of PCBs could be destroyed in superheated and supercritical water under oxidative and non-oxidative alkaline conditions. They also evaluated the formation of polychlorinated dibenzofurans (PCDFs) during PCB destruction.

Kubatova et al. [74] investigated the dechlorination of aliphatic organochlorine compounds, such as lindane, dieldrin, tetrachloroethane, trichloroethene, and polyvinyl chloride (PVC) in superheated water. In another study, Marrone et
al. [83] reported that the dechlorination of methylene chloride was much faster using superheated water rather than using supercritical water.

Johnson *et al.* [75] used SWE as a tool to remove the carboxylic, aliphatic and carbohydrate types of organic carbon from humic soil. They also used steam at 150-250 °C but found that liquid water removed more carbon from soil because of many polar functional groups of the humic organic matter that readily associate with water. They later extended their work to effect rapid compositional and functional changes to peat organic matter by using superheated water treatment [76]. These studies suggested that superheated water effects the deoxygenation reactions of soil organic matter that mimic those of the geologically slow, natural diagenesis processes.

Superheated water oxidation (SWO) has been utilised as a process of waste disposal that made use of oxygen and superheated water. Toxic and hazardous organic materials, such as phenolic compounds, could be removed from wastewater by using this method. It could also be an effective treatment for wastewater that has a chemical oxygen demand (COD) that is too low for incineration and too high for biological treatment. On-line coupled SWE and SWO equipment was used by Kronholm *et al.* [77] to extract PAHs from a soil sample and then to destroy them with potassium persulfate as oxidant. They also examined the on-line coupling of SWE with oxidation in supercritical water (SCWO) to oxidise (destroy) the extracted PAHs from contaminated soil [78]. Technically they found that the SWE-SCWO coupling method was capable of safely and effectively extracting PAHs from real soil and oxidising them.

Superheated water also has been reported to efficiently degrade explosives, such as TNT, HMX, and RDX from highly contaminated soil using a static system at 275 °C for an hour [79]. Based on the results, a pilot scale remediation of the aged contaminated soils was designed and built to clean 4-6 kg of soil by using 4 L of superheated water.
Yang et al. [80] utilised the SWE approach to determine the effect of temperature on the solubility and partitioning behaviour of organic pollutants from gasoline and diesel fuel in water. The ability of this method to determine partitioning behaviour was demonstrated at ambient temperature by a comparison of toluene solubility to literature values. The partitioning coefficients of fuel components, such as benzene, toluene, ethylbenzene and xylenes (BTEX), PAHs and n-alkanes between liquid and water was determined at temperatures ranging from ambient to 250 °C. and pressures from 1 bar to 50 bar. They found that raising the water temperature has a significant effect on enhancing toluene solubility, while water pressure has no significant effect. The partitioning of both BTEX and PAHs from liquid fuel into water was greatly increased at higher temperature.

A study of different types of sorbent used for extractions from aqueous samples, was reported by Yang et al. [81]. They extracted several analytes, such as chlorophenols, amines, n-alkanes and PAHs, by using different sorbents including: Florisil, glass beads, and alumina for normal phase packings, and silica bonded C18 and polymeric XAD-2 resins for reversed phase (RP) packings. Elution from RP packings required water that was ~100-150 °C hotter than for elution from normal phases. Aromatic solutes required ~50 °C hotter water for elution from XAD-4 than from C18 packings. This pattern demonstrated that water could most easily disrupt dipole interactions (Florisil and alumina), while higher temperature water was required to interrupt Van der Waals attractions (silica bonded C18), and even higher temperature water was needed to overcome the π-electron interactions (XAD-4) between solutes and solid sorbents.
1.2 Superheated Water Chromatography (SWC)

In the past few years, many studies of superheated water chromatography (SWC) have been reported, which mainly focus on testing the feasibility of using superheated water as the mobile phase for reversed-phase separations. When Hawthorne and co-workers [9] found that the extraction of analytes ranging from polar to non-polar analytes by using pure water were selectively dependent on temperature, this inspired researchers at Loughborough to use superheated water as a mobile phase for RP-HPLC [84]. As superheated water at selected temperatures can imitate a mixture of organic solvent and water in RP-HPLC, it has been employed as a mobile phase in the RP-PHLC of moderately polar and non-polar analytes [84-104]. In addition to improving the separation process and reducing analysis time, temperature programming can be used to mimic a gradient elution/solvent programming as in conventional HPLC [124, 125].

The use of this superheated water chromatographic system has resulted in effective separations for a wide range of aromatic analytes, homologue compounds, pharmaceuticals, and carbohydrates, on a few types of stationary phases such as, ODS silica, polystyrene-divinylbenzene (PS-DVB), X-Terra, zirconia-PDB, and porous graphitic carbon (PGC) [84-104]. All these columns, except ODS silica columns, seem to be suited to this technique because they did not suffer from the possible hydrolysis of the bonded phase, or the dissolution of the support material itself in a high temperature aqueous environment. In contrast, ODS silica columns have been reported to be unstable when used at high temperatures (>80 °C) even though they also gave good results, comparable to the others [86]. Superheated water chromatography (SWC) has been shown to be compatible with conventional HPLC detectors such as UV/Vis and fluorescence detection since it is similar to conventional HPLC at high temperature. UV/Vis detection has been reported to be the most feasible to be coupled on-line to the superheated water system.
1.2.1 Applications of Superheated Water Chromatography (SWC)

The separation of aromatic analytes ranging from polar analytes, such as amides and phenols, followed by less polar ketones and aldehydes by using superheated water at temperature up to 210 °C was first reported by Smith and Burgess [85, 86]. The separations were carried out by using a polystyrene-divinylbenzene (PS-DVB) column which showed very high retention for non-polar analytes and moderate retentions for moderately polar analytes. In each case the retention factors systematically decreased as the temperature increased. The applicability of this approach to pharmaceuticals has been demonstrated by the separation of a mixture of five barbiturates at 200 °C [85]. They also demonstrated that superheated water could resolve analytes on the basis of their hydrophobicities by separating a series of paraben homologues at 210 °C [85, 86]. Furthermore, a series of studies was carried out using an ODS-bonded phase silica column which gave good results comparable to that of PS-DVB column even though they were expected to be less stable [86]. With these ODS silica columns, the analytes could be eluted at considerably lower temperatures (≈40-50 °C) than from the PS-DVB column. This agreed with a conventional RP-mode that retention is weaker when using ODS-silica than PS-DVB columns. It was suggested that prolonged use of ODS-silica column may result in some loss of retention and that the stability of these silica based bonded phases may be limited.

Yarita et al. [87] then evaluated the stability of the PS-DVB stationary phase by carrying out a durability test, in which superheated water was passed through the PS-DVB column for 144 hours. They proved that reproducible separations of phenols in a temperature range of 100 – 150 °C could be achieved in a long time with the PS-DVB column unlike the ODS column. Burgess [84] has made a stability (under superheated water conditions) comparison among a few types of columns including zirconia-PBD, silica-ODS, PS-DVB and PGC. As expected, polymeric PS-DVB columns were found to be the most stable. On the other hand, the silica ODS column was
the least stable due to the dissolution of silica-based material at high temperature. Meanwhile, a zirconia-PBD column which was believed to have a similar physical properties to a silica-based column gave good separation and was less retentive compared to a PS-DVB column. PGC, which is made of an inert graphite material, was more promising but it gave poor separations with long tailing peaks. Smith et al. [88] then demonstrated a SWC separation of anilines on a PGC column at 182 °C.

Smith and Burgess used a UV/Vis detector in their work, but Miller and Hawthorne [89] then introduced the use of GC flame ionisation detector (FID) in superheated water chromatography. This overcomes previous attempts to use FID with HPLC using aqueous/organic eluents, which required the removal of interfering organic components from the mobile phase, with the possible loss of volatile analytes. They applied temperature programming in their work to improve separations of a variety of alcohols, hydroxy-substituted benzenes and amino acids, and decrease analysis times. By programming the temperature from 120 °C to 150 °C, the polarity of water was reduced and the separation of seven alcohols, ranging from relatively more polar (methanol) to less polar (butanol) was achieved. The use of FID and temperature programming in SWC was also reported by Smith and Burgess [88, 90, 92]. They applied this method to a range of analytes using PS-DVB and PGC columns. The SWC-FID system was further improved in terms of signal stability during temperature programmed operation, by Ingelse et al. [91]. They first optimised the FID in the flow injection analysis (FIA) mode in conjunction with ODS silica and carbon columns. In this FIA mode, they found that with water flow rates of 100 µL min⁻¹ or higher, the use of a wide bore FID jet resulted in an improved stability of the FID signal but the FID sensitivity was decreased. Therefore, with water flow rates of 50 µL min⁻¹ or lower, the standard FID jet was applied instead of the wide-bore jet. The coupling of SWC-FID in the split mode was then investigated by Yang et al. [93] by connecting a Tee union between the separation column and the FID system to split the water flow. They used PGC (Hypercarb) and PS-DVB...
(PRP-1) columns to separate several carbohydrates, carboxylic acids, and amino acids, and found that the FID system was very stable at total flow rate up to 1.24 mL/min.

Yang et al. [94] investigated the retention behaviour of some aromatic compounds, including phenols, anilines and alkylbenzenes, in a SWC. The retention times for non-polar compounds, such as benzene, toluene, ethylbenzene and m-xylene (BTEX) were generally longer than those of chlorinated phenols and anilines. However, all their retention factors \((k)\) were reduced by raising the water temperature. They also found that a lower solubility of the solute corresponds to a longer retention of this solute and the solubility enhanced by heating the water. Furthermore, they demonstrated the separations of polar and non-polar analytes by using both normal (alumina) and reversed-phase (Nucleosil C18 and PRP-1) columns. The retentions of these analytes were shortest on alumina, moderate on Nucleosil C18 (silica bonded), and longest on PRP-1 (PS-DVB) column.

Wilson [95] examined the conditions required to separate five model drugs using superheated water on a range of stationary phases at different temperatures up to 225°C. Among the stationary phases were porous graphitic carbon (PGC); two types of polymer-based materials: PLRP-S and Oasis; two types of zirconia-based materials: ZirChrom PBD, a polybutadiene-zirconia and ZirChrom CARB, a graphitised carbon-zirconia phase; and two types of silica-based materials: BDS Hypersil and X-Terra. In general, carbon (PGC and ZirChrom CARB) and polymer-based phases required the highest temperature (>100 °C) to elute all five analytes. However, the ZirChrom PDB stationary phase was not very retentive, hence needed lower temperature to separate the analytes. On the other hand, silica-based materials also gave good separation at elevated temperatures, unfortunately the BDS-Hypersil phase did not prove to be robust and degraded in a relatively short time. In contrast to the Hypersil phase, all the analytes could be separated by an X-Terra phase up to 165 °C with excellent peak shape.
Zirconia columns coated with polybutadiene (PBD) have attracted other researchers to further investigate their stability in superheated water conditions. McNeff et al. [96] compared the separation of five phenol compounds using a zirconia-PBD column with an acetonitrile/water mobile phase at 30 °C and 80 °C, and with superheated water mobile phase at 200 °C. This column was reported to be stable up to 200 °C and compared to a polymeric column, it was more efficient. Hence, water temperatures up to 200 °C were also favoured by Fields et al. [97], to evaluate the chromatographic characteristics (retention, efficiency and selectivity) of testosterone and related compounds using a zirconia-PBD column compared with conventional HPLC. The separation efficiencies were found to be comparable between both SWC and conventional HPLC using the zirconia-PBD column. Yang et al. [98] then studied the temperature effect on peak width and column efficiency in SWC using a zirconia-PBD column, as well as Zorbax RX-C8, PS-DVB, and Hypersil ODS columns. They found that the retention time and peak width of several polar and chlorinated compounds are reduced with increasing temperature from 60 °C to 160 °C. On the other hand, the column efficiency was either improved or almost unchanged with increasing the temperature from 60 °C to 120 °C, but decreased when the temperature was further raised to 160 °C.

A capillary scale SWC has been claimed by Kephart and Dasgupta [99], to be capable of separating both polar and non-polar compounds (six benzene derivatives) on polybutadiene and elemental carbon modified zirconia packings, in approximately 2 min. The low flow rates and the small thermal mass in the capillary allow rapid temperature ramps and greatly facilitate radial heat transfer, therefore faster separation. The system has been shown to operate at temperatures as high as 370 °C and pressures up to 11,000 psi. In another study, Yan et al. [100] introduced a high temperature ultrafast liquid chromatography (HTUFLC) and applied it with superheated water. By using superheated water at a temperature of 120 °C and a flow rate of 12 mL/min,
the separation of phenolic compounds on the PS-zirconia column was accomplished in less than 30 seconds.

Hu et al. [101] used pure water as a mobile phase and raised the temperature from ambient up to 65 °C to study temperature effects on retention behaviour of hydrophobic analytes: nucleosides and their bases on an octadecylsilane (ODS) column. Their work showed that the partitioning behaviour of the hydrophobic analytes between the stationary and mobile phases was extremely dependent on the temperature of the column.

As with extraction, adding organic modifier to superheated water has also gained interest in separation, in order to reduce the high temperatures. The influence of temperature in the range of 75-180 °C on the solvation properties of superheated water was studied by Pawlowski and Poole [102] on a porous polymer sorbent PLRP-S column. Water containing 1% (v/v) of acetonitrile (ACN) was used as the mobile phase to separate 25 varied compounds. It was shown that the elution strength of 1% ACN/water at 180 °C corresponded to 15-25% ACN/water, 25-35% propanol/water, or 50-60% methanol/water, at ambient temperature. Kondo et al. [103] employed superheated water modified with dimethyl sulfoxide (DMSO) to separate polar, relatively polar and non-polar analytes (hydroxybenzenes, phenol derivatives, BTEX, and PAHs) by using an ODS2 column. They found that, by increasing the temperature of the eluent, less DMSO was required to achieve similar retention for a given solute. Less polar solutes such as BTEX and PAHs required greater temperature increase to achieve the same retention resulted by 1% decrease in DMSO concentration in the eluent. Jones and Yang [104] further examined the separation of non-polar compounds (PCBs, PAHs, benzene, toluene, and xylene) using superheated water modified with methanol at temperatures from 21 to 140 °C. They found that a 1% increase in methanol concentration was approximately equal to an increase in temperature of 4-5 °C. Hence, the addition of methanol into the water greatly
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reduced the high temperatures that are normally required to separate non-polar compounds.

Chienthavorn and Smith [105, 106] investigated the potential of buffered superheated water as a mobile phase for RP-HPLC to suppress or control ionisation of analytes in order to improve peak shapes and reproducibility. They used a temperature gradient from 70-190 °C to separate a series of sulphonamides on a PS-DVB column at pH 3, 7 and 11. Teutenberg et al. [107] also used phosphate buffered superheated water to separate six anticancer drugs on a PS-DVB column, by adjusting the pH from 11.5 to 3.5. The best separation was achieved at a pH of 3.5 and a temperature of 150 °C.

As the properties of deuterium oxide (heavy water) are very similar to those of water, it could also be employed as a mobile phase in RP-HPLC. In addition, it should provide an ideal eluent for coupling on-line separations to NMR spectroscopy since no strong signals would be seen in the NMR spectrum resulting from the proton containing solvents used as the chromatographic eluents, which could otherwise interfere with the spectra of the analytes. Smith et al. [126] first demonstrated the coupling of superheated deuterium oxide (D₂O) HPLC to NMR for the separation of barbiturates on a PS-DVB column, used in both the on-line and stop-flow modes of direction. A methanolic extract of powdered ginger has also been successfully separated by D₂O on an X-Terra C18 column using temperature gradient from 50 to 130 °C at 4 °C/min [108, 127]. On-line and off-line HPLC-NMR was applied for the analysis of vanillin, dihydroferulic acid, zingerone and ferulic acid. The potential for expanding this system to HPLC-NMR-MS so that both NMR and mass spectra could be obtained from the same chromatographic separation was then investigated [105, 128, 129]. Louden and Handley [130] demonstrated the coupling of superheated deuterium oxide (D₂O) HPLC with on-line characterisation via a combination of diode array UV, NMR, FT-IR spectroscopy, and MS for the analysis of ecdysteroids in plant extracts. This
combination of spectrometers enabled the on-flow collection of UV, NMR, IR and mass spectra for the steroids.

1.3 SWE Coupled to Separation Techniques

The rapid development of SWE has led to the direct combination of this technique with subsequent analysis i.e., on-line SWE. Until recently, on-line SWE has been coupled only with conventional chromatographic methods. The key to combining SWE with a chromatographic method is the interface between the two techniques.

In most of the SWE methods, solvent trapping was used to collect the extracted analytes by liquid-liquid partitioning, using methylene chloride or chloroform [9, 10, 23, 30, 41, 43, 44, 58, 59]. To minimise or eliminate the consumption of the organic solvents for sample concentration, sorbent trapping was developed and set on-line with the extraction column. A solid-phase extraction (SPE) cartridge or a small guard column containing a sorbent material served as the accumulator trap to collect the superheated water extracts. By using the sorbent trapping method, SWE can be coupled with LC or GC, either in an off-line [11,14, 19, 21, 46, 47, 131] or on-line [132-138] arrangement. In an off-line coupling of SWE with HPLC [131], the sorbent trap was removed from the extraction apparatus after analyte collection and then coupled to a HPLC system for analysis. Off-line superheated water extraction is simpler to perform because only the extraction step must be understood, and the extract can be analysed by any appropriate method. On the other hand, on-line SWE [132-138] requires an understanding of both the SWE and the chromatographic conditions, and the sample extract is not available for analysis by a different method.
1.3.1 SWE Coupled On-line with Conventional Chromatographic Techniques

Li *et al.* [132] demonstrated an on-line coupling of SWE with HPLC for the determination of caffeine, nitrotoluenes, PCBs, chlorophenols, and anilines in spiked samples. They used a silica-bonded C18 small column as the sorbent trap which also served as the interface between SWE and HPLC. SWE and HPLC analysis could be performed simultaneously using the on-line system. The extraction mode and HPLC mode could also be conveniently switched by using shut-off valves. Compared to the off-line coupling system [131], the on-line system was more convenient to operate. A similar approach has been used for analysing herbicides in soil. Crescenzi *et al.* [133] analysed traces of polar and medium polar contaminants in soil by coupling on-line a hot phosphate-buffered water extraction apparatus to a liquid chromatography/mass spectrometer (LC/MS) system via a C18 trap. They found that the recoveries obtained at extraction temperature of 90 °C were better than those obtained with Soxhlet extraction.

Hyotylainen and co-workers directly coupled superheated water extraction with a LC-GC system for the determination of polyaromatic hydrocarbons (PAHs) [134] and brominated flame retardants (BFRs) [135] in sediment samples. In the first approach, the extracted analytes were adsorbed into a solid-phase trap which also served as the LC column to remove most of the interfering matrix components and the fraction containing the PAHs was transferred to the GC system. Whereas in the second approach, they used a solid-phase trap to collect the extracted analytes containing the BFRs and eluted them to an LC column where they were cleaned, concentrated and fractionated before transfer to the GC system via an on-line interface. The SWE-LC-GC methods for both PAHs and BFRs proved to be sensitive, selective and repeatable. The clean-up system, including a Tenax trap and a normal-phase HPLC column, allowed the use of FID detection. Limits of
detection were improved compared to traditional methods, and in addition, the amounts sampled could be as low as 10-100 mg of sediment.

Hyotylainen and Riekkola [120] then introduced the on-line combinations of superheated water extraction (SWC) and microporous membrane liquid-liquid extraction (MMLLE) with GC. MMLLE which is used as replacement for the solid-phase trap, is a continuous process via a hydrophobic membrane. The extract obtained with MMLLE is typically cleaner than that obtained with the solid-phase trap and thus can often be transferred directly to the GC via a large-volume injection technique. This made the system simpler than the previously described SWE-LC-GC method [134, 135]. Another benefit was that lengthy drying of the solid-phase trap was avoided, leading to shorter analysis times and improved reliability for most volatile analytes. Losses of volatile compounds can occur during drying in the SWE-solid-phase trap combination. However, they discovered that the sensitivity of SWE-LC-GC was better because the solid-phase trapping was more efficient than the membrane extraction. The used of MMLLE with SWE of PAHs from sediment and soil samples was further compared with solvent trapping (liquid-liquid extraction, LLE) and SPE trapping by Luthje et al. [121], in terms of recovery, efficiency and selectivity. In their research, they claimed that solvent trapping gave the best recovery but SPE trapping was the most efficient, whilst MMLLE enhanced the selectivity relative to LLE and SPE.

Luque-Garcia and Luque de Castro [136] developed four analysis steps (SWE, filtration, pre-concentration, and separation) sequentially in a continuous method for the determination of acid herbicides in different types of soil. The subsequent steps for quantitation for the extracted analytes were assisted by a flow-injection manifold that acted as an interface between the devices where filtration, pre-concentration, individual chromatographic separation and UV detection were performed. A similar approach with the additional of post-column derivatisation and fluorescence detection was also demonstrated to extract N-methylcarbamates from different fruits and
vegetables [137]. In another work, Morales-Munoz et al. [138] also used a flow injection manifold as an interface between the extractor and fluorescence detector to allow the real time monitoring of the PAHs extracted from environmental solid samples.

Apart from the usual on-line SWE method, an on-line extraction with superheated water by using microwave has also been demonstrated by Luque-Garcia et al. [139]. This method known as microwave-assisted water extraction was also coupled to continuous filtration, pre-concentration and chromatographic separation with UV detection as reported earlier.

1.3.2 SWE Coupled with aqueous RP-LC and SWC Techniques

The results obtained by all these on-line coupling systems showed that they worked well. However, the growth of such approaches has appeared slow, as researchers prefer to use the simpler, off-line approaches to understand the fundamentals of SWE and establish simple SWE methodology for general laboratory use. The coupling of SWE to SWC can be more interesting since there will be no major involvement of organic solvent and will be therefore cheaper as well as easier to handle compared to the coupling of SWE-HPLC methods mentioned earlier. Young et al. [5] first reported the use of only water in the coupling method by interfacing SWE with WRP-LC (water-only reversed phase liquid chromatography) to separate a series of aromatic analytes. The separations were carried out using a mobile phase of 100% water at room temperature with a very low retentivity of stationary phase. Unfortunately, most of the extract could not be passed to the LC system. Bone and Smith [140] demonstrated the trapping of a dilute aqueous solution of analyte onto a SPE polymeric sorbent and its subsequent release as a concentrated solution, by using superheated water. In preliminary study [141] (detailed later as part of this thesis), we demonstrated that superheated water extraction (SWE) can be coupled directly to superheated water
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chromatography (SWC) via a solid phase trap. A small guard column packed with polymeric sorbent (polystyrene divinylbenzene) was set on-line with the extraction cell for trapping pharmaceutical compounds extracted from a spiked sand sample. The trapped analytes were thermally desorbed by superheated water and were passed to PS-DVB analytical column to be separated by temperature programmed SWC. A quite similar approach but with an off-line arrangement was subsequently reported by Lamm and Yang [142] for the analysis of anilines and phenols from sand samples. After the extraction and the trapping, the trap was removed and then connected to the SWC system.

1.4 Present Study

In most of the SWE coupling methods reported, significant volumes of organic solvent are still needed to elute the analytes from the sorbent trap and also for the separation processes. As superheated water can be used for both extraction and separation, the present studies have looked at the prospect of on-line coupling of superheated water extraction to superheated water chromatography (SWE-SWC).

The novel aspects of the proposed approach to on-line SWE-SWC are: (a) the use of water as an extractant, as the mobile phase, as well as in sample clean-up and concentration, will avoid completely the use of organic solvent at any stage, (b) the solid-phase trap which is used to collect the extracted analytes can also be used as a clean-up step for the removal of any unwanted polar materials from the extract prior to chromatographic analysis, (c) polar washing prior to SWE (cold water extraction) and non-polar washing prior to chromatographic separation (warm water extraction) can be employed in this system, and finally (d) this on-line coupling can be applied to analyse solid samples from the extraction to the chromatogram automatically, thus reducing analysis time compared to the off-line coupling.
Unlike any other on-line coupling system, only one pump is needed to pump the water through the whole coupled SWE-SWC system. The pump must be a high-pressure pump, capable of compressing the water to the required pressure. A heating oven such as a GC oven is the most appropriate way to heat the column in each step: extraction, desorption, as well as separation to the selected temperature. In some applications, temperatures above 200 °C must be used to achieve efficient extraction and this requires special extraction vessels, valves and sealing materials. Since the materials must withstand high temperatures and pressures, stainless steel is the common material employed for columns, frits and also the capillary tubing. In addition, separation at high temperatures also requires a thermally stable stationary phase.

A previous study in our laboratory [140] demonstrated that a superheated water extract could be trapped from a water carrier flow onto a cooled SPE cartridge, and then released as a concentrated solution, by using the same water flow simply by rapidly raising the temperature of the trap. In the present study, the on-line coupled SWE-SWC equipment will be constructed in the laboratory using simple switching valves and a solid-phase trap as the interface between the extractor and the chromatograph. Initially, this on-line system will be used as a method to extract analytes from a spiked sand sample and to trap the analytes onto the ambient solid-phase trap and finally to separate them directly, under superheated water conditions. Alternatively, the analytes can be released and assayed selectively by manipulating the temperatures. By increasing the release temperature in steps, less polar analytes can be analysed sequentially. The analytes will be separated by a temperature-programmed SWC. At this preliminary stage, a small HPLC guard column packed with polymeric sorbent (polystyrene divinylbenzene) similar to the packing material of PLRP-S analytical column is used as the sorbent trap and model compounds of drugs and antioxidants (paracetamol,
phenacetin, salicylamide, caffeine, methyl paraben, and ethyl paraben) are used as the analytes.

The on-line SWE-SWC method will then be applied to samples with more complicated matrix, such as compost. In order to extract triazine herbicides from a spiked compost sample, the on-line method will be further developed by the inclusion of two clean-up steps (prior to extraction and prior to separation). Two different kinds of compost samples: ericaceous compost, representing compost with high organic content (100% peat) and seed compost, with lower organic content (only up to 60% peat + sand), are chosen as the sample matrices to carry out the SWE of the triazines and directly assay by SWC. The following triazine herbicides are evaluated: atrazine, simazine, propazine, ametryn and terbutryn. The previous analysis methods for the triazines will be described in the next chapter.

The final aim of this work is to validate the on-line coupling SWE-SWC system in order to develop a quantitative analytical method for the extraction from real environmental samples of a group of typical herbicides used in agriculture. The validation of the on-line coupling system method will be assessed by the evaluation of the recoveries of triazine herbicides extracted from spiked compost samples. A comparison of the on-line system with the off-line system and traditional solvent extraction (shake-flask method) will also be carried out.
CHAPTER TWO
CHAPTER TWO

PREVIOUS ANALYSIS METHODS
FOR TRIAZINE HERBICIDES

2.0 Introduction

Herbicides are commonly used for suppressing unwanted species in crops. One family of common herbicides that have risen in popularity and usage over the last decade are the triazines [143]. They form a wide group of substances that belong among the most common agrochemicals applied for pre- and post-emergence weed control in food crops, such as corn, apple, grapes, etc. Triazine herbicides generally degrade slowly, hence, they can be detected in the environment for a long time following their application. Triazine herbicides and their metabolites or degradation products can persist for months in some soils and seasonal carry-over can cause phytotoxicity problems in crop rotation. The persistence of the triazine herbicides and their metabolites in soils, waters and plants as well as animal materials is therefore considerable, from several months for the parent compounds to many years for their degradation products, which are often more toxic.

Due to their toxicity and persistence, we need to be aware of the ecological and health hazards of their use. Most pollution with triazines occurs in environmental water and soil. Therefore monitoring for the presence of triazine herbicides has become important and their presence especially in water for human consumption must be limited. Atrazine, one of the most common triazine herbicides, is among the major pollutants of ubiquitous presence owing to its widespread use in agriculture as well as for other purposes [144]. However, despite has been banned in Italy and Germany, atrazine is still widely used around the globe [145, 146].
2.1 Structure and Characteristic of Triazine Herbicides

A great majority of triazine herbicides are derived from symmetric triazines (s-triazines), six-membered heterocycles with symmetrically located nitrogen atoms, that are substituted in positions 2, 4 and 6 (Figure 2.1) [147]. Triazines that have greater bio-activity generally contain halogenated (R1) and diamino (R2 and R3) functionalities.

The electron density resulting from the inclusion of the nitrogen in the ring and the diamino substituent groups imparts significant polarity to these compounds. The degree of polarity will change depending on the functional groups present at either the R1, R2 or R3 substitution sites. All nitrogen atoms in triazines contribute to a negative charge and therefore are able to form hydrogen bonds with the hydrogen atoms of water.

![Figure 2.1 Structure of a symmetric triazine compound](image)

The physico-chemical properties of s-triazine derivatives are primarily determined by the substituent in position 2 (R1); this is most often -Cl, -SCH₃ or -OCH₃, which divides the s-triazines into three groups: chloro-, thiomethyl- and methoxy- triazines, respectively. These s-triazines are weakly basic, poorly soluble compounds of a low polarity, stable both in the solid phase and in solution [147]. The basicity increases in order of Cl < SCH₃ < OCH₃ and also increases with an increasing number of hydrogen atoms in the
substituted amino groups and with increasing length and branching of the alkyl chain in positions 4 and 6 (R2 and R3).

2.2 Analysis of Triazines

The study and survey of the widespread distribution of triazine herbicides in the environment requires the availability of an efficient analytical method. The preparation of samples for the analysis of triazines in environmental solid samples, such as soil, sediment, or compost, is often the most time-consuming and error-prone step in their assay, due to the complexity of the sample matrices. Moreover, the concentration levels are too low to allow direct determination. The nature of the samples necessitates the use of clean-up and pre-concentration techniques to provide enriched sample extracts and eliminating matrix interferences as far as possible. At the moment, numerous research activities are aimed at developing simple sample preparation procedures that could be automated and coupled on-line with the final analytical measurement step [148, 149].

In most cases, the analysis of triazines relies on the use of liquid-liquid extraction (LLE) in which large amounts of organic solvents are consumed [150]. Nowadays, it is still common to use several hundred millilitres of solvents for the treatment of one sample and the method is therefore costly. The disposal of these solvents is sometimes difficult and incomplete. They can enter the atmosphere easily and some of them can be hazardous to the environment and the laboratory analysts.

Many new extraction techniques have appeared in the past few years, which can help to reduce the solvent consumption considerably, or can even achieve solvent-free extraction for the analysis of triazines. Among these techniques are solid-phase extraction (SPE) [148, 149, 151-155] and solid-phase microextraction (SPME) [156-159], supercritical fluid extraction (SFE)
Chapter 2

Previous Methods for Triazines

[160, 161], pressurised liquid extraction (PLE) [162, 163], microwave-assisted extraction (MAE) [164-167], and superheated water extraction (SWE) [21, 40, 41, 46, 47, 55, 132, 168].

2.2.1 Solid-Phase Extraction (SPE) and Solid-Phase Microextraction (SPME)

SPE [148, 149, 151-155] is a very important alternative to laborious and time consuming liquid-liquid extraction (LLE) for the extraction of triazines from liquid samples. Its advantages over LLE include the ability to pre-concentrate traces of analytes from very dilute aqueous solutions, decreased use of hazardous solvents, high extraction efficiency and easy automation of the procedure. On the other hand, SPME [156-159] is an alternative extraction method, which has the same benefits as SPE but is a totally solvent-free technique, that can be applied to numerous environmental samples.

Both SPE and SPME consist of two separate stages: an adsorption step (either analyte adsorption on sorbent stationary phase in the SPE method or analyte partition between the sample matrix and a polymeric stationary phase, which is coated on a fused-silica fibre in the SPME method); this is followed by a suitable desorption step. The desorption step consists of leaching with solvents for SPE, or thermal desorption in the inlet port of a gas chromatograph for SPME.

Octadecyl bonded silica (ODS) is the most commonly used sorbent for SPE. Various solvents and their mixtures have been tested for the desorption of triazines from the non-polar ODS phase [151, 154, 155]. Polystyrene-divinylbenzene resins have also been reported to be highly efficient for the pre-concentration of chloro- and thiomethyl-triazine herbicides from drinking water [149] and human urine [152] prior to reversed-phase liquid chromatography with UV diode array detection. Pichon et al. [153] compared
three different types of sorbents: C18 silica, styrene-divinylbenzene copolymer (PRP-1) and porous graphitic carbon (PGC), for the SPE of atrazine, simazine, and nine degradation products. They found that the more polar derivatives were not retained by either C18 silica or PRP-1.

Robust SPME methods enabling the simultaneously determination of a number of triazine herbicides have been developed for the analysis of water and soil samples. Determination of triazine herbicides from soil samples required the extraction of compounds from the soil prior to SPME. Several polymeric stationary phases are commercially available for coating the fused-silica fibres. Fernandez et al. [156] analysed seven selected herbicides (5 triazines, molinate and bromacil) in soil samples by applying SPME based on the use of carbowax/divinylbenzene fibre (CW-DVB). Polydimethylsiloxane (PDMS) coated fibre is most commonly used for the extraction of triazines [157] and it has been reported by Kumazawa et al. [159] that the extraction efficiency of PDMS for triazines in human body fluids was greater than the extraction efficiency of CW-DVB, polyacrylate (PA), or PDMS-DVB.

2.2.2 Supercritical Fluid Extraction (SFE)

SFE techniques [160, 161] have emerged in the past decade as an excellent tool to overcome the difficulties of solid sample extraction. SFE was distinguished from conventional techniques by the use of temperatures and pressures exceeding the critical values of the extracting phase. CO₂ is the most widely used supercritical solvent due to its high diffusity, low density and viscosity, thus allowing rapid and efficient extraction. However, the low dielectric constant of supercritical CO₂ hinders the extraction of polar and ionic analytes. On the other hand, the use of supercritical water is not very popular because it is too reactive, while other leaching agents, such as ammonia, freons or any organic solvents are not suitable, either being too expensive or environmentally suspect.
The extraction efficiency for triazines directly depends on their solubility in the supercritical fluid. Schutz et al. [160] compared the recoveries of atrazine, terbuthylazine, and the hydroxy metabolites, extracted from humic soil by supercritical CO₂ with those obtained by Soxhlet extraction. Both methods gave comparable high recoveries for atrazine and terbuthylazine. A modifier can also be mixed with the supercritical fluid to increase the triazine solubility. Jandra et al. [161] discovered that, an addition of 10% methanol as a modifier was necessary to extract atrazine and its polar hydroxy metabolites by SFE-CO₂ at a high pressure of 50 MPa.

2.2.3 Pressurised Liquid Extraction (PLE)

PLE [162, 163] is another extraction technique that has received wide attention in the past several years. PLE, known commercially as accelerated solvent extraction (ASE), is similar in approach to SFE but uses organic solvents rather than CO₂ or other supercritical fluids. PLE achieves rapid extraction with small volumes of organic solvents by using high temperatures and high pressures to maintain the solvents in liquid state. The extraction is normally performed by using the automated Dionex-ASE system.

Automated PLE with dichloromethane:acetone 65:35 v/v for the extraction of atrazine and its degradation products from soil has been reported by Hrdlicka and Dolinova [162]. The extract was cleaned-up by gel permeation chromatography and was assayed by HPLC. In another work, Guzella and Pozzoni [163] extracted triazines and chloro-acetanilides from agricultural soil, using methanol at 125 °C. The extract was assayed by GC-NPD/MS.
2.2.4 Microwave-Assisted Extraction (MAE)

In recent years, MAE [164-167] has developed into a good alternative to traditional extraction methods and has become a popular routine technique in environmental analysis due to its rapidity and its ability to handle multiple samples simultaneously. MAE uses polar organic solvents in contact with solid samples heated in a microwave oven to extract the analytes. Microwave heating is very efficient and involves two mechanisms: ionic conductance and dipolar rotation. The principle of this technique is based on absorption of microwave energy which raises the temperature (and pressure), allowing the diffusion of the compounds from the matrix to the surrounding solvent [166].

Xiong et al. [164] compared water, methanol, acetone-hexane (1:1), and dichloromethane for the extraction of triazines from soils, and indicated that water was as efficient as the organic solvents. MAE with water near its boiling point (95 – 98 °C) has been reported to extract selectively atrazine and its principal metabolites from agricultural soils [165]. It is difficult to concentrate the aqueous extract after MAE because of the high boiling point of water, therefore, Shen and Lee [166] combined MAE with SPME to determine triazine herbicides in soil samples. Water containing 1% methanol was employed as the extractant and the triazines were analysed by GC-MS.

2.2.5 Superheated Water Extraction (SWE)

Extraction using superheated water is a promising alternative to other sample pre-treatments, as water is the ideal solvent for the establishment of clean methods. The polarity, surface tension, and viscosity are greatly reduced under superheated water conditions yielding a solvent that is very similar in properties to those organic solvents, such as methanol and acetonitrile, but which is tunable with temperature [81]. The versatility and tunability of superheated water makes it an ideal solvent for the extraction of triazine
herbicides from complicated matrices, such as soil, sediment or compost [21, 40, 41, 46, 47, 55, 133, 168]. Typically, the sample was extracted under static conditions, where the water was held in the extraction cell for controlled time periods to allow sufficient contact between water and the solid sample for efficient extraction. Alternatively, dynamic or continuous flow-through techniques can be used.

The key factors in the extraction process are the triazines' solubility in the superheated water, desorption of the triazines from the matrix surface and finally, diffusion of the desorbed triazines into the superheated water. The solubilities of triazines, such as atrazine, cyanazine, and simazine, have been measured in pure and modified superheated water by Curren and King [40].

SWE has been shown by Crescenzi et al. [46] to be an efficient alternative for the extraction of multiresidue herbicides including triazines from soil. The herbicides were extracted with water at 90 °C and collected on-line by a SPE cartridge. Next, they observed that phosphate-buffered water, pH 7.5, heated at 100 °C extracted much larger amounts of terbutylazine and its metabolites from a naturally aged soil compared to water alone at the same temperature [47]. Following this, they developed an on-line coupled hot phosphate-buffered extraction with liquid chromatography / mass spectrometer (LC/MS) system to determine 13 selected pesticides including triazines from soil [133].

Konda et al. [168] employed SWE to extract six selected organic pesticides from soil, which included triazines such as atrazine and diazinone. Diazinone which is more polar than atrazine produced a lower recovery than that of atrazine because significant decomposition occurred at 105 °C in water. Static SWE along with SPE was employed by McGowin et al. [21] for the analysis of PAHs, triazines and other pesticides in municipal solid waste compost. The optimum temperature for the extraction of pesticides was lower than for PAHs so they were not extracted together in the same procedure. It was suggested that dynamic extraction would improve the SWE method.
rather than the static extraction. Richter et al. [41] employed SWE to extract pesticides (including triazines) with a broad spectrum of polarities from soil, and collected them by using solvent trapping (dichloromethane) before determination by GC-MS. SWE has also been performed on animal tissue, for the isolation of the atrazine from beef and swine kidney [55]. The inclusion of 30% ethanol (v/v) at 100 °C resulted in complete recovery of atrazine from beef kidney.

2.3 Determination of the Triazines by Conventional Chromatographic Techniques

Gas chromatography (GC) and liquid chromatography (HPLC) are the most common techniques for the determination of triazines. With GC, s-triazines can be detected with flame ionisation detection (FID) [154, 169], but a more sensitive and selective response is obtained when using the nitrogen-phosphorus detector (NPD) [170, 171], because of the presence of nitrogen atoms in the analytes. Meanwhile, GC-MS [172] has the obvious advantage of the possibility of identifying the triazines and their degradation products. Unfortunately, GC fails with compounds that are non-volatile and highly polar, such as hydroxy-s-triazines, because they decompose under the experimental conditions, and therefore, are not amenable to direct analysis, consequently derivatisation is required.

On the other hand, HPLC provides direct determination without derivatisation of both non-polar s-triazines and their polar degradation products. Reversed phases are the most often employed for the separation of s-triazines degradation products. Determination of seventeen chloro- and methylthio-triazines and their metabolites by RP-HPLC has been reported by Beilstein et al. [173]. Extensive clean-up procedures for the soil extracts are needed in order to remove matrix materials which caused high background signals in the UV detector. UV photometric detection, preferably, diode array detector, is
quite sensitive due to the ability of triazines to strongly absorb UV light [149, 152, 155, 173-175]. Amperometric detection, even though less sensitive, it is more selective for the analysis of hydroxy derivatives in complex matrices [176]. HPLC has also been coupled with MS detection in order to elucidate the structures of triazine residues [148, 160].

2.4 Determination of the Triazines by Superheated Water Chromatography (SWC)

So far there are no reports in the literature of the application of SWC in the analysis of triazine herbicides. Therefore, this study explores the potential of superheated water to extract and separate three chloro-triazines (atrazine, simazine and propazine) and two thiomethyl-triazines (ametryn and terbutryn). The chlorine atom and the thiomethyl group are positively charged, but the thiomethyl group is more positive than the chlorine atom, therefore favour weaker hydrogen bonds. Thus, the thiomethyl-triazines are less polar and hence less soluble in water compared to the chloro-triazines.

The separation of these triazines under superheated water conditions will be discussed in chapter 5, followed by the optimisation of their extraction and separation in chapter 6, and finally, the quantitative analysis of these triazines will be discussed in chapter 7.
CHAPTER THREE
CHAPTER THREE

EXPERIMENTAL

3.0 Introduction

This chapter describes the chemical/reagents, apparatus and instruments used throughout the work. It also includes the instrumentation set-up for the on-line SWE-SWC coupling system as well as the operation for this coupled system.

3.1 Chemicals and Samples

3.1.1 Deionised Water

Deionised water used throughout this work as a mobile phase and extraction solvent was purified via an Elga Maxima HPLC purification unit (Elga Ltd. Wycombe, Bucks, UK). The measured resistance of the water was 18.2 MΩ cm⁻¹.

3.1.2 Solvents

All solvents used were HPLC grade and were supplied by Fisher Scientific (Loughborough, UK)

3.1.3 Daytona Sand

Daytona sand with 40 – 100 mesh was obtained from Fisher Scientific (Loughborough, UK) and was used for spiked studies in the analysis of the
pharmaceutical compounds. The sand was cleaned using methylene chloride followed by acetone, then was dried in an oven at 100 °C for about an hour.

3.1.4 Compost

Two types of compost were used as sample matrices in the analysis of triazine herbicides. The composts were ericaceous compost and seed compost from B&Q (Loughborough, UK).

3.1.5 Pharmaceutical Compounds

Paracetamol, salicylamide, caffeine, phenacetin, methyl paraben and ethyl paraben were analytical grade supplied by Sigma-Aldrich (Gillingham, Dorset, UK). Stock solutions (1 mg/mL) of individual standards and standard mixtures were prepared by dissolving accurate amounts of pure standards in acetonitrile. Working standard solutions were obtained by further dilution of the stock solutions with deionised water.

3.1.6 Triazine Herbicides

Atrazine, simazine, propazine, ametryn and terbutryn were analytical grade obtained from Supelco, Bellefonte, USA. Stock solutions (1 mg/mL) of individual standards and standard mixtures were prepared by dissolving accurate amounts of pure standards in acetone. Working standard solutions to be used as calibration standards and spiking solutions were made up each week by further dilution of the stock solutions with deionised water. All the solutions were stored at 4 °C.
3.2 Instrumentation

3.2.1 SWE Coupled On-line with SWC

The initial design of the on-line coupling system of SWE-SWC is shown in Figure 3.1. It consisted of three parts: 1) extraction, 2) trapping and 3) separation. Figure 3.2 shows the layout and switching operation of this on-line coupling system (described later in section 3.3.2).

A Shimadzu LC-10AD HPLC pump (Shimadzu Corporation, Kyoto, Japan) was employed to pump the water through the whole system, as an extraction solvent as well as the mobile phase. The water was sonicated for 15 min and de-oxygenated constantly with nitrogen to prevent any solute oxidation or corrosion to the system. A Rheodyne 7125 injection valve with 20 µL loop was employed to inject standard solutions.

The first part was consisted of a pre-heating coil (1 m x 0.17 mm i.d. stainless steel tubing) and an extraction cell (5 cm x 4.6 mm i.d. stainless steel cell fitted with stainless steel frits, 2 µm pore size and 2 mm in thickness). Both were placed inside a GC oven (series 104, Pye Unicam, Cambridge, UK) controlled by an oven programmer. A stainless steel cooling coil (1 m x 0.17 mm i.d.) was connected at the outlet of the extraction cell and ran outside the first oven to a second oven (Packard model 437A, Illinois, USA) containing a pre-heating coil and a trapping column, a stainless steel guard column (2 cm x 2.0 mm i.d.) fitted with stainless steel frits, packed manually with 5 µm polystyrene divinylbenzene (Polymer Lab., Shropshire, UK). This exit of the trapping column lead through a second cooling coil to a third pre-heating coil and a separation column, PLRP-S column (150 x 4.6 mm i.d.) packed with 5 µm polystyrene divinylbenzene (Polymer Lab., Shropshire, UK) mounted in a third GC oven (series 104, Pye Unicam, Cambridge, UK). The final cooling coil was immersed inside an ice-water bath to cool the mobile phase before...
the Jasco model 875-UV spectrophotometric detector (Jasco, Spectroscopic Co., Ltd., Tokyo, Japan). Two Rheodyne six-port switching valves (model 7010) were employed to change flow routes (through or by-pass) for the extraction cell and the trap column. The coupling system was completed by a Jasco model 880/81 back pressure regulator (Jasco, Spectroscopic Co., Ltd., Tokyo, Japan) maintained at 35 kg cm\(^{-2}\), and a Viglen computer Pentium II with Varian Star software (Viglen Ltd., Middlesex, UK) for data analysis.
Figure 3.1: Schematic diagram of the on-line coupling system of superheated water extraction (SWE) with superheated water chromatography (SWC)
Figure 3.2: Layout and switching operation of superheated water extraction (SWE) coupled on-line with superheated water chromatography (SWC)
The on-line coupling system was further developed to cater for more complicated sample matrices, whereby extra two six-port switching valves were added between the extraction cell and the trap, and also between the trap and the separation column (Figure 3.3, described later in section 3.4.2), which allowed the water flow to be passed to waste for the clean-up steps. The real picture of this on-line SWE-SWC system is shown in Figure 3.4.
Figure 3.3: 3D-Diagram of the on-line coupling of superheated water extraction (SWE) with superheated water chromatography (SWC), with the addition of waste switching to allow for clean-up.
Figure 3.4: Picture of the on-line coupling system of superheated water extraction (SWE) with superheated water chromatography (SWC)
3.3 SWE and SWC Methods of Pharmaceutical Compounds

3.3.1 Preparation of Spiked Sand

Dried clean sand, weighing approximately 1.5 g was packed into the extraction cell and 10 µl of a sample mixture (containing 1 mg/mL of each of paracetamol, phenacetin, salicylamide, caffeine, methyl paraben and ethyl paraben) was spiked onto it.

3.3.2 Analytical Procedure of Pharmaceuticals

The on-line operation of all the three processes: extraction, desorption, and separation is explained by reference to Figure 3.2.

The extraction cell, loaded with the spiked sample was placed in oven-1. The extraction oven was first heated at 120 °C for 5 min. After 5 min, both switching valves (valve-1 and valve-2) were switched into the inject position to pass water through the extraction cell and the trap column at a flow rate of 1 mL/min. The trap column packed with 200 mg PS-DVB in oven-2 was at ambient temperature so that the extracted analytes would be trapped. Any unretained sample would elute directly into the analytical column (PLPR-S, PS-DVB) in oven-3, these were assayed immediately using a temperature program from 100 °C to 175 °C at a rate of 10 °C/min. Then, after 5 min, the extraction cell was switched out of system. When the assay of the unretained sample had been completed, the temperature of oven-3 was returned to 100 °C. Valve-2 was turned into the load position to by-pass the trap while the temperature of oven-2 was raised to 150 °C. The trapped analytes were then fully released at 150 °C by passing the water flow through the trap and direct to the analytical column. The released analytes passed to the analytical column and were focussed at 100 °C. They were immediately
chromatographed using a gradient temperature from 100 °C to 175 °C at a rate of 10 °C/min and detected by UV detection at 254 nm.

To demonstrate selective fractionation of the sample extract, the experiment was repeated but this time the trapped analytes were released by raising the temperature of oven-2 in stages. The analytes were released selectively in sequential temperatures: ambient, 70 °C, 90 °C and 110 °C. At each stage, the released fraction was focussed on the cool analytical column at 75 °C and was chromatographed by temperature programming oven-3 from 75 °C to 185 °C at a rate of 15 °C/min. During the assay, the trap was taken out of the water flow by switching valve-2 into the load position and the trap was kept at a constant temperature.

3.4 SWE and SWC Methods of Triazine Herbicides

3.4.1 Preparation of Spiked Compost

Compost was ground and sieved to a size smaller than 1 mm. 10 g of the compost was then spiked at a desired volume of a sample mixture (containing 1 mg/mL of each atrazine, simazine, propazine, ametryn and terbutryn) and was thoroughly wetted with approximately 30 mL of acetone. The slurries were mixed and shaken for a few minutes to homogenise them and air-dried overnight at room temperature to allow evaporation of the solvent.

3.4.2 Analytical Procedure of Triazines

Approximately 0.5 g portion of sieved spiked ericaceous compost or 1.0 g portion of sieved spiked seed compost, was placed into a stainless steel extraction cell. 2 μm stainless steel frits were located at both inlet and outlet of the extraction cell. The cell was put inside an extraction unit oven and a
1.0 m x 0.17 mm i.d. stainless steel pre-heating coil was connected to its inlet port.

The analytical procedure can be divided into four steps and described by reference to Figure 3.3.

1. **Cold extraction**
   The sample was first extracted at ambient temperature by flowing the water through the extraction cell at a flow rate of 2 mL/min for about 10 min. Valve-1 (extraction valve) and valve-2 were switched on so that the extracted polar compounds were sent into a waste beaker (cold wash).

2. **Superheated water extraction**
   The water flow was stopped by switching off valve-1 while the extraction oven was heated at 170 °C. The water was then passed through the extraction cell at 1 mL/min. Valve-2 was now switched off and valve-3 (trapping valve) was switched on to let the water pass through the trap column (50 x 4.6 mm i.d. unbonded X-Terra column), which was at ambient temperature. At the same time, valve-4 was also switched on so that any untrapped components were sent into another waste beaker. After 5 min, the extraction oven was switched out of the water flow and cooled by opening its door and returning its temperature to ambient.

3. **Clean-up and pre-concentration**
   When the extraction and trapping was complete, valve-1 was switched off and the water flow by-passed the extraction cell. The trap oven was heated up to 60 °C and held for 10 min so that any highly polar compounds were released out to waste by the warm water (warm wash).

4. **Superheated water chromatographic separation**
   After the warm water wash, valve-4 was switched off so that the flow from the trap passed to the analytical column (100 x 2.1 mm i.d. Hypercarb...
The temperature of the trap column was then increased up to 200 °C and the water flow was passed through the trap to fully release the triazine compounds. After 3 min, valve-3 was switched off so that the water flow by-passed the trap and directly passed to the analytical column. The released triazines were focussed on the analytical column at 170 °C and were assayed immediately using a temperature gradient from 170 °C to 250 °C, at 10 °C/min and detected at 222 nm.

3.5 Solvent Extraction Method of Triazines

Approximately 5.0 g of spiked compost was placed inside a centrifuge tube and 15 mL of acetone was added. The mixture was shaken vigorously for 5 min and sonicated for 30 min. The suspension was then centrifuged at 3000 rpm for 10 min. The clear supernatant solvent was separated by filtration and evaporated in a water bath at 50 °C. Finally, the dry residue was reconstituted in 0.5 mL of acetonitrile. 10 μL of the solution was assayed by direct injection onto the analytical column and the triazines were separated using the same temperature gradient as in section 3.4.2.
Early studies [132-138] demonstrated that superheated water extraction (SWE) systems could be coupled directly to assay procedures. However, these methods still used a large amount of organic solvents to elute the analytes from the trap and as the mobile phase in the assay. Young and co-workers [5] were the first researchers to use water on its own in both extraction and separation methods, by interfacing SWE with WRP-LC (water-only reversed phase liquid chromatography). However, most of the extract was not transferred to the WRP-LC system. More recently, Lamm and Yang [142] reported the off-line coupling system of SWE and SWC.

The aim of the present study was to set up a separation using superheated water linked directly to an extraction with a trapping column. To demonstrate the capability of the on-line system, six pharmaceutical compounds: paracetamol, phenacetin, salicylamide, caffeine, methyl paraben, and ethyl paraben were used as model compounds. A preliminary report of this work has been published [141].

4.1 Establishment of SWC Separation Conditions for the Pharmaceutical Compounds

Most previous separations using superheated water have examined either polar or low molecular weight compounds [84-102]. A series of simple pharmaceuticals: paracetamol, phenacetin, salicylamide, caffeine, methyl
paraben and ethyl paraben, was therefore selected as the model compounds in this study. Initially, aspirin was also included, but because it tended to hydrolyse very rapidly at high temperature, it was not used further. In earlier studies, Wilson [95] reported SWC separations on different types of stationary phase of a range of drugs which included paracetamol, phenacetin and caffeine, whilst the SWC separation of the alkyl parabens was demonstrated by Smith and co-workers [85, 86] on a polystyrene divinylbenzene (PS-DVB) column.

Column durability has been a major limitation on the development of superheated water chromatography (SWC). In this study, two types of column were tested: a polystyrene divinylbenzene (PS-DVB) column which is thermally stable but not very efficient, and an amide C16 column with good efficiency but which has never been previously reported for use at elevated temperatures though it is stable in 100% ambient water.

The chromatograph was first set up for the superheated water assay. The flow rate was set constantly at 1 mL/min. 10 μL of a 1 mg/mL sample mixture of the pharmaceutical compounds was injected at different isothermal temperatures and with temperature programming, which involved a gradual raising of the temperature of the entire column during the run. Temperature programming is applied to imitate gradient elution of RP-HPLC in order to get better separation. As temperature can affect retention, selectivity, and peak shape, the separations at isothermal temperatures were compared with the separations using temperature gradients in terms of the efficiency and the resolution. In order to ensure that there was a temperature equilibrium established between the stationary phase and the water mobile phase, a 10 min equilibration was always used after the desired oven temperature was reached, before injection.
Chapter 4 Qualitative Analysis of Pharmaceuticals

4.1.1 Polystyrene Divinylbenzene (PS-DVB), PLRP-S Column

PLRP-S is the trade name of a polymeric reversed phase packing based on polystyrene divinylbenzene (PS-DVB) units. It is hydrophobic and hydrolytically stable from pH range 1 to 14 [177]. PS-DVB columns are very retentive towards polar compounds and often show lower efficiencies than octadecylsilane (ODS) columns, particularly with methanol as a room temperature eluent [178]. The lower column efficiency may be due to a slow intra-particle sorption rate, specifically slow diffusion of solute molecules within the polymer matrix. Despite of this limitation, PS-DVB column was still selected in this study because early work in SWC [85, 86] had shown that it is stable up to 200 °C. The decision to use a PS-DVB column was further influenced by the fact that most of the available conventional ODS bonded stationary phases might be prone to degradation above temperatures of 120 °C [86].

At 170 °C (Figure 4.1A), the separation of all the six model compounds (paracetamol, phenacetin, salicylamide, caffeine, methyl paraben and ethyl paraben) was obtained in 26 min with good resolution but not as efficiently or as rapidly as with gradient temperature from 60 °C to 185 °C at 15 °C/min (Figure 4.1B). By separating the compounds using a temperature gradient, the efficiency was much improved with narrower peaks and less peak tailing (Table 4.1). However, the baseline drift increased due to the high rate of ramping temperature. From these results, it is evident that temperature programming can give better results compared to isothermal temperature conditions.
Figure 4.1 Chromatograms showing the separation of the pharmaceuticals/model compounds on PS-DVB (150 x 4.6 mm) column at (A) temperature 170 °C, and (B) temperature gradient 60 °C – 185 °C, 15 °C/min. Experimental conditions: mobile phase, 100% water; flow rate, 1.0 mL/min; detection, 254 nm; Peaks: 1. paracetamol, 2. salicylamide, 3. caffeine, 4. methyl paraben, 5. phenacetin, 6. ethyl paraben.
Table 4.1 Comparison of chromatographic parameters for the isothermal separation at 170 °C and with temperature gradient, 60 °C – 185 °C, 15 °C/min, on a PS-DVB column (conditions as in Figure 4.1).

<table>
<thead>
<tr>
<th>PHARMACEUTICAL COMPOUNDS</th>
<th>ISOTHERMAL TEMPERATURE</th>
<th>( t_R ) (min)</th>
<th>( w_{1/2} ) (sec)</th>
<th>( N )</th>
<th>( k )</th>
<th>( R_s )</th>
<th>Tailing (5.0%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Paracetamol</td>
<td>170 °C</td>
<td>2.69</td>
<td>11.09</td>
<td>1172</td>
<td>0.79</td>
<td>-</td>
<td>2.06</td>
</tr>
<tr>
<td>2. Salicylamide</td>
<td></td>
<td>5.29</td>
<td>17.59</td>
<td>1804</td>
<td>2.52</td>
<td>6.4</td>
<td>1.67</td>
</tr>
<tr>
<td>3. Caffeine</td>
<td></td>
<td>7.39</td>
<td>30.85</td>
<td>1145</td>
<td>3.93</td>
<td>3.1</td>
<td>1.86</td>
</tr>
<tr>
<td>4. Methyl paraben</td>
<td></td>
<td>11.23</td>
<td>28.95</td>
<td>3004</td>
<td>6.49</td>
<td>4.5</td>
<td>1.64</td>
</tr>
<tr>
<td>5. Phenacetin</td>
<td></td>
<td>22.38</td>
<td>59.79</td>
<td>2797</td>
<td>13.92</td>
<td>8.9</td>
<td>1.35</td>
</tr>
<tr>
<td>6. Ethyl paraben</td>
<td></td>
<td>26.30</td>
<td>65.80</td>
<td>3190</td>
<td>16.53</td>
<td>2.2</td>
<td>1.39</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PHARMACEUTICAL COMPOUNDS</th>
<th>GRADIENT TEMPERATURE 60 °C – 185 °C, 15 °C/min</th>
<th>( t_R ) (min)</th>
<th>( w_{1/2} ) (sec)</th>
<th>( N )</th>
<th>( k )</th>
<th>( R_s )</th>
<th>Tailing (5.0%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Paracetamol</td>
<td></td>
<td>8.05</td>
<td>17.06</td>
<td>4449</td>
<td>4.37</td>
<td>-</td>
<td>1.35</td>
</tr>
<tr>
<td>2. Salicylamide</td>
<td></td>
<td>11.58</td>
<td>15.95</td>
<td>10523</td>
<td>6.72</td>
<td>7.5</td>
<td>1.15</td>
</tr>
<tr>
<td>3. Caffeine</td>
<td></td>
<td>12.98</td>
<td>22.20</td>
<td>6828</td>
<td>7.66</td>
<td>2.6</td>
<td>1.31</td>
</tr>
<tr>
<td>4. Methyl paraben</td>
<td></td>
<td>15.38</td>
<td>18.17</td>
<td>14311</td>
<td>9.26</td>
<td>4.2</td>
<td>1.10</td>
</tr>
<tr>
<td>5. Phenacetin</td>
<td></td>
<td>17.22</td>
<td>25.73</td>
<td>12521</td>
<td>12.59</td>
<td>8.0</td>
<td>1.08</td>
</tr>
<tr>
<td>6. Ethyl paraben</td>
<td></td>
<td>21.91</td>
<td>29.12</td>
<td>11301</td>
<td>13.61</td>
<td>2.0</td>
<td>1.10</td>
</tr>
</tbody>
</table>

Key: \( t_R \), retention time (min); \( w_{1/2} \), width at half height; \( N \), theoretical plates (efficiency); \( k \), retention factor; \( R_s \), resolution.
4.1.2 Discovery Amide C16 Column

In the last few years, embedded polar phases have become increasingly popular, particularly for highly aqueous eluents. These include the Discovery C16 Amide phase, which incorporates an amide linkage at the base of a C16 bonded phase [179]. By placing this polar functional group deep in the bonded phase, unique selectivity is generated. Recent reports [6, 179] have shown that the embedded polar phases provide stable and reproducible analyte retention times even in 100% aqueous mobile phases when C18 phases collapse. Because of these special characteristics of the amide column, they were also investigated in this study.

The separations obtained at 130 °C (Figure 4.2A) and with a temperature gradient from 70 °C to 160 °C at 10 °C/min rate (Figure 4.2B) resulted in good efficiency and resolution with symmetrical peaks (Table 4.2). However, after a few assays, the performance of the column started to deteriorate. In order to investigate this problem, the inlet part of the column was opened and a huge gap in the packing material was observed. The life time of the Amide column at elevated temperature (>100 °C) was probably shortened because the superheated water dissolved the silica base of the column. With dissolution of the silica base, active silanols were created and the stationary phase was washed off by the mobile phase. From these results, although the Amide column was a highly efficient column, it was less thermally stable than the PS-DVB column. Therefore, it is not suitable for extended superheated water chromatography.
Figure 4.2 Chromatograms showing the separation of the pharmaceuticals on Amide C16 column at (A) temperature 130 °C, and (B) temperature gradient 70 °C – 160 °C, 10 °C/min. Experimental conditions: mobile phase, 100% water; flow rate, 1.0 mL/min; detection, 254 nm; Peaks: 1. paracetamol, 2. salicylamide, 3. caffeine, 4. methyl paraben, 5. phenacetin, 6. ethyl paraben.
Table 4.2 Comparison of chromatographic parameters for the isothermal separation at 130 °C and with temperature gradient, 70 °C – 160 °C, 10 °C/min, on an Amide C16 column (conditions as Figure 4.2).

<table>
<thead>
<tr>
<th>PHARMACEUTICAL COMPOUNDS</th>
<th>ISOTHERMAL TEMPERATURE 130 °C</th>
<th>GRADIENT TEMPERATURE 70 °C – 160 °C, 10 °C/min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$t_R$ (min)</td>
<td>$W_{1/2}$ (sec)</td>
</tr>
<tr>
<td>1. Paracetamol</td>
<td>3.31</td>
<td>6.39</td>
</tr>
<tr>
<td>2. Salicylamide</td>
<td>5.12</td>
<td>8.84</td>
</tr>
<tr>
<td>3. Caffeine</td>
<td>7.30</td>
<td>8.31</td>
</tr>
<tr>
<td>4. Methyl paraben</td>
<td>11.11</td>
<td>14.46</td>
</tr>
<tr>
<td>5. Phenacetin</td>
<td>15.21</td>
<td>19.99</td>
</tr>
<tr>
<td>6. Ethyl paraben</td>
<td>23.27</td>
<td>35.19</td>
</tr>
</tbody>
</table>

Key: $t_R$, retention time (min); $W_{1/2}$, width at half height; $N$, theoretical plates (efficiency); $k$, retention factor; $R_s$, resolution.
4.2 Sorbent trap Coupled On-line with Superheated Water Chromatography (SWC)

The use of a sorbent trap to trap analytes from aqueous solutions has been widely employed in off-line and on-line arrangements with HPLC. For example, superheated water extract was trapped on a solid phase extraction (SPE) cartridge [47] and the analytes were then eluted for the assay with an organic solvent. In addition, in previous work in this laboratory [140], it was shown that analytes could be trapped from an aqueous solution onto a SPE cartridge and then could be released as a concentrated solution simply by raising the temperature using the same water flow, thus avoided completely the use of organic solvents. The present work takes the next stage so that the trapping can be coupled directly to the SWC system. The first step was to determine whether a good focussing of the analytes could be achieved in the trap. Therefore, a sample was directly injected into the trap column at ambient temperature, then the trapped analytes were released by thermal desorption and finally assayed by SWC.

Compatibility should also occur between the sorbents in the trap column and in the analytical column. In theory, the retention of the analyte on the trap should be ideally similar to, or lower than that on the analytical column for a perfect coupling so that the sample is eluted from the trap and focussed on the analytical column [180]. Since the assay of the pharmaceutical compounds with SWC was successful on a PS-DVB column, a PS-DVB packing was also used in the trap in this study. Figure 4.3 shows a block diagram of the first stage of the coupling method.
Initially only one switching valve was used to either direct the water flow into the trap or to by-pass the trap and go straight to the analytical column. 1.0 metre pre-heating coils were used in the system to ensure thermal equilibration between the incoming mobile phase and the columns, so that unacceptable peak broadening can be avoided. The pre-heating coil was therefore connected to the inlet of each column and was placed together with the column in the oven. Earlier work in this laboratory [84] had shown that 1.0 metre length of the capillary stainless steel tubing was adequate as the pre-heating coil. 1.0 metre stainless steel cooling coils were then used to cool the eluent after each heated processes i.e., desorption and separation. The cooling coil was thus connected to the outlet of each column and was placed outside of the oven. The elevated and potentially variable temperature of the eluent emerging from the analytical column prior to UV detection might affect the light path through the detection flow cell and result in an unstable baseline. Hence, the cooling coil before the UV detector was immersed inside
an ice-water bath to maintain the stability of the baseline. The back-pressure regulator was used to prevent the liquid water from boiling above its normal boiling point when temperatures above 100 °C were employed.

10 µL of a 1 mg/mL standard solution of the pharmaceutical compounds was injected into the trap at ambient temperature. Any sample which was not retained passed directly to the analytical column which was programmed at temperature gradient of 100 – 175 °C, 10 °C/min and the assay started immediately. During this run, the switching valve was turned off so that the water would by-pass the trap. After the assay of the unretained analytes had been completed, the temperature of the analytical column was reduced back to 100 °C. The trap oven temperature was then raised up to 150 °C to release the trapped analytes. The released analytes were then passed to the analytical column and were assayed using the same temperature gradient.

Figure 4.4 shows the chromatogram of the pharmaceutical compounds, not trapped at ambient temperature (Figure 4.4A) and released from the trap at 150 °C (Figure 4.4B). With the trap at ambient temperature, only one peak was observed and was identified as paracetamol which could not be trapped by the trap column due to its high solubility in water. For each of the compounds, the octanol-water partition coefficients (K_{ow}) which have been correlated to water solubility are given in Table 4.3 as log P. In an attempt to trap the paracetamol, the trap column was immersed in ice water, but the paracetamol was still eluted from the trap column. On the other hand, the compounds which were trapped and then released at 150 °C, included all the remaining analytes as in Figure 4.4B.
Chapter 4  
Qualitative Analysis of Pharmaceuticals

Table 4.3: Octanol-water partition coefficient (log P) for the pharmaceuticals (data taken from on-line version of SRC’s Log Kow)

<table>
<thead>
<tr>
<th>PHARMACEUTICAL COMPOUNDS</th>
<th>FORMULA</th>
<th>MOLECULAR MASS</th>
<th>log P (Log Kow)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Paracetamol</td>
<td>C₈H₉NO₂</td>
<td>151.2</td>
<td>0.46</td>
</tr>
<tr>
<td>2. Salicylamide</td>
<td>C₇H₇NO₂</td>
<td>137.1</td>
<td>1.28</td>
</tr>
<tr>
<td>3. Caffeine</td>
<td>C₈H₁₀N₄O₂</td>
<td>196.2</td>
<td>-0.07</td>
</tr>
<tr>
<td>4. Methyl paraben</td>
<td>C₉H₁₆O₃</td>
<td>152.2</td>
<td>1.96</td>
</tr>
<tr>
<td>5. Phenacetin</td>
<td>C₁₀H₁₃NO₂</td>
<td>179.2</td>
<td>1.58</td>
</tr>
<tr>
<td>6. Ethyl paraben</td>
<td>C₉H₁₀O₃</td>
<td>166.2</td>
<td>2.47</td>
</tr>
</tbody>
</table>

Figure 4.4  
Chromatograms showing the separation at temperature gradient 100 °C – 175 °C, 10 °C/min of the pharmaceuticals (A) not trapped at ambient temperature and (B) trapped and then released at 150 °C. Experimental conditions: mobile phase, 100% water; flow rate, 1.0 mL/min; detection, 254 nm; Peaks: 1. paracetamol, 2. salicylamide, 3. caffeine, 4. methyl paraben, 5. phenacetin, 6. ethyl paraben
4.3 SWE Coupled On-line with SWC via Solid-phase Trap (SWE-trap-SWC)

The first step shows that it was feasible to couple the solid-phase trap column on-line with SWC system and a good focussing of all the analytes, except paracetamol was achieved in the trap. Therefore, the coupling method was further developed by linking a SWE system directly to the SWC system, whilst the solid-phase trap served as the interface between the two systems as well as to collect and pre-concentrate the sample extracts. At this stage, two switching valves were needed for the operation of the extraction cell and the trap column, so that the water flow can pass through or by-pass the extraction cell and/or the trap column. Figure 4.5 shows a block diagram of the SWE system coupled directly to the SWC system with the trap as the interface. Detailed figures of the on-line coupling system are shown in chapter 3.

Figure 4.5 Superheated water extraction (SWE) coupled directly to the superheated water chromatography (SWC) via a trap column.
1. water pump  2. injector  3. UV detector  4. back-pressure regulator
EC: extraction cell   TC: PS-DVB trap column  AC: PS-DVB analytical column  V1, V2: switching valves-1 & 2  P1, P2, P3: pre-heating coils
C1, C2, C3: cooling coils
Approximately 1.5 g of clean sea sand was loaded inside the extraction cell. 10 μL of a 1 mg/mL of the sample mixture was then spiked onto the sand and the extraction cell was sealed carefully to ensure no leakage would occur when it was pressurized. When the extraction cell had been placed inside the oven, the temperature was raised to 120 °C. After 5 min, water at a flow rate of 1 mL/min was passed through the cell straight into the trap column (at ambient temperature) and finally into the analytical column (programmed at temperature gradient of 100 °C – 175 °C, 10 °C/min). After another 5 min, switching valve-1 was then turned off to switch the water flow to by-pass the extraction cell. The extracted analytes were trapped by the sorbent trap at ambient temperature and any unretained sample was eluted from the trap into the analytical column and assayed immediately. After the assay of the unretained sample had been completed, the water flow was stopped and the analytical column was cooled to 100 °C. In order to release all the trapped analytes simultaneously, the trap oven was then heated at 150 °C and water was passed through the trap and directly into the analytical column so that all the released analytes were assayed immediately using a temperature gradient of 100 °C – 175 °C, at 10 °C/min. Switching valve-2 was then turned off during the assay so that the water flow by-passed the trap when it was cooled down.

Figure 4.6 shows the chromatograms of the pharmaceutical compounds obtained by direct injection and compared with those obtained by on-line coupling method. As anticipated, paracetamol was lost (Figure 4.6C) because it was not retained in the sorbent trap even by ambient water during the SWE process, as evidenced in Figure 4.6B. The retention time of the analytes obtained by on-line SWE-SWC was slightly shorter due to a faster mass transfer. This probably because the sample extract released by thermal desorption was actually introduced into the analytical column at higher temperature mobile phase compared to ambient temperature by direct injection.
Figure 4.6 Chromatograms showing the separation of the pharmaceuticals at temperature gradient 100 °C – 175 °C, 10 °C/min via (A) direct injection, (B) not trapped at ambient temperature and (C) trapped and released at 150 °C. Experimental conditions: mobile phase, 100% water; flow rate, 1.0 mL/min; detection, 254 nm; Peaks: 1. paracetamol, 2. salicylamide, 3. caffeine, 4. methyl paraben, 5. phenacetin, 6. ethyl paraben
Slight peak broadening (< 6%) was observed for the first three peaks in the on-line method (Table 4.4). This might be attributed to the additional dead volume introduced into the system by both switching valves 1 & 2 (prior to the extraction cell and prior to the trap column) and associated connection tubings, or lack of focusing when the extract reached the analytical column.

Table 4.4  Comparison of peak width at half height ($w_h$) before (direct injection) and after the coupling of SWE-SWC (conditions as Figure 4.6).

<table>
<thead>
<tr>
<th>PHARMACEUTICAL COMPOUNDS</th>
<th>DIRECT INJECTION $W_h$ (sec)</th>
<th>ON-LINE SWE-SWC $W_h$ (sec)</th>
<th>% PEAK BROADENING</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Paracetamol</td>
<td>42.34</td>
<td>-</td>
<td>5.6</td>
</tr>
<tr>
<td>7. Salicylamide</td>
<td>22.62</td>
<td>23.88</td>
<td></td>
</tr>
<tr>
<td>8. Caffeine</td>
<td>36.98</td>
<td>37.54</td>
<td>1.5</td>
</tr>
<tr>
<td>9. Methyl paraben</td>
<td>27.39</td>
<td>28.80</td>
<td>5.1</td>
</tr>
<tr>
<td>10. Phenacetin</td>
<td>49.88</td>
<td>47.38</td>
<td>-</td>
</tr>
<tr>
<td>11. Ethyl paraben</td>
<td>53.30</td>
<td>52.65</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 4.5 gives a comparison of both direct injection and on-line SWE-SWC methods (Figure 4.6) relative to five chromatographic parameters: retention time ($t_r$), efficiency ($N$), retention factor ($k$), selectivity ($\alpha$), and resolution ($R_s$). Compared to the direct injection, the on-line method sustained only a small amount of loss in each of the parameters; 10.9% loss in efficiency, 5.0% loss in retention factor, 1.2% loss in selectivity, and 11.1% loss in resolution.
Table 4.5 Comparison of chromatographic parameters for the separation via direct injection and via on-line coupling of SWE-SWC, assayed at temperature gradient, 100 °C – 175 °C, 10 °C/min (conditions as Figure 4.6).

<table>
<thead>
<tr>
<th>PHARMACEUTICAL COMPOUNDS</th>
<th>DIRECT INJECTION (Figure 4.6A)</th>
<th>ON-LINE SWE-SWC (Figure 4.6C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$t_R$ (min)</td>
<td>$N$</td>
</tr>
<tr>
<td>1. Paracetamol</td>
<td>7.75</td>
<td>669</td>
</tr>
<tr>
<td>2. Salicylamide</td>
<td>17.06</td>
<td>11356</td>
</tr>
<tr>
<td>3. Caffeine</td>
<td>19.06</td>
<td>5305</td>
</tr>
<tr>
<td>4. Methyl paraben</td>
<td>23.42</td>
<td>14589</td>
</tr>
<tr>
<td>5. Phenacetin</td>
<td>31.44</td>
<td>8444</td>
</tr>
<tr>
<td>6. Ethyl paraben</td>
<td>35.65</td>
<td>8930</td>
</tr>
</tbody>
</table>

Key: $t_R$, retention time (min); $N$, theoretical plates (efficiency); $k$, retention factor; $\alpha$, selectivity; $R_s$, resolution.
In order to improve the efficiency in less retention time, the inlet needs to be cooled to focus the analytes and a faster thermal gradient is then needed to elute them. Figure 4.7 shows the chromatogram of the extracted sample assayed at temperature gradient from 60 °C to 185 °C, at a rate of 15 °C/min. The efficiency was much improved with good selectivity and resolution (Table 4.6).

![Chromatograms showing the separation of the extracted analytes at temperature gradient 60 °C – 185 °C, 15 °C/min, (A) not trapped at ambient temperature and (B) trapped on PS-DVB trap column and released at 150 °C. Experimental conditions: mobile phase, 100% water; flow rate, 1.0 mL/min; detection, 220 nm; Peaks: 1. paracetamol, 2. salicylamide, 3. caffeine, 4. methyl paraben, 5. phenacetin, 6. ethyl paraben]
Table 4.6 Chromatographic parameters of the separation using a temperature gradient, 60 °C – 185 °C, at 15 °C/min (conditions as Figure 4.7)

<table>
<thead>
<tr>
<th>PHARMACEUTICAL COMPOUNDS</th>
<th>( t_R ) (min)</th>
<th>( N ) (theoretical plates)</th>
<th>( k )</th>
<th>( \alpha )</th>
<th>( R_s )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Paracetamol</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2. Salicylamide</td>
<td>12.09</td>
<td>22501</td>
<td>7.06</td>
<td>1.14</td>
<td>3.5</td>
</tr>
<tr>
<td>3. Caffeine</td>
<td>13.54</td>
<td>11538</td>
<td>8.03</td>
<td>1.20</td>
<td>4.9</td>
</tr>
<tr>
<td>4. Methyl paraben</td>
<td>15.93</td>
<td>18542</td>
<td>9.62</td>
<td>1.36</td>
<td>8.2</td>
</tr>
<tr>
<td>5. Phenacetin</td>
<td>21.09</td>
<td>11287</td>
<td>13.06</td>
<td>1.08</td>
<td>5.0</td>
</tr>
<tr>
<td>6. Ethyl paraben</td>
<td>22.69</td>
<td>12560</td>
<td>14.13</td>
<td>2.0</td>
<td></td>
</tr>
</tbody>
</table>

Key: \( t_R \), retention time (min); \( N \), theoretical plates (efficiency); \( k \), retention factor; \( \alpha \), selectivity; \( R_s \), resolution.

4.4 Selective Fractionation

The elution power of water can be tuned via temperature whereby the polarity of water can be decreased by sequentially raising the extraction temperature [9]. Hence, the fractionation of the extracted compounds according to their polarity was possible. It should be possible to perform selective fractionation by trapping all the extracted analytes and releasing them sequentially by increasing the temperatures in stages.

In order to carry out the selective fractionation, the experiment in section 4.3 was repeated but this time the thermal desorption of the trapped analytes was performed in a set of incrementally increasing temperatures. At each thermal desorption step, the eluted sample was assayed by a thermal gradient of the
analytical column from 75 °C to 185 °C at a rate of 15 °C/min. During each thermal desorption step, only switching valve-2 was turned on, and during each assay, both switching valves 1 & 2 were turned off so that the water flow by-passed both extraction and trap columns. Each release temperature yielded a separate fraction of the retained analytes (Figure 4.8B-E) and can be compared with the chromatogram obtained by direct injection at the same thermal gradient (Figure 4.8A). As before, paracetamol was not retained by the trap even at ambient temperature (Figure 4.8B). When the temperature was increased to 70 °C, salicylamide and caffeine were completely released in less than 15 min, and additional thermal desorption time up to 30 min yielded no increase in removals (Figure 4.8C). Further temperature increment up to 90 °C released most of methyl paraben (Figure 4.8D), while the remainder was released together with phenacetin and ethyl paraben at 110 °C (Figure 4.8E).
Figure 4.8: Separations on PS-DVB column at temperature gradient from 75°C to 185°C, 15 °C/min via (A) direct injection, and sequential elution of the trapped analytes at different temperatures: (B) ambient temperature, (C) 70 °C, (D) 90 °C and (E) 110 °C. Experimental conditions: mobile phase, 100% water; flow rate, 1.0 mL/min; detection, 254 nm; Peaks: 1. paracetamol, 2. salicylamide, 3. caffeine, 4. methyl paraben, 5. phenacetin, 6. ethyl paraben
4.5 Summary

An affordable, environmentally friendly on-line coupling system using superheated water eluent has been successfully demonstrated by directly coupling superheated water extraction (SWE) with superheated water chromatography (SWC) via a solid phase trap as the interface. The initial stage of this on-line coupling system involves the qualitative analysis of six selected pharmaceutical compounds (paracetamol, salicylamide, caffeine, phenacetin, methyl paraben, and ethyl paraben). A spiked study which extract a standard solution of these compounds from sand was performed. The information gained from this model system can then be applied to the extraction of a real environmental sample.

The chromatogram resulted by the on-line coupling method shows that only a small percentage of peak broadening occurred to some of the peaks, and therefore only a small percentage of loss in efficiency. However, the loss did not affect the separation and this is compensated by the high sensitivity of the on-line method because all the extracted analytes were transferred into the analytical column.

Selective fractionation was successfully demonstrated by this on-line coupling method when sequential elution of the trapped analytes was performed in a series of increasing temperature and the eluted analytes were separated by superheated water chromatography with gradient temperature.

Preliminary results seem very promising and further work in the next chapter will concentrate on a more complicated real sample matrix. This will give more accurate information about the efficiency of the extraction and separation by using the on-line SWE-SWC method. Clean-up steps may need to be included when using real sample due to matrix interferences.
CHAPTER FIVE
CHAPTER FIVE

DETERMINATION OF TRIAZINE HERBICIDES BY SUPERHEATED WATER CHROMATOGRAPHY (SWC)

5.0 Introduction

As superheated water has been used successfully in the previous chapter for the separation of model compounds, such as drugs and anti-oxidants, it was decided to examine the superheated water separation of the triazine herbicides with an aim of eventually examining SWE of the triazines from spiked samples in a coupled system. During the past 25 years, much emphasis was placed on chromatographic methods for the determination of triazine herbicides, mainly gas chromatography (GC) [154, 169-172] and liquid chromatography (HPLC) [149, 152, 155, 173-175]. However, so far SWC has never been tried, despite superheated water being shown to be feasible as the mobile phase for reversed-phase separation of many polar and relatively non-polar compounds.

The present study examines the efficiency and the selectivity of the separation of five selected triazine herbicides (Figure 5.1); using different types of columns under superheated water conditions. The triazine compounds which include three chloro- and two thiomethyl-triazines used in the analysis are listed below:

<table>
<thead>
<tr>
<th>Compounds</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Simazine</td>
<td>Cl</td>
<td>NHCH₂H₅</td>
<td>NHC₂H₅</td>
</tr>
<tr>
<td>2. Atrazine</td>
<td>Cl</td>
<td>NHCH₂H₅</td>
<td>NHCH(CH₃)₂</td>
</tr>
<tr>
<td>3. Propazine</td>
<td>Cl</td>
<td>NHCH(CH₃)₂</td>
<td>NHCH(CH₃)₂</td>
</tr>
<tr>
<td>4. Ametryn</td>
<td>SCH₃</td>
<td>NHCH₂H₅</td>
<td>NHCH(CH₃)₂</td>
</tr>
<tr>
<td>5. Terbutryn</td>
<td>SCH₃</td>
<td>NHCH₂H₅</td>
<td>NHC(CH₃)₃</td>
</tr>
</tbody>
</table>
Chapter 5

Determination of Triazines by SWC

Figure 5.1 Structures of the triazine herbicides.

(Ameyrn)

b.p.: 345 °C
m.p.: 88 °C

(Simazine)

b.p.: 307.5 °C
m.p.: 226 °C

(Terbutryn)

b.p.: 349.1 °C
m.p.: 104 °C

(Atrazine)

b.p.: 313 °C
m.p.: 173 °C

(Propazine)

b.p.: 318.5 °C
m.p.: 213 °C

(Key: b.p., boiling point; m.p., melting point)
Chapter 5  
Determination of Triazines by SWC

5.1 Selection of the Analytical Column

In order to obtain a good separation of the five selected triazines with superheated water as the mobile phase, an appropriate column that is stable at high temperature is required. Since the triazines studied are structurally related, the required column must also be highly selective. Polymer, silica and carbon columns were investigated in a search for the best conditions.

5.1.1 Polystyrene Divinylbenzene (PS-DVB) Column

Polystyrene divinylbenzene (PS-DVB) columns have been employed in many SWC studies due to their thermal stability [85-89, 93-95, 98]. In chapter 4, the polystyrene divinylbenzene column has been used successfully for the separation of pharmaceutical compounds under superheated water conditions. Polystyrene divinylbenzene packing has been used in solid phase extraction for the analysis of triazine herbicides [151, 152], however, to our knowledge, its usage in the separation of the triazines by conventional HPLC has not been previously reported. Therefore, a study to separate all the five triazines with SWC was carried out using the PS-DVB column.

The PS-DVB column used to separate the triazine herbicides was the PLPR-S column with 5 μm particles. The polymeric stationary phases are very retentive since they generate additional interactions with the π electrons of the analyte and therefore tend to require high temperature for elution [81]. Figure 5.2 shows the separation of two homologous triazine herbicides: atrazine and simazine at 190 °C. Both peaks were tailed severely and the separation was less efficient than that reported earlier for phenols [85-87]. The peak tailing was caused by the particularly strong electronic (n-n) interactions between the polar groups on the analytes and the polymer surface. In order to improve the peak shape, higher temperatures were employed but the separations still failed to give good peak shapes. Due to these poor results, no further separation of the triazine compounds was carried out with this column type.
Chapter 5

Determination of Triazines by SWC

Figure 5.2  A chromatogram showing the separation of 2 triazines (25 ppm) on a PS-DVB column (150 x 4.6 mm i.d.) at 190 °C. Experimental conditions: mobile phase, 100% water; flow rate, 1.0 mL/min; detection, 254 nm; Peaks: 1. simazine, 2. atrazine.

5.1.2 Discovery Amide C16 Column

In conventional HPLC, silica based columns have been widely used for the separation of the triazine herbicides [149, 151, 154, 155, 160, 165, 174, 176]. These columns are popular because they offer excellent performance in terms of efficiency and their usage is well understood. However, most silica based columns are thermally unstable and therefore not suitable for superheated water separations. Despite this, Discovery Amide C16 column was tested for the separation of the triazines because of its unique selectivity and reported suitability in 100% water eluents [6, 179]. Moreover, the Amide C16 column has been used previously for the SWC separation of pharmaceutical compounds (see chapter 4) and produced excellent efficiency and selectivity. In conventional HPLC, the Amide C16 column provided excellent resolution, peak shape and reproducibility for the determination of triazine herbicides [181].
Figure 5.3A shows that the SWC separation of the five triazine herbicides at 150 °C, was, however, not very successful with only moderate efficiency and poor resolution between peaks 3 and 4. However, the separation was better than the separation using the PLRP-S column (Figure 5.2) and it seemed promising. Unfortunately, when the temperature was increased to 160 °C (Figure 5.3B), the retention times were decreased and the last three peaks became seriously distorted. When the inlet part of the column was opened, a huge gap in the packing was observed indicating that the stationary phase had dissolved in the superheated water mobile phase. Hence, due to the instability of this column at temperatures higher than 100 °C, no further separation could be done.

![Chromatograms showing the separation of 5 triazines (25 ppm) on a Discovery Amide C16 (150 x 4.6 mm i.d.) column at temperature (A) 150 °C and subsequently (B) 160 °C. Experimental conditions: mobile phase, 100% water; flow rate, 1.0 mL/min; detection, 254 nm; Peaks: 1. simazine, 2. atrazine, 3. ametryn, 4. propazine, 5. terbutryn.](image)
5.1.3 X-Terra Column

In the past few years, there have been many attempts to find a silica-based column that would be more stable to hydrolytic attack at high temperatures and high/low pH. One of them is the hybrid silica, X-Terra column, which has proven to be stable at high temperature up to 165 °C [95]. The X-Terra phase represents a silica bonding chemistry in which the monomers and polymerization process are controlled to create a particle with the right organic/inorganic balance [182]. As a consequence, the stationary phase exhibits fast and highly efficient separations with high stability. An X-Terra C18 column has been successfully used in SWC for the separation of drugs [95] and ginger extracts [108] but it has not been reported for the analysis of the triazine herbicides, even in conventional HPLC. Normally, X-Terra columns have a C18 bonded phase which can make them highly retentive. However, the unbonded X-Terra material contains numerous Si-Me groups and thus could act as a weakly retentive C1 phase which should be ideal for SWC.

Hence, the third column selected in this work was an unbonded X-Terra column with 3.5 μm particles. The back-pressure on the 3.5 μm column was higher than 5 μm column of the same dimension, therefore a shorter column was used. As expected, the X-Terra was the least retentive of the columns tested and therefore, separation of the triazine herbicides at 160 °C (Figure 5.4) resulted in a very fast analysis time and thus, poor resolution. Since X-Terra is a silica-based column, lower temperature than 160 °C should be still adequate to disrupt the Van der Waals interactions and prolong the analysis time in an attempt to get better resolution. However, the attempt to separate them at lower temperature failed due to a serious peak broadening and poor resolution.
5.1.4 Retention Behaviour of the Triazines on Silica-based Columns

Since Amide C16 and un bonded X-Terra columns are both silica-based columns, the triazines were separated in the same retention order. In comparison with simazine, atrazine contains a more hydrophobic substituent, isopropyl instead of ethyl, on the nitrogen atom in position 2 (Figure 5.1), and thus the retention order on both columns is based on this difference in hydrophobicity. Apart from this, the two columns could not provide enough resolution due to their limited selectivity toward polar analytes. The chromatograms (Figure 5.3A & 5.4) show that peak 3 (ametryn) and peak 4 (propazine) overlapped. Therefore, in order to resolve these two compounds, a more selective and thermally stable column should be employed.

Figure 5.4  A chromatogram showing the separation of 5 triazines (25 ppm) on an un bonded X-Terra (50 x 4.6 mm i.d.) column at 160 °C. Experimental conditions: mobile phase, 100% water; flow rate, 1.0 mL/min; detection, 254 nm; Peaks: 1. simazine, 2. atrazine, 3. ametryn, 4. propazine, 5. terbutryn.
5.1.5 PGC Hypercarb Column

In earlier work, carbon-based columns have been used to separate the triazine herbicides. For example, Mao and Carr [183] succeeded in selectively separating ten triazine herbicides at 60 °C by using a carbon-coated zirconia column with 30:70 v/v acetonitrile:water as the mobile phase. A porous graphitic carbon (PGC) column has also been used to confirm a separation of selected triazine herbicides in a conventional HPLC [153]. A previous study demonstrated that the PGC stationary phase is stable under superheated water conditions over a long period of time and temperatures as high as 220 °C [84]. A wide range of compounds, included anilines [88], alcohols [84, 108], drugs [95] and carbohydrates [93], has been successfully separated on a PGC column using superheated water as the mobile phase.

Therefore, we selected a Hypercarb PGC column with 5 μm particles for this study. All the five triazines were separated under superheated water conditions and good separation was finally obtained in less than 10 min using a temperature gradient (Figure 5.5) with good efficiency and selectivity (Table 5.1). In the next chapter, we will examine the optimisation of the separation of the triazines on the PGC column.
Figure 5.5 A chromatogram showing the separation of 5 triazines (25 ppm) on PGC (100 x 2.1 mm i.d) column with temperature gradient 160 °C to 260 °C, 15 °C/min. Experimental conditions: mobile phase, 100% water; flow rate, 1.0 mL/min; detection, 254 nm; Peaks: 1. propazine, 2. atrazine, 3. simazine, 4. ametryn, 5. terbutryn.

Table 5.1: Chromatographic parameters obtained from the separation of triazine herbicides on a PGC column at temperature gradient from 160 °C to 260 °C, 15 °C/min (based on Figure 5.5).

<table>
<thead>
<tr>
<th>TRIAZINE COMPOUNDS</th>
<th>CHROMATOGRAPHIC PARAMETERS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$t_R$</td>
</tr>
<tr>
<td>1. Propazine</td>
<td>6.48</td>
</tr>
<tr>
<td>2. Atrazine</td>
<td>6.87</td>
</tr>
<tr>
<td>3. Simazine</td>
<td>7.26</td>
</tr>
<tr>
<td>4. Ametryn</td>
<td>8.75</td>
</tr>
<tr>
<td>5. Terbutryn</td>
<td>9.31</td>
</tr>
</tbody>
</table>

Key: $t_R$, retention time (min); $N$, theoretical plates (efficiency); $k$, retention factor; $\alpha$, selectivity; $R_s$, resolution.
5.1.6 Retention Behaviour of the Triazines on the PGC Column

The triazines may be more retained on the PGC phase compared to the silica base columns because PGC is reported to be much more hydrophobic [184]. In addition, there is a significant electronic ($\pi$-$\pi$) interaction between the polar groups on the triazine and the carbon surface. The closer the triazine’s polar group can get to the carbon surface, the stronger are the $\pi$-$\pi$ interactions. Both hydrophobic and electronic interactions contribute to the strong retention of triazines on the PGC column.

Apart from its thermal stability, the PGC column is also efficient in resolving all five triazines due to its high selectivity towards structurally-related compounds. The flat homogeneous surface of PGC is responsible for its unique selectivity to geometrical isomers and the resolution of closely related peaks [184]. The carbon phases are considered locally flat and thus provide an adsorption-like retention mechanism. The selectivity of PGC is completely different to silica or polymeric phases [185]. Retention is determined by the strength of interaction with analytes and the surface of the PGC, specifically the molecular area of the analyte in contact with the PGC surface. On PGC, retention often increases as the polarity of the analyte increases. This effect is described as “polar retention effect on graphite” or PREG [186].

Kriz et al. [187] reported that PGC column shows significantly increased discrimination for compounds based on the number of methyl substituents present compared with silica-based column. In this study, the retention selectivity of hydrophobic components was demonstrated by the separation of the homologue series of triazines with the same functional group, Cl, but with alkyl groups, in increasing order, i.e. propazine > atrazine > simazine. The structure of simazine (Figure 5.1) shows that the molecule is rather flat. Addition of the substituted methyl group close to the triazine ring interferes with the planar centre of the molecule. This inhibits the polar groups on the molecule from getting close to the carbon surface, thus decreasing the $\pi$-$\pi$
interaction which leads to the decrease in retention. Furthermore, the more planar molecule was more retained because it can be more easily accommodated to the flat surface of the PGC. This is the reason why propazine was eluted first followed by atrazine, then, simazine (Figure 5.5), unlike the hydrophobic separation on the silica-based columns [183]. Figure 5.6 shows that on the PGC column, the retention (log k) in the homologue series decreases with the addition of a methyl group. This also means that retention on PGC decreases with an increase in the solute’s hydrophobicity. In contrast, on a silica-based column (Figure 5.7 from reference [183]), the retention increases linearly upon addition of a methyl group, which is a typical characteristic of hydrophobic selectivity in the reversed phase mode.

![Figure 5.6 A plot of log k for a triazine homologue series (chloro-triazines) versus the number of carbon atoms in alkyl groups on PGC column. Compounds: 1. simazine, 2. atrazine, 3. propazine.](image-url)
Figure 5.7 A plot of log k for a triazine homologue series (chlorotriazines) versus the number of carbon atoms in alkyl groups on ODS column. Compounds: 1. simazine, 2. atrazine, 3. propazine (Data taken from ref. [183]).

5.2 Summary

In this study, the Hypercarb PGC was found to be the most suitable column to separate the triazine compounds in superheated water condition due to its stability at very high temperatures and also its high selectivity towards structurally-related compounds. Both the silica-based columns, Amide C16 and X-Terra, were not selected further because of their limited selectivity, which caused the peaks of ametryn and propazine to overlap. Moreover, the Amide C16 column is not stable at high temperature though it appeared to have a better selectivity than the X-Terra column. The triazines were more retained on the PGC column as a result of both hydrophobic and electronic (π-π) interactions. The retention behaviour on this column also differed from
silica-based column due to its unique retention mechanism. The optimisation of the separation will be discussed in the next chapter.
CHAPTER SIX

ON-LINE COUPLING OF SWE AND SWC OF TRIAZINE HERBICIDES

6.0 Introduction

Once the SWC method had been developed for the assay, the study examined the coupling of the separation to the extraction and the optimisation of the overall assay. In the development of the on-line superheated water extraction-chromatography (SWE-SWC) method, the SWE, the trapping and pre-concentration, and the SWC separation was optimised separately, then the final optimisation was carried out with the whole on-line coupled system. Preliminary experiments in chapter 4 using the on-line SWE-SWC method, were aimed at separating six pharmaceutical compounds (paracetamol, salicylamide, caffeine, phenacetin, methyl paraben and ethyl paraben) extracted from spiked sand. We demonstrated that the on-line coupling system worked well. In the present work, we applied the similar coupling system with the inclusion of two clean-up steps to determine five triazine herbicides (simazine, atrazine, propazine, ametryn and terbutryn) extracted from a model matrix of spiked compost.

The order used for optimising the steps was as follows: first, the superheated water chromatographic (SWC) separation of the triazines using UV detection was optimised; then the variables affecting the pre-concentration and desorption steps were studied; and finally the superheated water extraction (SWE) itself was optimised in detail, together with the clean-up steps. After the optimisation of each individual step had been completed, the work focussed on the application of the whole on-line SWE-SWC system.
6.1 Optimisation of Superheated Water Chromatography (SWC)

In a search for the best conditions for the SWC separation of all five triazine herbicides, Hypercarb PGC was selected as the analytical column as explained in chapter 5. In order to obtain an appropriate separation of the triazines with the PGC column, the experimental variables such as temperature and flow rate of the mobile phase, as well as the detection wavelength were optimised. 25 μg/mL samples of a standard mixture solution of the five triazine compounds were injected directly onto a Hypercarb PGC (porous graphitic carbon) analytical column and were separated at selected elevated temperatures. An injection volume of 10 μL was preferred to obtain a quantifiable absorbance signal.

Figure 6.1 shows a block diagram of the SWC system. A 1.0 metre stainless steel pre-heating coil was connected to the inlet of the column and was placed together with the column in the oven to ensure thermal equilibration between the incoming mobile phase and the column. Since the UV detector could not tolerate high temperature, the hot eluent coming from the column outlet was cooled by a 1.0 metre exit tubing and it was then wrapped with a set of copper cooling fins. The cooling fins normally could disperse the heat well enough to cool down the eluent, in order to maintain the stability of the baseline. The back-pressure regulator was used to maintain the pressure of the mobile phase in the SWC system.

Figure 6.1  Superheated water chromatography (SWC) system.
1. water pump  2. injector  3. UV detector  4. back-pressure regulator
AC: PGC analytical column  P1: pre-heating coil  C1: cooling coil
6.1.1 Temperature of Mobile Phase

Different isothermal temperatures and temperature gradients were employed to achieve a good separation with high efficiency in a reasonable analysis time. The separation of the triazines isothermally at a temperature of 245 °C (Figure 6.2A) was compared with the separation using a temperature gradient from 160 to 260 °C at 15 °C/min (Figure 6.2B), both at the same detection wavelength, 254 nm.

Isothermal temperatures less than 200 °C were insufficient to separate all the five triazines since the PGC column is very retentive. At 245 °C, (Figure 6.2A), the separation was accomplished in less than 5 min, however, the first three triazines (the chloro-triazines) were incompletely resolved. On the other hand, separation with a temperature gradient, (Figure 6.2B), gave a better efficiency and resolution compared to the isothermal temperature. The analysis time can be reduced by increasing the initial temperature but this affects the resolution of the first three peaks. Hence, in our work, all the assays with superheated water employed a temperature gradient with different ranges and rates depending on the performance of the PGC column. Initially, the temperature gradient employed was from 160 °C to 260 °C, at 15 °C/min but the resolution was not very good. Therefore, the ramping rate was reduced to 10 °C/min but the initial temperature was increased to 170 °C in order to keep the analysis time short. However, if the PGC column showed signs of deterioration, the temperature gradient was altered slightly to maintain the resolution.
Figure 6.2 Chromatograms showing the separation of triazines on Hypercarb PGC (100 x 2.1 mm i.d) column at (A) 245 °C and (B) 160 °C to 260 °C, 15 °C/min. Experimental conditions: mobile phase, 100% water; flow rate, 1.0 mL/min; detection, 254 nm; Peaks: 1. propazine, 2. atrazine, 3. simazine, 4. ametryn, 5. terbutryn.
6.1.2 Flow Rate of Mobile Phase

The influence of the flow rate of the mobile phase was studied over a range of 0.3 mL/min to 2.0 mL/min for the 2.1 mm i.d. PGC column. Since the PGC column is very retentive, lower flow rates such as 0.3, 0.4 or 0.5 mL/min were not appropriate due to the long analysis times and may also cause peak broadening. Whereas very high flow rates were also not advisable because a high back-pressure developed within this narrow bore column. The best separation was obtained at a flow rate of 1 to 1.3 mL/min, which is equivalent to 3.0 mL/min for a conventional, 4.6 mm i.d. column. These high flow rates were possible because elevated temperatures reduce the viscosity of the mobile phase [81].

6.1.3 Wavelength of Detection

Most triazines exhibit absorption maxima in aqueous solutions around 220 to 225 nm and/or 255 nm, while their hydroxy derivatives absorb at lower wavelengths, ~215 nm [188]. The more polar triazines, such as the chloro-triazines tend to absorb more at the lower wavelengths. Hence, the initial detection wavelength of 254 nm was changed to 222 nm in order to obtain higher sensitivities for the chloro-triazine compounds. Figure 6.3 shows that the peak areas for the three chloro-triazines increased (compared to Figure 6.2B) while maintaining the peak areas of the thiomethyl-triazines. Therefore, 222 nm was selected as the detection wavelength in future work.
Figure 6.3  A chromatogram showing the separation of triazines on PGC (100 x 2.1 mm i.d) column at temperature gradient from 160 °C to 260 °C, 15 °C/min. Experimental conditions: mobile phase, 100% water; flow rate, 1.0 mL/min; detection, 222 nm; Peaks: 1. propazine, 2. atrazine, 3. simazine, 4. ametryn, 5. terbutryn.

6.2 Selection of the Sorbent Trap Material

The key to combining SWE with SWC is the interface between the two techniques. A trap column was therefore placed before the analytical column (Figure 6.4). 10 µL of 25 µg/mL of a standard solution of the triazines mixture was injected onto the trap column at ambient temperature and released by heating, then collected on the analytical column for 3 min and separated as described previously in section 6.1.
Hence, the main issue in this step of the on-line coupling method is the packing material in the trap. Firstly, sorbent trap materials that can retain the triazine compounds were investigated as a method for the enrichment from a dilute aqueous extracted solution (pre-concentration) and simultaneous elimination of matrix interferences (clean-up).

A few types of sorbent were tested for the solid phase trap: Hypersil ODS (octadecylsilyl silica), unbound X-Terra silica, and Hypercarb PGC (porous graphitic carbon). When the sample was injected onto the trap at ambient temperature, it was found that all the triazines were retained in all of these traps at ambient temperature with no breakthrough. Even though all the three sorbent materials were capable of trapping and retaining the triazines at ambient temperature, the main problem was to find the right temperature to release the trapped triazines from the sorbents and transfer them into the SWC system. From the separation process (see section 6.1), it was found that the thiomethyl-triazines (ametryn and terbutryn) had longer retentions and
thus required higher temperature to thermally desorb them from the trap, which might damage the sorbent material in repeated use. It is therefore necessary to ensure the thermal stability of the sorbent trap material during the thermal desorption of the analytes.

As the PGC column had proved to be thermally stable in superheated water conditions [84], PGC sorbent was therefore first tested to be used as the trap material. When PGC column was used as the analytical column in the assay, an isothermal temperature of 245 °C (see Figure 6.2A) was required to separate all the five triazines due to its strong retentivity. Therefore, temperatures higher than 200 °C were expected to fully release all the five triazines from the PGC trap. Hence, 250 °C was tested as the release temperature. Figure 6.5 shows the chromatogram of the triazines released at 250 °C from the PGC trap.

![Chromatogram](image)

Figure 6.5 A chromatogram showing the separation of triazines released from PGC trap at 250 °C, separated on PGC (100 x 2.1 mm i.d) analytical column with gradient temperature from 130 °C to 220 °C, 15 °C/min. Experimental conditions: mobile phase, 100% water; flow rate, 1.3 mL/min; detection, 222 nm; Peaks: 1. propazine, 2. atrazine, 3. simazine, 4. ametryn, 5. terbutryn.

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Unfortunately at this high temperature, the chloro-triazines tended to decompose, especially atrazine and simazine. The degradation products appeared to be more polar than the triazine compounds, thus they were released and separated earlier than the triazines. It has been reported [153] that generally the degradation of the triazines depends on several factors, such as hydrolysis, photolysis and microbial activity. The degradation rates decreased in the order methoxy- < chloro- < thiomethyl-triazines. In this work, the effects of high temperatures and humic media in the sample matrix appear to lead primarily to dealkylation reactions in position 4 and 6, thus converted the chloro-triazines to the dealkylated metabolites. Figure 6.6 [189] shows the potential dealkylation reactions of atrazine, simazine, and propazine to the dealkylated metabolites: de-ethylatrazine (DEA), de-isopropylatrazine (DIA), and dide-alkylatrazine (DDA).
Figure 6.6: Reported dealkylation reactions of atrazine, simazine, and propazine to de-ethylatrazine (DEA), de-isopropylatrazine (DIA), and dide-alkylatrazine (DDA) [189].
Another possible pathway for the degradation is the dechlorination reaction, reported by Pacakova [147], in which hydrolysis of triazines in strongly acidic and basic solutions, especially at elevated temperatures, formed hydroxy derivatives. Since the chloro-triazines have been found to be thermally unstable compared to the thiomethyl-triazines [21], they could be degrading through hydrolysis of the substituent (Cl) in position 2, under superheated water conditions. According to Cai et al. [190], chemical hydrolysis is considered to be the predominant degradation pathway for atrazine into the environment. In addition, oxidation reactions caused by the existing dissolved oxygen in the system might also degrade the chloro-triazines. Although the water used as the extractant and the mobile phase had been sonicated and was de-oxygenated constantly with nitrogen, it was impossible to remove all the dissolved oxygen from the system. Hence, oxidation reactions still could occur to the chloro-triazines even though it should not be very serious. **Figure 6.7** [191] shows the potential hydrolysis/dechlorination and oxidation reactions of atrazine, as the other two possible degradation pathways.

![Figure 6.7: Reported dechlorination and oxidation reactions of atrazine [191]](image-url)
To overcome or to minimise the degradation problem, because silica based sorbents such as Hypersil ODS, are less retentive than PGC, we anticipated that the trapped triazines could be released at a lower temperature. **Figure 6.8** shows the chromatogram of the triazines released from Hypersil ODS trap at 150 °C. Unfortunately, the last triazine compound, terbutryn was still not released at this temperature and yet decomposition of the chloro-triazines still occurred. It was not appropriate to release the triazines at higher temperature because Hypersil ODS is not normally stable at temperatures higher than 80 °C [86]. Therefore no further desorption studies were carried out with Hypersil ODS trap.

![Chromatogram](image)

**Figure 6.8** A chromatogram showing the separation of triazines released from ODS trap at 150 °C, separated on PGC (100 x 2.1 mm i.d) analytical column with gradient temperature from 130 °C to 220 °C, 15 °C/min. Experimental conditions: mobile phase, 100% water; flow rate, 1.3 mL/min; detection, 222 nm; Peaks: 1. propazine, 2. atrazine, 3. simazine, 4. ametryn.
Another alternative was to use a thermally stable hybrid silica, such as unbonded X-Terra silica column, which is thermally stable up to 165 °C [95] and is less retentive than the PGC. A small 50 mm X-Terra column was used as the solid phase trap and it proved to be suitable, without any significant degradation of the analytes or of the sorbent material itself. All the triazines were released and no visible degradation peaks were observed in the chromatogram. Even though the desorption temperature, 200 °C was quite high for X-Terra column, it was only heated for 3 min before it was cooled to ambient temperature (see later in section 6.3). Therefore, the column could survive in this condition for a long time despite its reported stability up to only 165 °C and it could be used repeatedly.

6.3 Optimisation of the Pre-concentration and Desorption Steps

Once a suitable sorbent trap had been selected, the conditions of trapping and thermal desorption were then optimised. In order to optimise the pre-concentration and the desorption steps on the unbonded X-Terra sorbent trap, a few important variables with respect to the desorption process were studied, such as mode, temperature, flow rate and period of desorption.

6.3.1 Confirmation of Trapping Efficiency

10 μL of a 25 μg/mL standard solution of the triazines mixture was injected into the X-Terra trap column, which was coupled on-line to the SWC system (Figure 6.4). The analytes were trapped at ambient temperature, while the water was eluted from the trap and passed to the PGC analytical column. The PGC column was then chromatographed immediately using a temperature gradient from 160 °C to 260 °C, at a rate of 15 °C/min. No analyte peaks were observed in the chromatogram (see Figure 6.11A), except a solvent peak, demonstrating that all the triazines were fully trapped.
The temperature of the PGC analytical column was then returned to 160 °C, and the trapped triazines were released by thermal desorption and chromatographed immediately with the same temperature programming.

### 6.3.2 Desorption Mode

Two operational modes were tested to carry out the desorption with superheated water: (a) dynamic desorption, in which the analytes were removed in a moving stream of water mobile phase at a fixed desorption temperature; and (b) static desorption, in which the water flow was stopped and the trap was heated to a fixed desorption temperature for a specific period of time, then the water flow was started to remove the analytes from the sorbent.

Initially, a static mode combined with a dynamic mode was employed. Once the temperature of the trap oven was raised up to the selected desorption temperature, it was maintained for five minutes without any flow. The water flow was then flushed through the trap to elute the released triazines. However with this combination mode, even at temperatures less than 200 °C, all the three chloro-triazines; simazine, atrazine and propazine decomposed into more polar degradation compounds. Therefore, only the dynamic mode was used for the desorption, whereby the trap oven was first heated up ballistically to the selected desorption temperature without any water flow. As soon as the desorption temperature was reached, the water flow was started to release the trapped triazines from the sorbent trap and elute the released triazines into the analytical column, to be assayed immediately using a temperature gradient. No significant decomposition was observed to any of the triazines with this dynamic mode. This probably works because in dynamic mode, the chloro-triazines were washed from the heated zone immediately at the desorption temperature and therefore, they were not exposed to this high temperature for sufficient time to cause the degradation.
6.3.3 Desorption Temperature

The most challenging part of the study was to find the most suitable temperature that would release the trapped analytes from the sorbent trap without any degradation of the analytes or of the sorbent material. Temperature is the most significant parameter because it is expected to have a greater effect than any other parameter on the solubility and mass transfer in the thermal desorption process [192].

Once the triazines had been trapped by the X-Terra column at ambient temperature, the trap oven was heated up to the selected temperature. The water flow was then passed through the trap to release the trapped analytes and they were assayed immediately with a temperature gradient from 160 °C to 260 °C at 15 °C/min. When 180 °C was employed as the desorption temperature with the dynamic mode, only two triazines: atrazine and simazine were released (Figure 6.9A), while the other three triazines: propazine, ametryn and terbutryn, were subsequently released at 200 °C (Figure 6.9B).
Figure 6.9 Chromatograms showing the separation of the triazines released from X-Terra trap in dynamic mode at (A) 180 °C and (B) 200 °C, separated on PGC (100 x 2.1 mm i.d) analytical column with gradient temperature from 160 °C to 260 °C, 15 °C/min. Experimental conditions: mobile phase, 100% water; flow rate, 1.0 mL/min; detection, 222 nm; Peaks: 1. propazine, 2. atrazine, 3. simazine, 4. ametryn, 5. terbutryn.
The longer retention of propazine on the X-Terra trap, but its faster elution from the PGC column, probably reflects differences in the retention mechanism on these two media. This effect is in contrast to the previous pharmaceuticals study (see chapter 4), in which there was a sequential release on increasing desorption temperature which was matched by the elution order on a similar column material.

In order to release all the five triazines simultaneously, a minimum temperature of 200 °C (Figure 6.11B) must be employed, because even at 190 °C, the last triazine, terbutryn failed to be released (Figure 6.10). Dynamic desorption was used at this high temperature to avoid any decomposition. It was confirmed by comparison to the direct injection chromatogram (Figure 6.11C). To determine whether there are any analytes remaining in the trap, hot water at 250 °C was flushed through the trap straight to the assay immediately after the run. The resulting chromatogram showed no significant analyte peaks, demonstrating that the thermal desorption at 200 °C was complete.

![Figure 6.10 Chromatograms showing the separation of the triazines released from X-Terra trap in dynamic mode at 190 °C, separated on PGC (100 x 2.1 mm i.d) analytical column with gradient temperature from 160 °C to 260 °C, 15 °C/min. Experimental conditions: mobile phase, 100% water; flow rate, 1.0 mL/min; detection, 222 nm; Peaks: 1. propazine, 2. atrazine, 3. simazine, 4. ametryn.](image-url)
Figure 6.11  Chromatograms showing the separation of the triazines released from X-Terra trap at (A) ambient temperature, (B) 200 °C and (C) direct injection, separated on a PGC (100 x 2.1 mm i.d) analytical column with gradient temperature from 160 °C to 260 °C, 15 °C/min. 

Experimental conditions: mobile phase, 100% water; flow rate, 1.3 mL/min; detection, 222 nm; 
Peaks: 1. propazine, 2. atrazine, 3. simazine, 4. ametryn, 5. terbutryn.
6.3.4 Desorption Period

The effect of the desorption period, that is the period during which the sorbent is flushed by the mobile phase at the maximum temperature was also studied. Peak areas might increase with increasing desorption period, indicating that the desorption of analytes from the sorbent was a slow process.

We determined that 3 min was sufficient to release all the triazines at 200 °C with good separation though longer periods probably will not affect the separation. However, we did not want to heat the X-Terra column at 200 °C for a longer time because this will shorten its life-time.

The sequence therefore, was that the trap oven was heated ballistically from ambient temperature to 200 °C in approximately 2.0 min without any water flow, then the water flow was switched on to release the trapped triazines. The released triazines were passed to the analytical column which was programmed at 160 °C to 260 °C, 15 °C/min and the assay was started immediately. After 3 min, the trap was switched out of the water flow and it was cooled by opening the trap oven door and programming the oven temperature back to ambient.

6.3.5 Desorption Flow Rate

Band broadening and peak tailing might be observed if the flow rate during the desorption flow rate was too high. However, in our work, the desorption flow rate was fixed at the same flow rate of the separation. In this coupled system, desorption and separation were run simultaneously, therefore the same flow rate was used for both processes. The flow rate was already optimised earlier during the chromatographic separation, in a range of 1 to 1.3 mL/min.
6.4 Superheated Water Extraction (SWE)

Once the trap and assay had been optimised, they were followed by the extraction process. An extraction cell was therefore placed before the trap as in Figure 6.12.

![Diagram of extraction setup]

Figure 6.12 Superheated water extraction (SWE) coupled directly to the superheated water chromatography (SWC) via a trap column.
1. water pump  2. Injector  3. UV detector  4. back-pressure regulator

EC: extraction cell  TC: X-Terra trap column  AC: PGC analytical column  V1, V2: switching valves-1 & 2  P1, P2, P3: pre-heating coils  C1, C2, C3: cooling coils

The SWE of the triazines was carried out using two types of sample matrices: ericaceous and seed compost. The ericaceous compost consists of 100% peat material whereas the seed compost consists of a mixture of peat (up to 60%) with ground limestone and sand. These composts were selected as typical soil like matrices which contain organic matter and therefore might be more realistic than sand, which is often used in extraction studies elsewhere.

The compost was spiked with 20 µg/g of the five triazines mixture. 0.5 g of spiked ericaceous compost and 0.1 g of spiked seed compost were used for
the extractions. The extraction from compost would give more accurate information about the extraction efficiency of the SWE method since real adsorption of the analytes to the active sites on the matrix could have occurred.

Various sample properties such as the nature of the matrix, porosity, and size, may affect the extraction rate [192]. Extraction rate increases with decreasing particle size and thus with increasing sample surface area. Pre-treatment of the sample/matrix, such as grinding and sieving, is important. Therefore, the sample of compost was ground and sieved to < 1 mm in size before spiking. 10 g of sieved compost was spiked with 200 μL of a 1000 mg/L of stock triazines solution and thoroughly wet with 30 mL of acetone. The sample was then air-dried overnight at ambient temperature to evaporate the acetone, giving a dried concentration of 20 μg/g of each triazine.

6.4.1 Optimisation of the Clean-up Steps

The initial design of the SWE-SWC coupling system for the spiked sand study in chapter 4, did not include any provision for clean-up steps. However, when real samples, such as compost, were used in the extraction, pre-treatments before the extraction and before the assay were necessary to assist the assay process. The raw extract also needed to be cleaned-up before it was transferred to the trap or assay in SWC system due to the huge amounts of interferences found from the sample matrix, especially from the ericaceous compost. Moreover, the PGC analytical column particularly tended to adsorb any active compounds onto the surface which affected its efficiency. Therefore, it was vital to eliminate the matrix interferences as completely as possible during the extraction. Hence, the on-line coupling system was further developed (Figure 6.13 & 6.15), whereby additional valves were included to allow two clean-up steps using water to be included.
6.4.1.1 Polar Wash prior to Thermal Extraction

In order to get rid of any polar matrix components, which might shorten the life time of the trap column and disturb the assay, a cold water extraction at ambient temperature was employed before performing the extraction of the triazines at an elevated temperature. Ambient water was passed through the compost in the extraction cell for 10 min at a flow rate of 2 mL/min and the flow was directed to waste through switching valve-3 (V3), placed immediately after the extraction trap (Figure 6.13). This cold extraction is therefore termed as a polar wash. None of this wash was passed to the trap or the analytical column. The polar wash can be employed at a higher flow rate and for longer time than the actual extraction because the ambient water was shown not to extract any of the triazine compounds from the compost.

Figure 6.13  SWE coupled directly to the SWC with the inclusion of polar wash.

1. water pump;  2. Injector;  3. UV detector;  4. back-pressure regulator;  EC: extraction cell;  TC: X-Terra trap column;  AC: PGC analytical column;  V1, V2, V3: switching valves-1, 2 & 3;  P1, P2, P3: pre-heating coils;  C1, C2, C3: cooling coils.
After 10 min, the water flow was stopped and the extraction oven was heated at 170 °C. The water was then passed again through the extraction cell and this time the extract was passed directly to the ambient trap column by switching on valves 1 and 3 (V1 & V3). After 5 min, the extraction oven was switched out of the water flow and cooled by opening its door and returning its temperature to ambient. The trapped analytes were then released by heating the trap oven at 200 °C and chromatographed on the PGC column immediately from 160 °C to 260 °C, at 15 °C/min (Figure 6.14). When only a polar wash at ambient temperature was employed, it was clearly insufficient to remove the less polar interference peaks from the sample, which caused difficulties in the determination of the analyte peaks. It was therefore decided to examine a sequential series of extractions of the trap at increasing temperature.

Figure 6.14 A chromatogram showing the separation of total extract of trap from spiked ericaceous compost (after polar wash). Experimental conditions: analytical column: PGC (100 x 2.1 mm i.d) with temperature gradient from 160 °C to 260 °C at 15 °C/min, mobile phase, 100% water; flow rate, 1.3 mL/min; detection, 222 nm.
6.4.1.2 Non-polar Wash prior to Separation

In this on-line coupled SWE-SWC method, an ambient sorbent trap was used to trap the analytes from the dilute aqueous extract. This trap could also be used in the clean-up process. The idea was to remove the major part of the remaining interfering compounds from the extract by utilising a warm or hot water wash of the trap that would remove the interferents but leave the triazines. The hot water extraction of the compost yields abundant amounts of humic acids from the compost, as evidenced by the yellow-brown colouring of the extraction liquid. Therefore, switching valve-4 (V4) was placed after the trap so that the extract from the trap can be passed to waste or passed directly to the analytical column (Figure 6.15).

Figure 6.15 Superheated water extraction (SWE) coupled directly to the superheated water chromatography (SWC) with the inclusion of polar wash and non-polar wash.

- 1. water pump;
- 2. Injector;
- 3. UV detector;
- 4. back-pressure regulator;
- EC: extraction cell;
- TC: X-Terra trap column;
- AC: PGC analytical column;
- V1, V2, V3, V4: switching valves-1, 2, 3 & 4;
- P1, P2, P3: pre-heating coils;
- C1, C2, C3: cooling coils.
It was important to optimise the temperature for this wash. Too high a temperature in this process might degrade the most polar analytes, and too low a temperature would not help in reducing the remaining interferences. In studies of the thermal desorption of the triazines in section 6.3, we observed that at temperatures up to 100 °C, none of the triazines were released from the trap. Therefore, a non-polar wash could be employed at any elevated temperatures up to 100 °C. Hence, a non-polar wash of the trap was employed at 60 °C for 10 min and the effluent was passed to waste. It was thought to be not advisable to wash for a much longer time at the elevated temperature because the chloro-triazines might be partially eluted during the washing, resulted in a lower recovery. Since the flow rate during the clean-up process was independent from any of those desorption or separation processes, it was optimised at 1 mL/min. After 10 min, the trap was switched out of the water flow and the temperature was quickly raised up to 200 °C. The water flow was then passed through the trap for 3 min to release the trapped triazines and passed them to the analytical column, which was programmed from 160 °C to 260 °C, at 15 °C/min and the assay started immediately.

Figure 6.16 shows the chromatogram of the extract after the clean-up steps (polar and non-polar washes). When compared to the chromatogram in Figure 6.14, most of the interference peaks have been successfully removed and thus the analyte peaks could now be determined.

This wash was called non-polar wash because the clean-up process was employed at an elevated temperature in order to get rid of any relatively non-polar unwanted substances. Without this non-polar wash, the extract of the spiked ericaceous compost was acidic with a pH of 3.8 compared to pH 4.8 with a non-polar wash. This shows that the non-polar wash was capable of removing an amount of humic acids which remained in the sample extract.
Figure 6.16 A chromatogram showing the separation of the extract from spiked ericaceous compost, after the polar and non-polar washes. Experimental conditions: analytical column: PGC (100 x 2.1 mm i.d) with gradient temperature from 160 °C to 260 °C at 15 °C/min, mobile phase: 100% water, flow rate: 1.3 mL/min, detection: 222 nm. Peaks: 1. propazine, 2. atrazine, 3. simazine, 4. ametryn, 5. terbutryn.

6.4.2 Optimisation of the SWE

The physico-chemical state of water in the extraction cell, instrumental parameters (mode, temperature, time, and flow rate of extraction) as well as sample characteristics (concentration and polarity of the analytes, nature of the matrix, and presence of interferences) all influence the SWE efficiency. However, for the optimisation of the SWE in this work, only the instrumental parameters were taken into account.
For the optimisation of the extraction parameters, ericaceous compost was initially used as the sample matrix. 0.5 g of ericaceous compost spiked with 20 µg/g of each triazine was loaded inside the extraction cell and a cold extraction (polar wash at ambient temperature) was first performed. Then, the oven temperature was raised to the selected temperature and the water was flushed into the extraction cell. Once the extraction was completed, the extraction cell was by-passed and water flowed directly to the trap. This allowed the extraction cell to cool down off-line and a replacement sample could be inserted while assay continued. The trapped extract was washed with water at 60 °C for 10 min and the effluent was passed to waste. The triazines were then released once the oven temperature was raised up to 200 °C and the released triazines were passed to the analytical column which was programmed a temperature gradient starting from 170 °C to 250 °C, at 10 °C/min. The assay was started as soon as the extract reached the analytical column. After 3 min, the trap was by-passed and water flowed directly to the analytical column.

6.4.2.1 Extraction Mode

Two operational modes were tested for the extraction of the triazines from the compost: the static mode, where the extractant was held in the extraction cell for controlled time period at an elevated temperature to allow sufficient contact between the water and the solid sample and this cell was then flushed with water; and the dynamic mode, in which the extractant flowed continually through the sample. The variables that affecting the extraction mode are the extraction temperature as well as the extraction time.

Initially a 10 min static extraction combined with a 5 min dynamic extraction was performed at an extraction temperature of 200 °C. The result shows that all the chloro-triazine peaks disappeared from the chromatogram (Figure 6.17B).
Figure 6.17 Chromatograms showing the separation of triazines by (A) direct injection, and extracted at 200 °C with static mode, from (B) spiked ericaceous compost, and (C) non-spiked compost, Experimental conditions: analytical column: PGC (100 x 2.1 mm i.d) with gradient temperature from 140 °C to 210 °C at 5 °C/min, mobile phase, 100% water; flow rate, 1.3 mL/min: detection, 222 nm. Peaks: 1. propazine, 2. atrazine, 3. simazine, 4. ametryn, 5. terbutryn.
The degradation products resulted from the decomposition of the chloro-triazines are very polar and they might have not been trapped, therefore, they were washed from the trap during the non-polar wash at 60 °C. In addition, static extraction of the ericaceous compost at extremely high temperature (≥ 200 °C) causes non-polar substances to co-elute with the thiomethyl-triazines, which produced the broad background peaks observed in Figures 6.17B and 6.17C. At this stage, the huge amount of interferences contributed by the large amount of peat material from the ericaceous compost caused a contamination to the PGC analytical column. Even though the column had been washed with stronger organic solvents, such as acetone and di-butyl ether, its performance was still not as good as before. Therefore, the separation process had to be re-optimised, whereby the temperature was programmed from 130 °C to 160 °C, ramping at 2 °C/min and then raised to 220 °C, ramping at 5 °C/min, whilst the flow rate was lowered to 0.9 mL/min. However, this resulted in a longer analysis time.

Hence, static extraction at extremely high temperatures must be avoided. Even at less than 200 °C, using extraction temperature with a static mode, the chloro-triazines were still degraded which resulted in lower recovery and this was expected from the study of the thermal desorption from the trap column (see section 6.3). Dynamic extraction was then studied and it was found that no significant degradation was observed even when extraction temperatures up to 170 °C were employed for 5 min (Figure 6.18).
Figure 6.18  Chromatograms showing the separation of triazines by (A) 10 µL direct injection onto the separation column, and 3 mL extracted from (B) 0.5 g spiked ericaceous compost and (c) non-spiked compost, at 170 °C for 5 min with dynamic mode. Experimental conditions: analytical column: PGC (100 x 2.1 mm i.d) with gradient temperature from 130 °C to 160 °C at 2 °C/min and up to 220 °C at 5 °C/min, mobile phase, 100% water; flow rate, 0.9 mL/min; detection, 222 nm. Peaks: 1. propazine, 2. atrazine, 3. simazine, 4. ametryn, 5. terbutryn.
6.4.2.2 Extraction Temperature

The most substantial part with the extraction using superheated water was the optimisation of the temperature used to extract all the five triazine compounds from the compost. In SWE, the extraction mechanism of the analytes is a contribution of both thermal desorption and the solvating effect of the superheated water [192]. The solvating properties of superheated water are greater at temperatures where the solubility is sufficient for extraction but not so high which may lead to the decomposition of the analytes. Increase in temperature accelerates thermal desorption of the compounds and strongly affected the solubility.

From the thermal desorption study in section 6.3, temperatures less than 100 °C were not sufficient to release the triazines from the trap, therefore only temperatures above 100 °C were examined to extract all five triazine compounds from the compost in a dynamic mode. It was found that the chloro-triazines: atrazine, simazine and propazine, which are relatively more polar than the thiomethyl-triazines: ametryn and terbutryn, could be extracted at a lower temperature than 150 °C. However, for total removal of all the five triazines, temperatures ≥ 150 °C were necessary. Unfortunately, at 200 °C, the chloro-triazines would decompose. Therefore the extraction temperatures were optimised at 170 °C (Figure 6.18) in order to ensure that all the triazine compounds were extracted without any significant degradation.

6.4.2.3 Extraction Time

Over times ranging from 3 to 10 min at 170 °C with dynamic extraction, the amount of triazines extracted did not change significantly. However, longer extraction (> 10 min) led to a decrease in the extraction efficiency, especially for the chloro-triazines. To investigate this problem, the extract was not passed into the trap, instead it was collected manually and the fractions
collected in different times were compared. In 3 to 5 min, the colour of the extract was light yellow with a pH of 6.7 to 3.8. As the time increased up to 15 min, the colour became darker and the pH decreasing to 2.2. This explained that, probably with longer extraction time, more humic acids were extracted and co-eluted with the triazines. Therefore, in order to extract the triazines from the compost without much interferences, 5 minutes was sufficient at the optimised temperatures.

### 6.4.2.4 Extraction Flow Rate

The flow rates of the water during the extraction were studied in a range of 0.5 mL/min up to 2.0 mL/min. It was observed that at a flow rate above 1.0 mL/min, as the temperature increased, a high back-pressure gradually developed. This happened because at high flow rate, all the analytes and the unwanted compounds were extracted rapidly which blocked the lower frit of the extraction cell. Frits with 2 μm pore size were used at the inlet and the outlet of the extraction cell to prevent any particulate inside the compost from entering the switching valve or the trap column.

Meanwhile it was noticed that the flow rate of the superheated water did not affect the extraction process between the studied range of 0.5 – 1.0 mL/min. Thus, a flow rate of 1.0 mL/min was selected as the optimum value. In order to avoid the possibility of plugging of the extraction cell by fine solid particles from the complex sample matrices, the lower frit of the extraction cell was regularly cleaned with methanol or renewed after a few extractions.
6.4 On-line SWE-SWC of Triazines from a Compost Sample

When all these steps were brought together, the overall on-line coupling method therefore proposed (by reference to Figure 6.15) as follows:

1. Sample compost was **ground**, **sieved** to less than 1 mm in size and **spiked** with 20 µg/g of a triazines mixture, and then placed in an extraction cell.

2. **Polar wash prior to the extraction** (clean-up step 1). Removal of the very polar organic compounds, such as fulvic and humic acids from the compost by cold extraction, with 20 mL ambient water for 10 min and the extract was passed to waste.

3. **SWE process.** The extraction oven was heated and extraction of the triazines from the compost with superheated water was carried out at 170 °C for 5 min, and the extract was passed to the trap at ambient temperature.

4. **Th extract was pre-concentrated** on an appropriate sorbent trap, and effluent from trap was passed to waste.

5. Extraction cell was then by-passed and **non-polar wash prior to the assay** (clean-up step 2) was carried out by increasing the temperature of the trap to 60 °C and flushing with 10 mL of water for 10 min. The effluent was passed to waste. Warm water at 60 °C was needed to remove any remaining relatively non-polar unwanted particles as completely as possible without eluting any of the desired analytes.

5. **Thermal desorption process.** The trapped triazines were released when the temperature of the trap oven reached 200 °C and the released
triazines were eluted by water flow to the analytical column. The trap was then by-passed after 3 min.

6. **Superheated water chromatographic (SWC) separation** was started as soon as the eluted extract reached the column, using a temperature gradient from 170 °C to 250 °C, at 10 °C/min and UV detection at 222 nm (Figure 6.19).

Figure 6.19B shows the chromatogram for the separation of the extract from 0.1 g of spiked seed compost. Seed compost which is more sandy and therefore more porous produced a chromatogram with a smooth baseline and less background interference, whereas with 0.5 g of spiked ericaceous compost, higher background interference was observed (Figure 6.18B) which affected the measurement of the recovery as well (see chapter 7). The mixture of sand and peat (up to 60%) in seed compost means less organic material compared to ericaceous compost (containing 100% of peat), therefore less interferences. In addition, the smaller amount of seed compost used in the extraction decreased the presence of interferences in the chromatogram (Figure 6.19B).

The resolutions of the triazines obtained from the on-line coupling method (Figure 6.19B) are lower than those obtained by direct injection (Figure 6.19A) due to the difference in the temperature during the sample introduction onto the analytical column. In the on-line coupling method, the sorbent trap was heated at 200 °C to release the trapped triazines, which was higher than the initial temperature (170 °C) of the separation, whereas in direct injection, the sample was introduced at ambient temperature. Even though a cooling coil was employed after the trap prior to the separation, it seems that it was not adequate to cool the released sample to ambient temperature. Therefore the sample was actually introduced at higher temperature than at ambient temperature.
Figure 6.19 Chromatograms showing the separation of triazines by (A) 10 μL direct injection onto the separation column, and 3 mL extracted from (B) 0.1 g spiked seed compost and (c) non-spiked seed compost, at 170 °C for 5 min with dynamic mode using both polar and non-polar washes. Experimental conditions: analytical column: PGC (100 x 2.1 mm i.d) at temperature gradient from 170 °C to 250 °C at 10 °C/min, mobile phase, 100% water; flow rate, 1.0 mL/min; detection, 222 nm. Peaks: 1. propazine, 2. atrazine, 3. simazine, 4. ametryn, 5. terbutryn.
6.5 Summary

In this study, we demonstrated that the on-line coupling system of SWE with SWC could be used successfully for the determination of triazine herbicides in a complicated matrix, such as compost. Five triazine herbicides: atrazine, simazine, propazine, ametryn and terbutryn were investigated with two types of sample matrices: ericaceous compost and seed compost. The SWE of spiked compost samples could be more difficult to perform than the SWE of spiked sand samples because of stronger matrix interactions and more interferences. In order to avoid as completely as possible the interferences found from the sample matrix, two clean-up steps with water were included in this on-line coupling system: polar wash prior to extraction (cold extraction at ambient temperature) and non-polar wash of the trapped extract prior to assay (wash at elevated temperature). The seed compost produced a better chromatogram with less background interference than the ericaceous compost sample. This was due to the nature of the seed compost which is more sandy and therefore more porous. In addition, it has less organic material (up to 60% of peat) and a smaller sample amount was used compared to the ericaceous compost.

The whole on-line coupling of SWE-sorbent trap-SWC system was developed after each of the mentioned steps: the SWC separation, the thermal desorption of the trapped analytes, the SWE, and the clean-up process, has been optimised individually. Temperature is of prime importance in ensuring the efficiency of each step. The extraction was successful at 170 °C for 5 min and the desorption of the trapped analytes was performed at 200 °C for 3 min, while the separation was carried out with PGC column at temperature gradient from 170 °C to 250 °C, at 10 °C/min with UV detection at 222 nm.

In both extraction and thermal desorption steps, dynamic mode was preferable. In static SWE, the extracted analytes were not removed until the extraction period was complete and therefore the possibility for thermal
degradation of the chloro-triazines and reactions were higher. The degradation of the chloro-triazines in particular, was expected to occur under the superheated water conditions, with three possible pathways for the degradation: dealkylation, dechlorination (hydrolysis) and oxidation. However, under the selected superheated water conditions at the optimum parameters, there was no obvious degradation of the triazines. Thus, it can be concluded that the on-line coupled SWE-SWC method with efficient clean-up steps and at optimised conditions provides good sensitivity and selectivity for the qualitative analysis of the triazines in complicated matrix samples. In the next chapter, we will discuss the quantitative analysis of these triazines.
CHAPTER SEVEN
CHAPTER SEVEN

QUANTITATIVE ANALYSIS OF TRIAZINE HERBICIDES

7.0 Introduction

Since the on-line coupling of superheated water extraction (SWE) with superheated water chromatography (SWC) produced a satisfactory separation of triazine herbicides (propazine, atrazine, simazine, ametryn and terbutryn), we next investigated whether a quantitative determination could be performed and thus validate the performance of the method. The validation of the on-line coupling system method was assessed from the evaluation of the recoveries of triazine herbicides extracted from the spiked compost samples. The relative standard deviation (RSDs) of the results based on five replicate spikes were used to assess the repeatability of the method.

Calibration curves of compost samples spiked with the triazines, over a range of different concentrations were obtained to indicate whether the method was quantitative for these herbicides. Determination of the calibration curve was based on the peak areas from the measurement of the samples at four concentrations over a tested range. The limit of detection (LOD) of each triazine by using the on-line coupling method was obtained from the calibration. It was defined as three times the standard error of the calibration divided by the slope.

We also compared the on-line system with an off-line SWE system using superheated water chromatography (SWC) and conventional liquid chromatography (LC) to confirm that there were no losses during the chromatographic stage. Solvent extraction followed by SWC or LC was also included as the traditional systems to compare with both the on-line and off-line systems.
7.1 Method Validation

The performance of the coupling SWE-SWC method was validated by evaluating firstly the detection limits (LOD) of the five triazines based on their calibration curves. The evaluation was then followed by assessing the percentage of recovery and the repeatability, which was calculated as the relative standard deviation (RSD).

7.2 Linearity and Limit of Detection (LOD)

LOD is the smallest concentration of analyte that gives a measurable response. The linearity of the calibration is a measure of how well the calibration plot of detection response vs. concentration approximates to a straight line. This was measured for each of the triazines over the concentration range tested. A high correlation coefficient ($r^2$) value indicates good correlation between the triazine concentrations and peak areas, thus giving a good linearity of the calibration curve.

7.2.1 Linearity and LOD of Standard Solutions

The linearity of the superheated water chromatography (SWC) method using a clean new Hypercarb PGC analytical column was determined by injecting directly a standard solution of the triazines mixture into the column with superheated water as the eluent. The separation was carried out using a temperature gradient from 170 °C to 250 °C, 10 °C/min at a flow rate of 1.0 mL/min with UV detection at 222 nm. The standard calibration curves (peak area vs. concentration) obtained for all the triazines were linear (correlation coefficients, $r^2 > 0.99$) over the concentration range studied, from 5 to 50 μg/mL, with triplicate runs for each concentration (Table 7.1).
Table 7.1: Correlation coefficient, $r^2$, of peak area vs. concentration of triazines (mean of 3 injections) in standard solution at a range of four concentrations from 5 to 50 $\mu$g/mL, by direct injection of 10 $\mu$L into a clean PGC column and also into the same PGC column after it had been used in SWC and SWE. Separation conditions as in section 7.2.1.

<table>
<thead>
<tr>
<th>TRIAZINE COMPOUNDS</th>
<th>$r^2$ (clean PGC column)</th>
<th>$r^2$ (PGC column after SWC/SWE study)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Propazine</td>
<td>0.9984</td>
<td>0.9963</td>
</tr>
<tr>
<td>2. Atrazine</td>
<td>0.9963</td>
<td>0.9951</td>
</tr>
<tr>
<td>3. Simazine</td>
<td>0.9972</td>
<td>0.9949</td>
</tr>
<tr>
<td>4. Ametryn</td>
<td>0.9983</td>
<td>0.9979</td>
</tr>
<tr>
<td>5. Terbutryn</td>
<td>0.9925</td>
<td>0.9085</td>
</tr>
</tbody>
</table>

7.2.2 Method LOD of the Triazines in Seed Compost

At this preliminary stage, the on-line coupling of the SWE-SWC method was evaluated by using spiked samples at a high levels of concentration, within the $\mu$g g$^{-1}$ (mg kg$^{-1}$) range. The linearity of this coupling method using the same Hypercarb PGC analytical column as in section 7.2.1, was studied with standard solutions of the triazines mixture spiked onto seed compost at five different concentrations: 3, 6, 9, 12 and 15 $\mu$g/g. The sample was cleaned using ambient water (polar wash), prior to extraction. The triazines were then extracted at 170 °C and trapped onto an X-Terra column at ambient temperature. The trapped extract was cleaned with warm water at 60 °C (non-polar wash). The trapped triazines were then released at 200 °C and
they were transferred on-line into the PGC column to be assayed using the same temperature gradient as in section 7.2.1. For each of the triazines, except terbutryn, the repeatability (RSD) in three replicates at each concentration was less than 5% except at 12 μg/g. The calibration of simazine (Figure 7.1) shows that one of the set of the peak areas at 12 μg/g, appeared to differ significantly from the others in the set. This result was considered as a potential outlier.

Unfortunately, neither Dixon's test nor Grubb's test based on the three points would confirm the value as an outlier and justified rejection. However, according to Miller & Miller [193], the practice of making three measurements and rejecting the one which differs most from the other two should be avoided. Therefore, a more reliable estimate of the value is obtained by using the middle (median) of the three values, which is shown in Figure 7.2. This

![Figure 7.1: Individual results using on-line SWE-SWC. Calibration of simazine from a spiked seed compost sample based on three replicates at each concentration ranging from 3 to 15 μg/g.](image_url)
practice was also applied for the calibration of the other triazines which gave correlation coefficients of higher than 0.99 except for terbutryn which was 0.9373 (Table 7.2).

![Graph showing calibration curve]

Figure 7.2: Calibration of simazine from a spiked seed compost sample based on the median value of three replicates at each concentration ranging from 3 to 15 μg/g, using on-line SWE-SWC.
Table 7.2: Correlation coefficient, $r^2$ and limit of detection (LOD) of triazines in spiked seed compost samples at 5 different concentrations from 3 to 15 $\mu$g/g (based on median of 3 samples), by using an on-line coupling SWE-SWC method. Separation conditions as in section 7.2.1.

<table>
<thead>
<tr>
<th>TRIAZINE COMPOUNDS</th>
<th>Correlation coefficient $r^2$</th>
<th>Limit of detection LOD ($\mu$g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Propazine</td>
<td>0.9901</td>
<td>1.4</td>
</tr>
<tr>
<td>2. Atrazine</td>
<td>0.9927</td>
<td>1.1</td>
</tr>
<tr>
<td>3. Simazine</td>
<td>0.9948</td>
<td>1.0</td>
</tr>
<tr>
<td>4. Ametryn</td>
<td>0.9916</td>
<td>1.2</td>
</tr>
<tr>
<td>5. Terbutryn</td>
<td>0.9373</td>
<td>2.4</td>
</tr>
</tbody>
</table>
Unlike the other triazines, the variability of terbutryn at each concentration was significant with RSD of more than 10%, and therefore the correlation coefficient, \( r^2 \) of the calibration for all the individual samples was only 0.7902 (Figure 7.3). The low correlation coefficient contributed to a high detection limit of terbutryn (Table 7.2).

![Figure 7.3: Individual results for terbutryn using on-line SWE-SWC.](image)

Figure 7.3: Individual results for terbutryn using on-line SWE-SWC. Calibration based on three replicates at each concentration of spiked seed compost ranging from 3 to 15 μg/g.

To investigate this problem in more detail, a standard solution of the triazines mixture was directly injected at four different concentrations from 5 to 50 μg/mL into the PGC column, which had been used for the assays of the sample extracts. The results in Table 7.1 on the used column, based on the average of the three injections at each concentration, show good correlation for all triazines except terbutryn. The correlation coefficient, \( r^2 \) of the standard
terbutryn injected on the used column, based on the individual injection was only 0.9085 (Figure 7.4). Thus, it appeared that the result of terbutryn was not reliable based on the performance of the used PGC column, and this might affect the measurement of the recovery.

![Graph showing calibration of terbutryn](image)

**Figure 7.4**: Calibration of terbutryn based on individual direct injection of standard solution of triazines at concentrations ranging from 5 to 50 \( \mu \text{g/mL} \) into a used PGC column.

### 7.2.3 Method LOD of Triazines in Ericaceous Compost

To determine if the sample matrix had an effect, the experiment was repeated by employing ericaceous compost, which contain more organic material (100% peat). However, due to the huge interferences from the ericaceous compost which appeared to rapidly contaminate the PGC column and affected the repeatability of the on-line SWE-SWC method, the linearity and LOD could not be determined. Instead, off-line SWE was performed by manually
collecting 3 mL of the extracts (the extraction, desorption and clean-up procedures as in section 7.2.2) and injected 10 μL onto a Nova Pak C18 analytical column with 50% acetonitrile/water as the mobile phase. With this C18 column, only four of the triazines could be investigated, because ametryn tended to overlap with propazine (Figure 7.5).

Figure 7.5: A chromatogram showing the separation of triazines on Nova Pak C18 (200 x 4.6 mm i.d.) column at ambient temperature. Experimental conditions: mobile phase, 50% acetonitrile/water; flow rate, 1.0 mL/min; detection, 222 nm. Peaks: 1. simazine, 2. atrazine, 3. ametryn, 4. propazine, 5. terbutryn.

Linearity was observed for all the triazines studied over a range of concentrations from 3 to 15 μg/g with correlation coefficients, $r^2$, between 0.9832 to 0.9857 (Table 7.3). With the ericaceous compost, it was expected that the LODs for all the triazines studied would be higher than the LODs obtained from seed compost because more interferences were found from the
ericaceous compost. Furthermore, the actual concentrations based on that only 10 µL from 3 mL of extract was used.

Table 7.3: Correlation coefficient, $r^2$ and limit of detection (LOD of triazines in spiked ericaceous compost samples at a range of concentrations from 3 to 15 µg/g, by using off-line coupling SWE-LC method, (based on mean of 3 samples). Extraction conditions as in section 7.2.2 and separation conditions as in Figure 7.5.

<table>
<thead>
<tr>
<th>TRIAZINE COMPOUNDS</th>
<th>Correlation coefficient $r^2$</th>
<th>Limit of detection LOD (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Propazine</td>
<td>0.9844</td>
<td>3.2</td>
</tr>
<tr>
<td>2. Atrazine</td>
<td>0.9832</td>
<td>3.6</td>
</tr>
<tr>
<td>3. Simazine</td>
<td>0.9857</td>
<td>3.4</td>
</tr>
<tr>
<td>4. Terbutryn</td>
<td>0.9852</td>
<td>3.5</td>
</tr>
</tbody>
</table>

From this study, the problems mainly came from the use of ericaceous compost which clearly gave a lot of interferences. Because the PGC column was particularly susceptible to contamination, its performance was easily affected by the interferences and therefore affected the results especially for terbutryn. If the contamination was not very serious, the PGC column could be cleaned with a strong organic wash using acetone and di-butyl ether or with acid base wash [194]. However, it seemed that the contamination caused by the ericaceous compost was serious because the washing was not very successful and therefore, the performance did not come back to normal.
The best way was to minimise the use of ericaceous compost in the on-line method since the whole extract goes to the analytical column.

7.3 Recovery Studies

A series of studies was then carried out to examine the recovery of samples in the coupled system, with separate extraction and chromatography assays. The analytical procedure below shows schematically the overall processes that have been carried out in the recovery studies.

**Analytical procedures:**

- **Solvent extraction by using acetone**
- **Cold extraction** → **Waste 1**
- **SWE** → **Waste 2**
- **Trap, clean-up & pre-concentration**
- **Off-line** → **3 mL collected & 10 μL injected**
- **On-line**
- **SWC on PGC**
- **HPLC on Nova Pak C18**
Recovery of each triazine was assessed from the extracted amount by measuring the peak area recovered after the extraction and comparing the result with that obtained for a standard solution by direct injection. Percentage recovery was calculated based on the ratio of the extracted amount with the amount of the triazines spiked onto the compost. Sample amount and analytes concentration, sample matrix, temperatures and method used, all affected the recoveries resulted.

### 7.3.1 Effect of Sample Amount and Analytes Concentration

The capacity of the extraction cell used in this work was not the same for different kinds of sample matrix. 1.0 g of seed compost could be filled into the extraction cell, whereas for the ericaceous compost, only 0.5 g was possible. With the off-line SWE method, the full amount of each compost sample was used in order to obtain readable peaks. However, with the on-line SWE-SWC method, only a small amount of sample was needed because the whole extracts was injected into the separation column. Therefore, the amount of the seed compost was decreased 10 times (0.1 g) due to the high sensitivity. For the ericaceous compost, the on-line SWE-SWC method was initially applied to 0.5 g of the spiked ericaceous compost, but since the ericaceous compost kept on contaminating the PGC column, no further studies could be carried out. On the other hand, the sample amount used for solvent extraction was higher than those used for both the off-line and on-line SWE systems.

The effect of the concentration of the triazines spiked onto the seed compost samples was studied at 20, 25, 30 and 50 μg/g. It was observed that the increasing of the concentration did not improve the recoveries. The percentage recoveries obtained with 20 and 25 μg/g were about the same, however, the recoveries were lower when the concentrations were further increased to 30 and 50 μg/g. This might be because the sorbent trap had
been overloaded at the higher concentrations, thus caused a breakthrough. Breakthrough can occur in the trap due to insufficient retention of analytes or by exceeding the capacity of the sorbent. 20 μg/g was therefore selected as the spiked concentration. It was also shown that trap breakthrough is dependent on the temperature of the water entering the trap. When superheated water extraction was employed, a 1.0 metre cooling capillary prior to the trap ensured that the temperature of the water reaching the trap was near ambient.

7.3.2 Effect of Sample Matrix

Recoveries can also depend on the nature of the matrix. Ease of separation will vary according to whether analytes are deposited in, adsorbed on or chemically bonded to the sample matrix [192]. Also relevant are the location of the analytes and the porosity of the sample. This on-line system was therefore assessed by employing two different kinds of sample matrices: seed compost which consists of a mixture of peat (up to 60%) and ground limestone with sand, and ericaceous compost which consists of 100% peat material. Each compost was originally ground and sieved before it was spiked with 20 μg/g of the triazines. 0.5 g of spiked ericaceous compost and 0.1 g of spiked seed compost were used for the extraction. The organic matter (peat material) in both types of compost consists of humic and non-humic substances but differs. The humic acids are amorphous, three-dimensional, polymeric, acidic substances of high molecular mass and aromatic structure [195]. The non-humic substances would consist of carbohydrates, proteins, fat, waxes and other low-molecular-mass compounds associated with humic acids.

The recoveries of the triazines extracted from these two types of compost: samples were compared based on the results obtained by the on-line SWE-SWC method, at extraction temperature of 170 °C (Table 7.4). 170 °C was
selected because it was the best temperature at which efficient extraction of all the analytes was obtained (see later in section 7.3.3). The extracted triazines were trapped onto an unbounded X-Terra column at ambient temperature and were thermally desorbed at 200 °C. The whole extract was then transferred directly into a PGC analytical column and was chromatographed immediately using a temperature gradient.

Table 7.4: Mean of percentage recoveries (%RSD) of triazines extracted at 170 °C from spiked seed compost and spiked ericaceous compost, each with a concentration of 20 μg/g of the triazines, by using on-line SWE-SWC method.

<table>
<thead>
<tr>
<th>TRIAZINE COMPOUNDS</th>
<th>SAMPLE MATRICES</th>
<th>( \text{SEED COMPOST} ) (n=5)</th>
<th>( \text{ERICACEOUS COMPOST} ) (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Propazine</td>
<td>60 (5.8)</td>
<td>52 (25)</td>
<td></td>
</tr>
<tr>
<td>2. Atrazine</td>
<td>60 (8.2)</td>
<td>32 (16)</td>
<td></td>
</tr>
<tr>
<td>3. Simazine</td>
<td>63 (10.9)</td>
<td>25 (11)</td>
<td></td>
</tr>
<tr>
<td>4. Ametryn</td>
<td>102 (5.1)</td>
<td>78 (12)</td>
<td></td>
</tr>
<tr>
<td>5. Terbutryn</td>
<td>103 (10.5)</td>
<td>75 (16)</td>
<td></td>
</tr>
</tbody>
</table>

The chloro-triazines are more polar than the thiomethyl-triazines, therefore they eluted at the beginning of the chromatogram, in the region where humic substances abound. When hot water was used as the extractant, it was also capable of extracting large amount of humic acids from the compost as seen by the yellow-brown colouring of the waste extract after the clean-up step at
60 °C (non-polar wash). At higher temperatures, more humic acids would be extracted from the matrix. The two clean-up steps prior to the assay were insufficient to get rid of all these interferences. This is one of the reasons that probably contribute to the poor recoveries of the chloro-triazines especially in ericaceous compost: due to the interferences (humic acids) which co-eluted with these analytes. With the on-line method, the whole bulk of extract was directly injected into the SWC system, which meant that more chances of interferences would occur.

From the chromatograms (Figure 7.6), we observed that more interferences were found from ericaceous compost and lower recoveries of the chloro-triazines compared to the recoveries from the seed compost. Moreover, the extracts from the ericaceous compost were dark brown in colour and dirtier unlike the extracts from seed compost which were light yellow.

Ametryn and terbutryn gave high recoveries from the seed compost, but low recoveries from the ericaceous compost sample. The low recoveries might also be because ametryn and terbutryn were adsorbed more strongly on the matrix active sites, which would be higher in ericaceous compost and could have affected the extraction. It has been discovered that triazines' adsorption in soil or compost was mainly correlated to the organic content [196]. Ericaceous compost largely consists of peat material (a kind of partly decomposed plant material) which contributes to high organic content. Generally, the higher the organic content in the sample matrix, the stronger the triazines might be adsorbed. Furthermore, it has been reported [197] that usually, the thiomethyl-triazines can be adsorbed in the sample matrix more strongly than the chloro-triazines. The recovery of terbutryn obtained by solvent extraction using acetone was even lower (Table 7.5b), indicating that solvent extraction method (shake-flask method) with 100% acetone was not adequate to extract terbutryn from the ericaceous compost due to the stronger matrix interactions. In practice, water at very high temperature was more efficient as the extractant for terbutryn.
Figure 7.6A Chromatograms showing the separation of triazines extracted at 170 °C for 5 min with dynamic mode, from 0.5 g ericaceous compost spiked with 20 μg/g of triazines, with PGC (100 x 2.1 mm i.d) column at temperature gradient from 130 °C to 160 °C at 2 °C/min and up to 220 °C at 5 °C/min. Experimental conditions: mobile phase, 100% water; flow rate, 0.9 mL/min; detection, 222 nm. Peaks: 1. propazine, 2. atrazine, 3. simazine, 4. ametryn, 5. terbutryn.

Figure 7.6B Chromatograms showing the separation of triazines extracted at 170 °C for 5 min with dynamic mode, from 0.1 g seed compost spiked with 20 μg/g of triazines, with PGC (100 x 2.1 mm i.d) column at temperature gradient from 170 °C to 250 °C at 10 °C/min. Experimental conditions: mobile phase, 100% water; flow rate, 1.0 mL/min; detection, 222 nm. Peaks: 1. propazine, 2. atrazine, 3. simazine, 4. ametryn, 5. terbutryn.
One further possible reason for the overall poor recoveries from ericaceous compost, is that triazines are susceptible to hydrolysis in acidic or basic aqueous environments, although are relatively stable under neutral conditions. Even though the superheated water was neutral, the ericaceous compost was very acidic and therefore the extracts had gave a pH of 3.8. This could possibly cause the hydrolysis of the triazines which was enhanced by the elevated temperature.

The RSD values of all the triazines in ericaceous compost were also poor with the average of 16%. The results were not very repeatable because the PGC analytical column became less efficient, after a few assays had been carried out. In every case, after an on-line SWE was completed, the efficiency was poorer, thus the recovery was lower. It seemed that the triazines were not well retained in the column because it was suspected that an enormous amount of unwanted non-humic substances could be sticking strongly to the stationary phase of the column, presumably cellulose, lignin, fat or waxes from plant cells, which had been co-extracted with the triazines. A very strong organic wash or acid-base wash [194] had to be performed regularly. Because of this problem, on-line SWE-SWC at higher temperatures than 170 °C was avoided due to the possibility of extracting more of these non-humic substances from the matrix in addition to the triazines. In on-line SWE-SWC, the whole extract was injected into the PGC analytical column and the PGC column tended to adsorb unwanted species easily, hence, we tried to minimise the use of ericaceous compost as a sample matrix. Subsequently, off-line SWE followed by SWC also failed with this ericaceous compost because the unwanted substances interfered with the dilute extract and the peaks of the analytes could not be seen in the chromatogram.

Compared to the ericaceous compost, the seed compost, which is more sandy and contains less peat material (only up to 60%), permitted better recoveries of the triazines and the results were more repeatable (Table 7.4), due to the porosity of the sample matrix. It has been reported by Kronholm
that recoveries were usually better with porous samples and with analytes located at the surface. In addition, less organic matter in less sample amount means less interferences which led to higher recoveries.

7.3.3 Effect of Temperature

The dependence of recovery on temperature of extraction was determined using the off-line SWE methods (as in analytical procedure section 7.3) at different extraction temperatures, from 150 °C up to 210 °C. Percentage recoveries and RSDs of the triazines were calculated based on five replicates. The off-line SWE method for the spiked seed compost was followed by SWC using the PGC column. However, the similar method failed with the assay for spiked ericaceous compost since the ericaceous compost tended to contaminate the PGC column. Therefore the assay for the spiked ericaceous compost was carried out by conventional LC using Nova Pak C18 column and 50% acetonitrile/water as the mobile phase. All the results were tabulated in Table 7.5a and 7.5b and were compared with the solvent extraction method whereby the triazines in the spiked compost were extracted using cold acetone, followed by centrifugation and filtration, trap to dryness and re-dissolving in acetonitrile. As mentioned earlier, ametryn was not examined by LC on the C18 column as it overlapped with propazine.

Using a spike level of 20 µg/g for the five triazines investigated (atrazine, simazine, propazine, ametryn and terbutryn), an increasing temperature of extraction from 110 °C up to 170 °C, caused an increase in the recoveries. However, when the extraction temperature reached 190 °C, the recoveries for all the chloro-triazines (simazine, atrazine and propazine) started to decrease, unlike the thiomethyl-triazines (ametryn and terbutryn) whose recoveries kept on increasing as the temperature rose.
Table 7.5a: Mean of percentage recoveries of triazines (%RSD) of spiked seed compost with a concentration of 20 μg/g, obtained by off-line SWE-SWC and solvent extraction-SWC, n=5.

<table>
<thead>
<tr>
<th>TRIAZINE COMPOUNDS</th>
<th>OFF-LINE SWE-SWC % Recovery (%RSD)</th>
<th>SOLVENT EXT-SWC % Recovery (%RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>170 °C</td>
<td>190 °C</td>
</tr>
<tr>
<td>1. Propazine</td>
<td>68 (6.1)</td>
<td>56 (8.9)</td>
</tr>
<tr>
<td>2. Atrazine</td>
<td>67 (5.0)</td>
<td>54 (6.6)</td>
</tr>
<tr>
<td>3. Simazine</td>
<td>66 (6.8)</td>
<td>50 (7.8)</td>
</tr>
<tr>
<td>4. Ametryn</td>
<td>91 (3.9)</td>
<td>97 (4.0)</td>
</tr>
<tr>
<td>5. Terbutryn</td>
<td>105 (3.4)</td>
<td>128 (3.3)</td>
</tr>
</tbody>
</table>

Table 7.5b: Mean of percentage recoveries of triazines (%RSD) of spiked ericaceous compost with a concentration of 20 μg/g, obtained by off-line SWE-LC and solvent extraction-LC, n=5.

<table>
<thead>
<tr>
<th>TRIAZINE COMPOUNDS</th>
<th>OFF-LINE SWE-LC % Recovery (%RSD)</th>
<th>SOLVENT EXT.-LC % Recovery (%RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>150 °C</td>
<td>170 °C</td>
</tr>
<tr>
<td>1. Propazine</td>
<td>103 (10.8)</td>
<td>113 (16.5)</td>
</tr>
<tr>
<td>2. Atrazine</td>
<td>78 (2.3)</td>
<td>89 (4.7)</td>
</tr>
<tr>
<td>3. Simazine</td>
<td>76 (5.7)</td>
<td>86 (9.0)</td>
</tr>
<tr>
<td>4. Terbutryn</td>
<td>39 (6.9)</td>
<td>52 (4.9)</td>
</tr>
</tbody>
</table>
Use of hot water as extractant or mobile phase inherently poses a danger that the high temperature might decompose thermally unstable or thermolabile compounds. In previous work, McGowin et al. [21] reported that the recoveries of the chlorine-containing triazines, such as atrazine and propazine, decreased significantly when the extraction temperature was raised from 110 °C to 250 °C, whereas the recovery of the thiomethyl-triazine, ametryn was affected less by temperature. Curren and King [40] suggested an approach for the thermally unstable chloro-triazines, such as simazine, atrazine and cyanazine, so that the extractions could be performed at lower temperatures. They used co-solvents, such as urea or ethanol, in conjunction with temperature to reduce the hydrogen bond density of water in order to enhance the solubility of the chloro-triazines and thus reduce the extraction temperature.

This is probably the main reason why the recoveries of all the chloro-triazines from seed compost obtained by the off-line SWE-SWC system or solvent extraction-SWC were not quantitative (Table 7.5a). However, with the SWE-conventional LC system, from ericaceous compost, the recoveries were better because the assay was done in ambient temperature (Table 7.5b). These results were further compared with the recoveries obtained by solvent extraction, which were definitely better, especially with solvent extraction-conventional LC method, when no elevated temperatures were involved.

The thiomethyl-triazines (ametryn and terbutryn) are more thermally stable, and did not decompose in SWE even at higher temperatures and thus produced higher recoveries compared to the solvent extraction (Table 7.5a). However, the recovery of terbutryn extracted by the off-line SWE-SWC system seemed to be unreliable because it was well above 100% especially at 210 °C extraction temperature, and yet the RSD was less than 5%. To investigate this problem, the recoveries of the triazines extracted from spiked seed compost at 210 °C were also studied by using the on-line SWE-SWC method. This time both the recovery and RSD value of terbutryn were high.
Chapter 7  
Quantitative Analysis of Triazines

(Table 7.6), indicating that the effect of the interferences at 210 °C was more severe with the on-line method. To make matter worse, the recoveries of all the chloro-triazines were much lower than those obtained with the off-line method at the same temperature. This suggests that with the on-line SWE-SWC method at 210 °C, both groups were effected by temperature and interferences which caused poor recovery and repeatability.

Table 7.6: Mean of percentage recoveries of triazines (%RSD) of spiked seed compost with a concentration of 20 μg/g, obtained by on-line SWE-SWC using 210 °C extraction temperature.

<table>
<thead>
<tr>
<th>TRIAZINE COMPOUNDS</th>
<th>% Recovery (%RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=3</td>
<td></td>
</tr>
<tr>
<td>1. Propazine</td>
<td>18 (7.4)</td>
</tr>
<tr>
<td>2. Atrazine</td>
<td>15 (6.3)</td>
</tr>
<tr>
<td>3. Simazine</td>
<td>18 (9.6)</td>
</tr>
<tr>
<td>4. Ametryn</td>
<td>108 (10.5)</td>
</tr>
<tr>
<td>5. Terbutryn</td>
<td>144 (12.5)</td>
</tr>
</tbody>
</table>

To further investigate this problem, a blank sample (non-spiked compost) was run at these different temperatures. None of the target analytes were detected in a blank of the seed compost sample, although a peak at the same retention time as terbutryn appeared when the extraction temperature reached 210 °C (Figure 7.7). This meant that terbutryn could not be quantified at this temperature because its peak was enhanced by a matrix interference. Nevertheless, the error, which occurred significantly in the
calibration data of the standard terbutryn (see Figure 7.4), might also contributed to the apparent high recovery of terbutryn.

Figure 7.7: Chromatograms showing the separation of triazines after SWE at 210 °C from (A) spiked seed compost and (B) non-spiked seed compost. Experimental conditions: analytical column, Hypercarb PGC (100 x 2.1 mm i.d) at temperature gradient from 170 °C to 250 °C at 10 °C/min; mobile phase, 100% water; flow rate, 1.0 mL/min; detection, 254 nm. Peaks: 1. propazine, 2. atrazine, 3. simazine, 4. ametryn, 5. terbutryn
The structure of the interference peak in Figure 7.7B could be identified by LC-MS if needed in future. However, this temperature was not normally used as low recoveries of the chloro-triazines would result.

7.4 Method Comparison

From the recoveries obtained from the seed compost, the coupling of SWE with SWC method, either off-line or on-line, at an appropriate extraction temperature, seemed to give quite similar performances. The recoveries obtained by both methods at 170 °C extraction temperature, were plotted in Figure 7.8 and were compared with the solvent extraction-SWC method.

![Graph showing percentage recoveries of triazines](image)

**Figure 7.8:** Percentage recoveries of triazines obtained from spiked seed compost with a concentration of 20 μg/g by different methods.
From this plot, it was obvious that both of the coupled methods gave lower recoveries of the chloro-triazines (propazine, atrazine and simazine) but higher recoveries of the thiomethyl-triazines than the solvent extraction method. It was confirmed that a significant decomposition of the chloro-triazines occurred in SWE when compared to the direct injection chromatogram. On the other hand, the thiomethyl-triazines (ametryn and terbutryn) are more thermally stable, therefore they did not decompose in SWE. As suggested by Curren & King [40], a modifier can be added to the water to reduce the extraction temperature, hence minimise the possibility of decomposition towards the chloro-triazines. This might increase the recoveries, but at the expense of extracting more matrix components that could interfere the assay. However, it was not tested in this study.

Compared to the solvent extraction method, at the optimum extraction temperature with simple clean-up steps, quantitation is easier and more reliable in both the off-line and on-line SWE-SWC methods because the amount of co-extracted substances reaching the column is minimised, thus reducing the interfering compounds as well as the background. The extract of seed compost using SWE was light yellow unlike the organic solvent extract, which was dark brown, indicating that more interferences in the latter extracts. However, more precautions must be taken when higher temperatures are employed in SWE because the humic substances in the matrix might degrade and co-extracted with the analytes. The main advantage of the on-line method is that all the extracted triazines are delivered to the analytical column giving improved sensitivity compared to the off-line method (Figure 7.9A & 7.9B). Hence, smaller samples sizes could be used. Moreover, the efficiencies of the separations achieved using the two methods were very similar (Table 7.7).
Figure 7.9: Chromatograms showing the separation of triazines extracted from spiked seed compost after polar and non-polar washes, via (A) off-line SWE-SWC (10 μL from 3 mL) and (B) on-line SWE-SWC (3 mL), at 170 °C for 5 min with dynamic mode. Experimental conditions: analytical column: PGC (100 x 2.1 mm i.d) at temperature gradient from 170 °C to 250 °C at 10 °C/min; mobile phase, 100% water; flow rate, 1.0 mL/min; detection, 222 nm. Peaks: 1. propazine, 2. atrazine, 3. simazine, 4. ametryn, 5. terbutryn
Table 7.7: Comparison of chromatographic parameters via off-line SWE-SWC and via on-line SWE-SWC based on Figure 7.9.

<table>
<thead>
<tr>
<th>TRIAZINE COMPOUNDS</th>
<th>OFF-LINE SWE-SWC</th>
<th>ON-LINE SWE-SWC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$t_R$ (min)</td>
<td>$W_h$ (sec)</td>
</tr>
<tr>
<td>1. Propazine</td>
<td>6.61</td>
<td>9.16</td>
</tr>
<tr>
<td>2. Atrazine</td>
<td>7.18</td>
<td>9.71</td>
</tr>
<tr>
<td>3. Simazine</td>
<td>7.75</td>
<td>8.82</td>
</tr>
<tr>
<td>4. Ametryn</td>
<td>9.81</td>
<td>10.17</td>
</tr>
<tr>
<td>5. Terbutryn</td>
<td>10.45</td>
<td>11.59</td>
</tr>
</tbody>
</table>

Key: $t_R$, retention time (min); $W_h$, width at half height; $N$, theoretical plates (efficiency); $k$, retention factor; $\alpha$, selectivity; $R_s$, resolution.
This study (Table 7.7) showed that the coupling system of the SWE-SWC method that was developed, worked well for the extraction and analysis of triazine herbicides with good efficiency and selectivity, though decomposition would occur for the chloro-triazines in hot water. In the on-line method, the retention times were slightly shorter, probably, because the sample extract was actually introduced at higher temperature into the separation column as a consequence of the thermal desorption at 200 °C, even though had been cooled by the cooling coil after the trap. In the off-line method, the sample extract was introduced at ambient temperature. However, there was no significant change in efficiency.

Even though recoveries obtained by both off-line and on-line SWE-SWC systems were about the same (Figure 7.8), there were several benefits of the on-line system in comparison with the off-line system. The on-line method provides higher sensitivity compared to the off-line method (Figure 7.9) and solvent extraction system as the whole extract goes to the assay. Due to the greater sensitivity, the analyte identification then becomes more reliable and it is also highly suited for samples in which the amounts of analytes are very low. The on-line coupling system does not require sample handling in-between the whole process, and therefore it is highly suitable to automate for multi-analyte analysis and high sample throughput.

7.4.1 Comparison with Previous Work

The limits of detection (LODs) for the five triazines (simazine, atrazine, propazine, ametryn and terbutryn) obtained in this work (see Table 7.2) ranging from 1.0 to 2.4 µg/g, were higher than those obtained by other traditional methods typically with determination using HPLC and UV detector [165, 174]. Since this study was mainly focussed on the initial stage of the development of the on-line SWE-SWC method, the determination was carried out at a high concentration level, µg/g instead of ng/g. Battista et al. [174]
determined the LODs of the triazines in soil sample (clay+silt+sand) via HPLC using an LC-18-DB reversed phase column and a guard column, with UV detection at 220 nm. The mobile phase was acetonitrile-phosphate buffer (10 mmol/L; pH 6.7; 38:62, v/v). They obtained low LODs of 0.8 to 3 ng/g for simazine to terbutryn respectively, but the method required an exhaustive procedure of extraction and isolation which required two cartridges with a large volume of organic solvents being used for the mobile phase and clean-up process. However, the higher LODs of the five triazines obtained in this on-line SWE-SWC method were compensated for by the simplicity of the on-line method which gave short analysis time, and was cheap and environmental friendly. Future work with this on-line SWE-SWC method should be applied for the analysis of herbicides at trace level of concentration in real environmental sample.

The benefits and limitations of both on-line and off-line coupling systems are compared with the traditional extraction system (shake flask method) employed in this work (Table 7.8).
Table 7.8: Comparison of off-line and on-line SWE-SWC systems for analysis of triazines in spiked seed compost sample with traditional system (solvent extraction-SWC).

<table>
<thead>
<tr>
<th></th>
<th>Traditional System</th>
<th>Off-line System</th>
<th>On-line System</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Analytical procedure</td>
<td>Solvent extraction with acetone. Centrifugation and evaporation of extract and assayed manually.</td>
<td>SWE with solid phase trap. Extract collected and assayed manually.</td>
<td>SWE - solid phase trap - SWC. On-line transfer of extract from trap directly to assay.</td>
</tr>
<tr>
<td>2. Analysis time</td>
<td>~ 2 hours</td>
<td>~ 60 min</td>
<td>~ 40 min</td>
</tr>
<tr>
<td>3. Manual work</td>
<td>intensive</td>
<td>average</td>
<td>minimal</td>
</tr>
<tr>
<td>4. Consumption of organic solvent</td>
<td>maximum ~ 15 mL</td>
<td>If needed, minimum. (however did not use in this off-line study)</td>
<td>none</td>
</tr>
<tr>
<td>5. Sample amount</td>
<td>maximum 5 g</td>
<td>average 1.0 g</td>
<td>minimum 0.1 g</td>
</tr>
<tr>
<td>6. Sensitivity</td>
<td>satisfactory</td>
<td>good</td>
<td>excellent</td>
</tr>
<tr>
<td>7. Automation</td>
<td>not possible</td>
<td>not possible</td>
<td>possible</td>
</tr>
<tr>
<td>8. Interference</td>
<td>more</td>
<td>less</td>
<td>less (with efficient clean-up steps)</td>
</tr>
</tbody>
</table>
7.5 Summary

With the growing need for the validation of herbicides/pesticides analysis methods in environmental samples, methods that required little or no organic solvent are attractive options. The current methods of herbicides analysis are typically solvent and labour intensive with the much time and effort spent on sample pre-treatment, which is therefore costly.

In this work, the method developed utilises superheated water extraction (SWE) along with a solid phase trap, coupled either off-line or on-line with superheated water chromatography (SWC), and can be used for quantitation of triazine herbicides in spiked composts. The quantitation is easier with the inclusion of clean-up steps and when the optimum extraction temperature is employed. The method developed was validated by evaluating accuracy (percentage recovery), repeatability (relative standard deviation, RSD), and limit of detection (LOD). From the results obtained, this on-line SWE-SWC method has the potential for the application of trace analytes in real environmental samples.

In general, extractions of triazines in seed compost samples carried out at 170 °C by both off-line and on-line SWE-SWC systems gave better results than those obtained at other temperatures and the values are quite comparable to each other. With the off-line system, the recoveries were between 68 and 105%, with RSDs between 3.4 and 6.8%, whereas with the on-line system, the recoveries were between 60 and 103%, with RSDs between 5.1 and 10.5%. The poor recoveries were due to the thermally labile character of the chloro-triazines (propazine, atrazine and simazine). The higher the temperature, the lower the recoveries of the chloro-triazines obtained due to the analytes degradation and also possibly the degradation of humic substances.
On the other hand, the extraction of triazines in ericaceous compost by on-line SWE-SWC yielded poor recovery and repeatability, mainly caused by the interferences that co-extracted with the analytes. The Hypercarb PGC column in the SWC system tended to adsorb unwanted components easily. That is why with the ericaceous compost, the efficiency of the PGC column decreased with use which affected the recovery and the repeatability of the on-line method. Unlike the PGC column, the C18 column used in conventional LC system was not easily contaminated by the interferences, therefore the assay of the ericaceous extract was easier, however, it could not resolve all the five triazines. The recoveries of the chloro-triazines were better in ambient temperature, but the recovery of terbutryn from the ericaceous matrix was poor, both by SWE and solvent extraction, possibly due to stronger matrix interactions. This is because the ericaceous compost has high organic matter content which led to strong adsorption of terbutryn into the compost.

On the whole, on-line SWE-SWC is a more convenient method compared to off-line SWE-SWC because it is faster, more sensitive, less manual work and less sample amount required. In addition, it is possible to automate for multi-analyte analysis and high sample throughput. Unfortunately, there is more risk to interferences because the whole sample extract was directly injected into the SWC system which may decrease the recovery. In order to minimise those interferences, the extraction conditions must be adjusted to optimum values especially the temperature and also the amount of sample must be reduced. With the on-line system, the amount of sample can be as low as 10 to 100 mg. The clean-up steps performed in this on-line method are very useful to get rid of the matrix interferences as much as possible prior to the assay.
CHAPTER EIGHT

Conclusions and Future Work
An on-line coupled system of superheated water extraction (SWE) with superheated water chromatography (SWC) has been successfully constructed by using simple switching valves and a solid-phase trap as the interface between the extractor and the chromatograph. The principle of this laboratory-made on-line system is based on the altered physico-chemical properties of water at elevated temperatures and pressures. The application of this on-line system in the analysis of pharmaceuticals and triazine herbicides was demonstrated.

8.1 Analysis of Pharmaceutical Compounds

In this study, superheated water extraction (SWE) directly linked to superheated water chromatography (SWC) is shown to be a feasible analytical technique for the rapid and reliable qualitative analysis of pharmaceutical compounds (paracetamol, salicylamide, caffeine, phenacetin, methyl paraben, and ethyl paraben) from spiked sand samples. Only a small percentage of loss in efficiency was observed when compared with direct injection. However, the loss did not affect the separation and this was compensated by the high sensitivity and rapidity of the on-line method.

8.1.1 Selective Extraction

Selective fractionation by trapping all the extracted analytes and releasing them sequentially by increasing the temperatures in stages has been successfully demonstrated with these pharmaceuticals, even though their
polarities are not markedly different from each other. This shows that selective extraction can easily be carried out with compounds which have different polarities, ranging from polar to non-polar, by programming the water temperatures.

8.2 Analysis of Triazine Herbicides

The further development of this on-line SWE-SWC with the inclusion of clean-up steps (polar and non-polar washes) enabled quantitative analysis of triazine herbicides (atrazine, simazine, propazine, ametryn and terbutryn) from two types of complicated sample matrices: ericaceous compost and seed compost, in a closed system. This eliminated the manual sample pretreatment procedure that is often time consuming, environmentally unfriendly and liable to generate errors. The two clean-up steps with water: polar wash prior to extraction (cold extraction at ambient temperature) and non-polar wash of the trapped extract prior to assay (wash at an elevated temperature) were very useful to avoid as completely as possible the interferences found from the sample matrix.

8.2.1 Interferences

The seed compost, with a lower organic content (only up to 60% peat), produced a good chromatogram with less background interference and gave good results of recoveries comparable to the off-line method. In contrast, the sample matrix with higher organic content (100% peat), i.e. ericaceous compost, gave poor results with high background interference of chromatogram, which also caused a problem to the PGC analytical column. The Hypercarb PGC column in the SWC system tended to adsorb unwanted components easily. That is why with the ericaceous compost, the efficiency of
the PGC column decreased with use which affected the recovery and the repeatability of the method.

Hence, two kinds of interferences were identified in this case:

1. Humic substances which formed a characteristic hump in the chromatogram of triazines extracted from the ericaceous compost.

2. Non-humic substances such as, carbohydrates, lipids, or waxes, which contaminated the PGC column and affected its efficiency. These relatively non-polar substances were co-extracted with triazines at high temperatures but they were strongly retained / adsorbed by the PGC column.

In the analysis of phenylurea and triazine herbicides in soil samples, Ferrer et al. [198] applied a styrene-divinylbenzene (SDB-XC) disc in order to remove the humic and fulvic acids from the aqueous soil extract by ion-exchange interactions. The humic substances remained bound onto the SDB-XC disc with 85% retention, even during methanol elution of the trapped herbicides, resulting in reducing the interference from the humic acid peak in the LC chromatogram. Thus, in this on-line SWE-SWC, a SDB-XC disc can be placed in the extraction cell with the similar function. The SDB-XC was shown to be stable at water temperatures up to 250 °C [12]. Hence, the solid-phase trap column used in this on-line system can probably be replaced by SDB-XC disc so that the huge amount of humic acids, particularly from the ericaceous compost, can be removed from the sample extract.

If lipids or any hydrophobic substances caused the change in the column performance, washing the column with non-polar solvents such as, dichloromethane or chloroform might be able to reduce the contamination.
8.2.2 Thermal Degradation

Because the chloro-triazines are thermally labile compounds, they tended to decompose at temperatures more than 170 °C. In order to avoid the decomposition, modifier e.g. ethanol or urea [40] could be added into the superheated water. Extraction can be performed at lower temperatures if co-solvents are used in conjunction with adjustment of temperature to reduce the hydrogen bond density of water. However, problems may be encountered due to the extraction of co-extractives when using modified superheated water.

8.3 Improving the On-line SWE-SWC System

The overall system can be fully automated using computer-controlled valves and a simple program for switching them at the appropriate intervals. To simplify the on-line system, an automatic switching valve (Figure 8.1) can be used to replace the manual switching valves. Furthermore, extraction and assay can be carried out simultaneously if a multi-solvent delivery unit is used instead of a single pump.

Figure 8.1 Analytical Laboratory Automated Switching Valves
The on-line coupling system can also be automated for multi-analyte analysis and high sample throughput. Although, there is no commercial SWE instrument available in the market, a Dionex ASE system (Figure 8.2) can still be used for temperatures up to 200 °C.

Figure 8.2  Dionex ASE system coupled to automated SPE

8.4 Potential Applications

Because of the high sensitivity of this on-line SWE-SWC system due to the whole extract is delivered to the analytical column, only a small amount of sample is needed. Since the LODs of the triazines by this on-line method were determined at high level of concentration, the values could not be used to measure the exact sensitivity of this method. However, the results show potential and future work with this on-line method should be applied for the analysis of herbicides at trace level of concentration in real environmental samples, such as soil or sediment. This can also be applied for the analysis of aged environmental samples.
Superheated water extractions of natural products, such as: essential oils, antibiotics and antioxidants from plants have been widely applied due to the growth of interest in nutraceuticals. Plants have been used as medicines for centuries, however, in order to commercialise the botanical drugs, methods that required little or no organic solvent are attractive options. Isolation, separation and purification can be a lengthy and expensive process, hence, on-line SWE-SWC method could be the best option. Even though some of the botanical compounds may degrade in hot water, there should be always an alternative to overcome this problem. Moreover, since they are polar compounds, very high temperatures are normally not required.
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PAPERS AND PRESENTATIONS
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Papers:


Poster Presentations:

• R. Tajuddin and R. M. Smith*, *SWE coupled on-line with SWC*, presented at 26th International Symposium on High Performance Liquid Phase Separations and Related Techniques, Montreal, Canada, June 2nd – 7th, 2002


