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Theory of Solvation and its Application to the Supercritical Fluid Extraction/Supercritical Fluid Chromatographic Analysis of Pharmaceuticals.

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A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS OF THE UNIVERSITY OF NORTHUMBRIA AT NEWCASTLE FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN COLLABORATION WITH PFIZER CENTRAL RESEARCH.
(SANDWHICH, KENT, U.K.)

FEBRUARY 1996

To my Parents

Mr & Mrs. M. Zakir Khundker.

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Abstract

The main objectives of this PhD project were to relate analyte solubility in supercritical carbon dioxide via molecular structure and also to investigate the factors that influence the solubility and extraction of analytes in a supercritical fluid extraction (SFE) when using carbon dioxide as the solvent.

The polarity of an analyte was selected as the key parameter to developing a means of estimating steroid solubility in supercritical carbon dioxide. Polarity can be estimated by the hydrophobicity term, log P (based on partition coefficients), and also of the solubility parameter, δ,. The use of partition coefficient in conjunction with a calculated solubility parameter was demonstrated as a reasonable means of estimating steroid solubility in supercritical carbon dioxide. Experimental determination of the solubility of several steroid compounds with a range in polarities in supercritical carbon dioxide was carried out in order to correlate solute polarity to the solute solubility. A chromatographic method was also investigated based on capacity factor measurements for the prediction of steroid solubility in supercritical carbon dioxide.

The application of supercritical fluid extraction (SFE) with carbon dioxide and modified carbon dioxide for the extraction of four antifungals from an animal feed matrix has been investigated. The SFE experiments were designed to optimize extraction conditions for the extraction of the antifungals from the animal feed to allow for the evaluation of the most significant variables influencing extraction. A method was also developed for the analysis of the SFE animal feed extracts by packed-column supercritical fluid chromatography. The modification of the mobile phase with polar modifier was necessary to elute the antifungals. The procedure provided an alternative separation selectivity to the existing reversed phase high performance liquid chromatography techniques with much shorter analysis time.

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Chapter 1.0 Introduction and Background to Supercritical Carbon Dioxide

1.1. Development of Supercritical Fluids

Historically, Hannah and Hogarth[1] first reported the unique solvent properties of supercritical ethanol. They discovered that the concentration of cobalt chloride in supercritical ethanol was higher than the value predicted by vapour pressure calculations. These properties remained unnoticed, except for a few references in the geological literature,[2-4] until Booth and Bidwell's review in 1948.[5] Zhuze and co-workers[6] reported a process for determining lanolin from wool grease in 1958, but the real starting point for the use of supercritical fluids as an extraction media was demonstrated by Zosel[7] at the Max Planck Institute for Coral Research. Since that time, supercritical fluid extraction has found widespread use in numerous areas in chemical engineering. The use of supercritical fluids as chromatographic mobile phases was first demostrated by Klesper et al.[8] These scientists used supercritical chlorofluoromethanes as mobile phases for the separation of nickel porphyrin isomers. Klesper et al. continued to develop SFC with packed columns by applying the technique to polystyrene oligomer separations.[9] These developments were followed by Sie et al.[10-15] who investigated the use of carbon dioxide, isopropanol, n-pentane and other materials as mobile phases with packed columns while applying the technique to the analysis of polycyclic aromatic hydrocarbons, antioxidants, dyes and epoxy resins. Giddings et al.[16-17] demonstrated the migration of carotenoids, sugars, nucleosides, amino acids, polypeptides, various polymers and other solutes using what was described as "high pressure gas chromatography" with carbon dioxide or ammonia as the mobile phase. The work of Giddings in the late 1960's, which showed various potential application areas, caused supercritical fluids' future to appear promising. In 1981, a further important development was demonstrated when fused silica capillary column SFC was introduced by Novotny and Lee.[18] Nowadays supercritical fluids are extensively used in extraction and chromatography.

1.2 Supercritical Fluids

All substances can exist as solids, liquids or gases. A substance becomes supercritical fluid when it is maintained at temperatures and pressures above the critical point, Cp; this is illustrated in the form of a phase diagram (Figure 1.1).[19]

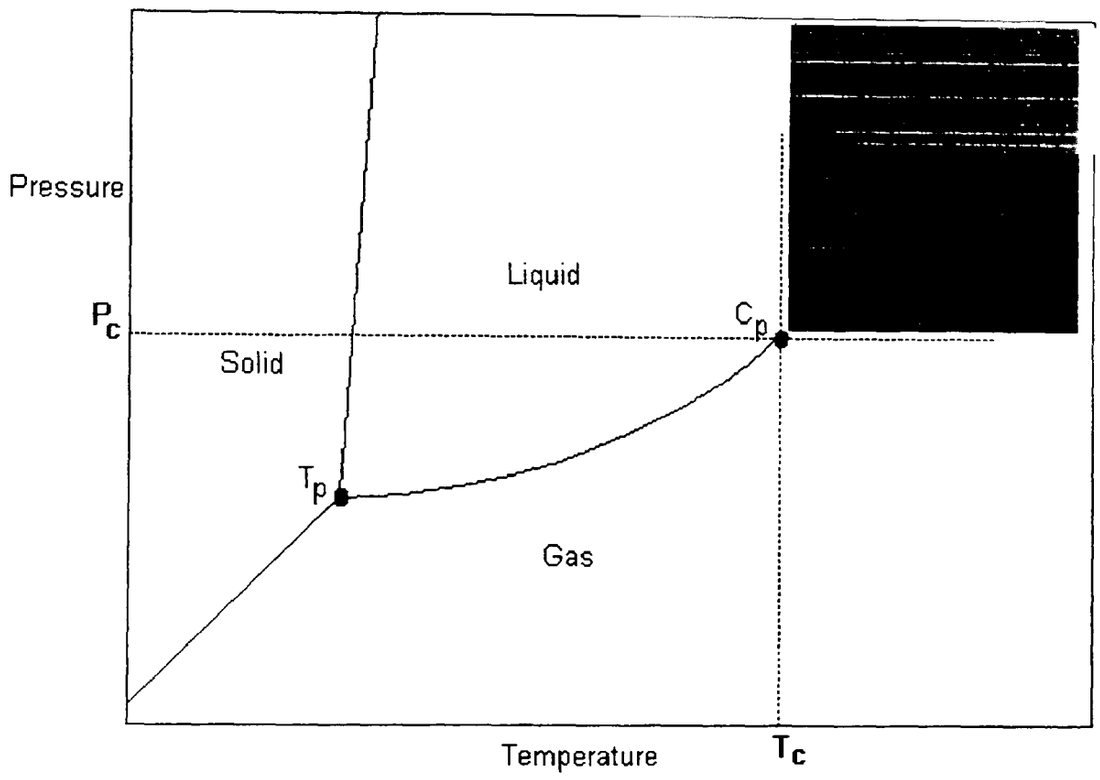


Figure1.1 Phase diagram for a single substance.[19]

The critical point is defined by a critical temperature, T_c, and a critical pressure, P_c, above which the substance is neither a gas nor a liquid, but has properties of both. At temperatures and pressures above the critical point the substance does not condense or evaporate to form a liquid or a gas, but is a fluid with properties changing continuously from gas-like to liquid-like as the pressure increases. Equilibrium conditions between gas and liquid are represented by a solid black curve in Figure 1.1. Below the T_c and P_c gas and liquid states co-exist whenever the pressure and temperature conditions are such that they are in equilibrium. At the critical point (Cp)

the densities between the two phases become identical and the distinction between gas and liquid no longer exists, and the substance becomes a supercritical fluid.

The physical and chemical properties of supercritical fluids, such as solvating power, diffusion and viscosity are all a function of density. At constant temperature, the density and hence the solvent strength increases as the pressure increases. The greater the density then the greater the solvating power of the supercritical fluid. The density changes at a maximum rate near the critical point. In addition the solubility depends on density and increases with pressure. Small pressure differences have a marked effect on density close to the critical point. While raising pressure increases the density, raising temperature decreases the density. Figure 1.2 shows the density isotherm for carbon dioxide at several temperatures.[19]

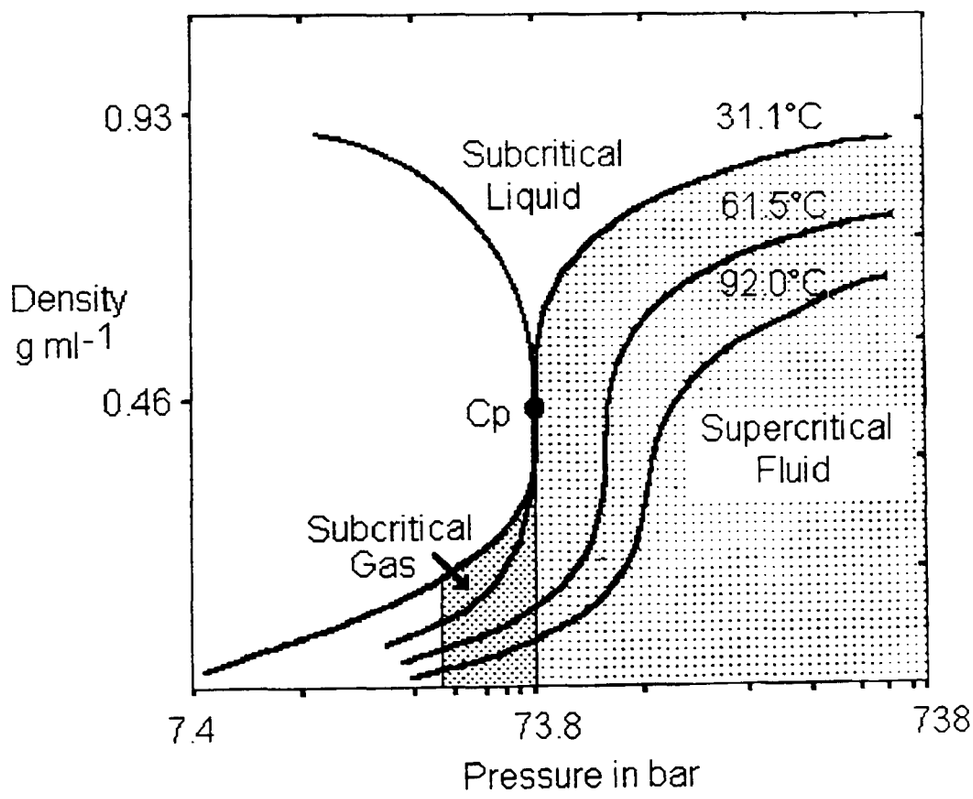


Figure 1.2 Phase diagram for carbon dioxide at several temperatures.[19]

In the region allocated just above the critical pressure, relatively small changes in pressure result in a fairly large changes in density. For example, at 35°C an increase

in pressure of only 20 bar from 80 to 100 bar will result in a density increase from 0.451 g ml^{-1} to 0.725 g ml^{-1}. However, a further increase of 100 bar results in a much smaller density increase, and carbon dioxide at 35°C and 200 bar has a density of 0.872 g ml^{-1}. However the rate of increase in density is less for higher temperature isotherms. This is because the density of a supercritical fluid is function of both pressure and temperature.

Table 1.1 Physical Property values for gases, supercritical fluids and liquids.[20]

	Density (10^3 kg m^{-3})	**Viscosity (mPa. s)**	**Self-diffusion Coefficient (10^4 m^2s^{-1})**
Gas 30°C,1 atm	(0.6-2) x 10^3	(1-3) x 10^2	0.1-0.4
Supercritical fluid			
Near T_c P_c	0.2-0.5	(1-3) x 10^{-2}	0.7 x 100^{-3}
Near T_c, $4P_c$	0.4-0.9	(3-9) x 10^{-2}	0.2 x 10^{-3}
Liquid 30°C,1 atm	0.6-1.6	0.2-0.3	(0.2-2) x 10^{-5}

Table 1.1 shows some approximate values of important properties: density, viscosity, and diffusion coefficients. The diffusivity of a supercritical fluid is somewhere between that of a gas and a liquid, the viscosity is similar to that of a gas while the density is close to that of a liquid.

The high diffusivity of a supercritical fluid allows it to penetrate porous materials, while the liquid-like densities enable supercritical fluids to dissolve analytes from a solid matrix. The density of a supercritical fluid is easily controlled by pressure and

temperature enabling the solvation characteristics of a supercritical fluid to be tailored for particular applications. In addition, their low viscosity provides favourable flow characteristics aiding mass transfer of analytes from the extraction cell to the collection system.

Table 1.2: Critical parameters of selected substances useful as supercritical fluids.[20]

Fluid	T_c (°C)	P_c (Atm)	Density (g/ml)
Carbon Dioxide	31.1	72.9	0.47
Nitrous Oxide	36.5	71.7	0.45
Ammonia	132.5	112.5	0.24
n-Pentane	196.6	33.3	0.23
n-Butane	152.0	37.5	0.23
n-Propane	96.8	42.0	0.22
Sulphur Hexafluoride	45.5	37.1	0.74
Xenon	16.6	58.4	1.10
Methanol	240.5	78.9	0.27
Water	374	227	0.0.34

The two parameters which are important when considering the selection of supercritical fluid are the critical temperature and pressure. Ideal supercritical fluids should possesses low T_c and P_c allowing them to be used with commercially available pumps and ovens. Their usefulness is determined by their critical parameters, solvating properties, toxicity and availability. Carbon dioxide is unique amongst the list of supercritical fluids shown in table 1.2 for effective supercritical fluid extraction or chromatography. Its low critical temperature allows extractions to be carried out at low temperatures, thereby permitting extractions to be performed on thermally labile compounds. Modest compression of carbon dioxide produces a profound change in its

fluid density as shown on figure 1.2 due to the high non-ideality exhibited by this fluid. It is also non-flammable, non-toxic and easy to dispose of, properties which facilitate its use in a laboratory environment.

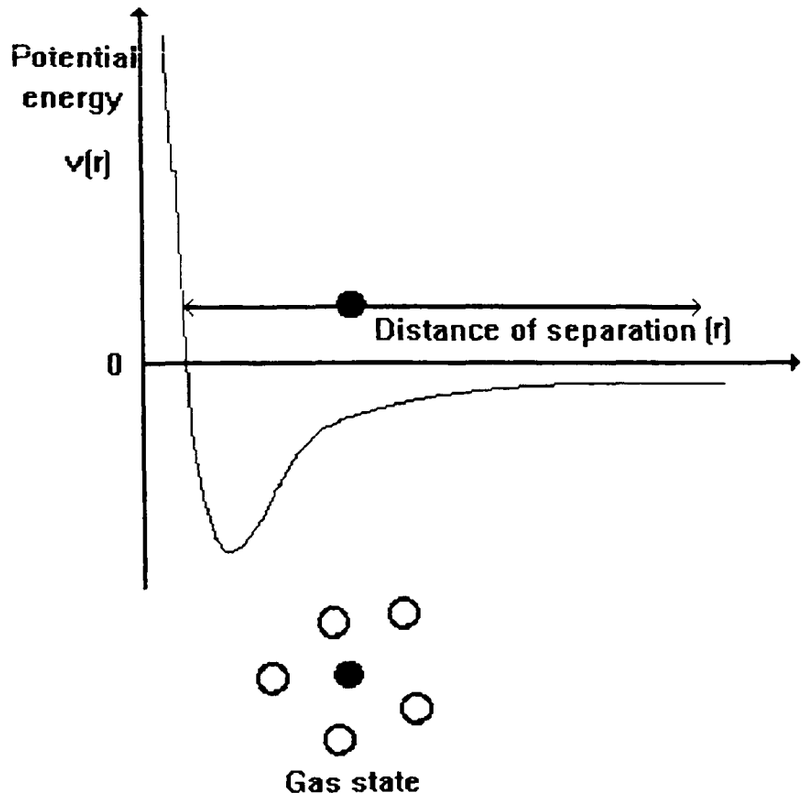
1.3 Fundamental Properties of Supercritical Fluids Relevant to Extraction and Chromatography

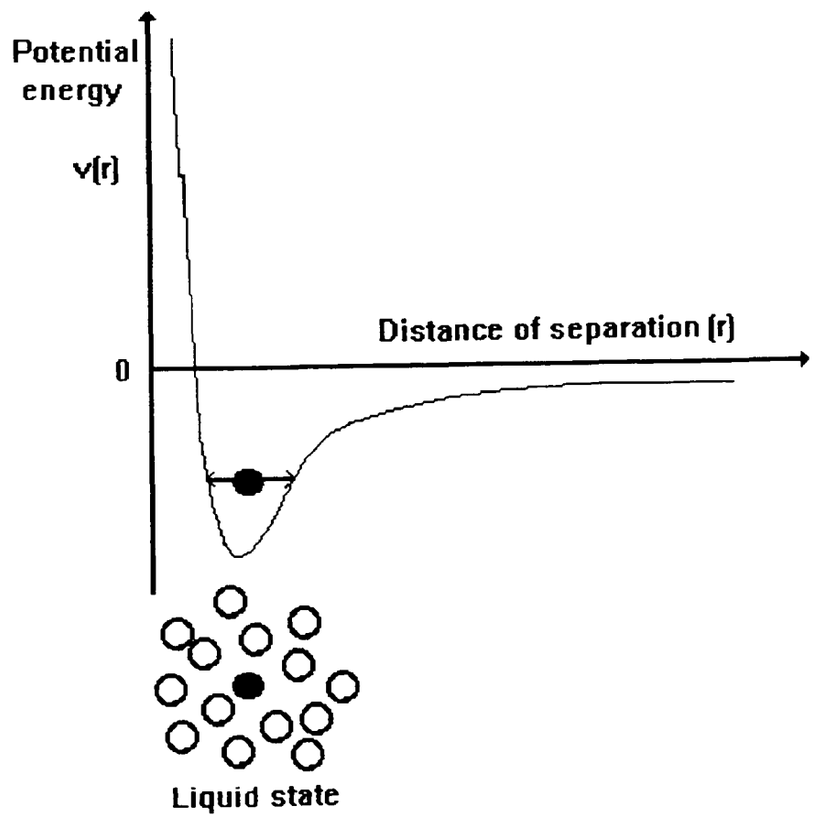
1.3.1 Introduction

The principal factor that governs the properties of a solvent can be explained in terms of intermolecular forces. The power of such forces strongly depends on the distance between molecules. In a liquid solvent, the density of the liquid is constant; the molecular distances change so little that only a little change in solvent properties results. In the case of a supercritical fluid, the kinetic energies of molecules are high due to a higher temperature than the critical temperature. The molecular distances and hence the density can be controlled mechanically by controlling the pressure from a gas-like value to a liquid-like value without condensation to liquid. This unique behaviour enables the properties of a supercritical fluid to be controlled. [21]

1.3.2 Supercritical Fluids as Solvents

A supercritical fluid must have solvating power if it is to be used as an extraction solvent or a chromatographic mobile phase. The solvating power of a supercritical fluid is enhanced when it is compressed; the density of the fluid approaches that of a liquid. Figure 1.3 demonstrates the behaviour of molecules in gas, liquid and supercritical states in terms of the intermolecular potential and the average molecular energy.[21]


Potential
energy
v(r)
Distance of separation (r)
0
Gas state


Potential
energy
v(r)
Distance of separation (r)
0
Liquid state

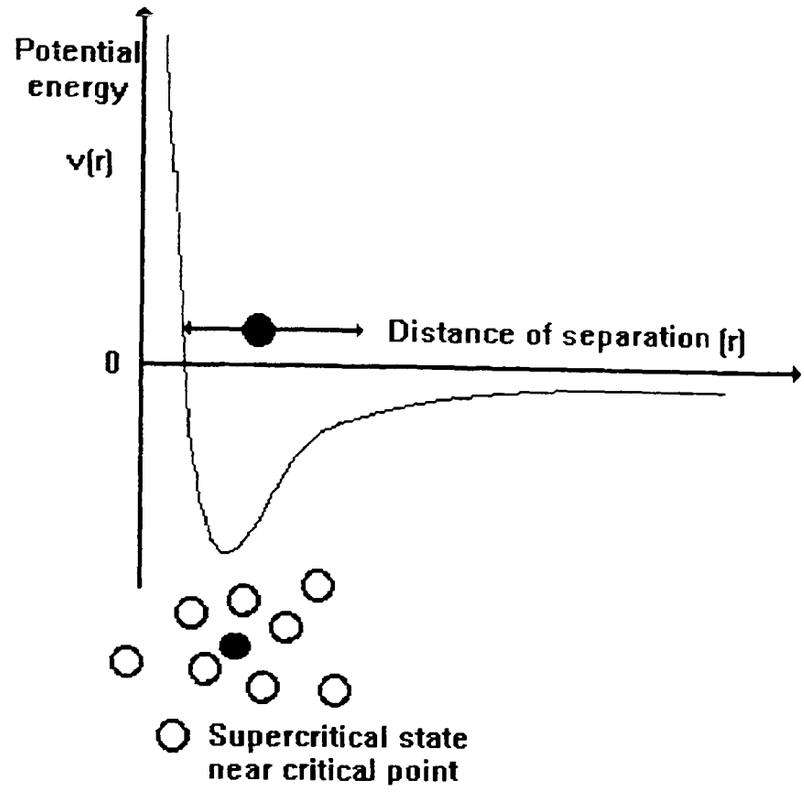


Figure 1.3 The behaviour of molecules in gas, liquid and supercritical states in terms of the intermolecular potential and the average molecular energy.[21]

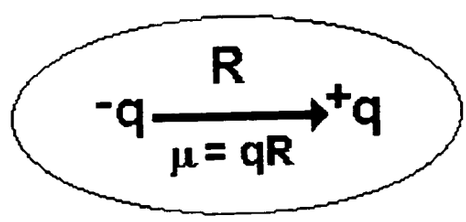
An energetically lower state is preferred at low temperatures. In the liquid state at low temperatures, each molecule feels the attractive intermolecular potential. Most molecules are then trapped in the potential well. The depth of the potential well is usually larger than the average kinetic energy per molecule, moving around only in a small region surrounded by adjacent molecules. On the contrary, in the gaseous state, most molecules at high temperatures can move freely over the attractive "potential well" due to the large average kinetic energy resulting in expanding the free volume of the system.[21]

In the supercritical fluid state near the critical temperature, some molecules can move freely and some are trapped to form weak clusters. This is due to the kinetic energies of each molecule fluctuating around the average value. Clusters are formed when the molecular kinetic energy is smaller than the attractive energy between adjacent

molecules. These clusters also rapidly change in size and constitution due to molecular collisions. When a solute molecule is introduced into the supercritical fluid and the solute-solvent attractive interaction is larger than the solvent-solvent interaction, the solute molecule is surrounded by the solvent molecules to form a cluster. This is due to the attractive potential energy surrounding the solute molecule being larger than the average kinetic energy of the solvent (supercritical) molecules. The formation of clusters around a solute molecule is considered a major cause of enhanced solubility in supercritical fluids.

1.4 General Solubility Theory

When two charges q and -q are separated by a distance R, they constitute an electric dipole moment, μ. A polar molecule is a molecule with a permanent electric dipole moment that arises from the partial charges on atoms linked by polar bonds. Non-polar molecules may acquire a dipole moment in an electric field on account of the distortion the field causes in their electronic distributions and nulear positions. Similarly, polar molecules may have their existing dipole moments modified by the applied field.[19]



Dipole moments can reveal important information through their role in intermolecular forces and in their contribution to the ability of a substance to act as a solvent for ionic solids. When a polar solvent such as water dissolves an ionic solute it plays two roles: Firstly, strong attractive electrostatic forces (to opposite charged ions) reduce the free energy of the system, and secondly there is a reduction in the coulombic interactions between the ions in the solution. When a molecule with no permanent

dipole moment is surrounded by a polar solvent the attractive forces do not exist and the molecule is said to be insoluble. In other words the attraction of water to itself is much grater than to non polar molecules and as a result water squeezes out the non polar molecules.

1.4.1 Intermolecular Forces

The electrostatic interactions between molecules can often be described in terms of the van der Waal forces of attraction. These attractive forces can be sub-divided into:

1. The London, or "dispersion" force between non-polar atoms and molecules as well as polar molecules.[19] These forces are the weakest of all chemical forces of attraction. In the dispersion interaction, an instantaneous dipole on one molecule induces a dipole on the other molecule, and the two dipoles then interact to lower energy. They are dependent on temperature and increase with the molecular volume as well as the number of electrons. Dispersion forces are responsible for the condensation of the nonpolar substances into liquids and solids as well as for the solubility of nonpolar substances into nonpolar liquids.

2. Dipole/dipole interactions: The Keeson or "orientation" force, which exists between any pair of polar molecules each possessing permanent dipoles.[19] These interactions are responsible in part for the association of polar liquids and also explain the condensation of polar substances from the gaseous state to liquids and solids at low temperatures.

3. Dipole/induced-dipole interactions: The Debye, or "induction forces" which exist between a molecule with a dipole moment and any other molecule by virtue of the dipole induced in the second molecule by the permanent dipole.[19] The strength of the

interaction depends on the dipole moment of the polar molecule and the polarizability of the second molecule.

The total attractive interaction energy between molecules is therefore the sum of all three contributions discussed above. It should be noted here that because carbon dioxide is a symmetrical molecule it does not possess a permanent dipole moment. It does however have a permanent quadrupole i.e. two dipole moments back-to-back. This makes an important contribution to the intermolecular forces, but operates over a shorter range than dipole-dipole interactions.

1.4.2 Repulsive Forces and Total Interactions

When molecules are squeezed together, the nuclear and electronic repulsions and the rising electronic kinetic energy begin to dominate the attractive forces.

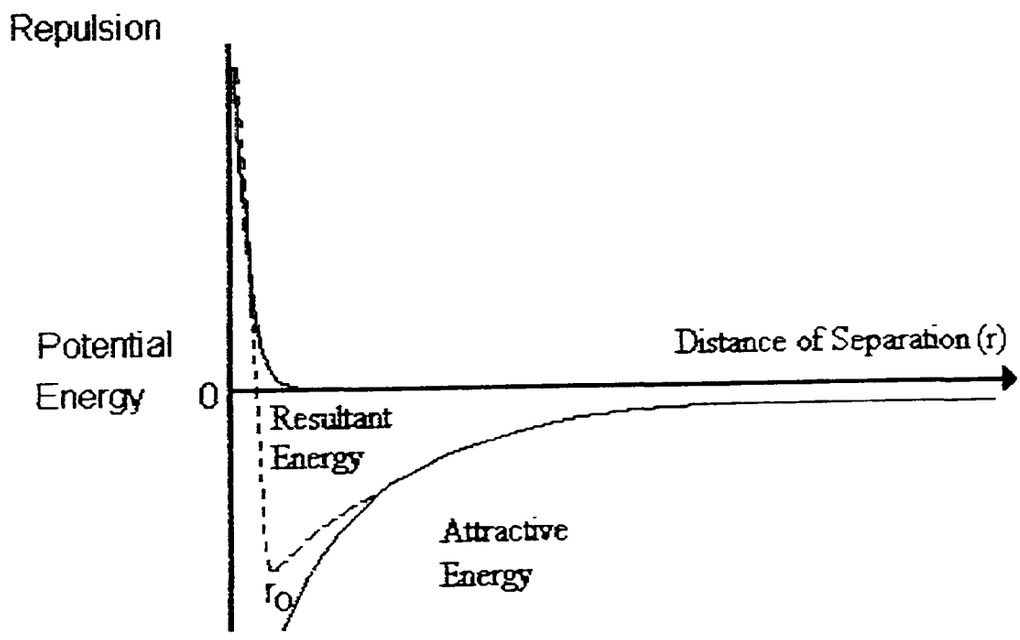


Figure 1.4 is the Lennard Jones expression plot of repulsive and attractive terms.[19] The sum curve (dashed line) has it's minimum at r_0, which is equivalent to the nearest two molecules can approach without being repelled from one another.

As well as van der Waal forces of attraction there are Coulombic forces of attraction which exists between ions. These forces are proportional to the charges on the ions, and inversely proportional to the distance between them. Coulombic forces are omnidirectional and can operate over a longer range than other intermolecular forces.

1.4.3 Solubility Parameter Theory

This theory is based on the equation of state proposed by Van der Waals. The equation was derived as an approximation for non-ideal gases taking account of both the attractive and repulsive forces between particles.[19] The repulsive forces assume that the particles behave as small but impenetrable spheres. As a result an approximate volume (nb) is given to the particles. Thus, the perfect gas law can be modified to.[19]

$$P(V - nb) = nRT \qquad 1.1$$

Where P is the pressure, V is the volume, T is the temperature, n is the amount of substance and R is the gas constant.

The attractive forces hold the particles together which results in an overall reduction in pressure. This reduction of pressure is determined by the frequency of collisions with the walls and the impulse of each collision. The actual pressure drop is approximately proportional to the square of the density. Therefore, the reduction of pressure can be written as $a(n/V)^2$, where a is a constant characteristic of each gas. Van der Waals equation of state is,

$$P = nRT \Big/ (V - nb) - a(n/V)^2 \qquad 1.2$$

or when expressed in terms of molar volume $V_m = V/n$,

$$P = RT/(Vm - b) - a/V^2 n \qquad 1.3$$

a/V_m^2 is the internal pressure of the gas.

The method for estimating the solubility of a solute in a supercritical solvent applies the van der Waal equation of state for non-ideal gases as its foundation.

Solubility parameter was first introduced by Hildebrandt and Scott[22] for a regular solution. It is expressed in terms of the cohesive energy density c, leading to the following equation;

$$\delta = \sqrt{C} = \left(\frac{\Delta E_v}{V}\right)^{0.5} = \left[\frac{D(\Delta H_v - RT)}{M}\right]^{0.5} \qquad 1.4$$

Where, ΔEv is the energy of vaporisation, V is the molar volume, ΔH_V is the heat of vaporisation , M is the molecular weight of the solute or the fluid and D is the density.

The Hildebrandt Solubility parameter theory applied to a supercritical fluid cannot relate to the energy of vaporisation (ΔEv) as vaporisation does not take place under supercritical conditions. Using the cohesive energy density from van der Waals equation (note that the cohesive energy density is approximately equal to the enthalpy of vaporisation per unit volume), and assuming that the solubility parameter for a liquid and a gas at liquid density are similar, Giddings extended this theory to supercritical fluids by the following equation:

$$\delta = 1.25 P_c^{0.5}\left[\frac{\rho}{\rho_{liq}}\right] \qquad 1.5$$

Where P_C is the critical pressure, ρ is the supercritical fluid density and ρ_{liq} is the liquid density of the gas.

If the liquid's solubility parameter is also known then the above equation can be simplified to,

$$\delta = \delta_{liq}\left[\frac{\rho}{\rho_{liq}}\right] \qquad 1.6$$

The reduced solubility parameter is a term which relates the solubility parameter of the solute to that of the supercritical solvent and takes the form,

$$\Delta = \frac{\delta}{\delta_o} \qquad 1.7$$

where δ is the solubility parameter of the supercritical fluid and δ_o is the solubility parameter of the solute.

Solubility parameter theory provides an estimation of solute solubility in a supercritical fluid. It is fairly simple to use and can give estimates quickly. To solubilize a solute compound, the solubility parameter of both the solute and the solvent need to have similar values. Therefore, if a supercritical fluid is to be used as a solvent, the pressure of the fluid must be higher than the critical pressure, where the density becomes similar to that of liquid.

1.5 Theory of Dissolution Relating to Analyte Solubility in Supercritical Fluids

The dissolution process can be explained by two endothermic and one exothermic processes:[23]

1. The solid solute molecules interact with neighbouring molecules. For dissolution the molecules are separated into isolated component particles. The energy for this endothermic process is called the lattice energy and is smallest when the solute molecules are non-polar. The energy generally increases in the order of increasing intermolecular force: non-polar substances< polar substances< hydrogen-bonded substances < ionic substances.

2. The solute component particles are separated from each other and enter the solvent. The solvent molecules interact with each other, endothermic energy is necessary in order to form a cavity to accommodate the solute molecules in the solvent. The energy

necessary for this process is also larger in the order of increasing intermolecular interaction in the solvent: non polar solvents < polar solvents < hydrogen-bonded solvents. At the same time, the energy is also larger when the volume of the solute molecule is larger. This is due to more intermolecular bonds between the solvent molecules being broken in order to accommodate more room.

3. The solute molecules once dispersed in the solvent then interact with the neighbouring solvent molecules and the interaction is exothermic. The energy released is larger in order of increasing interaction: both solvent and solute molecules are non polar < one is non-polar and the other is polar < both are polar < the solute particles are solvated by the solvent molecules.

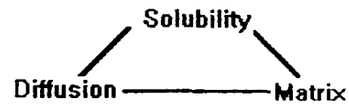
The overall enthalpy change is exothermic if the energy loss in the first and second steps is smaller than the energy gain in the third step. Thus when the solute particles are strongly bonded to each other, the solute is only soluble in a solvent in which the solute-solvent interactions are large. When solvent molecules are highly associated to each other, the solute dissolves well in solvent that will result in strong solute-solvent interactions after the component particles are dispersed.

On the basis of the above we can rationalise the solubility of organic molecules in supercritical carbon dioxide as follows:

Polar molecules tend to have low solubility in supercritical carbon dioxide because the energy required to isolate individual molecules (i.e. the lattice energy) is relatively high and is not compensated by solute-carbon dioxide interaction as these are relatively low (CO_2 being only able to interact by dispersion forces and dipole-dipole interactions). The formation of a cavity in the CO_2 is a relatively low energy process due to the relatively weak interaction between CO_2 molecules. Hence non-polar molecules will have high solubility in supercritical carbon dioxide.

1.6 Basic Principles of SFE

There are three controlling factors that governs the rate of extraction in supercritical fluid extraction (SFE), as can be seen in the following triangle:[24]



Firstly the solute must be reasonably soluble in the supercritical fluid for the extraction to be successful. This is particularly true at the begining of the extraction where the rate of extraction is much higher. Control of solubility allows stepwise extraction to be carried out. Secondly, the solute must be transported efficiently by diffusion from the interior of the matrix. Thirdly, the matrix effect, which means that analytes are locked into the structure of the matrix or strongly bound to the surface of the matrix. The rate of removal from the matrix using SFE is a function of its solubility in the fluid media and the rate of mass transport of the solute out of the sample matrix.

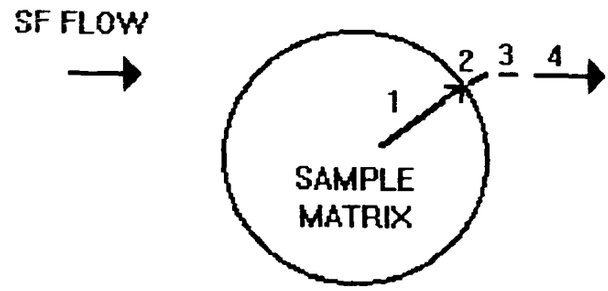
To understand the impact of matrix on extraction rates, four major mass transport mechanisms should be considered (figure 1.5):[20]

analyte diffusion through the internal volume of the sample

surface desorption of the analyte

diffusion of the analyte through a surface boundary layer

transport in the bulk supercritical fluid phase



1 = Diffusion through matrix

2 = Desorption from the surface

3 = Diffusion through 'SF' surface

4 = Transport in the SF flow

Figure 1.5 Mass transport steps for the SFE of an analyte from a porous matrix particle

The diffusion process may simply be normal diffusion of the solute or it may involve diffusion of the fluid into the matrix. It should be noted that some matrices swell on exposure to supercritical fluids, thus facilitating the mass transport of the analyte from within a sample matrix.

Surface desorption of an analyte by a supercritical fluid is often an important step in SFE involving difficult sample matrices. For certain analyte-matrix combinations, the solvent power of a supercritical fluid may not be sufficient for a rapid or complete extraction. The use of a cosolvent is often required for aiding acceleration of desorption of an analyte from the surface of the sample matrix. Diffusion of the analyte through a surface boundary layer can also influence analyte extraction. Many solid samples will promote condensation of a surface layer of the dense extraction fluid at the fluid-solid interface. The density of the adsorbed surface film will partly depend on the pressure applied to the supercritical fluid. The development of a condensed fluid film at the surface of the sample matrix can aid recovery of certain analytes through competitive adsorption at the sample interface as well as inhibit the

transport of the analyte into the fluid phase. Finally, the transport of the analyte in the bulk fluid phase is governed primarily by the diffusion coefficient of the analyte in the fluid medium. As mentioned previously, the diffusion coefficient of solutes in supercritical fluids are intermediate between those that they exhibit in liquid and gaseous media. This factor is independent of the sample matrix.

1.7 Kinetic Model for SFE

In the dynamic mode of extraction, the volume of the supercritical fluid is above the solubility limits of the analytes being extracted. In situations like this the rate of diffusion out of the matrix is rate limiting. This fact has become the basis for kinetic models of SFE. The most recent models proposed are the 'hot-ball model' by Bartle *et al.*[25] (1991) and the model proposed by Pawliszyn (1993) based on mass transfer chromatographic theory.[26]

The Hot-Ball Theory

The hot-ball theory derives its name from the analogy of the mathematical solution of dropping hot spherical balls into cold water. The model ignores solubility limitations and assumes that the matrix particles are uniform spheres. The distribution of the solutes through the matrix at the beginning of the extraction is also assumed to be uniform. Assuming the above conditions, the model requires that differential Fourier equations[27] must be solved.

The hot-ball theory predicts that the natural logarithm of the mass of solute in the sample matrix after the extraction (m) divided by the initial mass (mo) of solute in the sample matrix versus time should have the relation derived in Figure 1.6. [24]

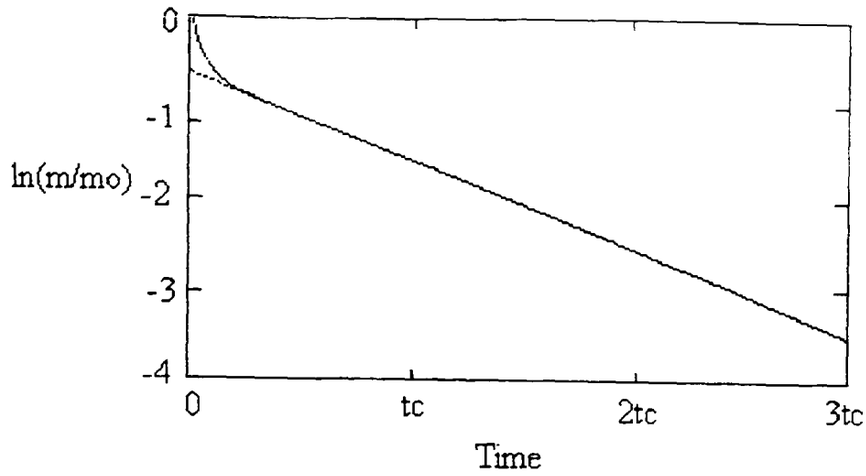


Figure 1.6 Theoretical curve for the hot-ball SFE model.[24]

There are two characteristic parameters on the curve. The first is the time which is related to the radius of the sphere by Equation 1.8, where r is the radius and D is the diffusion coefficient. The second is the intercept of the linear portion, -I, which has a value 0.5 (actually -0.4977) for a sphere.

$$t_c = r^2 / \pi^2 D \qquad 1.8$$

The graph takes the form of a straight line with slope $-1/t_c$ with the linear portion of the graph beginning at $0.5t_c$. The initial non-linear part of the graph is explained by extraction from mainly the outer part of the spherical matrix. When there is no more solute on the surface the extraction becomes an exponential decay. This model has been used to predict the time required for total solute extraction for several matrices,[28] although deviations from the theoretical curve have been observed particularly with respect to the intercept, -I. Bartle *et al.*[25] have attributed these discrepancies to irregular spheres and non uniform solute distributions found in sample matrices. The hot-ball theory has been adapted successfully for thin films[29] where the geometry of the matrix is known.

Chromatographic Mass Transfer Kinetics

The Pawliszyn model[26] for the extraction of analytes is based on well established mass transfer kinetics, the chromatographic elution process and convolution theory. Assuming a matrix particle consisting of an organic layer on an impermeable core, and that the analyte is adsorbed onto the surface of the core, the model considers the individual mass transfer steps involved in transporting the analyte from the core into the bulk supercritical fluid. Each of the mass transfer steps is described individually and related to their contribution to the height equivalent to one theoretical plate (HETP).[30] Pawliszyn's model uses five different contributions in calculating the overall HETP. The selection of the individual components is designed to reflect the extraction process as accurately as possible using the available theory. Therefore, there is a contribution to account for slow desorption kinetics from the solid core. Also there is a contribution for diffusion through the organic part of the matrix. . The analyte migrates in and out of a pore structure of the matrix during the elution. This can be described as resistance to mass transfer in the fluid, associated with the porous nature of most matrices. Resistance to mass transfer is also the fourth contribution, and accounts for eddy diffusion caused by the random pathway of analytes flowing through the sample. The final contribution is from an obstruction factor which is characterised by the structure of the matrix.

Chapter 2.0 Application of Supercritical Fluid Extraction and Supercritical Fluid Chromatography in the Pharmaceutical Industry

2.1 SFE in the Pharmaceutical Industry

Supercritical fluid extraction (SFE) is widely perceived as a technique for the extraction of low to moderately polar compounds. The application of SFE as an alternative to distillation and conventional solvent extraction has been considered by many industries including the food, petroleum, synthetic fuel and environmental industries.[31-40] With increasing concern over the use of chemical solvents in the manufacture of pharmaceuticals, as well as the need for high quality products, alternatives to energy intensive and costly extraction schemes have been sought. Pharmaceutical compounds are usually: a) highly polar and present at trace levels, b) non-volatile and standards for recovery and reproducibility are rigorous, and c) matrices are usually quite active and may contain co-extractives. The currently used sample preparation techniques for analysis include solid phase extraction, liquid-solid extraction, Soxhlet extraction, and liquid-liquid extraction. Recent studies have shown that the use of supercritical fluids as an extraction medium provides a powerful alternative to traditional extraction methods.[41-42] Efficient and selective isolation of specific analytes from complex matrices can often be accomplished by using supercritical fluids for extraction. The benefits of SFE include faster analysis and reduction in sample handling. Due to the high solute diffusivity in the less dense supercritical fluid compared to a liquid, the extraction is faster. Other advantages of SFE for pharmaceutical compounds include protection from degradation by light, heat, or oxygen; high loadability of samples and possibility of trace analysis; elimination of hazardous and/or expensive solvents; and, analysis of aqueous samples (serum, urine, and saline solution) without introducing organic solvents. The gentle extraction conditions used in SFE compared to more traditional extractions such as Soxhlet extraction, also provide greater assurance against chemical reaction not taking

place during the extraction. This ensures that isolated analytes are representative of the original sample. Carbon dioxide is the most popular choice for extraction of target analytes from solid or liquid pharmaceutical matrices. Although carbon dioxide is a non-polar molecule, the lone pairs of electrons on the oxygen atoms (quadruple moment) allow the solvation of many polar compounds. Polar organic solvents can be added to carbon dioxide as entrainers for enhancing extraction efficiencies. However, it should be noted that the solubility of analytes in the supercritical fluid or modified supercritical fluid does not always ensure its extractibility. The location of the analyte in or on the matrix is critical, and the interaction of the analyte with active sites on the matrix must be disrupted. These features can greatly lengthen the extraction time, require higher extraction temperatures and higher modifier concentration. In addition to carbon dioxide, other supercritical fluids such as nitrous oxide can also be used as extraction fluids. Other than the nature of the extraction fluid, operating conditions and parameters of extraction also influence extraction efficiencies. These include extraction density, extraction temperature, extraction time, fluid flow rate, the inertness of the internal surface of the system, as well as the arrangement and conditions of solute trapping. A cold solute trap is usually used in SFE in order to enhance the efficiency of trapping the extracted analytes.

The following sections represent a review of the current literature available on the SFE of pharmaceuticals.

2.1.1 Spiked Animal Feed

In the pharmaceutical industry, long-term feeding studies of chemical substances involves incorporating the target analytes into animals feed prior to administration. Analytical methods are required to monitor the dose level, to verify dosage uniformity throughout the sample matrix and to test for analyte stability in the feed as part of toxicological investigations. Schneiderman et al.[43] and Messer and Taylor[44] were

among the first to report on the applicability of using SFE as a quantitative method for extracting drugs from animal feed matrices. Locke reported extracting menadione (Vitamin K3) from spiked rat chow using supercritical carbon dioxide at 8000 psi and 60°C. Quantitative extraction was achieved within 20 minutes. Collection of the analyte was achieved by trapping the expanding supercritical carbon dioxide into a 6 x 0.25 in i.d. stainless steel tube filled with silica gel. The silica gel was subsequently washed with 10 ml methylene chloride. Menadione was determined in the extract without any cleanup using reversed phase HPLC with reductive mode electrochemical detection. The lowest concentration of menadione extracted quantitatively was 20 µg/g of feed. An average recovery of 90.5% with 2.2% RSD was reported at the 1 mg/g level.

Messer and Taylor[44] reported the applicability of extracting a hypolipidermic drug, 4- trifluoromethyl-2-biphenyl carboxylic acid quantitatively from a spiked rat feed matrix at a level of 1 %. Extraction was performed with supercritical carbon dioxide off-line and solid phase trapping followed by solvent rinsing was utilized. An optimized method for quantitative extraction of the pure drug was initially developed with high reproducibility. The parameters investigated for extraction of the pure drugs were as follows: the density of CO_2, the number of CO_2 thimble volumes, collection trap (C-18 vs stainless steel) and rinse solvent (50% acetonitrile / 50% water and 100% acetonitrile). The studies were carried out in triplicate with 100% CO_2 and the trap temperature, the chamber temperature and the nozzle temperature were kept constant. Triplicate 100 mg samples of spiked animal feed containing 1% drug were next extracted using the optimized extraction conditions. The average recovery obtained for these extractions was only 68.8% with a RSD of 20.6%, despite the fact that acceptable recoveries and RSDs were obtained with the pure drug. The results obtained lead to the authors believe that the problem was with the sample itself rather than the poor solubility or extractibility of the analyte in the supercritical fluid. To investigate the inhomogeneity in the "crystalline matrix" causing the poor

reproducibility, the animal feed was spiked with the drug in a different manner. Rather than mixing the drug mechanically as a crystalline substance on a large scale, the drug was first dissolved in methylene chloride and a slurry with the pure ground feed was prepared. The slurry was then mixed, allowed to dry and was used as the "solvent matrix". The dried solvent matrix was then thoroughly mixed. Triplicate 100 mg samples of the "solvent matrix" were extracted using the optimized extraction conditions. The average recovery obtained for these extractions was 84.0% and the RSD of 2.09%. This study showed that the "crystalline matrix" was not homogeneous and since the matrix size was only 100 mg, the sample for each replication was not always representative. The authors concluded that the less than quantitative recoveries may reflect a matrix effect which might be eliminated with the addition of a modifier in the extractant phase, where the modifier function would be to compete with the drug for the active sites of the matrix. In this particular example of SFE of a polar drug from an animal matrix, the use of a modifier such as methanol would probably have resulted in complete recovery.

Messer et al.[45] reported routine extraction of atovaquone [trans-2-[4-(chlorophenyl) cyclohexyl]-3-hydroxyl-1-2-napthoquinon] from rat feed. Initial experiments involved extraction of atovaquone from an inert Celite matrix to establish the extraction profile of the drug in the absence of the matrix. Specifically, after increments of time the dynamic extraction was interrupted, the collection trap was rinsed and the resulting solution was analysed. Spiked test samples were prepared by spiking 0.20 ml of a 1.00 mg/ml solution of atovaquone in methylene chloride onto a Celite bed inside the extraction cell. The samples were left overnight at ambient conditions to allow solvent evaporation. The atovaquone spiked samples were then subjected to a five step extraction. Supercritical carbon dioxide was used as the extraction solvent at a flow rate of 2 ml/min throughout the extraction protocol. The pressure was held at 350 bar and oven temperature at 50°C producing a supercritical fluid density of 0.9 g/ml. The trap consisted of stainless steel spheres (100 μm diameter) held at 5°C during

extraction and 40°C during rinsing. The temperature of the variable restrictor was kept constant at 55°C during extraction and rinsing. The stainless steel trap was rinsed with 1 ml of acetonitrile for the first 4 steps and 3 ml of acetonitrile for the last step. The five steps of extraction carried out were as follows: Step 1. Equilibration (vessel pressurised), 2 minutes; dynamic 2 minutes, steps 2 and 3, dynamic 3 minutes, step 4 dynamic, 4 minutes; step 5 dynamic, 8 minutes. This gave a total dynamic extraction time of twenty minutes. The results showed a recovery of 67.1 % in the first step and an additional recovery of 16.7% in the second step, giving >80% recovery of avequone in the first five minutes of the extraction. The total recovery obtained for the extraction profile was 95.4% indicating high solubility of atovaquone in supercritical carbon dioxide in a reasonable amount of time. It also showed that the stainless steel trap was able to hold the analyte during the extraction process and effectively delivered the analyte upon application of the rinse solvent. To study the effect of a modifier, a second set of extractions was performed with 2 % methanol modifier. Experimental conditions were kept the same with the exception of the increasing the trap temperature to 70°C to ensure that the methanol peering in the trap was in a gaseous state. The results obtained under these conditions showed that the extraction recoveries for the combined initial two steps was only 61.8% for methanol-modified CO_2, compared with >80% for the extraction with 100% CO_2. The lower recovery was thought to be due to poor collection efficiency. Consequently, pure CO_2 was used for extracting the spiked drug. Atovaquone was studied at six levels ranging from 0.0335 to 1.1208%. Sample sizes for extraction were either 250 or 500 mg depending on the spike level. Extraction were performed in triplicate and >95% recovery with <5% RSD was obtained for all matrices except for the 0.0335% level, which was 89%. The authors concluded that the SFE results were comparable to conventional extraction results.

Euerby et al.[46] reported the SFE of a novel corticosteroid tipredane at a level of 10 ppm from rodent diet. A number of collection methodologies were investigated by the authors to obtain maximum recovery of the analyte and it was concluded that the

direct collection from the back pressure regulator (BPR) through a short length of tetrafluoroethylene tubing into a calibrated flask containing 1 ml of methanol, stoppered with a small piece of cotton wool to trap any escaped analyte gave the best results. The authors investigated the extraction of the tiperadane with and without the presence of ethanol modifier. The flow rate of ethanol was altered, keeping the CO_2 flow rate constant to determine the optimum polarity of the SF and the optimum collection efficiency. The optimum extraction conditions employed were as follows: pressure, 200 kg/cm^2, oven temperature 70°C, CO_2 flow rate, 3.0 ml/min, ethanol flow rate, 0.3 ml/min, and BPR temperature 40°C and an extraction time of 2 minutes. The authors compared the optimized extraction with the more conventional extraction procedures of Soxhlet and ultrasonic agitation. The average extraction recovery obtained with SFE was 85% with an RSD of 6% compared with 92% obtained from Ultrasound (RSD 3%) and 94% Soxhlet extraction (RSD 6%). The authors concluded that the advantage of SFE is that the extraction is rapid and no preconcentration step was required prior to HPLC analysis. The effect of total flow rate of both CO_2 and ethanol on the extraction recovery was investigated in this particular case. It was found that the maximum extraction reovery (85%) was achieved at a CO_2 flow rate of 3 ml/min and increasing the flow rate further resulted in a loss of extraction recovery. This was probably due to a inefficient trapping of the extracted analyte and complete recovery could probably be achieved with a better collection system.

Williams et al.[47] presented a comparison of supercritical, subcritical, hot pressurized and cold solvent extraction of propanolol, tamoxifen and two experimental drugs, ZM 95527 and ZM 169369 from rodent diet. The experimental conditions employed for the supercritical and subcritical fluid extraction were CO_2-MeOH (85/15 v/v or 83/17 v/v) at 70°C and 17.25 MPa. Recoveries obtained by different extraction methods were compared and it was found that hot, pressurised MeOH at 2.0 ml/min and 70°C was more efficient for the recovery of ZM 95527, less efficient for the recovery of tamoxifen, but not significantly different for the recovery of propanolol and ZM

169369. The recoveries obtained by the subcritical method were intermediate between the SFE and the hot, pressurised MeOH. The authors concluded that although the efficiency of these methods was similar to conventional extraction with acidified MeOH, the precision obtained by the solvent extraction method was superior to the other methods described.

2.1.2 Formulations

This is the largest category of pharmaceutical analysis involving the mostly varied group of complex matrices, for example ointments, syrups, capsules, infusions, creams and tablets. Richter et al.[48] reported the use of chloroform modified CO_2 for extraction of an antihistamine drug from a transdermal patch as a replacement for conventional liquid extraction. In the matrix itself, the drug was suspended in a gel under an adhesive. Quantitative extraction data was achieved with an RSD of 2.7%. A similar study was also reported[49] for the extraction of nitroglycerin from transdermal patches, where CO_2 fluid was modified with tributyl phosphate (75/25, vol/vol). Methanol was used as the collection solvent after extraction at 450 atm with CO_2 at 75°C for 30 minutes. The recovery achieved was 99.1% with an RSD of 2.8%.

Extraction of triamcinolone from dermatological patches was also attempted by Edwardson and Gardner.[50] Extractions were carried out with CO_2 modified with 0, 5 or 10% methanol at 80°C for 5 minutes. Recoveries of only 10% were achieved. However, the authors did not investigate a longer extraction time, different extraction temperature or modifier which may have resulted in improved extraction recovery of triamcinolone. The SFE of misoprostol, a synthetic prostogladin from a hydroxypropyl methyl cellulose dispersion was reported by Roston.[51] Sample components were extracted and subsequently precipitated in the cryofocussing trap during the decompression step. Experimental conditions used were 70°C, CO_2 density

of 0.8 g/ml and extraction time of 4 minutes. Maximum extraction efficiency was reported to be only 65%.

Dean and Lowdon[52] reported extraction of megestrol acetate from a tablet formulation. Preliminary investigations were first performed by extracting the drug from an inert Celite matrix to establish optimized experimental conditions for extraction from the tablet matrix. Optimized extraction conditions were first developed with 100% CO_2 at a temperature of 55°C, density 0.81 g/ml and a flow rate of 2 ml/min. Subsequent extraction from a tablet formulation using the optimized extraction conditions gave a poor recovery. The tablets were ground to aid the rate of extraction. A 160 mg tablet was weighed, ground to a powder and 20 mg sub-samples were extracted over increasingly longer periods at the optimum conditions described previously. The results obtained indicated a maximum recovery of 70.4% after 20 minutes. Further extractions were carried out with 10% modified methanol-CO_2 using the same optimized conditions and a maximum recovery of 92.8% was achieved after 20 minutes of extraction time. The authors also presented a direct comparison of SFE with a United States Pharmacopoeia (USP) monograph method for the analysis of megestrol acetate from the tablet formulation and concluded that the USP method provided the analyst with more precise determinations of megestrol acetate from a tablet matrix.

SFE from tablet matrices was also explored by Anderson et al.[53] All major ingredients from davron tablets were extracted with supercritical carbon dioxide and chromatographed with a coupled SFC system. All major active ingredients were identified along with binders and excepients used in the tablet formulation, but quantitative recoveries were not reported. Ibuprofen tablets were extracted qualitatively by supercritical carbon dioxide (pressure 5000 psi, temperature 50°C) without any excessive grinding by Nair et al.[54]

Anklam and Muller[55] reported extraction of caffeine and vanillin from tablet formulation using supercritical carbon dioxide at 50°C and 350 bar. Tablets were ground to a powder and subsamples of 100 mg were mixed with 1 g of sand, moistened with 100 µl of water prior to extraction. A 5 minute static and 20 minute dynamic extraction time was employed. The recovery achieved for vanillin and caffeine were 97.7 % and 98.5% with an RSD of 3.1% and 1.4%. The authors concluded that the SFE results were comparable to Soxhlet extraction results. Scalia et al.[56] reported the extraction of vitamin A, vitamin E and their esters in tablet preparations using SFE and HPLC. Tablets were powdered and subsamples (100-150 mg) were mixed with sea sand (3 ml) and extracted with supercritical carbon dioxide at 40°C and 200 atm for 15 minutes. Quantitative extraction data was achieved (> 95.6%) with an RSD of 1.7% and 3.9% for Vitamins A and E, respectively. The authors concluded that the SFE method was superior to the existing standard method. Howard et al.[57] successfully demonstrated the application of SFE as a sample preparation method for felodipine tablets. Static/dynamic mode SFE together with CO_2 with 8.7% MeOH modifier was used for extraction. The recoveries obtained by this method (98.6%) were comparable with traditional liquid extraction method (99.7%) and similar levels of drug degradation were observed for both methods. Lawrence et al.[58] demonstrated the application of SFE for the extraction of several benzodiapines (alprazolam, clorazepate, chlordiazepoxide, diazepam, oxezepam, tamazepam,triazolam) in solid dosage form. One quarter of a ground tablet or capsule sample of various weights was dispersed in 450 mg of Hydromatrix and extractions (5 minute static and 10 minute dynamic) were carried out with CO_2 modified with 2% MeOH at pressure 100 atm and temperature 65°C. The eluate was collected in 500 µl of MeOH which was evaporated to dryness before dissolution of the residue in 50 µl of MeOH. All dosage form extracts were free from matrix components and were analysed by GC-MS. Extraction yields which were sufficiently high to allow GC-FTIR analysis were only obtained from the dosage forms containing >1 mg of benzodiapeine.

Moore and Taylor[59] successfully demonstrated the application of SFE for the extraction of polymyxin B sulphate from cream and ointment matrices. Cream (200 mg) was extracted at 55°C and 300 atm for 45 minutes with 5% MeOH modified CO_2 (2 ml/min). For the ointment, a pressure of 450 atm and temperature 60°C were employed at the same flow rate. Average recoveries reported for the cream and the ointment were 108% and 136% with an RSD of 5% and 1.9%, respectively. Messer and Taylor[60] investigated the application of inverse analytical SFE and HPLC for the determination of acylvoir from 5% Zovirax ointment. The ointment sample (100 mg) was placed between the frits of a SPE tube, and the tube was subsequently transferred to the extraction cell. The extraction cell was kept at a temperature of 50°C and CO_2 modified with 2% MeOH was used as the extraction fluid at 0.9 ml/min to extract the hydrocarbon-based ointment base. Two portions of 0.01 M-NaOH were used for the ultrasonic extraction of the acylvor active component from the inner vessel. An average recovery of 99% with RSD 5.3% was reported for ointment containing at the 50 mg/g of acylvoir.

Mulchey et al.[61] reported the application of SFE for direct extraction of polar active ingredients from liquid matrices. The investigation involved the extraction of sulfamethazole and trimethoprim from Septa infusion. The active ingredients of Septa infusion contained 16 mg of trimethoprim and 80 mg of sulfamethazole per ml of infusion. The active components are dissolved in 40% propylene glycol, 10% ethyl alcohol, and 0.3% diethanolamine with 1% benzyl alcohol and 0.1% sodium metabisulphite added as preservatives. Extractions were performed in two ways. The first method involved extracting septra infusion liquid directly using a modified extraction vessel designed to bubble the supercritical fluid before exiting to the trap. This method however gave poorer yields as a result of restrictor plugging caused by precipitation of sulphamethoxazole when the solution pH was lowered by the introduction of CO_2. The second method involved spiking the active ingredient onto

Celite followed by extraction of the drug with 100% CO_2. This way quantitative recovery was achieved for both drugs when the active ingredients were spiked at <1.0 mg. Co-extractives of the drug were observed but did not interfere with the HPLC assay.

Hedrick and Taylor[62] reported a qualitative study of the hydrochloride salts of triprolidine and pseudoephedrine from an aqueous matrix. The analytes were found to be insoluble in supercritical CO_2. A molar excess of tetrabutylammonium hydroxide was added to 3 ml of 1 mg/ml solution of both compounds in order to extract the free bases formed with 100% CO_2. Qualitative static extraction results showed that the free bases were extractable from water and chromatography could be achieved under supercritical conditions.

2.1.3 Tissue-Serum-Faeces-Urine-Milk

The rapid and accurate measurement of ultratrace levels of drugs and their metabolites in biological matrices plays a major role in the pharmaceutical development process. These measurements of drugs and metabolites provides information for the mechanism of action in pharmacology and toxicology studies, as well as for clinical development.[63] Ramsey et al.[64] reported using supercritical carbon dioxide to extract four veterinary drugs, trimethoprim, hexestrol, diethylsilbestrol and denestrol from freeze dried pig-kidney. The use of on-line SFE-SFC-MS was demonstrated for qualitative analysis of the drugs from the matrix. Portions of powdered kidney (1 g) were mixed with methanol (10 ml), spiked with relevant amounts of each drug, sonicated for an hour to allow homogenous mixing and then the solvent was dried under nitrogen stream. Extraction was carried out, with 100% CO_2 for 8 minutes and then with CO_2 modified with 20% methanol for 0.5 minutes at a flow rate of 4 ml/min. During the extraction, drugs were retained in an amino-bonded column while the endogenous material was passed to waste. The eluted

drugs were detected with high specificity by tandem MS. The authors concluded that even though the method showed great potential, the detection limits were not sufficient to meet the stringent controls on drug residues in meat for human consumption.

The solubilization of 5 veterinary sulpha drugs in supercritical fluid media and their extraction from spiked chicken liver and swine muscle samples were investigated by Cross et al.[65] CO_2, modified with 10-20 % methanol was required and the authors concluded that increasing extraction pressure, time and % modifier all facilitated greater solubilisation, as does the use of small-diameter extraction cells. The effect of temperature varied from drug to drug, but in all cases recoveries were maximized at low temperatures. It was found useful to immobilise the water in the animal tissue by the addition of diatomaceous earth to the extraction vessel. The authors reported recoveries in the mid 90% range at moderate pressures in less than 1 hour, with very little variation between the set of related sulphonamides. Greater solubilization of these drugs could probably be achieved with increasing severity of extraction conditions.

Liu and Wehmeyer[66] demonstrated the use of SFE for the direct extraction of drugs from plasma prior to analysis. The supercritical fluid was passed directly through the plasma samples spiked with either a neutral (flavone) or an acidic drug, ketorolac. The addition of an antifoam agent to the plasma prior to extraction was necessary to avoid restrictor plugging caused by denaturising of the plasma proteins by the supercritical fluid. The effluent from the extraction cell was bubbled through a small volume of methanol or into an empty tube to trap the extracted drug. The effect of extraction pressure and time on absolute recovery were investigated. The optimum extraction conditions used were as follows: pressure 30 MPa, temperature 60°C and an extraction time of 20 minutes. For spike levels of 25 ng /ml of ^{14}C-flavone and ^{14}C-ketoflavone recoveries obtained averaged 98% (RSD of 5.2%) and 80% (RSD 11.08%) respectively and for spike levels of 100 and 250 ng/ml of ^{14}C-flavone the

recoveries obtained averaged 85% (RSD of 5.8%) and 87% (RSD 3.8%), respectively. The authors concluded that the absolute recovery, selectivity, precision, and accuracy of SFE was comparable to those obtained by conventional liquid-liquid extraction.

SFE coupled with SPE using octadecylsilane cartridges has been subjected to selective isolation of ultratrace levels of a drug metabolite, mebervine alcohol, from dog plasma.[67] In these studies dog plasma was applied directly to the extraction cartridge and the cartridge washed to remove protein and then extracted under supercritical conditions using 5% methanol modified CO_2. The extracted metabolite of mebeverine, mebervine alcohol (MEBOH) was trapped by bubbling the effluent from the extraction cell through a small amount of 2-propanol. The effects of extraction pressure and temperature on analyte recovery were examined. ^{14}[C] MEBOH was used to examine the absolute recovery of the SFE/SPE approach. The addition of amine modifier to the fluid phase was required to aid extraction. For spiked levels of 50 ng/ml, ^{14}[C]MEBOH recoveries obtained averaged 95%. For 10 ng/ml spike level, recoveries dropped to 83%. It was reported that these extraction conditions also eluted unwanted material from the cartridges themselves. The accuracy of SPE-SFE was considered to be comparable to conventional SPE methods. The SPE-SFE procedure described by the authors was similar to the SPE procedure, the only difference being the use of a supercritical fluid to elute the analyte from the cartridge. Although the SFE-SPE method is less time consuming, the SPE method is probably a better choice in terms of cleaner extracts and improved extraction recoveries.

The use of SPE columns as a quantitative trapping device was demonstrated by Maxwell et al.[68] Three antimicrobial drug residues were extracted at 600 bar at expanded gas flow rates of 3-4 ml/min. Residues were eluted from the SPE column by off-line analysis. An integral metering valve-collector assembly reduced the loss of trace analytes in the tissue.

Vitamin K1 (phylloquinone) has been extracted[69] with CO_2 at 8000 psi and 60° C from commercial soy protein and milk based powered infant formulas. Quantitative extraction required only 15 minutes and extracts were free of co-extractives from the lipophilic materials. Recovery of vitamin K1 obtained from the milk-based powered formula was 95.6% with an RSD of 7.4%, and that from a soy protein-based product, 94.4%, with an RSD of 6.5%. Application of this method to liquid formulas by using a larger extraction chamber packed with Chromosorb W, to which 7 ml of liquid product was added, proved unsuccessful. Ndiomu and Simpson[70] reported the extraction of morphine from freeze dried serum samples spiked with 200 μg/ml. Recoveries of 96.7% with an RSD of 3.2% were obtained compared to 92.2% for SPE. In the same study, the authors also reported the successful analysis of morphine in placental samples.

Karlsson et al.[71] investigated the application of SFE recovery studies of budeosonide from blood plasma matrix. Plasma was spiked with [^{3}H]-budesonide and a 500 μl subsample was deposited onto a filter paper in the extraction thimble. Extractions were with pure CO_2 at 110°C for 30 minutes. The analytes were trapped on to a ODS bonded column during extraction. After extraction, the trap was washed with MeOH at 40°C and the system was depressurised. The budeosonide extracts were quantified using a liquid scintillation counter and the average recoveries reported were >80%.

Edder et al.[72] demonstrated the application of SFE for extracting codeine, ethylmorphine and morphine in hair of drug addicts. A sample (50 mg) of pulverised hair was extracted with CO_2/MeOH/triethylamine/H_2O (85/6/6/3) at 0.7 ml/min and 40°C for 30 minutes. After extraction, the solvent was evaporated and the samples were treated with 100 μl of pyridine and 100 μl of propionic anhydride at 60°C for 30 minutes. The solvent was evaporated and the residue was dissolved in ethyl acetate ready for analysis using GC-EIMS. The recovery obtained for morphine was 93.5%.

Comparisons of SFE results with those obtained with other extraction techniques were also presented.

2.1.4 Other Applications

The potential for SFE as a size reduction method as an alternative to milling and extraction of fermentation products were demonstrated by Larson and King.[73] In particular SFE was shown to be suitable for extraction of mevinolin, a metabolite of the fungus Aspergillus terreus from a freeze dried fermentation broth. Ndiomu and Simpson[70] demonstrated the potential of SFE for extracting drugs such as papaverine and quinone from plant material. The SFE of taxicin as anticancer drugs from dried needles of the English yew tree, Taxus baccata was demonstrated by Heaton et al.[74] The optimum extraction conditions for the extraction taxicin were: 10% methanol modified CO_2 at 50°C and 400 atmospheres for 100 minutes. The extraction efficiency of SFE was reported to be comparable with liquid solvents and methods were developed for quantitative and qualitative monitoring of the extracts.

Ma et al.[75] (demonstrated the extraction of Chinese herbal medicine, frankincense, myrrh and evodiarutaecarpa using supercritical carbon dioxide. The optimum extraction condition of 20 MPa, 50°C and 40 minutes extraction time yielded good extraction efficiency and selectivity, especially for the high molecular weight and oxygenated components.

Liu et al.[76] investigated the application of SFE to extract diosgenin following acid hydrolysis from tubers of *Dioscorea nipponica*. Samples of 0.05 g were extracted with pure CO_2 over a range of range of temperatures and pressures for different periods of time. Diosgenin determinations were carried out using capillary GC of trifluoroacetate derivatives. Highest recovery was achieved at 3100 psi and 44°C for

70 minutes, but >82% of this yields was extracted by 40 minutes. The yield were 33% lower using conventional light petroleum extraction.

The potential use of a supercritical fluid other than supercritical carbon dioxide for extraction of pharmaceutical compounds was demonstrated by Sauvage et al.[77] The comparative behaviour of nitrous oxide and carbon dioxide as the supercritical fluids was studied for extraction of a halogenated aromatic phenoxy derivative (HAPA) of an aliphatic alkane from a dog feed and a halogenated aromatic phenoxy derivative of urea (HAU) from a rodent feed. Results were obtained with pure supercritical fluids with high solvating power (density of 0.79 g/ml) and fluids modified with a polar modifier (methanol and acetonitrile). The results indicated that with modified nitrous oxide, the extraction time of HAU was reduced by a factor of 2. For the extraction of HAPA, the total extraction time required was only a few minutes compared to 70 minutes extraction time by the classical method.

The use of Freon-22 in supercritical fluid extraction and chromatography was demonstrated by Li et al.[78] For the extraction of a series of steroids, the extraction efficiency with Freon-22 was found to be significantly better with shorter extraction time compared to supercritical carbon dioxide.

Tables 2.1 summarises the results obtained by SFE.

Table 2.1 Summary of results obtained by SFE

Analyte	Matrix	Extraction Conditions	% Recovery	Reference
Menadione	rat chow	CO_2 at 8000 psi, 60°C and 20 mins	92.4% recovered with 2.2% RSD at the 1 mg/g level	43
Hypolipidermic drug	rat feed	CO_2; 0.9 g/ml, 50°C and 30 mins	84.2% revvovered with RSD 2.1%	44
Atovaquone	rat feed	CO_2 at 350 bar and 50°C	>95 % recovered with <5 % RSD	45
Tiperadane	rodent diet	10% EtOH modified CO_2 at 200 kg/cm^2, 70°C and 2 mins	85 % recovered with 6% RSD	46
Propanolol, Tamoxifen, ZM 95527 and ZM 169369	rodent diet	MeOH modified CO_2 at 70°C, 17.25 MPa	88.4% with an RSD of 12.9% for propanolol, 86.2% with an RSD of 8.1% for tamoxifen, 84.5% with an RSD of 12.1% for ZM95527 and 81.2% with an RSD of 12.2% for ZM 169369.	47
Antihistamine	trandermal patch	Chloroform modified CO_2	quantitative recovery, 2.7% RSD	48
Nitroglycerine	transdermal patch	tributyl phosphate modified CO_2 at 80°C and 5 mins	99.1% recovered with 2.8% RSD	49
Triamcinolone	dermatological patches	MeOH modified CO_2	10% recovered	50
Misoprostol	hydroxypropyl methylcellulose	CO_2 at 0.8 g/ml, 70°C and 4 mins	65 % recovered	51
Megestrol Acetate	tablet	MeOH modified CO_2 at 0.81 g/ml, 55°C and 20 mins	92.8% recovered	52

Davron	tablet	CO_2	qualitative	53
Ibuprofen	tablet	CO_2 at 5000 psi and 50°C	-	54
Caffeine and Vanillin	tablet	CO_2 at 350 bar, 50°C and 25 mins	97.7% recovered for vanillin with RSD of 3.1%, 98.5% recovered for caffeine with RSD of 1.4%	55
Vitamin A and E	tablet	CO_2 at 200 atm, 40°C and 15 mins	>95.6% recovered	56
Felodipine	tablet	MeOH modified CO_2	98.6% recovered	57
Benzodiapine	tablet/capsule	MeOH modified CO_2 at 100 atm, 65°C and 15 mins	quantitative	58
Polymyxin B sulphate	cream/ointment	MeOH modified CO_2 at 300 atm, 55°C and 45 mins for the cream, MeOH modified CO_2 at 450 atm, 60°C and 45 mins for the ointment	108%recovered for the cream with 5% RSD and 136% recovered for the ionment with 1.9% RSD	59
Acylvoir	ointmrnt	MeOH modified CO_2 at 50°C	99% recovered with RSD 5.3% at 50 mg/g level	60
Sulfamethoxazole and trimethoprim	septa infusion	CO_2 at 250 atm, 50°C and 100 ml CO_2 for direct analysis, 0.85 g/ml, 60°C, 2 ml/min from Celite.	direct from infusion: sulfamethoxazole recovery 5%. After immobolization onto Celite: 113% recovered with 14% RSD for sulfamethoxazole and 85% recovered with 10% RSD for trimethoprim	61
triprolidine and pseudoephedrine	aqueous	CO_2	qualitative	62

Veterinary drugs	pig-kidney	CO_2 only and MeOH modified at 302 bar and 75°C	qualitative	64
Veterinary sulpha drugs	liver and swine samples	MeOH modified CO_2	recoveries ranged from 27 to 97%	65
Ketorolac and flavone	plasma	CO_2 at 30 MPa, 60°C and 20 mins	98% recovered for flavone with RSD 5.2%, 80% recovered for ketorolac with 11.1% RSD	66
Mebervine alcohol	dog plasma	MeOH modified CO_2 at 350 atm, 40°C and 10 mins	95% recovered at the 50 ng/ml spike level	67
Antimicrobial drug residue	liver	CO_2 at 600 bar	quantitative	68
Pollyquinone	soy protein and milk based infant formula	CO_2 at 8000 psi, 60°C and 15 mins	95.6% recovered for infant formula with RSD 7.4%, 94.4% recovered for soy protein with 6.5% RSD	69
Morphine	serum	CO_2	96.7% recovered with RSD 3.2%	70
Beudeosomide	plasma	CO_2 at 110°C and 30 mins	> 80% recovered	71
Cadeine, Morphine and ethyl morpjine	hair	MeOH, TEA, H_2O modified CO_2 at 3100 psi, 44°C and 70 mins	>93.5%	72
Mevinolin	fermentation broth	CO_2 and various modifiers (MeOH, t-butyl amine, acetic acid)	selective extraction investigated	73
Taxicin	needles of yew tree	MeOH modified CO_2 at 400 atm, 50°C and 100 mins	83% recovered	74
Chinese herbal medicine	plant	CO_2 at 20 M Pa, 50°C and 40 mins	quantitative	75

Diosgenin	tubers of Dioscoica	CO_2, range of temperature and pressure studied		76
Halogenated aromatic phenoxy derivatives	dog feed/rodent feed	MeOH/MeCN-modified CO_2,MeOH/MeCN-modified NO_2,CO_2 and NO_2 at 0.79 g/ml	quantitative	77
Steroids		CO_2 and Freon-22	quantitative	78

2.2 SFC of Pharmaceuticals

Pharmaceutical compounds are commonly polar, non-volatile and thermally or chemically labile. Techniques such as high performance liquid chromatography (HPLC) and gas chromatography (GC) are applied for the routine analysis of pharmaceuticals. Although supercritical fluid chromatography (SFC) has been known for more than twenty years, it has emerged as a viable technique only recently. SFC is a chromatographic technique which exhibits some characteristics of GC and liquid chromatography. Unlike GC or HPLC, where the mobile phase dominates the type of detection method employed, SFC utilises mobile phases with physical parameters that allow the implementation of either fluid phase or gas phase detectors. This multidetector compatibility makes SFC a very attractive separation method for analysing complex mixtures. Pharmaceutical industries can reap real benefits from SFC for analysis of thermally labile, polar and weakly chromophoric compounds not readily amenable for analysis using other techniques.

The application of SFC for pharmaceutical analysis is widespread and there has been numerous review articles published about the analysis of drugs. Bartle et al.[79] published a review on capillary SFC (cSFC) on different types of samples including analysis of five drugs while Wilson et al.[80] published a comprehensive on the use of SFC and SFE in the pharmaceutical industry. Niessen et al.[81], Wong et al.[82] and Xie et al.[83] published reviews on bioanalytical and biomedical applications of SFC which included analysis of drugs. Chester et al.[84] and Lubke[85] also published reviews on SFC and SFE where sections of the review included applications of SFC for drug analysis.

The majority of the published articles on SFC of drugs reported the analysis of test solutions from pure compounds (authors often fail to mention about the sample matrix). Some articles however, include real matrices such as blood plasma and

serum, urine, biological tissues and plants. In some cases, the details were given of the different pretreatment required such as derivatization or separation methods (extraction, centrifugation, precolumn cleanup treatments) prior to the analysis. Since in most papers concentration levels of the compounds analysed are omitted, no deductions can be made about the quantitative aspect of the analysis. The following sections presents examples of the application of SFC for the analysis of drugs in real matrices.

2.2.1 Urine-Tissue-Biological Fluid

The application of cSFC with FID detection was investigated by Later et al.[86] for the analysis of selected drugs in both standard solutions and complex mammalian urine extracts. The authors reported the qualitative analysis of adrenocortical steroids, (e.g. prednisone, prednisolone and methyl prednisolone) from an equine urine sample. These steroids are used as anti-inflammatory agents in veterinary applications. The steroid prednisolone was detected in an ethylacetate extract of an equine urine sample following a "therapeutic" dose (level not mentioned) of the compound. The chromatographic conditions employed for the analysis were supercritical CO_2 at 130°C and pressure programming from 125 atm after a 30 minute hold, to 245 atm at 50 atm/min, then to 320 atm at 10 atm/min. The chromatographic column used was a 18 m x 50 μm id methylpolysiloxane capillary column. The analysis time reported was 20 minutes.

The same authors also demonstrated the potential for identifying human urine extracts of 9-carboxy tetrahydrocannabinol by cSFC with FID. The peak obtained from a sample containing ca. 400 ng/ml of 9-carboxy tetrahydrocannabinol gave an excellent signal-to-noise ratio. Supercritical CO_2 was used as the mobile phase with density programming from 0.25 g/ml after a 15 minutes hold to 0.40 g/ml at 0.015 g/ml/min,

and then to 0.65 g/ml at 0.01 g/ml/min at a temperature of 120°C. The column employed for the separation was a 15 m x 50 μm id SE-33 column.

Crowther and Henion[87] briefly investigated the application of pSFC with MS for the detection of a crude acid extract of equine urine containing phenylbutazone and its metabolites. The authors do not mention the concentration levels studied and details of the detection limits or sensitivity were not provided. CO_2 modified with 10 % MeOH was used as the mobile phase and the chromatographic column employed was a 20 cm x 2.1 mm id diol column. The analysis time reported was ca. 6 minutes.

Wong and Dellafera[88] investigated the use of cSFC with FID for the analysis of phenobarbital from serum samples. Phenobarbital was chosen due to its therapeutic application as an antieleptic, as well as its potential abuse resulting in overdose cases. Barbitone was used as an internal standard. The serum samples containing the drug were extracted using both liquid-liquid extraction and SPE prior to analysis by SFC. Chromatography was carried out with a 10 m SB-methyl-10 column (50 μm id) along with a 5 m fused silica column (50 μm id) to provide a retention gap. Chromatography was obtained with CO_2 as the mobile phase at a temperature of 120°C and both density gradient, from 0.25 to 0.6 gm/ml (0.02 gm/ml/min), and pressure programming, from 100 to 300 atm (20 atm/min), was employed. The use of SPE (solid phase extraction) was necessary to prepare the samples to ensure adequate column life. The procedure was used to estimate a patients phenobarbital concentration at a level of 20 mg/L.

Niessen et al.[89] demonstrated pSFC of the mitomycin C in plasma samples with UV detection at 360 nm. The plasma samples containing the drug were injected onto a short precolumn (10 mm x 3.2 mm id, packed with the stationary phase material) prior to analysis. After washing and drying the precolumn, the drug was desorbed using a mixture of CO_2 and methanol (88/12, v/v). Chromatography was obtained

with a Rosil C_{18} column (5 μm, 150 x 4.6 mm id). Other columns investigated were silica and aminopropyl but both failed to elute mitomycin C. The use of a C_8 column was also investigated but the peak shape obtained was not acceptable. The chromatographic conditions employed involved a mobile phase of CO_2-methanol (88/12, v/v) at a flow rate of 2 ml/min, pressure 30 MPa and a temperature of 50°C (the compound is unsuitable at temperatures above 60°C). These conditions eluted mitomycin C at ca. 2-3 minutes. The detection limit obtained was reported to be 5 ng/ml.

David and Novotny[90] successfully demonstrated the analysis of steroids androsterone and conjugated androsterone compound from both urine (spiked at 10 ng/ml) and plasma (spiked at 5 ng/ml) sample by cSFC. Chromatography was achieved on a 10 m x 50 μm id fused silica column coated with SE-33 (0.50 μm film thickness). Nitrous oxide was used as the mobile phase and pressure programming was operated over the range of 75 atm to 315 atm (8 atm/min) at a column temperature of 100°C. The analysis time reported for these compounds were in the order of 40-50 minutes.

Mount et al.[91] investigated the use of SFC with ECD for the analysis of the anti-malarial drug mefloquinone in blood samples. D,L-erythro-a-(2-piperidyl)-2,8,-bis(trifluromethyl)-4-quinolinemethanol was used as the internal standard and the method incorporated partitioning into methyl tert.-butyl ether (MTBE) from an aqueous base, back extraction into dilute aqueous acid and final partitioning into MTBE from aqueous base. Chromatography was achieved on a 200 x 0.75 mm glass lined steel column packed with 7 μm Zorbax BP silica gel using supercritical pentane containing n-butylamine (0.15%) and methanol (1%) as mobile phase. n-Pentane was chosen rather than CO_2 based on the ease of addition of precise amounts of the modifiers to a liquid at room temperature and the reduced response of the ECD to the n-pentane compared to CO_2. The column was conditioned for 4-6 hours at 300° C and pressure 2100 MPa for 10-20 minutes at the beginning of each working day. For

analysis the column and transfer line were maintained at a temperature of 210°C with the ECD at 350°C. Under these conditions the retention times obtained for mefloquinone and the internal standard was 8 and 4 minutes, respectively. The detection limit obtained was 7.5 ng/ml in 0.1 ml blood samples with RSD of 3.8-6.5% and exhibited good linearity (linear range 100-450 ng/ml). Mount et al.[92] also reported the application of pSFC with ECD for the analysis of artemisinin in whole blood. The blood samples were prepared by liquid extraction and centrifugation prior to analysis. The chromatography was performed at 80°C on a 20 cm x 1 mm id. Deltabond cyano column with CO_2 at 17.2 MPa as the mobile phase. The detection limit obtained was 20 ng of artemisinin /ml of blood sample. The recovery reported by the authors for 480 ng of artemisinin /ml of blood was 81.6% with RSD of 10.3%. The main source of variation in the result was thought to be the poor reproducibility of injection.

Ramsey et al[93] reported the use of SFE with pSFC-MS-MS for veterinary drugs, sulphamethazole and trimethoprim in freeze dried pig kidney spiked at a level of 10 mg of drug /kg of kidney. The mobile phase composition used comprised of a gradient of MeOH with 100% CO_2 for 8 minutes followed by 20% MeOH modified CO_2 in 0.5 minutes and maintained for 10 minutes. The chromatographic column utilized was a 100 x 4.6 mm id amino column and the analysis was carried out at a temperature of 75°C. The authors concluded that although the detection limits (1 mg/kg) obtained by this method were not sufficiently low to meet the stringent controls on drug residues in meat for human consumption, there is potential for further development of the approach.

Perkin et al.[94] demonstrated the application of pSFC with MS for the analysis of sulphamethazine from a spiked porcine kidney extract. The chromatographic method employed comprised of a mobile phase containing 15% MeOH modified CO_2 at a

flow rate of 4 ml/min and a temperature of 75°C. The column employed for the separation was a 100 mm x 4.6 mm id amino column. The analysis time reported under these conditions was ca. 4 minutes.

Pinkston et al.[95] demonstrated the application of cSFC with MS detection for the analysis of mebeverine spiked from a dog plasma matrix. The spiked dog plasma samples were prepared for analysis by SPE. Chromatography was performed on a Dionex 10 m x 50 μm id SB methyl 100 column with a 0.25 μm film thickness. CO_2 was used as the mobile phase at 140°C and pressure programming from 10.1 MPa to 40.5 MPa at a rate of 10.1 MPa/min. The authors reported excellent recoveries (91-104%) for spiked plasma samples spiked with 5.95 to 59.5 ng mebeverine/ml of plasma. The RSD obtained were mostly 10%.

Wang et al.[96] demonstrated the application of pSFC with UV detection for the analysis of camazepam and its metabolites in a liver matrix. The liver microsomes were prepared from the treated (80 mg/kg) male rats (80-100 g). Camazepam and its metabolites were analysed after incubation with rat liver microcosmes and extraction of the products into $CHCl_3$. Both achiral column and chiral column were used for the analysis of camazepam and eight of its derivitives. Achiral separation was achieved with a 25 cm x 4.6 mm id amino column at a temperature of 30°C and pressure 150 bar. The mobile phase comprised of a gradient of CO_2 containing EtOH (13% EtOH for 4 minutes increased at 3%/min to 30% EtOH). Chiral separations were achieved on a 25 cm x 4.6 mm id Chiracel OD-H column at a temperature of 30°C and a pressure of 200 bar. The mobile phase used was CO_2 containing EtOH at a flow rate of 2.5 ml/min. No single EtOH concentration was suitable for resolving all the enantiomeric pairs in one chromatographic run. Isocratic mobile phase conditions were employed for the optimal resolution of each enantiomeric pair with the aim to obtain baseline resolution of all the compounds in the shortest possible time.

Smith et al.[97] briefly reported the separation of ranitidine and its four metabolites, isolated from pig and rat liver by SPE, using pSFC with UV. Separations were achieved on a 10 cm x 4.6 mm id cyanopropyl bonded silica column at a temperature of 71°C and pressure 3200 psi. The chromatographic mobile phase used comprised of CO_2/MeOH/aq.40% methyl amine/water (400/98/1/1, v/v). An analysis time of ca. 8 minutes was achieved under these conditions. In a further study, the authors also briefly reported the potential of pSFC for the separation of ranitidine and its metabolites present in rat urine, following SPE. The details of the detection limits, RSD or the sensitivity of the method were not provided.

Simmons et al.[98] demonstrated the application of pSFC with UV detection for the analysis of phenylbutazone and oxyphenylbutazone in serum. The spiked serum samples (20 μg/ml of serum) were prepared for analysis by SPE (ODS column). Chromatography was performed on a 25 cm x 1 mm id Deltabond ODS (5 μm) column operated at 85°C and pressure 170 atm with CO_2 containing 5% MeOH as the mobile phase at 240 nm. The recoveries reported for phenylbutazone and oxyphenylbutazone were 82% and 83.4%, respectively and the corresponding detection limits obtained were 0.1 and 1 μg/ml. The use of other columns such as silica and cyano were also investigated but failed to give acceptable chromatography.

Wong et al.[99] investigated the use of cSFC with FID for the analysis of cyclosporin, tacrolimus and rapamycin in blood. The blood was prepared for analysis by centrifugation with appropriate solvents and SPE (C_{18} column). Chromatography was performed on a 10 m x 50 μm id SB-biphenyl column operated at 70°C and pressure programming from 100 to 300 atm at a rate of 10 atm/min with CO_2 as the mobile phase. The quantitation of the cited drugs at therapeutic levels was not achieved due to low recoveries and sensitivity obtained with FID.

2.2.2 Formulations

Jagorta and Stewart[100] briefly reported the analysis of diazepam, chlorodiazepoxide and their related compounds in Valium tablet and Librium capsule form using cSFC. Each tablet or the capsule contained 10 mg of the active substance. Chromatography was achieved using a SB-cyanopropyl-50 (195 μm id and 0.25 μm film thickness) capillary column with FID detection. For analysis of diazepam and its related products, nordiazepam, 3-amino-6-chloro-1-methyl-4-phenyl-carbostyril and 2-methylamino-5-chlorobenzophenone a multi-linear pressure programme was employed with CO_2 as the mobile phase. The temperature gradient in addition with the pressure gradient resulted in baseline separation of all four compounds in under 24 minutes. For the analysis of chlorodiazepoxide and its related products, 7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one-4-oxide and 2-amino-5-chlorobenzophenone the chromatographic conditions employed with CO_2 as the mobile phase were as follows: multilinear pressure programme with 9 min hold at initial pressure of 200 atm, then 50 atm/min ramp to 300 followed by 20 atm/min ramp to 400 atm, then hold for 5 min at 400 atm. The oven programme was isothermal at 120°C. Under these conditions excellent separation of all the compound were achieved in under 20 min. Recoveries of 101-103% of the labelled amount were obtained for both compounds with RSD of 4.1% and 3.2% for diazepam and chlorodiazepoxide, respectively compared to RSD of 1.2% and 0.4% obtained from an established HPLC method. Jagota and Stewart[101] have also studied the use of cSFC with FID for the analysis of ibuprofen, ketoprofen and mefanamic acid in tablet or capsule form. Samples were prepared for analysis by dissolving individual tablet ot capsule with MeOH followed by sonication. Chromatography was carried out using CO_2 as the mobile phase at 130°C. The column investigated was a SB-biphenyl-30 (10 m x 50 μ m id, 30% biphenyl and 70 % methylpolysiloxane). Pressure programming was used to elute the compounds and the total analysis time recorded was under 20 minutes.

Jagota and Stewart[102] have also reported the separation of selected oestrogens (oestrone, equilin, alpha-oestradiol, beta-oestradiol and d-equilenin) and methyl testosterone in commercial dosage forms using cSFC with FID. Three different commercial dosage forms were investigated. They included one in which oestrogens were conjugated, one in which they were esterified, and finally, one containing only β-oestradiol. The oestrogens in the conjugated and esterified dosage were converted into 'free' oestrogens with acid hydrolysis and extracted with chloroform prior to analysis. Chromatography was carried out on a 10 m x 50 μm SB-cyanopropyl-50 column and carbon dioxide density gradient at a temperature of 73°C. The retention times obtained under these conditions were ca. 19 minutes. The authors concluded that there was no statistical difference in the SFC vs HPLC (table 1) assay results for oestradiol in the dosage form at the 95% confidence level.

Table 1 Comparison of SFC and HPLC data obtained for the analysis of Oestradiol tablet.

Labelled amount	Amount found (SFC)	Amount found (HPLC)
2.0 mg	1.99±0.036 mg	2.07±0.029 mg
2.0 mg	RSD 1.8% (n = 3)	RSD 1.4% (n = 3)

DiMaso et al.[103] reported the application of SFC for the analysis of sorbitan triolate in metered dose inhaler formulations. Sorbitan triolate is used to keep the drugs dispersed in the propellant. The sorbitan triolate was separated from other components from all formulations tested. Chromatography was achieved on a C-18 bonded packed column (5 μm Rexchrom, 100 x 2.1 mm id) with CO_2 as the mobile phase at 40°C and 2400 psi. UV detection was used for the analysis. The experimental formulation was designed to deliver 120 μg of sorbitan triolate per dose and the amonut found was 123 μg/burst. A linear calibration curve for peak height as a function of weight of sorbitan triolate was obtained between 0.5-10 μg injected on column. The detection

limit calculated as three times the peak-to-peak ratio baseline noise was 50 ng per injected volume and the RSD values obtained for the peak height was between 1.0-2.0%. The authors concluded that this methodology is currently being used to determine sorbitan triolate droplet size and distribution in metered-dose inhaler plumes.

Masuda et al.[104] studied coupled SFE and SFC for the determination of fat soluble vitamins, retinol and tocopherol in hydrophobic ointments. The sample preparation for the ointments involved the following. About 1 g of accurately weighed ointment was blended with 9 g of accurately weighed diatomaceous earth powder. A 20 mg subsample of the mixture was extracted and chromatographed under the conditions of the coupled SFE/SFC system. Supercritical carbon dioxide containing ethanol was used as the mobile phase. Chromatography was performed on a Finepak SIL-C18 (4.6 mm id x 150 mm, 5 μm ODS silica packing) at a pressure of 200 Kg/cm^2 and a temperature of 40°C. Detection was by UV at 284 nm. Typical recoveries reported for retinol and tocopherol were 102% and 101.5% respectively. The authors concluded that the methodology was viable for quantitative analysis of pharmaceutical preparation, including fat soluble vitamins.

Anton et al.[105] reported the application of SFC with UV and ELSD detection for the analysis of cromation in both cream and lotion form. Samples were prepared (weights were not mentioned) for analysis by dilution with THF (tetrahydrofuran) followed by filtration prior to injection. Chromatography was achieved on a 270 mm x 2 mm CN column (3 μm). CO_2 modified with MeOH was used as the mobile phase for the analysis of cromation cream. The cromation lotion contained two additional preservatives to that of cream formulation and required the addition of trifluroacetic acid and ammonium acetate to the mobile phase. The recovery obtained by the SFC method (98.8 % with RSD of 0.6%for cream and 99.9% with RSD of 0.6% for

lotion) was comparable to the existing LC method with faster analysis and fewer experimental steps.

Scalia and Games[106] demonstrated the analysis of commonly occurring bile acids, chenodeoxycholic acid (CDCA) in capsule and ursodeoxycholic acid (UDCA) in tablet form by pSFC with UV. Each tablet or the capsule contained 250 mg of active substance. Sample processing of the tablet and the capsule involved dissolution of individual tablet or capsule with MeOH and filtration prior to injection. Baseline separation was achieved was achieved using a phenyl bonded silica column with UV as the method for detection at 210 nm. Supercritical CO_2 modified with 15% MeOH was employed as the mobile phase at pressure 200 atm and temperature 40°C. The average recoveries obtained for CDCA and UDCA were 101.6% with RSD of 2.3% and 102.0% with RSD of 2.3%. The authors concluded that the proposed SFC method is well suited for the quality control assays of medicinal drugs containing chenodeoxycholic acid and ursodeoxycholic acid.

Karlsson et al.[107] reported a qualitative method employing pSFC with UV detection (240 nm) for the determinaion of a dihydropyridine drug in an emulsion matrix (1 mg/ml). The influence of various experimental variables such as type and ampount of modifier, flow rate and tempertaure on chromatographic performance was studied. Optimization of the chromatographic parameters was achieved using experimental design and chemometricial methods. Chromatography was achieved on a Hypersil (200 x 4.6 mm id) column comprising of a mobile phase of CO_2 containing 20% 2-propanol at a pressure of 200 bar at a temperture of 31°C.

Strode et al.[108] demonstrated the application of SFE-pSFC with ECD and UV for the analysis of felodipine from tablets. The extracted felodipine was dissolved in MeOH prior to analysis and carbazole was used as the internal standard. Chromatography was performed on a 25 cm x 4.6 mm id Hypersil Si column (5 μm) with CO_2/MeOH

(6%) as the mobile phase at 45°C. The calibration graph was linear from the concentration range of 18-5000 ppm and the limit of detection reported for both detectors was 34.4 pg.

2.2.3 Plants

Holzer et al.[109] demonstrated the applicability of cSFC with FID for the separation of eight pyrrolizidine alkaloids (PAs) of the retronecine and otonecine (senecionine, integerrimine, senkirkine, neosenkirkine, retrosine, otoserine, hydroxysenkirkine and anonamine) from flowering specimens of *Senecio anonymus*. The fresh plant material (weights not mentioned) was subjected to liquid extraction and filtration appropriately prior to analysis. SFC separations of the PAs was achieved on a 10 m x 50 μm id fused silica capillary, coated with a 0.25 μm film of SB-Biphenyl-30. The mobile phase used was carbon dioxide and linear pressure programming was performed from 100 to 280 atm at 3 atm/min, after an initial isobaric period of 20 minutes. Complete separation of the PAs of the retronecine and otonecine was achieved with a detection limit of 1 ng at a signal to noise ratio of 3.

Balesevich et al.[110] reported the qualitative analysis of indole alkaloids from the leaves of *Catharanthus Roseus* by packed column SFC/MS. Samples of fresh leaves (100 g) were prepared for analysis by extraction with MeOH followed by filtration. Separations were obtained on an amino bonded 5 μm Spherisorb 100 x 4.5 mm column with CO_2 and a 5-15% gradient of MeOH at 65°C. Under these conditions employed, a complete chromatogram was obtained within 8 minutes with 10 major components and an estimated 40 alkaloids being detected when a UV monitor was used. Overall, three alkaloids were predominant; catharanthine, vindoline and 3',4'-anhydrovinblastine. Sharp peak shapes and numerous near baseline separations were reported. Desacetoxyvindoline, vindorsine, vindoline and deacetylvindoline, complex Asipidosperma alkaloids differing only slightly from each other in their functional

groups, were easily separated from each other as well as the pharmaceutically important bisinodoles leurosine, 3',4'-anhydrovinblastine and vinblastine which chromatographed as a group away from the majority of the components. The use of a mass spectrometer in thermospray mode in conjunction with the UV monitor enabled an estimation of 60 alkaloids to be detected. When the chromatograph was coupled to a mass spectrometer in EI mode, high quality EIMS were obtained, which allowed the identification of several alkaloids.

Berry et al.[111] investigated the use of pSFC for analysis of ergot alkaloids in plant extracts of *Claviceps purpurea*. Gradient pSFC was employed for the analysis of the ergot alkaloids of the clavine (agroclavine, festuclavine, elymoclavine, noragroclavine I and II, norchanoclavine, chanoclavine I and II) with UV (280 nm). The alkaloids were chromatographed using an aminopropyl bonded stationary phase (Spherisorb, 5 µm, 100 x 4.6 mm id) and CO_2 modified with MeOH as the mobile phase. The chromatographic conditions used were 10% MeOH modified CO_2 at a flow rate of 3 ml/min, temperature 75°C and pressure 365 bar for 2.5 minutes. The flow rate was then increased to 5 ml/min and the % MeOH increased to 15% at 2.8 minutes and then 20% after 5 minutes. The total analysis time was ca. 11 minutes with the last compound (norchanoclavine II) eluting at ca. 10 minutes. The applicability of SFC-electron ionisation mass spectrometry (EIMS) for the analysis of *Claviceps purprea* was also illustrated at the same chromatographic conditions as before, except a column pressure of 396 bar was used.

The separation of four opium alkaloids (codeine, morphine, papaverine and thebaine) from poppy straw extracts by supercritical fluid chromatography using packed columns was demonstrated by Janicot et al.[112] The authors investigated the optimization of the separation of seven opium alkaliods in standard mixture using sub- and supercritical fluid chromatography prior to applying the procedure to poppy straw extracts. Chromatography was achieved using a silica (5 µm LiChrosorb Si 60)

bonded phase packed in 120 or 230 x 4.6 mm id stainless steel columns at an UV detection at 280 nm. The influence of several mobile phase compositions was demonstrated by varying the MeOH content, water content and investigating various amine modifiers. The retention of alkaloids was reduced with increasing MeOH content in the mobile phase. Addition of various amines (methyl, ethyl and triethylamine) led to a general decrease in retention and negligible changes in the selectivity. The effect of water in the mobile phase was studied both in the presence and absence of an amine modifier. With the silica column, a decrease in retention was noticed in the absence of an amine modifier and with increasing water content. The mobile phase composition determined for the analysis of these alkaloids on silica column was CO_2-MeOH-methylamine-water 83.37:16.25:0.15:0.23, (w/w). The analysis time obtained under these conditions was 11 minutes.

Yun-hua et al.[113] applied cSFC with FID for the determination of panaxadiol and panaxatriol in ginseng and its preparations. The sample preparation involved extensive liquid extraction of 0.1 g of ginseng powder (80 mesh) or an appropriate amont of its preparations. Methyltestosterone was used as the internal standard. The ginseng extracts were purified by a partition column and concentrated by an adsorption column and then analysed by SFC. Chromatography was achieved with CO_2 as the mobile phase at 120°C and pressure programming from 10 MPa to 35 MPa at 1 MPa /min. The recoveries of panaxadiol and panaxatriol reported were 96.3±2.82% (n=6) and 85.1±5.1% (n = 6) respectively.

Heaton et al.[74] reported the use of both cSFC and pSFC of SFE extracts of taxicins from yew extracts. The authors investigated two different capillary columns (biphenyl and carbowax) and two different packed columns (ODS and nitrile) at different chromatographic conditions. Under optimized conditions, the biphenyl colum resulted in only partial resolution of taxicin I and II, whereas the more polar carbowax column gave baseline resolution within 30 minutes. Similarly, the ODS packed column gave

broad, overlapping, poorly-resolved peaks but the more polar nitrile column gave greater selectivity with baseline resolution in 12 minutes.

Tables 2.2 summarises the results obtained by SFC.

Table 2.2 Summary of results obtained by SFC

Analyte	Matrix	SF Chromatographic Conditions	Comments	Reference
Prednisone, prednisolone, methyl prednisolone	urine	CO_2 at 130°C and pressure programming Column: 18 m x 50 μm methyl polysiloxane	qualitative	86
9 Carboxy tetrahydro cannobinol	urine	CO_2 at 120°C and density programming Column: 15 m x 50 μm SE-33	50 ng/ml detection limit	86
Phenylbutazone	urine	MeOH modified CO_2 Column: 20 cm x 2.1 mm diol	qualitative	87
Phenobarbital	serum	CO_2 at 120°C and density programming Column: 10 m x 50 μm SB-methyl	qualitative	88
Mitomycon C	plasma	MeOH modified CO_2 at 50°C and 30 MPa Column: 150 mm x 4.6 mm C_{18}	qualitative	89
Androsterone and cojugated androsterone	urine/plasma	NO_2 at 100°C and pressure programming Column: 10 m x 50 μm SE-33	qualitative	90

Mefloquinone	blood	n-pentane containing n-butylamine and MeOH Column: 200 x 0.75 mm Zorbax silica gel	qualitative	91
Artemisinin	blood	CO_2 at 80°C and 17.2 MPa Column: 20 cm x 1 mm Cyano	81.6% recovered with RSD of 10.3% at 480 mg/ml level	92
Sulphamethazole and trimethoprim	pig kidney	CO_2/MeOH gradient at 75°C Column: 100 x 4.6 mm Amino	1 mg/kg detection limit	93
Sulphamethazine	porcine kidney	MeOH modified CO_2 at 75°C Column: 100 x 4.6 mm Amino	qualitative	94
Meberine	dog plasma	CO_2 at 140°C and pressure programming Column: 10 m x 50 μm SB-methyl	91%-104% recovered for 5.95-59.5 mg/ml level with 10% RSD	95
Camazepam	rat liver	CO_2/EtOH gradient at 30°C and 150 bar Column: 25 cm x 4.6 mm Chiracil OD-H	qualitative	96
Ranitidine	pig and rat liver	MeOH/methylamine/water modified CO_2 at 71°C and 3200 psi Column: 100 x 4.6 mm Cyanopropyl	qualitative	97

Phanylbutazone and oxyphenylbutazone	serum	MeOH modified CO_2 at 85°C and 170 atm Column: 25 cm x 1 mm Deltabond ODS	82% phenylbutazone recovered and 83.4% oxyphenylbutazone recovered	98
Cyclosprin, tacrolimus and rapamycin	blood	CO_2 at 70°C and pressure programming Column: 10 m x 50 μm Biphenyl	qualitative	99
Diazepam and chlorodiazepoxide	tablet/capsule	CO_2 at 120°C and pressure programming Column: SB cyanopropyl-50	>100% recovered with 4.08% and 3.16% RSD	100
Ibuprofen, ketoprofen, menfoinic acid	capsule	CO_2 at 130°C Column: 10 m x 50 μm SB biphenyl-30	Quantitative	101
Oestrogens	tablet/capsule	CO_2 at 73°C and density programming Column: 10 m x 50 μm SB cyanopropyl	100% recovered with 1.8% RSD at 20 labelled tablet and capsule	102
Sorbitan triolates	meterd dose inhalers	CO_2 at 40°C and 2400 psi Column: 100 x 02.1 mm C_{18}	100% recovered with 1.0-2.0% RSD	103
Retinol and tocophenol	ointment	EtOH modified CO_2 at 40°C and 200 kg/cm^2 Column: 15 cm x 4.6 mm ODS	>100% recovered	104
Cromation	cream/lotion	MeOH modified CO_2 Column: 25 cm x 2 mm Cyano	98.8% recovered with 0.6% RSD for the cream and 99.9% recovered with 0.6% RSD for the lotion	105

Chenodeoxycholic acid (CDCA) and ursodeoxycholic acid (UDCA)	capsule/tablet	MeOH modified CO_2 at 40°C and 200 atm Column: Phenyl bonded silica	101.6% recovered with 2.3% RSD forCDCA and 101% recovered with 1.9% RSD for UDCA	106
Dihydropyridine	emulsion	2-propanol modified CO_2 at 31°C and 200 bar	qualitative	107
Felodipine	tablet	MeOH modified CO_2 at 45°C Column: 25 cm x 4.6 mm Silica	quantitative	108
Pyrrolizidine alkoloids	plant	CO_2 and pressure programming Column: 10 m x 50 μm SB biphenyl-30	qualitative	109
Indole alkaloids	plant	MeOH/CO_2 gradient at 65°C Column: 100 x 4.6 mm Amino	qualitative	110
Clavine alkaloids	plant	MeOH modified CO_2 at 75°C and 365 bar Column: 100 x 4.6 mm Amino propyl	qualitative	111
Opium alkaloids	plant	MeOH/methylamine/water modified CO_2 Column: 230 x 4.6 mm Silica	qualitative	112

Panaxadiol and panaxatiol	plant	CO_2 at 120°C and pressure programming Column: 10 m x 50 μm SB cyanopropyl	quantitative	113
Taxacins	yew tree	CO_2 at 120°C and pressure programming Column: Biphenyl and carbowax capillary MeOH modified CO_2 at 60°C Column: ODS and nitrile packed column	quantitative analysis with packed column	74

Chapter 3.0 Compounds of Interest

This chapter aims to give background information on the types of compound that have been used throught out the experimental protocol.

3.1 Aims of the Project

The initial objective of these studies was to assess the factors governing solubility and extraction of specific analytes in a supercritical fluid extraction (SFE) using carbon dioxide as the solvent. The solubility of an analyte in supercritical CO_2 gives an indication of the relative extractibility of the substance as a function of pressure and temperature which is an important consideration in designing operating conditions for SFE. Four different antifungals were determined from an animal feed using SFE/pSFC. The SFE experiments were designed to optimize extraction conditions for the extraction of specific analytes, such as antifungals, from an animal feed matrix to allow for the evaluation of the most significant variables influencing extraction. The pSFC experiments were performed to evaluate the potential of pSFC with CO_2 and polar modifier as the mobile phase for the analysis of the antifungals from an animal feed. Two different methods were also considered for the prediction of steroid solubility in supercritical CO_2. The first method involved the use of chromatographic retention measurements to predict the solubility of testosterone in supercritical CO_2. The second method involved the use of partition coefficients in conjunction with a calculated solubility parameter as a reasonable means of predicting analyte solubility in supercritical carbon dioxide.

The polarity of an analyte is the key factor that influences its solubility in supercritical CO_2. It is possible to estimate polarity from molecular structure alone. For this reason polarity was selected as the key parameter to developing a means of estimating solute solubility in supercritical CO_2. Experimental determination of the solubility of

several steroid compounds with a range in polarities in supercritical CO_2 was carried out in order to correlate solute polarity to the solute solubility. Polarity can be estimated by the hydrophobicity term, log P (based on partition coefficients), and also of the solubility parameter, δ, estimated using fragmentation constants. Estimation of log P through structure activity relationships was investigated with the emphasis on a rapid and reliable evaluation of the hydrophobicity. These investigations were carried out with an aim to relate analyte solubility in supercritical CO_2 via the molecular structure. This can provide an effective method development in SFE based on informed decisions about the solvent strength required to solvate the targeted analyte. A chromatographic method was also investigated based on capacity factor measurements for the prediction of testosterone solubility in supercritical CO_2. The capacity factor of a solute is at least qualitatively inversely related to the solvating power of the mobile phase for that solute, i.e. the more soluble it is in the mobile phase, the less it is retained. The objective of this investigation was to evaluate if the measurements of retention times can provide a rapid way of generating solubility data for testosterone.

In SFE, for extraction to be successful, the solute must be sufficiently soluble in the supercritical fluid. The rule of SFE is that the lower polarity analytes are soluble in greater quantities than those analytes with higher polarities. A matrix material, such as animal feed, associated with a particular analyte can have profound effects on the successful isolation of target analytes in any SFE experiment. The complexity of the extraction process is undoubtedly increased by the introduction of a sample matrix. It has been known through other researchers that the interactions between the analyte and sample matrix are the least understood area of the extraction process. In most cases the sample matrix is solid and the analytes are either adsorbed onto the surface or trapped within matrix particles. The addition of a polar modifier or a cosolvent to supercritical CO_2 often enable analytes to be efficiently extracted and chromatographed from difficult matrices. The application of methanol modifier with

supercritical CO_2 is demonstrated for the determination of four different antifungals from an animal feed.

3.2 Introduction to Steroids

The use of steroid hormones in therapy has grown tremendously in the last 20 years, since the discovery of semi-synthetic routes for the industrial-scale preparation of the most important natural hormones and numerous highly active derivatives. About 200 steroid hormone compounds and related materials are known to date, representing roughly about 10% of the world's total population of pharmaceuticals.[114-120]

Steroid hormones are naturally occurring or synthetic materials consisting of a cyclopentanoperhydrophenanthrene skeleton (figure 3.1) comprising of three fused cyclohexane rings (A, B, C) in a non-linear arrangement and a terminal cyclopentane ring (D). They are amongst the most widely used drugs today.

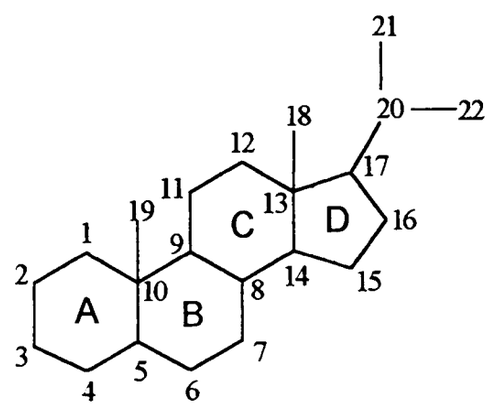


Figure 3.1 The basic ring structure of a steroid

The parent hydrocarbon related to the cyclopentanoperhydrophenanthrene nucleus is called cholestane. There are 17 carbons present in the fused ring (A, B, C and D) structure. Virtually all the steroid structures can be related to this backbone or its

derivatives. The biological activity of individual steroids will also depend on the attached substituents attached to the backbone ring structure. The inclusion of additional side chains on the parent compounds and modification of the existing side chains account for diversity of physiological activities of the numerous steroids.

The steroids hormones can be grouped according to their hormone activity and they generally fall into four major categories: androgens, progestogens, estrogens and corticosteroids.[116]

Androgens - Male hormones. Methyl groups attached to C-10 and C-13 (making 19 carbons) and the A ring is aliphatic. The most potent androgenic hormone is testosterone and its 4,5-dihydro metabolite.

Progestogens - Methyl groups on C-10 and C-13 and $COCH_3$ (e.g. megestrol acetate) on C-17 (making 21 carbons). The A ring is aliphatic.

Estrogens - Female hormones. Methyl group is attached to C-13 (making 18 carbons) and the A ring is usually aromatic. The primary hormone is 17 β oestradiol.

Corticosteroids - Similar to progestogens but with $COCH_2OH$ side chain on C-17 (e.g. cortisone). A hydroxyl or ketone group is on C-11.

Androgenic hormones are the male sex hormones. Their primary role is in the development of the male secondary sex characteristics. The most common androgenic hormone is testosterone and its 4,5-dihydro metabolite. Estrogenic hormones are female sex hormones secreted mainly by the ovary and are thought to be responsible for the secondary female sex character. In addition to estrogens, another important hormone of the ovary (or more exactly of the corpus leteum within it) is progesterone. Its main function is to prepare the mucous membrane of the uterus for the reception of the egg and, after fertilization, to stop ovulation.

Corticosteroids are produced in the body by the outer layer of the adrenal gland and the adrenal cortex. Their primary function is the regulation of some essential metabolic processes of the organism. They fall into two groups: glucocorticoids and mineralcorticoids. Glucocorticods, such as, cortisone and hydrocortisone have anti-inflammatory properties affecting protein, sugar and calcium metabolism, while mineralcorticoids, such as aldosterone, affect salt and water metabolism.

3.2.1 The Analysis of Steroid Hormone Drugs

To maintain high purity in the manufacture of steroid hormones the pharmaceutical industry requires a range of analytical techniques which are both qualitative and quantitative. The pharmaceutical analysis of steroids in the present state can be outlined by the following methods:

(1) Qualitative methods, identification - Although, some of the classical colour reactions[121] are still used for identifying steroids, the most important methods for this purpose are now infrared (IR) and UV spectroscopy, and the determination of the specific optical rotation and melting point of the parent hormone and/or its simple derivatives. An essential technique for the characterization of the quality of a steroid is thin layer chromatography, with semi-quantitative determination of the impurity spots.

Quantitative analysis - Spectrophotometric methods have been established for the quantitative analysis of steroid hormone drugs. Direct UV method is used widely, but its possibilities are limited. IR spectroscopy is barely used in quantitative analysis. The techniques most often applied are visible or ultraviolet methods based on chemical reactions. To solve problems, many new selective methods have been established where spectrometric and colorimetric methods are increasingly being combined with chromatographic separations.

As regards to other optical and the electrochemical methods, fluorimetry and polarography[122] have found application in relatively narrow fields. For direct determination of active hormones in biological fluids and in blood, more sensitive methods were required than the methods described above. The sensitivity requirements were met by the introduction of GC methods[123-127], particularly with the advantage of using selective detectors such as electron capture detection (ECD) and MS. However, the problem with GC is that some hormones may have to be derivatised in order to increase the volatility of the compounds. Recently, the use of HPLC[128-130] for the analysis of steroids has increased. This is due to the versatility offered by this technique where quantitative analysis can be carried out without being affected by the low volatility and the thermal stability of the steroid hormones.

3.3 Azoles

The discovery of azole in the early 1970's added impetus to the development of antifungal drugs; azole antifungals provided the prospect of broad-spectrum, potent agents which were found to be relatively well tolerated with patients.[131]

Azoles are used both topically and systemically to treat superficial and cutaneous mycoses of the type dermatophycoses, malasseziasis, onychomycosis and candidiasis involving skin or mucous membranes.[131] The general recommendations are that topical azole therapy should be used initially with progression to systemic therapy in patients unresponsive to or intolerant to topical applications. In the treatment of the systematic infections the azole antifungals offer a new dimension of therapy, particularly for systematic mycoses in immunosupperessed patients in whom poor clinical and mycological responses are often noticed. The azoles are also known for their expanded role in the prevention of fungal infection in specific populations of immunocomprised patients.

All of the azoles posses a five-member azole ring. Fluconazole possess two triazole groups, hence is designated as bis-triazole.

3.3.1 Fluconazole

Fluconazole, an orally active triazoyl agent, is representative of an important class of antifungal drugs for clinical use in superficial and systemic fungal infections. It is used for the treatment of superficial candidiasis, including orpharyngeal, oesophagel and urinary tract candidiasis and systemic candiasis, including disseminated disease, particularly in immunocompromised patients, including children.[132] A similar azole compound was also used (UK-47,265) for experimental purpose. Figure 3.2 shows the structure of fluconazole and figure 3.3 shows the structure of UK-47,265.

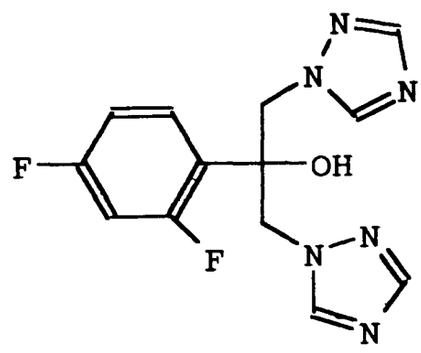


Figure 3.2 Structure of fluconazole

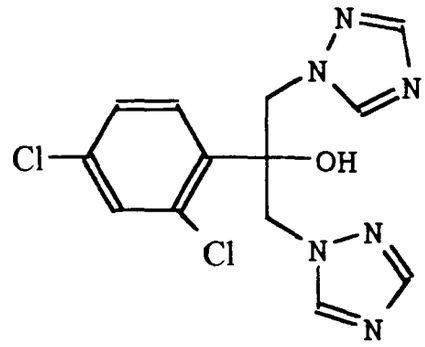


Figure 3.3 : Structure of UK-47,265

3.3.2 Tioconazole

Tioconazole, 1-{2-[2-chloro-3-thineyl)methoxy]-2-(2,4-dichlorophenyl)ethyl}-1H imidazole, is an imidazole drug commonly used as a broad-spectrum of anti-fungal activity including action against dermatophytes, malassezia furfur, and candida albicans.[133] Tioconazole is active against gram-positive bacteria. It is applied topically as a 1% cream, lotion, or powder in the treatment of tinea infections, pityriasis, versicolor, and candidiasis.

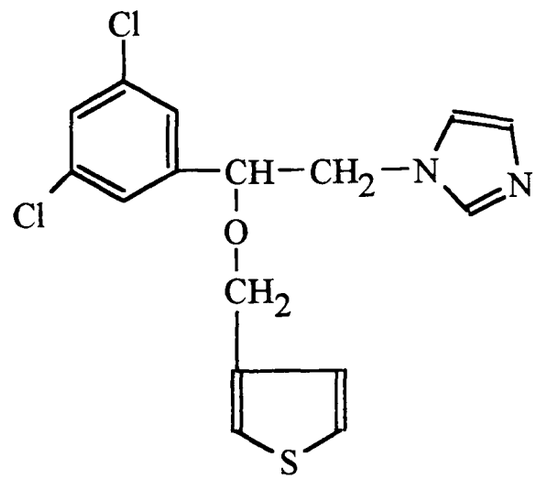


Figure 3.2 Structure of tioconazole

3.3.3 Hexaconazole

Hexaconazole [(RS)-2-(2,4-dichlorophenyl)-1-(1H-1,2,4-triazol-1-yl)-hexan-2-ol}, is a broad spectrum systemic fungicide with eradicant and protectant activity used for controlling a wide range of fungal diseases in cereal.[134] The compound is active mainly against basidiomycetes and ascomycetes where it is a potent inhibitor of ergosterol biosynthesis. As systemic fungicide it is transported in the xylem and there is no evidence of phloem activity.

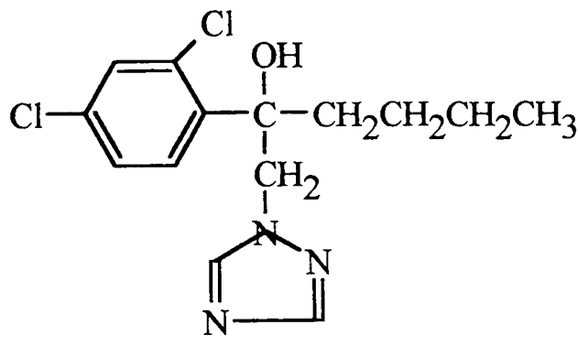


Figure 3.4 Structure of hexaconazole

3.4 The Determination of Drugs from an Animal Feed Matrix

The analysis of drugs from an animal feed is crucial as part of the chronic toxicity studies in the development stage of a drug.[135] Figure 3.5 shows a block diagram of a drug from screening to marketing.

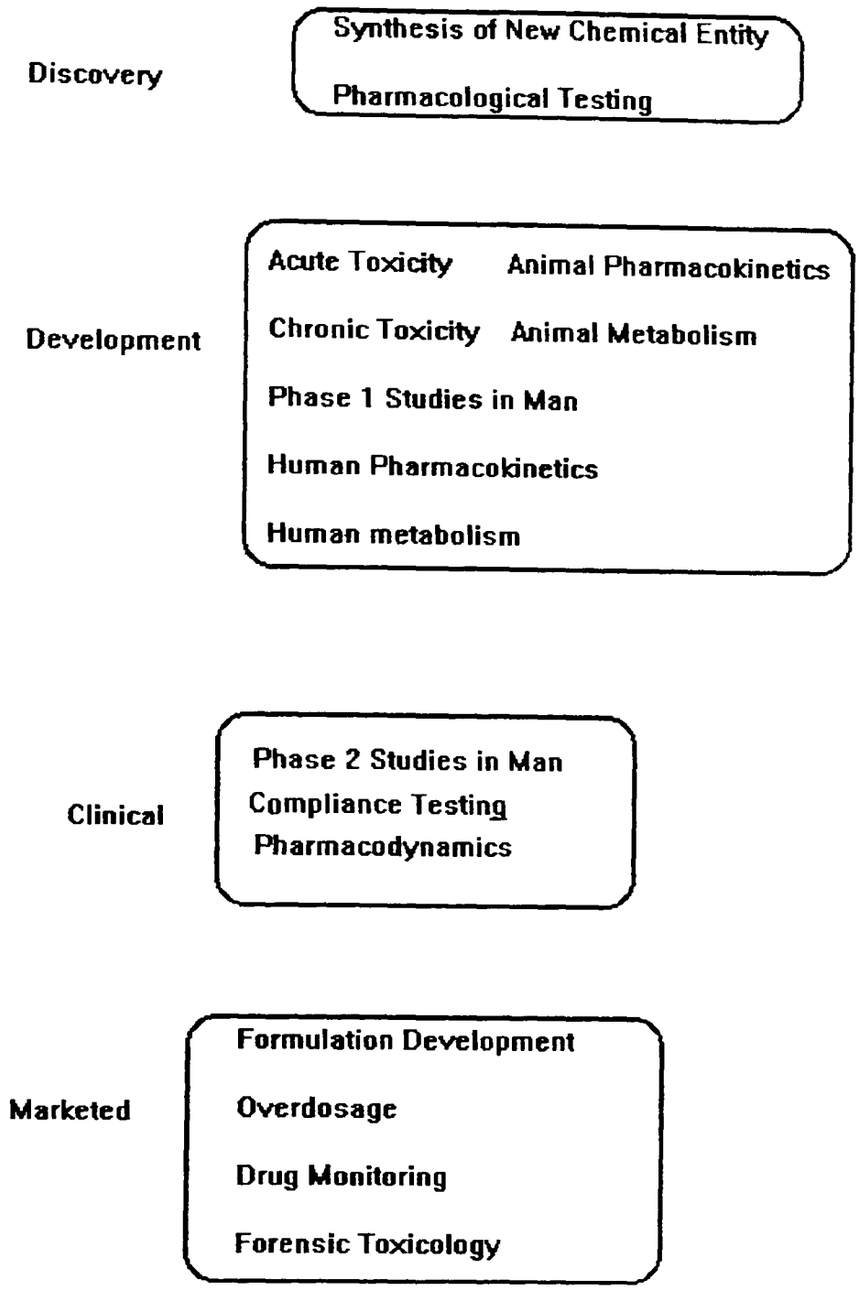


Figure 3.5 Flow diagram showing different stages from discovery to marketed stage for a drug.

Once the drug has been screened and shown a promising pharmacological effect the next step in drug research is generally to establish its safety by carrying out toxicology tests in animals. Initial toxicology tests involve the administration of increasing single doses of a drug to groups of animals (acute toxicology) to establish the toxic doses of single administrations. The next stage is called the chronic toxicity stage. In this phase it is impractical to dose large animals or an animal or an

individual basis everyday. In this situation the dose is introduced in animals' feed or drink. This method can be potentially error prone. The animals may not eat and drink to the same extent: if the food is not mixed correctly then the actual dose offered to the animals may be different; if the mix is not homogeneous, the animals may be selective in their eating, taking either consistently more, or consistently less, than nominal dose. A large error can also be introduced if it is necessary to mix the diet a long time ahead of the dosing time allowing the possibility of the drug decomposing in storage in contact with the diet.

The assay of the drug substances from the feed matrices is therefore necessary to monitor dosage levels, verify dose uniformity throughout the feed mix and confirm the drug's stability in both the short and long term. Animal feeds are complex mixtures of protein, lipids, glucicide, cellulose and mineral matter and if co-extracted the coextractives can interfere in the quantitative determination of the analyte of interest.[77] Extensive sample clean-up is also required to obtain a final sample sufficiently concentrated enough to be determined quantitatively. Various means of sample preparation are currently being investigated to extract the drug substances from animal feed prior to assay.[136-139] These methods include liquid-solid extraction, solid phase extraction, Soxhlet-type extraction and liquid-liquid extraction. The use of supercritical fluid extraction to determine analytes from the animal feed matrices has been investigated only briefly for several pharmaceutical compounds and was discussed in chapter 2 section 2.1.1. Chapters 8 and 9 focus on the use of supercritical fluid extraction for the determination of fluconazole, tioconazole, UK-47,265 and hexaconazole from the animal feed matrix.

Chapter 4.0 Instrumentation and Experimental Procedure

4.1 Characterisation of SFE and SFC Instrumentation

To perform SFE or SFC, the instrumentation required includes a pump, valves, detector cell, oven, a restrictor devices and extraction cell or separation column. The basic instrumentation for SFE/SFC can be built from either a HPLC or GC set up without any major modifications. The device that characterises a supercritical fluid instrument is the restrictor or back pressure regulator which is not found in HPLC or GC systems.

4.1.1 Restrictors

There are three different types of back-pressure device currently used in SFE and SFC:

1. Fixed restrictor (e.g linear, crimped, integral, fritted)
2. Mechanical -feed back regulator
3. Electrical-feedback regulator

Table 4.1 compares these types of the back-pressure devices and their characteristics.[140]

Type	Dead volume	Applications
fixed restrictor	<<nL	cSFC
mechanical-feedback regulator	>ml	large scale SFE
electrical-feedback regulator	~10 μl	SFE and pSFC

4.1.1.1 Fixed Restrictors

Fixed restrictors are the simplest form of restrictor consisting of a capillary tube having an appropriate diameter and length for the required back pressure and flow rate used.[140] They have a small dead volume to meet appropriate requirements for columns with an internal diameter of less than 50 μm. Several types of capillary restrictors have been used having linear, crimped, tapered, converging or diverging, integral and fritted ends.[141] The major advantage of these simple restrictors is cheapness, however, the restrictor to restrictor back pressure reproducibility is poor and the restrictors are prone to blockages due to the condensation of the solute along the tube length. Fixed restrictors are used mainly for capillary SFC and analytical SFE.

4.1.1.2 Mechanical-Feedback Regulators

This type of restrictor is used for large-scale SFE and packed SFC. It is a complex regulator consisting of a pressure sensing device and a needle valve.[142] The valve needs to be heated to prevent the supercritical fluid from being frozen in the flow path by the temperature reduction due to adiabatic expansion. The regulator controls the back pressure irrespective of the mass flow rate of the supercritical fluid making it more conveninent than fixed restrictors. The disadvantage of this type of regulator is high cost and large dead volume, a few mL to several tens of mLs, that does not allow fractionation of solutes in a fluid from an extraction cell in SFE.

4.1.1.3 Electrical-Feedback Regulator

This type of regulator consists of a needle valve and a pressure sensing device. The pressure signal controls the opening of the needle valve so that the pressure is maintained at the preset value.[143] In general, this type of valve controls the flow

resistance of the valve by changing the gap between the valve and the seat. Some regulators are equipped with an electronic pressure sensing device and a motor-driven needle valve. Satio et al.[144] developed a new back pressure regulator based on high speed switching of the fluid flow by periodically opening and closing the flow path of a solenoid valve. The regulator prevents blockages in the flow path which are possible because precipitated solutes and dry ice from the carbon dioxide are continually trapped and forced to pass through the valve. To maintain the required back pressure the valve is used with a pressure transducer.

4.1.2 Off-line SFE

SFE can be performed using two different approaches, off-line and on-line. In this chapter, the methodology of off-line extraction will be discussed where the analytes are extracted using SFE and collected in a device independent of the chromatography or any other instrumental analysis. In various stages of the experimental work, off-line SFE was performed and the extracts were analysed by a variety of methods. The final product of an off-line SFE experiment consisted of the extracts dissolved in a few millilitres of an appropriate solvent making it compatible with all conventional chromatographic techniques.

Off-line SFE involves relatively simple experiments and requires basic instrumentation. For SFE to be successful, the target analytes must be controlled through three key phases. Firstly, the analytes must be partitioned from the sample matrix into the bulk of the supercritical fluid. This step is governed by the physicochemical interactions of the analytes, sample matrix, and the supercritical fluid, as described in chapter 1 by the SFE extraction triangle. Secondly, during the extraction, the analytes must be swept away from the matrix and out of the extraction cell. Thirdly, the analytes must be efficiently collected from the supercritical fluid in a form that is compatible for subsequent analysis. Accuracy is required for all three

steps in order to obtain quantitative recoveries. The first step is involved in finding optimized SFE conditions that can best remove the target analytes from the matrix into the supercritical fluid. An understanding of the partitioning processes helps to choose appropriate experimental conditions including pressure, temperature and modifier. Steps two and three are essentially plumbing parameters. The fluid flow rate required to sweep the analytes from the extraction cell in step 2 helps to determine the pumping rate requirements, the maximum extraction cell size required and the type of restrictor used to control the fluid flow rate. Step 3 involving the collection of the analyte after the extraction is nearly always performed by depressurizing the supercritical fluid through a flow restrictor.

4.1.2.1 Instrumentation for SFE

Instrumentation for analytical-scale SFE is available from a number of suppliers, and there are several instrumental differences among manufacturers. The acceptance of SFE as an analytical technique is partially impeded by the lack of confidence that analytical SFE methods can be reproduced on various types of instrumentation.

In principle, the instrumentation for SFE consists of five basic components: (1) a source of fluid (CO_2 in this case), (2) a pressure controlled pump, (3) an oven for temperature control of the extraction cells, (4) a region for the decompression of the supercritical fluid, and (5) a collection region for trapping the analytes as they precipitate during the decompression step. Various levels of complexity can exist on each of the lateer parts and choices should be made relating to the type of extraction work that instrument is to be used for.

The pump is used to deliver the supercritical fluid to the extraction cell. Generally, the extraction pressures required are in the range of 100 to 680 atm or 1,500 to 10,000 psi. The pumping system chosen must be able to supply an uninterrupted supply of

fluid during the entire length of the extraction. Both syringe pumps and reciprocating pumps have been successful in meeting the requirements. The oven must be capable of uniformly heating the extraction cell as well as any associated connecting tubes. It is important to preheat the fluid before it reaches the cell, especially if high flow rates or large extraction cells are required. The extraction cell is constructed of an inert substance capable of withstanding high pressures. The region where the decompression takes place is important for the complete recovery of the extracted analytes. The most commonly used decompression devices are valves, back-pressure regulators, small i.d. orifices, or pieces of small i.d. capillary tubing, usually fused silica. During the decompression, a great deal of cooling takes place due to the Joule-Thompson effect. This cooling can often cause precipitation of the analyte and plugging the decompression region or restrictor. This is usually avoided by heating the decompression region to help prevent restrictor plugging. The collection region, like the decompression region, plays an important role in analyte recovery in SFE. A wide variety of methods for trapping analytes have been documented in the literature including collection in liquid solvents,[145-147] collection on solvent resin traps,[148-149] collection on cryogenically cooled surfaces,[150] collection directly into chromatographic columns via on-column or split/splitless injection ports.[151-154] The configuration of the trapping region is often determined by the modes of extraction, either on-line or off-line. Off-line SFE was performed with a Jasco SFE system throughout the whole experimental protocol and will be discussed next.

The Jasco SFE System

The basic instrumental parts of the Jasco system consists of the following:

Pumps: Jasco 880-PU intelligent HPLC pump, one with cooling jacket on pump head

Oven: Jasco 860-CO 1 cm extraction cell, pre-heating coil and rheodyne switching valve.

Back pressure regulator: Jasco 880-81

A schematic representation of the Jasco system is given in figure 4.1

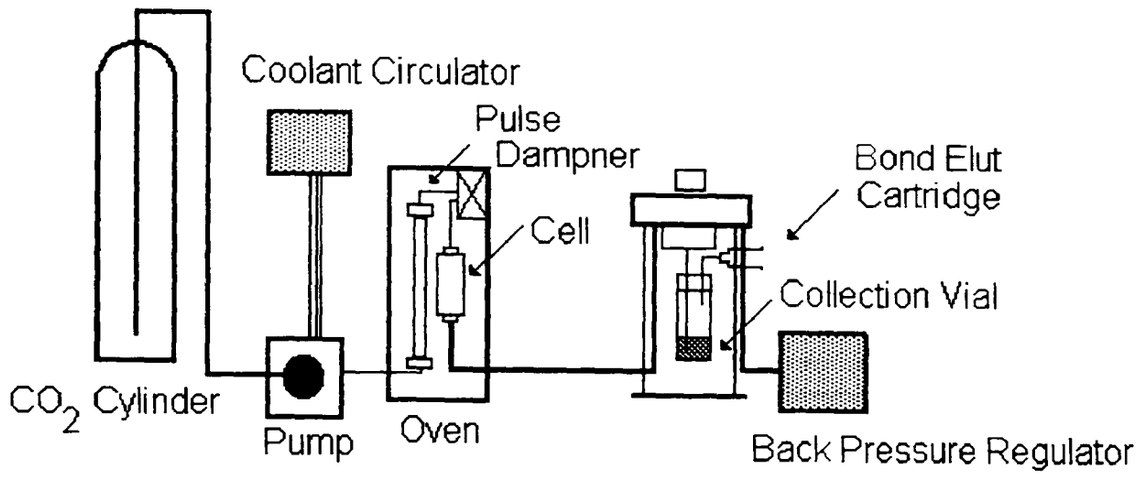


Figure 4.1 Schematic of a Jasco SFE system.

The supercritical fluid pump head is cooled by means of a jacket set between -5 to -10°C. A second pump is available for addition of modifier to the CO_2. Pressure is allowed to build up until the desired pressure and temperature is reached. The supercritical fluid flow is introduced into the extraction cell by manually switching a rheodyne valve. The back pressure regulator maintains the required pressure and hence the density of the supercritical fluid in the system. The supercritical fluid used, CO_2, a gas at ambient conditions, is vented from the collection device while the extracted analytes are retained. Screw cap glass vials (25 ml) with rubber septa at the top are introduced as the collection device. The metal pipe at the restrictor can pierce through the rubber septum. A hypodermic needle with a C_{18} Bond Elut cartridge also pierces through the septum in order to trap any escaping analyte and to allow CO_2 to vent to atmosphere. This collection system reduces the escape of analytes during extraction.

4.1.2.2 Dynamic and Static SFE

SFE can be performed using three different modes: 1. dynamic mode in which the supercritical fluid is continuously flowing through the extraction cell, 2. static mode in which the cell is pressurized with supercritical fluid and the extraction is allowed to proceed without any outflow of the supercritical fluid until the extraction is finished and 3. a combination of static followed by dynamic extraction. As far as the instrumentation is concerned, the only difference between these methods is the inclusion and use of additional valves near the inlet and outlet of the extraction cells for performing static extractions.

4.1.3 *Instrumentation for SFC*

Supercritical fluid chromatography (SFC) can be performed using either capillary columns or packed columns. In the last few years, research in SFC has led to two different approaches. Not only are there two different kinds of columns, but the stationary phases, the instrumentation and the typical applications are also different for the two forms of the SFC. Supercritical fluid chromatography instrumentation resembles both GC and HPLC equipment and because of this, it has been possible to take advantage of technological developments from both techniques. Capillary SFC was developed on principles based on capillary GC, where packed column SFC was based on the principles of conventional HPLC systems, including high-pressure pumps, stainless steel tubing, injection valves, and columns with few modifications, or none at all. A circulating bath or a refrigerating unit maintains a constant temperature for the supercritical fluid inside the pump reservoir.

4.1.3.1 Instrumentation for cSFC

Capillary SFC utilizes an open column which is a narrow, capillary tube, in which a film of a stationary phase is coated on the inside wall. The entire volume of the column, except that occupied by the stationary phase, is available for the flowing mobile phase. For a given stationary phase, three dimensions are important, the inner diameter of the column, the thickness of the stationary phase film and the length of the column. The diameter of the column affects the mobile phase flow through the column, the sample loadability, the permissible injection and detection volumes and the pressure drop over the column. All these effects have consequences for cSFC instrumentation. The basic components of an SFC include a pump, a computer for control and data collection, and finally the column.

As the temperature, pressure, and mobile phase composition of the supercritical fluid contribute to resolution and retention, the micro computer controls the pump by regulating the pressure/density ramping of the mobile phase. It can also control the oven temperature (if manufactured by the same company), injection mode, and possibly data acquisition, storage and manipulation. Normally the pumps are syringe pumps requiring a down time for refill after the reservoir has been emptied and they can deliver high and low flow rates with almost no pulsation. Injection of samples into the cSFE column requires a high pressure injection valve and is usually placed in a separately heated thermostatted unit, or kept at ambient temperature. The columns for cSFC have inner diameters of 50 μm and require syringe pumps to establish a stable flow. A restrictor is required to maintain supercritical conditions along the entire length of the column, as well as to control the flow rate. Depending upon the detector design, the restrictor is placed either immediately after or prior to detection. Detectors from either HPLC or GC can be used for SFC particularly with CO_2 as the mobile phase. The flame ionization detector has received the most interest for capillary SFC as it is both reliable and sensitive.

Carlo Erba Mega series SFC

All cSFC experimental work was performed with a Carlo Erba Mega Series cSFC (figure 4.2) and the instrumentation will be discussed next.

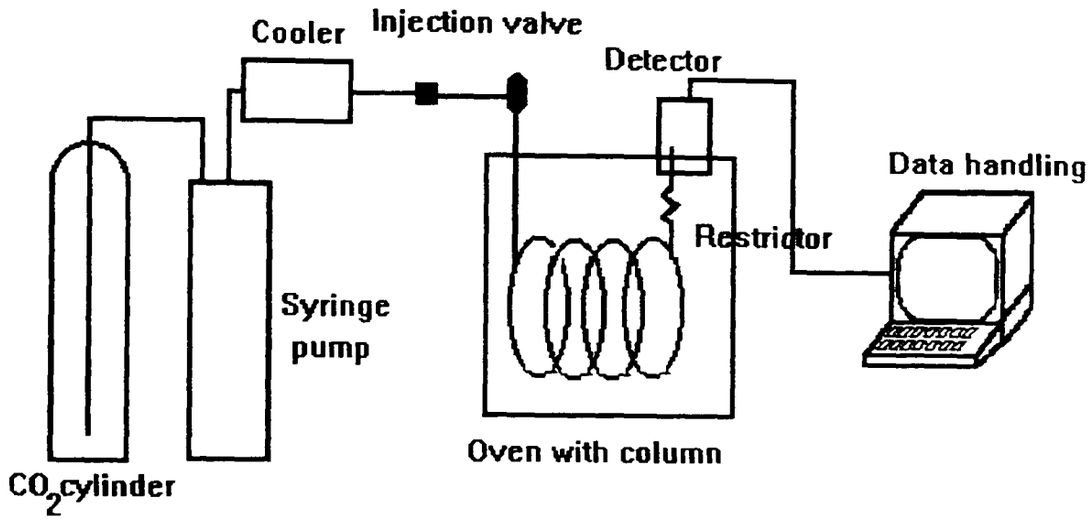


Figure 4.2 Schematic of a capillary SFC System.

The major components of the instrument consists of a Syringe pump, a GC oven, a FID and a recorder.

The syringe pump was capable of delivering pulse-free delivery of the mobile phase needed at the extremely low flow rates. The syringe pump discharge pressure was controlled with a micro computer. The control system was capable of programming the pressure or density of the mobile phase during a chromatographic experiment over a preselected pressure and/or density range. The sample was introduced into the mobile phase stream by a sample introduction valve. The mobile phase sweeps the sample onto the analytical column contained in the oven. The column was maintained at the desired mobile phase temperature in the temperature controlled chromatographic oven. A column/FID detector interface was placed between the column and the detector. This column detector interface reduced the column pressure

to atmospheric pressure in a smooth depressurization step. Finally, the detector signal was sent to the chromatographic data system.

4.1.3.2 Instrumentation for pSFC

Packed SFC utilizes a column similar to a HPLC column. The four relevant parameters in pSFC are the effect of particle size, the stationary phase, the column inner diameter and the column length. The instrumentation for a packed SFC system also is very similar to a HPLC system, except that there is a back pressure regulator, which pressurizes the mobile phase above its critical pressure, and a column heater is employed to keep the mobile phase above its critical temperature. The back pressure regulator maintains the system pressure through the detector for pSFC. Packed columns allow a much higher sample loading and can be adapted to a given detection volume by varying the column diameter without affecting the resolution making them compatible with UV detectors.

All pSFC was performed with a home made system which was built in the laboratory and will be discussed next. The basic components of the pSFC system consisted of the following:

Gilson 305 Master pump and slave pumps (HPLC pumps)

Dynamic Mixer

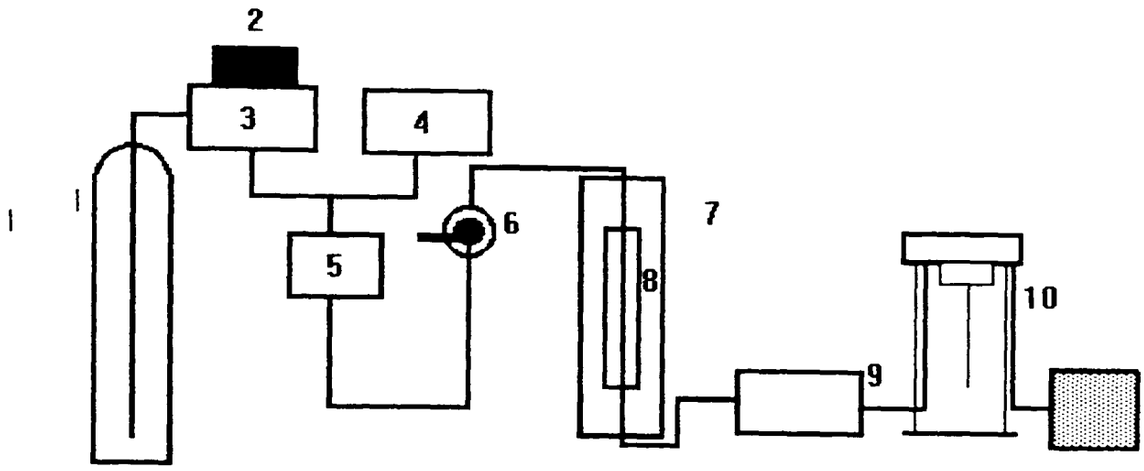
Manometric module

Oven

Jasco intelligent UV/Vis detector

Jasco back pressure regulator

In our laboratory, a pSFC has been constructed from a Gilson SFE system with a Jasco UV/Vis detector and back pressure regulator. Figure 4.3 shows a schematic diagram of the SFC system.



1. Gas cylinder, 2. Cooling jacket, 3. Master pump, 4. Slave pump,
5. Dynamic mixer, 6. sample introduction valve, 7. oven,
8. separation column, 9. detector and 10. back pressure regulator.

Figure 4.3 A schematic diagram of the SFC system.

The cooling of the reciprocating pump was achieved by means of a cooling jacket (-20 to -5°C) on the pump head to allow chromatographic operation of CO_2 as the mobile phase. An organic modifier was introduced into the eluent system by the second pump. Direct on-line mixing was achieved by the dynamic mixer. The temperature of the mixer fluid was kept at the desired chromatographic temperature by the use of the oven where the separation takes place. The injection valve is used for an introduction of the sample into the column. The effluent from the column flows into the detector and then into the back pressure regulator. The flow resistance of the back pressure regulator is automatically changed so that the system pressure is kept constant at the preset value. The pressure of the fluid is released by the back pressure regulator and the fluid containing the extracts flows out from the outlet as the ordinary gas.

4.2 Units Used for Pressure

At present, there are various units for measuring (a variety of units such as psi, atm, bar, kg/cm^2, and Pa) pressure. In spite of the recommendations for using the SI units, Pa is not yet well accepted by many scientists and engineers. Since specific equations require specific units, and data cited from the literature uses different units, it was decided to use all of these units as they appear in the original sources. For the reader's convenience, a unit conversion chart is given in table 4.2.

Table 4.2: Pressure unit conversion chart.

	kg/cm^2	atm	bar	MPa	psi
1 kg/cm^2	1	0.9678	0.9807	0.09807	14.22
1 atm	1.0332	1	1.0133	0.10133	14.70
1 bar	1.0197	0.9869	1	0.1	14.50
1 MPa	10.197	9.869	10	1	145
1psi	0.07031	0.06805	0.06895	0.006895	1

Chapter 5.0 : Estimating Steroid Solubility using Log P and Solubility Parameter Theory

The aim of this part of the thesis is to test the hypothesis that the hydrophobicity of a solute can be correlated directly to the solubility of that solute in the supercritical state. In supercritical fluid extraction (SFE), it is thought that the hydrophobic nature of a molecule can be closely related to its solubility in the supercritical phase. As with many pharmaceutical compounds, steroids have a widely varying hydrophobicities, ranging from completely insoluble in water to being highly soluble in water. It is expected that they will follow similar trends in supercritical CO_2 solubility. The use of partition coefficient in conjunction with a calculated solubility parameter as a reasonable means of estimating steroid solubility in supercritical CO_2 is demonstrated in this chapter.

5.1 Definition of Partition coefficients

The partition coefficient of a substance X is defined as the ratio of the concentrations of a solute equilibrated between two immiscible phases, typically an oil and an aqueous phase at a given temperature.

$$P = [X]_{org}/[X]_{aq}$$

Thus, from a qualitative standpoint, the partition coefficient can be defined as a measure of the solutes preference for the oil phase over aqueous phase, often referred to as lipophilicity or hydrophobicity. Hydrophobicity is normally measured using the logarithm of the partition coefficient in octanol-water systems (logP or $\log K_{ow}$). From a more fundamental standpoint, the partition coefficient may be described in terms of solutes cohesive and adhesive interactions with the respective solvent.[155]

Hence, partition coefficients can provide information on the intermolecular forces of the solute in solution.

5.2 Quantitative Structure-Activity Relations in Drug Design

Compared with a decade ago, tremendous progress has been made towards a rational basis for drug design. It is well known that the fundamental requirements for a particular kind of drug action are dependent upon the structure of the molecule and the location of the receptor. An effective drug has to satisfy three requirements. First, it must have a size, shape, and electron distribution complementary to that of the receptor. Second, it must have a lipophilicity-hydrophilicity balance that will ensure that it reaches the tissues where the receptor is located. Thirdly, it must accumulate selectively in the cells. These considerations lead to the establishment of relationships between the biological activity of a drug and its physical and chemical properties. These are called structure-activity relationships (SARs) and, if they can be expressed numerically, they become quantitative structure-activity relationships (QSARs).

SAR methods are nowadays of unquestionable importance in drug design. The objective of QSARs in drug design is to optimize the activity of drugs. QSAR models quantify biological activity in terms of molecular structure and provide useful forms of data reduction that assists in the rational design of compounds which appear optimum for therapeutic purposes. The molecular stucture coding in QSAR may take the form of quantum mechanical indices, topographical descriptors (chemical graph theory) or molecular fragments. The relationships between these mathematical models or descriptors is extrapolated to predict the activity of drugs.

Partition coefficients have been used to model the biological activity of both specific and non-specific drugs. Octanol water partition coefficients have been accepted as a favourable method of estimating the lipophilic character of organic compounds.[156-

[158] This arose from the assumption that the octanol phase may resemble organic material in lipid tissues of living organism.

The activity of both specific and non-specific drug activity are of interest in QSAR studies. Specific drug action may be based on the specific site receptor, whereas non-specific drug action can be based on the disruption of gross structural features of the body, such as a lipid membrane. Non-specific contributions can be obtained by direct measurement of the equilibrium concentration of a drug partitioned between two immisible phases, such as, octanol and water. The measurement of drug activity can be made more specific by using a fragmentation method proposed by Hansch and Leo,[159] where the interactions of the drug molecule with the biological systems are obtained by application of established thermodynamic principles based on additive correlations using substituent constants for component parts of the molecule.

QSAR models differ primarily in the manner in which the 'structure' (S) term is elucidated and quantified. Models follow the general form:[160]

$$A = f(S)$$

where S represents either an empirical or theoretical structural parameter for the total molecular structure or selected substructural fragments and A represents the magnitude of the measured dependent parameter (biological activity in QSAR).

There are two types of QSAR models : "Free - Wilson" or "Hansch". In the Free - Wilson model, the bioactivity is related to contributions from individual substituents on the drug molecule.[161] The QSAR studies then ascertain those substituents providing the greatest contributions. The Hansch-Leo approach is also known as the "linear free energy model" and S is encoded as combinations of various physico-chemical characteristics (hydrophobicity, molecular weight, refractivity, steric

influences) of the collection of structural analogs used in the study. Hansch and Leo approach is the most popular method for drug design as it is easier to screen novel molecular structures for drug activity by this method.

5.3 Measurement of Partition Coefficient

The experimental determination of LogP is governed by the acid-base behaviour of the compound concerned. Non-ionisable compounds are generally uncomplicated excepting that very large P values can cause difficulties when the aqueous phase concentration is so low that errors in assay can arise. A great number of pharmaceuticals, however, exhibit acid-base behaviour, and therefore partition into an organic solvent will be influenced by the compounds pKa.

The direct methods are straight forward, and are either 'batch' or 'dynamic' techniques. 'Batch' includes the 'shake flask method'[162-166] and the 'slow stirring'[167-168] method. The dynamic procedure involves a solvent extraction flow cell ('filter chamber'). All these direct methods require the compound concerned to have a chromophoric group (for spectrometric assay) or to be amenable to potentiometric titration. Care and precision are required in the measurement to obtain accurate values of P since it is a ratio and small errors in concentration measurement result in greater errors in P. For example, sufficient time must be allowed for the attainment of equilibrium (this can take up to several days); the aqueous buffer and octanol phases should be fully equilibrated with each other before the dissolution of the compound-otherwise volume changes result.

5.3.1 Shake Flask Method

The "shake Flask" method is probably the most widely used technique for measuring

log P. This method is however time consuming and requires a series of steps. Firstly, a known amount of solute is dissolved in the most appropriate phase of the solvent pair. The second phase is added next which involves adding usually 2 to 10 ml of octanol and 10 to 25 ml of water[162-166] and the solute is partitioned by shaking the two phases together. The shaking time reported for the procedure have been varied in the literature from 30 seconds to 12 hours[162-166] but approximately 30 minutes appears to be the most appropriate. The phases are separated under gravity for 2 to 12 hours followed by centrifugation (10 to 45 min at 1500 to 12000 rev/min[162-166]) and the concentration of the solute in both phases is determined by any available analytical technique (GC, HPLC, UV-Vis etc.).

5.3.2 Reversed Phase HPLC

RP-HPLC retention data are currently playing a significant role in the determination of log P. This method is based on the fact that a linear correlation exists between the retention of a compound in a reversed-phase chromatographic system and its partition coefficient.[167-171]

It has been implied that the partitioning of a chemical between organic and aqueous phases of a RP-HPLC column correlates with the partitioning which takes place between various phases of the environment.[168] It is also assumed that partition coefficients determined by the RP-HPLC method reflect true partitioning behaviour in biological systems due to the fact that the mobile and the stationary phases in the RP-HPLC resemble the aqueous and non-aqueous phases in biological systems, in contrast to the traditional octanol-water partitioning method. Octanol is an isotropic liquid in which the size and the shape of the solute molecules are not the determinants of the partitioning process. In contrast, biomembranes are anisotropic, and the molecular size and shape and the orientation of functional groups plays a role in the

partitioning process. Furthermore, the former is a static system, while the latter is the dynamic system. The RP-HPLC is both anisotropic and dynamic.[168,172]

RP-HPLC can be performed on commercially available analytical columns packed with a solid stationary phase consisting of a non-polar organic surface layer, such as C8 or C18 alkyl chains, covalently bound to silica patricles. The transportation of the solutes through the columns involves partitioning between the hydrocarbon stationary phase and the mobile phase consisting of water with an organic solvent. The solutes are eluted out of the column according to their hydrophobicity. The water soluble compounds are eluted first and the oil-soluble compounds are eluted last, in proportion to their hydrocarbon-water partition coefficient.

The chromatographic parameter commonly used for correlating octanol-water partition coefficient with measured HPLC data is the capacity factor, k'. The capacity factor ranging typically between 5 and 10 for a number of reference compounds is usually required to give a desired range of octanol-water partition coefficients. The log k' values of the reference compounds are plotted against their log octanol-water partition coefficient values and this correlation plot is used to estimate log partition coefficient values of the test compounds.

5.3.3 Available Methods for Calculating Octanol-Water partition Coefficients

Most of the experimental methods for the determination and estimation of octanol-water partition coefficients have only limited use for hydrophobic compounds and are also error prone. In addition, the experimental determination of many pharmaceutical compounds are often extremely difficult. In view of the experimental difficulties, a number of calculative methods have been developed based on mathematical treatment of the molecular structure for the rapid determination of logP. These molecular approaches can be divided into two categories. Firstly, those which use molecular

fragments and secondly, those based on an atomic approach. The former approach is preffered. The use of a fragmental approach has been viewed from two different prospectives. The first approach, described as a constructionist approach, was developed by Hansch and Leo whereas the later method, proposed by Rekker, uses a reductionist approach. In each case, the molecular fragments are used to derive a log P value. These molecular fragmental contributions are available in tabular form for each approach.

5.3.3.1 Hydrophobic Substituents Constants (Hansch-Fujita) Method

The first methodology for calculating logP was proposed by Fujita et al.[173] in 1964. Log P was considered to be an additive constitutive property and numerically equal to the sum of the logP of the "parent" solute plus a π term which represented the difference in logP between a particular substituent and the hydrogen atom which it replaced. Thus the π for substituent can be defined as

$$\pi(X) = \log K_{ow}RX - \log K_{ow}RH$$

where $\log K_{ow}RX$ is octanol-water partition coefficient of a derivative, $\log K_{ow}RH$ is octanol-water partition coefficient of a parent compound, and $\pi(X)$ is the hydrophobic substituent constant that can be defined as a contribution of substituent X to hydrophobicity when replacing hydrogen atom H by X (for example, $\pi(Cl) = \log K_{ow}C_6H_5Cl - \log {}_{ow}C_6H_6$; $\pi(Cl) = 2.84 - 2.13 = 0.71$).[159] A positive value of πmeans that relative to H, the substituent favours the octanol phase. A negative value of π shows its hydrophillic character relative to H. If two or more substituents are added to the parent compound, the equation takes a different form:

$$\log K_{ow}RX1X2.....Xn = \log K_{ow}RHH.....H + \Sigma\pi(X)n$$

Values of π for various substituents were determined on the basis of carefully measured octanol-water partition coefficient and tabulated.[167,173]

This proposed π system of Fujita et al.[173] was first applied only to substitution on an aromatic ring where the hydrogen atom being replaced was definitely of a hydrocarbon nature. Later this methodology was attempted by several other investigators where calculations were carried out with compounds where the hydrogen was clearly part of a polar moeity, such as a hydroxyl or amine. The results obtained were often prone to error. In addition, not all 'aromatic hydrogens' could be substitued without some correction factor, π for a substituent which is capable of hydrogen bonding is greater when it replaces a hydrogen on an electron-deficient ring than when replacing one of benzene's hydrogens.

5.3.3.2 Hydrophobic Fragmental Constants (Rekker) Method

The 'Fragmental' approach to the calculation of log P was pioneered by Rekker and his colleagues.[159] One of the approximations used in the hydrophobic substituents system is that the hydrophobicity of a hydrogen atom in the octanol-water system is close to zero, and thus a $\log K_{OW}$ of a compound can be obtained by summing the π constants. However, it was found at a later date that the hydrophobicity of a methyl group was significantly greater than that of a methylene group.[159] Following this, Rekker developed a new approach to calculate the $\log K_{OW}$ values based on this subsequent equation:[167]

$$\log K_{ow} = \sum_{1}^{n} a_n f_n$$

where a is a number of occurrences of a fragment, f, of the structural type, n. The hydrophobic fragmental constant, f, is the contribution of a constituent part of a

structure to the total hydrophobicity of a molecule.[157] A close relationship between this system and the previously described system can be demonstrated when considering their fundamental equations together:

$$\pi (X) = \log K_{owRX} - \log K_{owRH}$$

$$f_X = \log K_{owRX} - f_R$$

$$2f_H = \log K_{owH-H}$$

$$f_X = \pi (X) + f_H$$

By definition, adding the fragment constant of hydrogen to the π constant for any substituent gives the fragmental constant for that substituent.[173]

Careful study of the experimental $\log K_{ow}$ values enabled the determination of f constants for various fragments.[167,174] A number of fragments, such as CH_3, CH_2, CH, NH_2, NH, C_6H_5, OH, O, COOH, and COO were defined as primary fragments: they originate from regression analysis of partition coefficients and have known standard deviations. Inclusion of any fragments in regression analysis implies the availability of at least 4 or 5 different structures wth reliable experimental $\log k_{ow}$ values. Other fragments, defined as secondary fragments, were calculated on the basis of $\log K_{ow}$ values of "parent compounds" and f constants of primary fragments. A number of correction factors were also introduced to this system to account for various interactions between the functional groups.[165]

5.3.3.2 Leo-Hansch Fragmental Constants Method

Not long after Rekker's hydrophobic fragmental constant system, Leo-Hansch[159] developed a system using a very few carefully measured $\log K_{ow}$ values for simple structures to calculate the fragmental values for the most basic and useful constants, such as the fragmental value for the hydrogen atom or carbon atom. The fragmental value for the hydrogen atom was estimated from the assumption that in the H_2

molecule, the atom is not involved in any interactions and can be considered as an ordinary one. A carefully measured $\log K_{ow}$ value for H_2 is 0.45, therefore, $f_H = 0.22$ and is considered as the cornerstone of the system. The fragmental value for a carbon atom in an alkyl chain is another fundamental constant required in $\log K_{ow}$ calculations. The adopted approach was through the carefully measured $\log K_{ow}$ values of methane and ethane. The values obtained for $\log k_{ow} CH_4 = 1.09$ and $\log_{ow} CH_3CH_3 = 1.81$. Two routes are then available for calculating value of CH_3:

$$f_{CH_3} = \log K_{ow} CH_4 - f_H = 1.09 - 0.225 = 0.825$$

$$f_{CH_3} = 1/2 \log K_{ow} CH_3\text{-}CH_3 = 1/2[1.81] = 0.905$$

Rounding off the average value of 0.885 to 0.89 and the f_H value to 0.23 the following values are obtained

$$f_{CH_2} = f_{CH_3} - f_H = 0.66$$

$$f_{CH} = f_{CH3} - 2f_H = 0.43$$

$$f_C = f_{CH_3} - 3f_H = 0.20$$

Thus, in this method, the fragment constants $f_H = 0.23$ and $f_C = 0.20$ are regarded as the only fundamental ones required to calculate all alkane structures. The other factors (F) that affect the partitioning equlibrium in the more complex solutes are taken into account where simple summations of fragments fail. In the Leo-Hansch fragmental constant method, Rekker's basic equation is extended to:

$$\log K_{ow} = \sum_{1}^{n} a_n f n_n + \sum_{1}^{m} b_m F_m$$

where F represents a factor that affects the partitioning equilibrium in a solute where summation of fragments does not lead to acceptable results and b is the number of occurrences of factor F.Hansch and Leo proposed a set of correction factors in order to achieve correct calculation of $\log K_{ow}$ values.[159] Among these are corrections for

chain length, branching and unsaturation, ring size, aromaticity and hydrogen bonding.

5.3.3.4 Rekker's Revised f-System

In the previously described Rekker's f-system, a so called "magical constant" was introduced as a way of compensating discrepancies between experimentally determined log K_{ow} values and calculations achieved by fragment addition. It was noticed that this difference could be expressed in terms of a value 0.289, which was called the "magic constant". The Rekker's f-system equation was restated with the addition this constant:

$$\log Kow = \sum f + \sum kn \times C_M$$

where C_M is the magic constant and k_n is expressed as the frequency of C_M occuring in a structure under consideration.[174]

5.4 Calculation of log P Values for Steroids

The method adopted for calculating logP[175] in this thesis is a computer based approach, ClogP. The Hansch-Leo approach is implimented in the ClogP software package, based on the experimentally derived values for simple fragments within structures. Log P can then be obtained by dividing a compound into a series of these fragments, followed by simple summation of their hydrophobicity parameters.

5.5 Determination of Fedors Solubility Parameter in the Supercritical Phase

Solubility parameter theory was discovered over thirty years ago as a method for describing the solvent power of compressed gases.[22] The theory provides the means

to predict the quantitative behaviour of solutes in dense fluid supercritical solvents. There are several methods available for calculating the solubility parameter. These have been evaluated by King[176] who concluded that a 10% variance between different methods is commonplace. Perhaps the simplest method of calculating the solubility parameter is based on a group contribution method as proposed by Fedors.[177] The main advantage of this method is that it allows the estimation of the solubility parameter from only a knowledge of the solutes molecular structure. Structurally complex solutes can thus be calculated without prior knowledge of their thermodynamic properties. The Fedors method has been related to solubility in supercritical carbon dioxide by King and Fredrich[178] via the reduced solubility parameter proposed by Giddings.[179] King concluded that a large body of SFE data could be correlated, in a quantitative manner, to changes in the molecular structure.

The solubility parameter, δ_2, was described by Hildebrand in terms of thermodynamic properties on mixing of dilute solutions.[22] For a compound with low molecular weight this was found to be related the substances energy of vaporisation ΔEv at a given temperature, and the molar volume at that temperature V. The solubility parameter is related to these two physical properties by equation 5.1

$$\delta_2 = (\Delta Ev/V)^{0.5} \qquad 5.1$$

The heat of vaporisation is often measured to calculate the energy of vapourisation. The following equation relates the energy of vaporisation to the heat of vaporisation (equation 5.2).

$$\Delta Ev = \Delta Hv - RT \qquad 5.2$$

Where, R is the gas constant and T is the absolute temperature

The boiling point of a substance can be used to calculate the ΔHv using an empirical method proposed by Hildebrand [equation 5.3].

$$\Delta Hv = T_b^2 + 23.7\ Tb - 2950 \qquad 5.3$$

Where, T_b is the boiling point at standard pressure.

When a substance, such as a solid, possesses a very low vapour pressure, then difficulty arises in measuring either the energy of vaporisation or the boiling point of that substance. The Hildebrand method of estimating the solubility parameter is only practical if the numerical values T_b and T_c are accessible. This situation is uncommon for complex and novel compounds.

An alternative method for estimating solute solubility is based on the summation of atomic group contributions. The methods used in this thesis to calculate the solute solubility in the supercritical phase is that proposed by Robert Fedors.

By examining the vast amount of molar volume and energy of vaporisation information available for liquids Fedors found that a general system for estimating both the ΔEv and V could be set up. The system assumes, like the other systems, that the group contributions of individual functional groups on a molecule can be summed to give an overall estimate of the solubility parameter.

As explained above, the solubility parameter is calculated from the expression $\delta_2 = (\Delta E_i / V)^{0.5}$ where ΔE_i is the energy of vaporisation at a temperature of 25 °C and V is the corresponding molar volume. Fedors concluded that by examining a vast amount of data for simple liquids, both the ΔE_i and V values could be estimated for more complex molecules by adding the group contributions of the various fragments of the whole molecule. Using Fedors proposed method, the solubility parameter of a series of

steroids with ranging polarities was also calculated [table 5.1]. Table 5.2 shows an example calculation of solubility parameter by the Fedors Method for Testosterone.

Table 5.1 List of CLogP and solubility parameter values for different steroids.

Steroid	Solubility parameter	CLogP
megestrol acetate	10.44	3.904
betamethasone-17,21 dipropionate	11.42	2.872
clobetasone	12.09	1.907
clobetasol	12.66	1.919
hydrocortisone	14.09	0.66
prednisolone	13.04	0.444
pregnelone	10.72	3.907
testosterone	11.16	3.349
testosterone propionate	9.95	4.784
prednisone	13.48	-0.018
betamrthasone valerate	12.19	2.897
4-androstene-3,17 dione	10.3	2.887
betamethasone	13.83	0.806
testosterone acetate	9.94	4.255
cortisone acetate	12.08	0.7
beclomethasone dipropionate	11.53	3.108
clobetasol propionate	11.66	2.952

Table 5.2 Example calculation of solubility parameter for Testosterone.

Contribution	ΔEv per fragment	V per fragment	Fragment ΔEv for Testosterone	Fragment V for Testosterone
CH3-	1125	33.5	2250	67
CH2-	1180	16.1	9440	128.8
CH-	820	-1.0	3280	-4
HC=	1030	13.5	1030	13.5
C-	350	-19.2	700	-38.4
C=	1030	-5.5	1030	-5.5
C=O	4150	10.8	4150	10.8
OH	7120	10	7120	10
Ring Closure 5-6 atoms	250	16	1000	64
Conjugated double bonds	400	-2.2	400	-2.2
Total			30400	244

$$\delta 2 = (\Delta Ev/V)^{0.5} = 11.16$$

5.6 Steroid Solubility Prediction Using C log P and Solubility Parameter

A plot of solubility parameter versus C log P is shown in figure 5.1. This plot can be indicative of the probable solubility of steroids in supercritical carbon dioxide. It can be deduced from the plot that steroids (table 5.3) appearing on the top left hand corner are in the soluble region, the steroids appearing in the middle region are in the intermediate solubility region and the steroids in the bottom right hand corner are in the insoluble region.

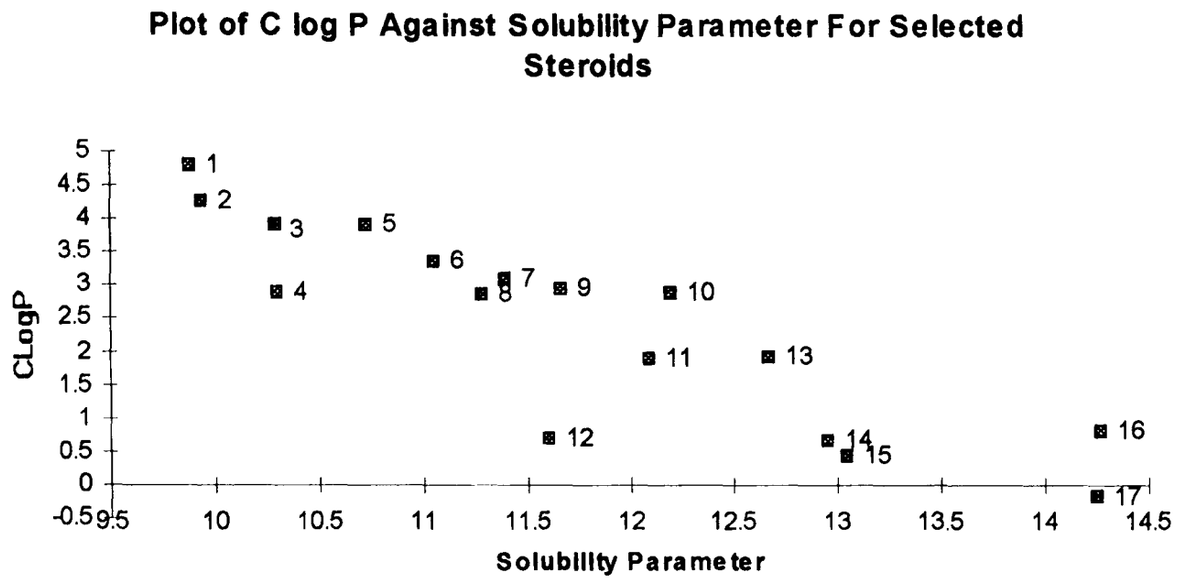


Figure 5.1 Plot of CLogP against solubility parameter for selected steroids.

Numbers represent the steroids 1 = testosterone propionate, 2 = testosterone acetate, 3 = megestrol acetate, 4 = 4-androstene-3,17-dione, 5 = pregnenolone, 6 = testosterone, 7 = beclomethasone dipripionate, 8 = betamethasone dipropionate, 9 = clobetasol propionate, 10 = betamethasone valerate, 11 = clobetasone, 12= cortisone acetate, 13 = clobetasol, 14 = hydrocortisone, 15 = prednisolone, 16 = betamethasone, 17 = prednisone

Table 5.3 Prediction of steroid solubility in supercritical CO_2 using solubility parameter and C log P.

Steroid	Predicted Solubility in Supercritical CO_2
megestrol acetate	soluble
betamethasone-17,21dipropionate	soluble
clobetasone	intermediate
clobetasol	intermediate
hydrocortisone	insoluble
prednisolone	insoluble
pregnelone	soluble
testosterone	soluble
testosterone propionate	soluble
prednisone	insoluble
betamrthasone valerate	soluble
4-androstene-3,17 dione	soluble
betamethasone	insoluble
testosterone acetate	soluble
cortisone acetate	intermediate
beclomethasone dipropionate	soluble
clobetasol propionate	soluble

5.7 Conclusion

A prediction of steroid solubility in supercritical carbon dioxide is described, based on the solubility parameter theory and the hydrophobic term, ClogP. This data is only qualitative and does not estimate supercritical conditions, such as temperature and pressure. The plot identifies trends in steroid solubility and later in the thesis the solubility of some of the selected steroids will be measured (chapter 7) which supports the trend indicated in this chapter.

Chapter 6.0 The Use of a Chromatographic Method to Predict Solubility of Steroids in Supercritical Carbon Dioxide

6.1 Introduction and Aims

One of the initial goals of the project was to attempt to find a relationship between solubility of an analyte in supercritical carbon dioxide and chromatographic retention. The aim of this study was to provide a simple relationship between the capacity factor and the solubility of testosterone in supercritical carbon dioxide. Testosterone was selected as it is a commonly used pharmaceutical compound and as it is more complex than other analytes commonly used for this type of research such as naphthalene. There was also literature solubility data available for testosterone at different temperatures and pressures.

The use of chromatographic retention data to predict physical chemical properties, such as, molar volume and solubility is well established in quantitative structure activity relationship (QSAR) studies.[180] Also, chromatographic retention data are easily available in the literature and they are also easy to generate. Chromatography, in itself, is a partitioning process and can be defined as a separation method based on different distributions through different interactions of solutes with a mobile phase and a stationary phase, which are in equilibrium. In the case where the mobile phase is a supercritical fluid, the chromatography is defined as supercritical fluid chromatography (SFC). The variable operating conditions for SFC provide the supercritical carbon dioxide mobile phase with sufficient variability in solvating ability. SFC retention measurements can provide important physiochemical information including the capacity factor of the component under investigation. The capacity factor is proportional to the partition coefficient of the analyte which in itself is an important factor which directly facilitates the modelling of extraction / separation.[181] Equilibrium properties, such as, partial molar volume and solubilities are also related to the partition coefficient.[180] Therefore by directly measuring the

capacity factor for a component an estimation of the analyte solubility in supercritical carbon dioxide can be obtained. Previous work[182] has shown that under selected conditions e.g. at constant temperature and for a particular column, the capacity factor for a particular solute is inversely proportional to its solubility in the mobile phase. This relationship can provide a rapid way of generating solubility data from capacity factor measurements providing the proportionality constant relating the two quantities are known.

6.2 Principles of Chromatographic Separation

Chromatography is essentially a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary, while the other percolates through it in a predefined direction. The chromatographic process occurs as a result of repeated sorption/desorption interactions during the movement of the sample components along the stationary bed, and the separation is due to the differences in the distribution constants of the individual sample components.

The column accommodates the two phases: the stationary phase, which is integral in the column, and the mobile phase which is transported through it. Separation is achieved because different sample components show different distributions over the two phases. Those solutes which are preferentially distributed in the stationary phase remain in the column longer than those that are distributed in the mobile phase. Consequently, individual solutes will be eluted from the column in order of their increasing distribution coefficients with respect to the stationary phase. The solutes are distributed between two phases to different extents because the molecular interactive forces between the solute molecules and those of the two phases are different for each individual solute. For those solutes distributed preferentially in the stationary phase, the forces between the solute molecules and stationary phase

molecules are much greater than the forces between solute molecules and those of the mobile phase. Conversely, solutes distributed preferentially in the mobile phase exhibit greater forces between them and the mobile phase than they do with the stationary phase.[183]

The density of the mobile phase is the most important parameter to influence the separation in SFC.[184] The influence of density on the solvent properties can be demonstrated using the concept of solubility parameter.[179] Using this concept, the solubility parameter of a solvent depends on its density and can vary from 0, up to 10 at high densities. To solubilise a substrate, the solubility parameter of the substrate and the solvent should be nearly equal. At low solvent densities, the solubility parameters of the mobile phase in the SFC are low compared to those of the substrate, but they increase proportionally with increasing density. Therefore, the capacity factor, k (the degree of retention of a solute), decreases at higher densities indicating higher solvent strength.

6.3 Retention Times and Capacity Factors

A solute, i, distributes itself over the two phases, mobile phase and stationary phase resulting in a total quantity, $q_{i.m}$, to be present in the mobile phase and a quantity, $q_{i.s}$, to be present in the stationary phase. The solute molecules which find themselves in the mobile phase will be transported through the column at the same speed (u) as the molecules of the mobile phase. However, this is only a fraction of all the solute molecules, so the average speed for all solute molecules will be only a fraction of u given by

$$v_i = [q_{i.m} / (q_{i.m} + q_{i.s})] \cdot u \qquad 6.1$$

where, v_i, is the migration speed, the average speed at which the solute band travels through the column.

The time, $t_{r.i}$, needed for the solute band to elute from the column is determined from the column length and average migration speed:

$$t_{r.i} = L / v_i \qquad 6.2$$

Similarly, the time which a mobile phase molecule will spend in the column is

$$t_o = L / u \qquad 6.3$$

where to is the hold-up time, mobile phase time, or unretained time.

Combination of the two equations gives,

$$t_{r.i} = (1 + q_{i.s} / q_{i.m}) \cdot t_o \qquad 6.4$$

by definition, the capacity factor (k_i) of the solute, i, is

$$k_i = q_{i.s} / q_{i.m} \qquad 6.5$$

and hence

$$t_{r.i} = (1 + k_i) \cdot t_o \qquad 6.6$$

The quantity, q, of the solute, i, in one of the phases is the product of the average concentration, c' of the solute, i, in that phase (where the average is taken along the length of the column) and the volume of that phase. Hence the capacity factor equation becomes

$$k_i = q_{i.s} / q_{i.m} = [c'_{i.s} / c'_{i.m}] \cdot V_s / V_m \qquad 6.7$$

The ratio $c'_{i.s} / c'_{i.m}$ is a constant if the distribution isotherm (a plot of $c_{i.s}$ vs $c_{i.m}$) is linear. The distribution coefficients (partition coefficient in terms of concentration (K_c)), may be defined as,

$$K_{c.i} = c_{i.s} / c_{i.m} \qquad 6.8$$

K_c may be independent of the solute concentration, but will always be a function of the temperature and pressure. If the distribution isotherm is linear, K_c will also equal the ratio of average concentrations in equation (6.3.7) and hence

$$k_i = K_{c.i} \cdot V_s / V_m. \qquad 6.9$$

Equation 6.3.9 relates retention in chromatography (k) to a thermodynamic parameter, the partition coefficient (K_c). The partition coefficient strongly depends on the operational parameters of SFC. The phase ratio V_s / V_m is a characteristic of the column and is one of the factors that determines the retention, k, in chromatography.

The phase ratio can be influenced by varying several parameters, for example, the type of column (packed or capillary), the column diameter etc.

6.4 The Effect of Operational Variables on Retention in SFC

In SFC, the typical effect of variables such as density (ρ), or pressure (P), temperature (T), and composition (χ) on retention is shown in equations 6.10-6.12, where β's are solute-dependent coefficients.[185] The first order coefficients are almost invariably negative for density and composition; they can be positive or negative for

reciprocal temperature, depending on whether the solute exhibit GC like or LC like behaviour i.e. whether the solute is volatile/poorly solvated or non-volatile/highly solvated. The second order coefficients are usually negative or smaller: they are often neglected over the relatively narrow, but practical range of densities or mobile phase composition typically employed for most samples. Assuming all other variables are constant, equations 6.10-6.12 show that the retention may be reduced by increasing density (or pressure), temperature (T), or the amount of stronger component in the supercritical fluid mixture (modifier). Since the retention of different solutes does not depend on these variables in precisely the same way (i.e. the first and/or second order coefficients in equations 6.10-6.12 are generally not the same for different solutes), a change in selectivity frequently accompanies a change in these variables, particularly temperature and composition of the modifier.

$$\ln k' = \beta_0 + \beta_1\rho + \beta_2\rho^2 \qquad 6.10$$

$$\ln k' = \beta_0' + \beta_1'/T \qquad 6.11$$

$$\ln k' = \beta_0'' + \beta_1''\chi + \beta_2''\chi^2 \qquad 6.12$$

From thermodynamic and other considerations, density has been shown to be more fundamental variable than pressure. For those supercritical fluids for which density can be predicted from pressure via an accurate equation of state, it is easy to attain an accurate and precise density by simply controlling the pressure and temperature.

6.5 *Experimental Section for the Capacity Factor Analysis of Steroids by cSFC*

Capacity factor measurements were obtained using a Carlo Erba capillary supercritical fluid chromatography system with a flame ionisation detector. The steroids under investigation were testosterone, megestrol acetate and pregnenolone (Figure 6.1). All steroids were provided in pure form by Pfizer Central Research, Sandwich, Kent. The sample preparation for these steroids involved simply dissolving

the pure steroids in MeOH. The final concentration of the analytes injected were 1000 ppm. The chromatographic columns used in this study included the following, a 50 m x 50 μm id biphenyl (0.25 μm film thickness) and a 10 m x 50 μm id SB-Cyanopropyl-50 (0.25 μm film thickness). The solutions of the samples were introduced onto the column using split injection.

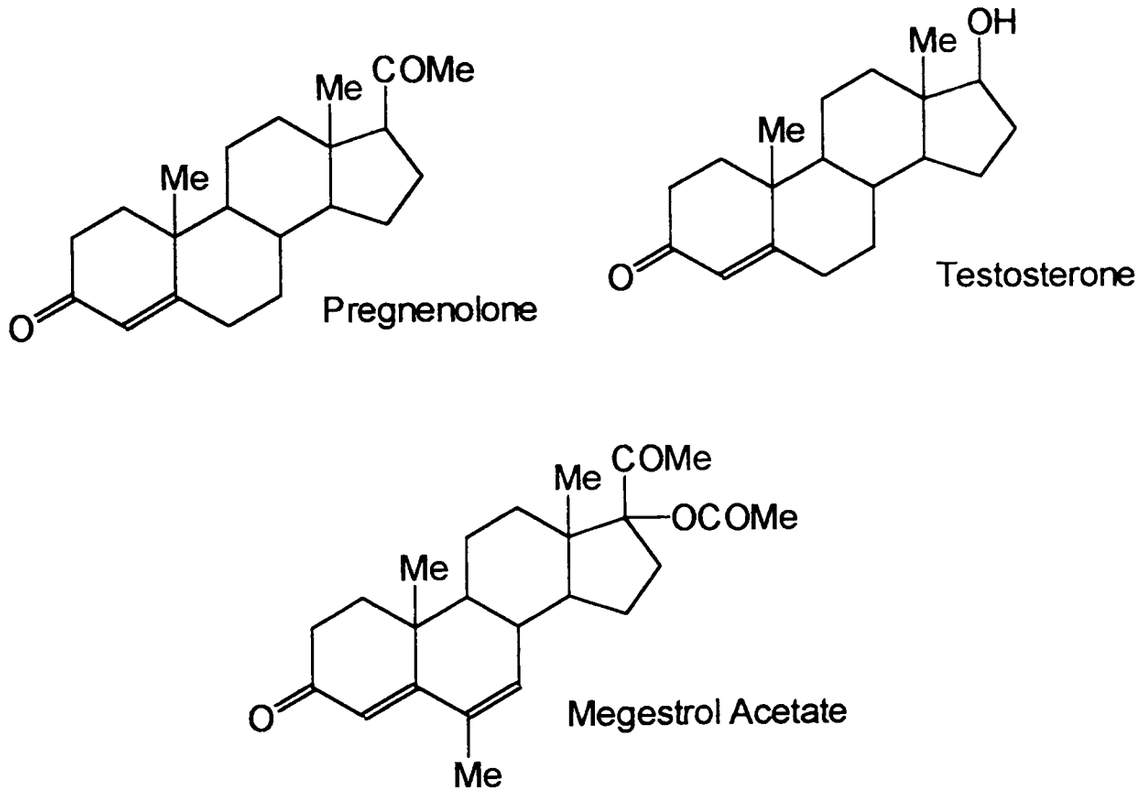


Figure 6.1 Structure of selected steroids.

6.6 Results and Discussion

Initially capacity factor measurements were determined (table 6.1) for testosterone using a biphenyl column at various temperatures and pressures.

Table 6.1 List of capacity factor measurements for testosterone at various temperatures and pressures using a biphenyl column.

Temperature (°C)	Density (g/ml)	Pressure (MPa)	Capacity factor (k)
55	0.817	24.18	0.56
55	0.772	20.75	0.90
55	0.718	17.32	1.66
55	0.671	15.63	3.03
55	0.629	13.97	5.50
80	0.772	32.6	0.51
80	0.750	30.4	0.60
80	0.718	27.6	0.81
80	0.671	23.9	1.37
80	0.629	21.6	2.15
90	0.772	37.2	0.37
90	0.718	31.5	0.62
90	0.671	27.2	1.04
90	0.629	24.6	1.12
90	0.519	19.4	1.49
100	0.772	41.3	0.33
100	0.718	35.5	0.50
100	0.671	30.6	0.84
100	0.629	27.6	1.24

In SFC, once the mobile and the stationary phase have been selected, the retention of a solute as measured by the capacity factor, k, is controlled by variables such as density or pressure, temperature and composition.[185] The initial study with testosterone involved investigating the relationship between capacity factor with density, temperature and a function of density / temperature (table 6.2) by regressing ln capacity factor against density, 1 / temperature and density / temperature. The derived equation obtained ($r = 0.93$, $r^2 = 0.86$) was as follows, enabling capacity factor for testosterone to be calculated providing the density and the temperature are known.

$$\ln k = -21.41 + [15.72 \times \text{density}] + [9511.52 / \text{temperature}] - [8365.67 \times \text{density} / \text{temperature}] \qquad \textbf{6.13}$$

In SFC, the degree of retention of a solute is also inversely proportional to the solvating power of the mobile phase for that solute, i.e. the more soluble a solute is in the mobile phase the less it will be retained.[182] On the basis of this, a similar relationship was derived between solubility with density, temperature and a function of density / temperature for testosterone using available literature solubility data[186] for testosterone at specific temperatures (35, 40, 45 and 55°C, table 6.3) and pressures. The derived equation ($r = 0.97$, $r2 = 0.95$) was of the form:

$$\ln \text{solubility} = 23.45 - [17.1 \times \text{density}] - [13356 / \text{temperature}] + [8639.9 \times (\text{density} / \text{temperature})] \qquad \textbf{6.14}$$

Table 6.2. List of ln capacity factor values for testosterone at specific density and temperature.

ln k	Density (g / ml)	1/temperature (K)	Density / temp. (g / ml.K)
-0.579	0.817	0.00305	0.00249
-0.105	0.772	0.00305	0.00235
-0.507	0.718	0.00305	0.00219
1.109	0.671	0.00305	0.00204
1.709	0.629	0.00305	0.00192
0.215	0.63	0.00270	0.00169
0.174	0.67	0.00270	0.00180
-1.109	0.77	0.00270	0.00206
-0.693	0.72	0.00270	0.00193
0.315	0.67	0.00283	0.00190
-0.211	0.72	0.00283	0.00204
-0.681	0.77	0.00283	0.00218
-0.511	0.75	0.00283	0.00212
0.765	0.63	0.00283	0.00178
-0.994	0.77	0.00275	0.00212
-0.478	0.72	0.00275	0.00201
0.039	0.67	0.00275	0.00185
0.113	0.63	0.00275	0.00174
0.398	0.52	0.00275	0.00143

Table 6.3. List of literature ln Solubility and 1/solubility data for testosterone at specific density and temperature.

ln solubility (literature values)	1/solubility (mole fraction)	density (g / ml)	1 / temp (K)	density / temp. (g / ml . K)
-14.187	1449275.0	0.329	0.00314	0.00103
-13.62	819672.0	0.554	0.00314	0.00174
-12.500	269542.0	0.671	0.00314	0.00211
-11.060	63452.00	0.724	0.00314	0.00228
-10.890	53476.00	0.761	0.00314	0.00240
-10.290	29377.00	0.791	0.00314	0.00249
-10.130	25619.00	0.814	0.00314	0.00256
-10.080	23958.00	0.834	0.00314	0.00262
-9.770	17507.00	0.857	0.00314	0.00269
-9.680	16008.00	0.873	0.00314	0.00274
-12.630	305810.0	0.671	0.00319	0.00214
-12.150	189753.0	0.744	0.00319	0.00238
-10.900	53967.00	0.770	0.00319	0.00246
-10.710	44743.00	0.796	0.00319	0.00254
-10.770	47348.00	0.796	0.00319	0.00254
-10.380	32341.00	0.830	0.00319	0.00265
-10.120	24863.00	0.861	0.00319	0.00275
-9.930	20467.00	0.882	0.00319	0.00282
-9.790	17937.00	0.889	0.00319	0.00284
-12.990	438596.0	0.658	0.00325	0.00214
-11.830	136799.0	0.748	0.00325	0.00243
-11.340	84034.00	0.786	0.00325	0.00255

-10.820	49776.00	0.811	0.00325	0.00263
-10.560	38700.00	0.832	0.00325	0.00270
-10.360	31646.00	0.851	0.00325	0.00276
-10.280	29189.00	0.870	0.00325	0.00282
-10.130	25107.00	0.882	0.00325	0.00286
-10.010	22207.00	0.898	0.00325	0.00292
-9.900	19889.00	0.908	0.00325	0.00295
-14.760	2564103.0	0.244	0.00305	0.00074
-13.870	1052632.0	0.373	0.00305	0.00114
-12.690	324675.0	0.524	0.00305	0.00159
-11.680	118483.0	0.630	0.00305	0.00192
-11.200	72886.00	0.672	0.00305	0.00205
-10.610	40535.00	0.719	0.00305	0.00219
-10.070	23624.00	0.752	0.00305	0.00229
-9.940	20721.00	0.775	0.00305	0.00236
-9.690	16160.00	0.802	0.00305	0.00244
-9.570	14265.00	0.820	0.00305	0.00250

Equation 6.13 was used to calculate the capacity factor for all available literature solubility data for testosterone at the four temperatures (35, 40, 45, and 55°C). All literature solubilities were then recalculated to take temperature and density into consideration using equation 6.14.

A direct relationship was then deduced between capacity factor (k) and solubility for testosterone by plotting ln capacity factor against ln calculated solubility (table 6.4). This can then be used as a solubility predictor for testosterone for any capacity factor value.

$$\ln \text{solubility} = (\ln k - 11.410)/ 1.085 \qquad \textbf{6.15}$$

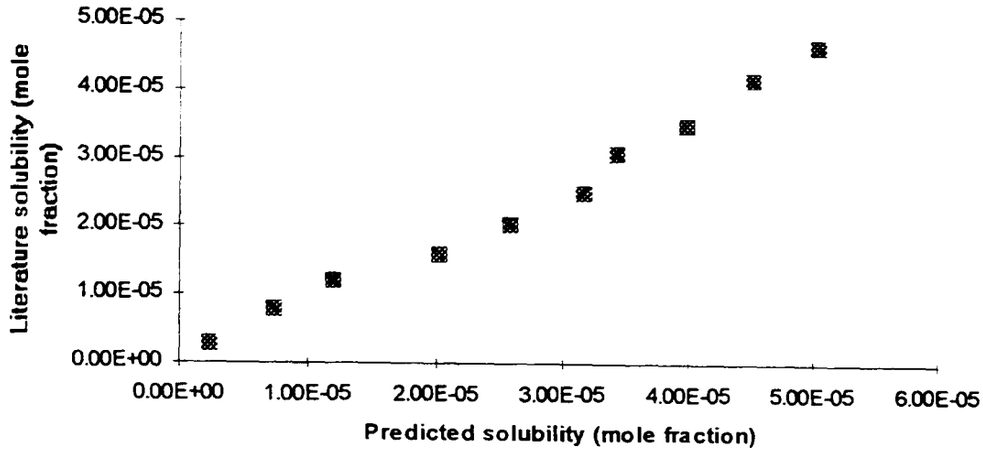
Table 6.4. List of ln k, ln calculated solubility (using equation 6.15) and ln literature solubility for testosterone.

ln capacity factor	ln calculated solubility	ln literature solubility
5.199	-15.015	-14.757
3.937	-13.823	-13.867
2.460	-12.427	-12.691
1.432	-11.448	-11.683
1.021	-11.059	-11.192
0.561	-10.625	-10.610
0.229	-10.320	-10.070
0.033	-10.108	-9.934
-0.261	-9.858	-9.690
-0.407	-9.692	-9.566
1.942	-12.707	-12.991
0.912	-11.722	-11.827
0.447	-11.306	-11.339
0.191	-11.032	-10.815
-0.049	-10.801	-10.564
-0.267	-10.594	-10.362
-0.484	-10.386	-10.282
-0.637	-10.254	-10.132
-0.804	-10.079	-10.008
-0.919	-9.969	-9.898
5.015	-15.237	-14.187
2.633	-12.971	-13.617
1.395	-11.793	-12.505

0.834	-11.260	-11.058
0.442	-10.887	-10.887
0.124	-10.585	-10.288
-0.119	-10.353	-10.127
-0.331	-10.152	-10.084
-0.574	-9.920	-9.770
-0.744	-9.759	-9.681
1.591	-12.173	-12.631
0.787	-11.406	-12.154
0.501	-11.133	-10.896
0.215	-10.860	-10.709
0.215	-10.860	-10.765
-0.159	-10.503	-10.384
-0.226	-10.177	-10.121
-0.450	-9.957	-9.927
-0.890	-9.833	-9.795

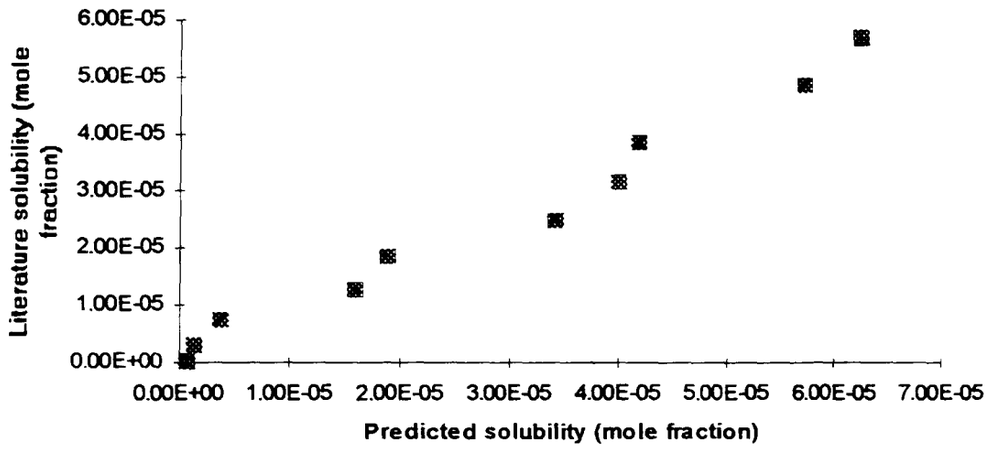
Solubility predictions were then carried out for testosterone at the four temperatures and compared with the literature solubility data. Figures 6.2-6.5 shows plots of literature solubility against predicted solubility. The correlation coefficient and the slope obtained for testosterone were 0.991 and 0.890 ± 0.096 at 35°C, 0.988 and 0.870 ± 0.123 at 40°C, 0.989 and 0.852 ± 0.101 at 45°C and 0.995 and 0.848 ± 0.066 at 55°C at 95 % confidence limits respectively. All plots show reasonable correlation between the literature and predicted solubility and these figures show that the slope and the correlation coefficient do not differ significantly from the "ideal" values of 1 and 1 respectively. Solubility isotherms were then constructed (figures 6.6-6.9) for testosterone at the four different temperatures.

Figure 6.2 Plot of Literature solubility vs Predicted solubility for Testosterone at 308°K



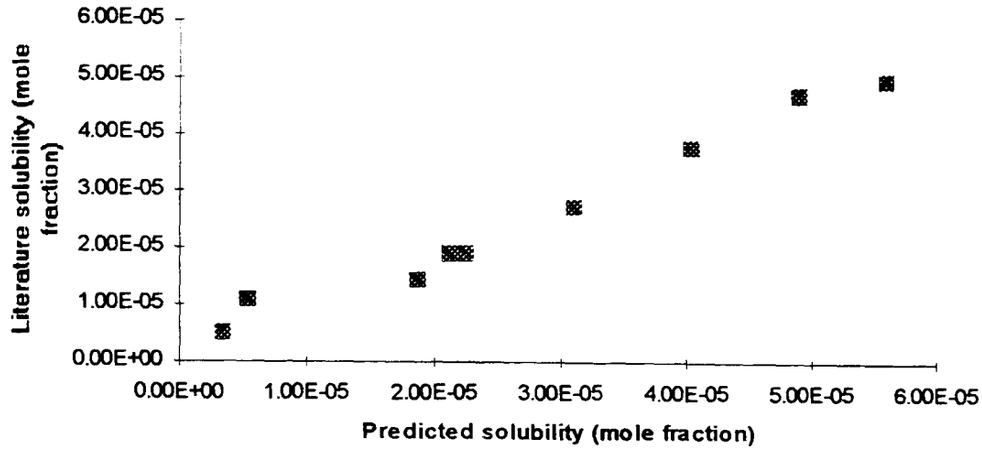
slope = 0.890 ± 0.096, r^2 = 0.991

Figure 6.3 Plot of Literature solubility vs Predicted solubility for Testosterone at 313°K



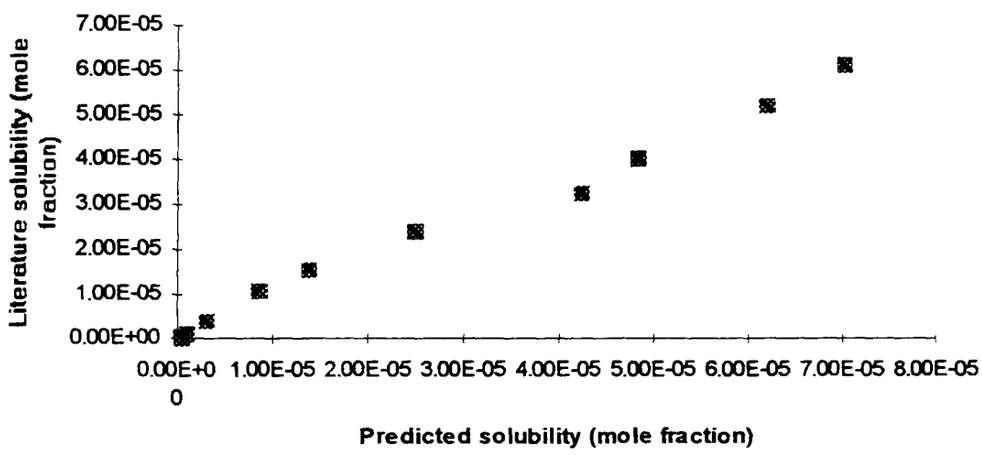
slope = 0.870 ± 0.123, r^2 = 0.988

Figure 6.4 Plot of Literature solubility vs Predicted solubility for Testosterone at 318°K



slope = 0.852 ±0.101, r^2 = 0.989

Figure 6.5 Plot of Literature solubility vs Predicted solubility for Testosterone at 328°K



slope = 0.848 ± 0.066, r^2 = 0.995

Figure 6.6 Solubility Isotherm for Testosterone at 308°K

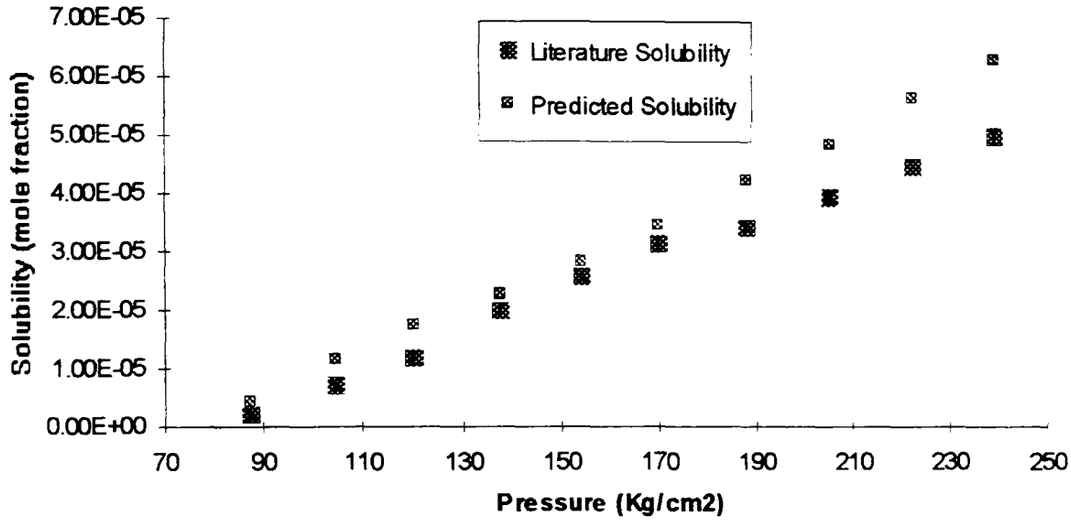


Figure 6.7 Solubility Isotherm for Testosterone at 313°K

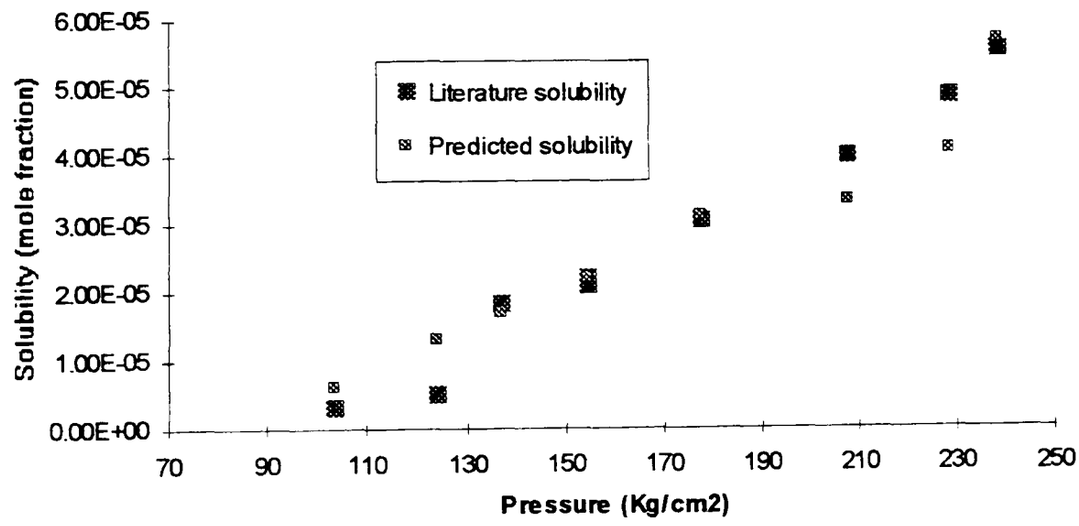


Figure 6.8 Solubility Isotherm for Testosterone at 318°K

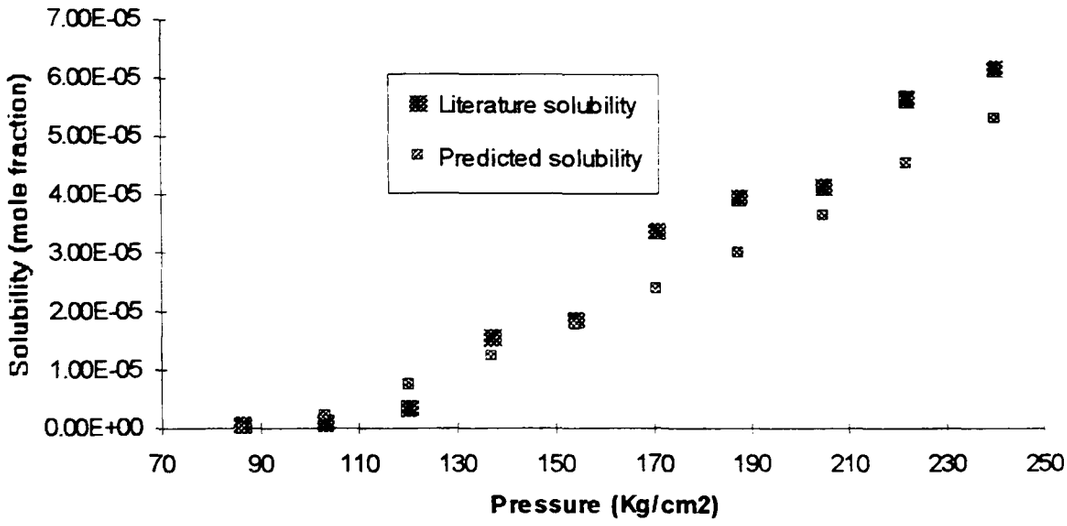
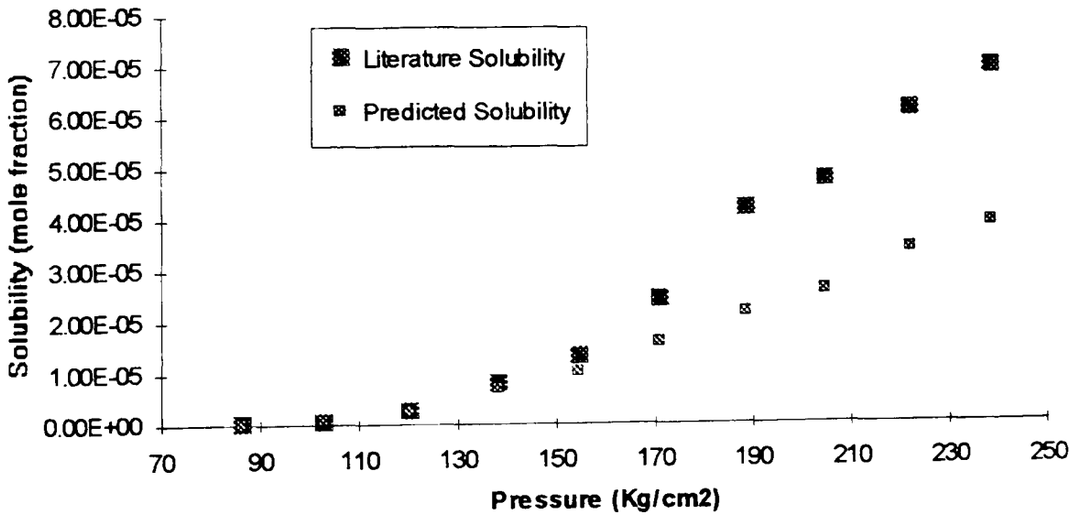


Figure 6.9 Solubility Isotherm for Testosterone at 328°K



All the data presented shows that the solubility model can be used as a reasonable solubility predictor for testosterone at the different temperatures. The validity of the proposed model was further checked by carrying out capacity factor measurement for testosterone at temperature 55°C. Solubility predictions were carried out (table 6.5) using equation 6.15 for both measured capacity factor and calculated capacity factor using equation 6.13 .

Table 6.5 Comparison of Predicted solubility against Literature solubility.

Density (g/ml)	**Measured capacity factor (k)**	**Solubility (mole fraction) using measured (k) (1×10^{-5})**	**Calculated capacity factor using eq. 6.13**	**Solubility (mole fraction) using calculated k (1×10^{-5})**	**Literature Solubility (mole fraction) (1×10^{-5})**
0.817	0.56	4.62	0.67	3.94	7.01
0.772	0.90	2.98	1.03	2.63	4.83
0.718	1.66	1.70	1.75	1.62	2.47
0.671	3.03	1.00	2.78	1.06	1.37
0.629	5.50	0.56	4.18	0.73	0.84

The results indicated that the model can be used as a solubility predictor for testosterone, although there is an increasing error at higher densities. This was expected due to the difficulties in measuring the capacity factor accurately as the testosterone peaks obtained were severely tailed and broad (figure 6.10-6.11) and this error was reflected in the solubility model. Also, at high densities testosterone becomes more soluble in the supercritical carbon dioxide mobile phase and hence

smaller capacity factor is obtained giving potentially greater error due to small retention times. The peak tailing may have been possibly caused by adsorption interaction in the column .The peak tailing behaviour arises when there are strong interactions between the solute and the stationary phase, but the solute/solute interactions are relatively weak. Initially, the amount of solute adsorbed onto the stationary phase increases rapidly as the concentration in the mobile phase is increased until a monolayer of solute molecules is formed on the adsorbent. As the interactions between solute molecules are weak, the adsorption ceases with the formation of the monolayer. The amount adsorbed remains constant despite the fact that the concentration in the mobile phase is increased further. Hence the distribution coefficient K_c is large at low c_m values but decreases as c_m increases. This means that the rate of migration is not the same across the band. and the center of the band which has a higher concentration, moves more rapidly catching up with the front leaving the tail behind.

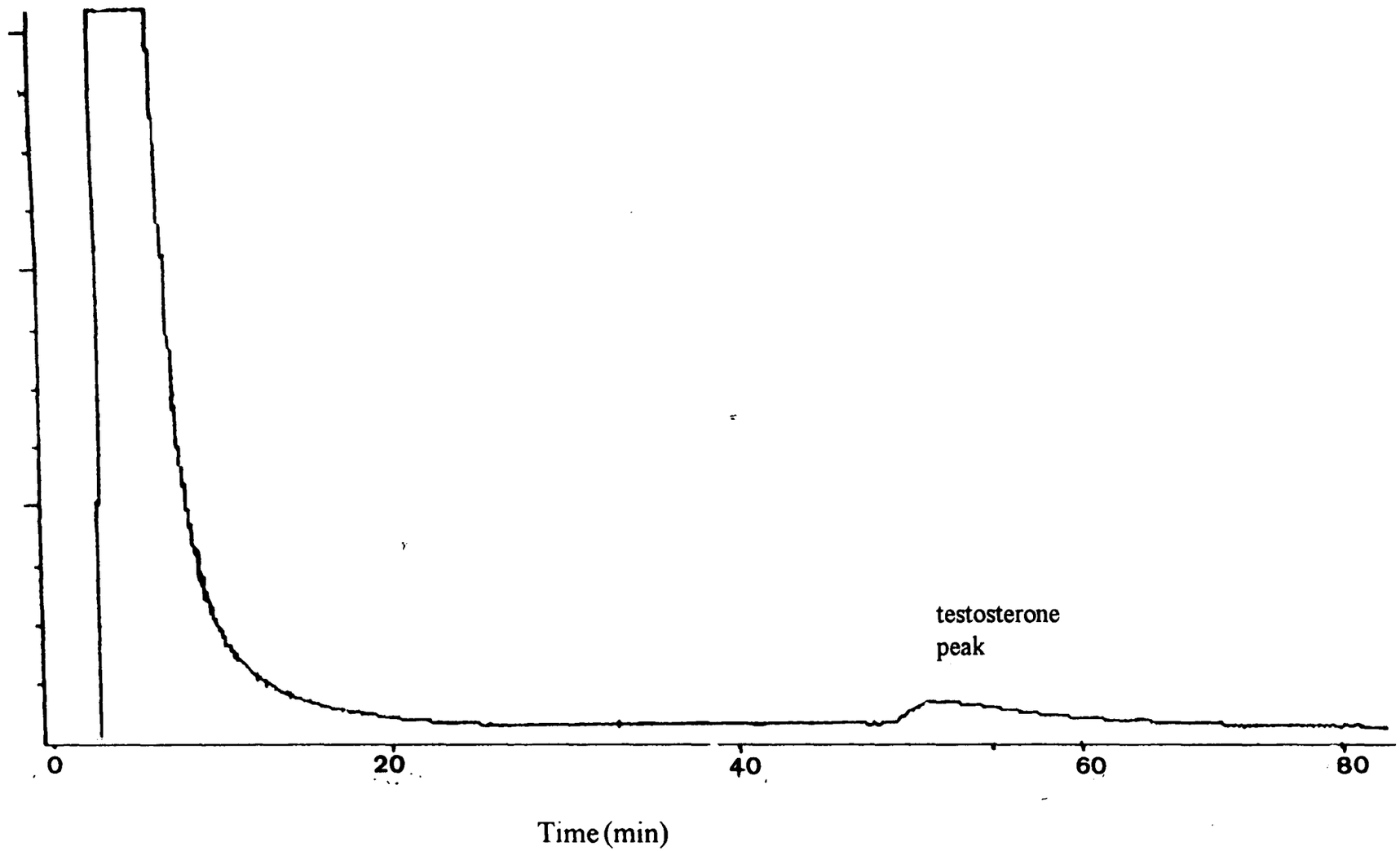


Figure 6.10 Example cSFC trace of testosterone obtained at 55°C and a density of 0.63 g/ml.

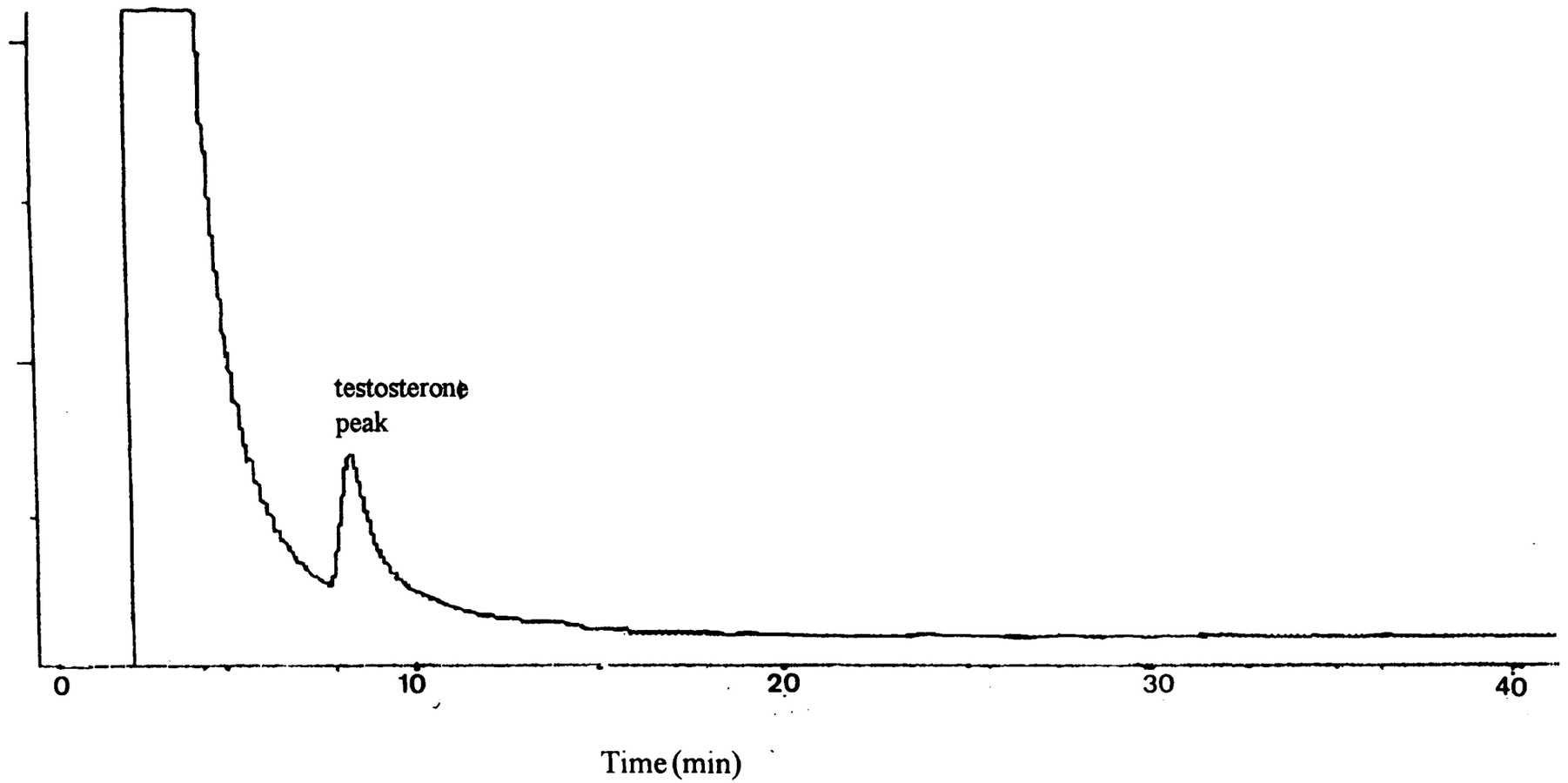


Figure 6.11 Example cSFC trace of testosterone at 55°C and a density of 0.772 g/ml.

The solubility values obtained were not absolute values and predictions of solubility can only be accounted for using the biphenyl column. An attempt was made to find a similar relationship between testosterone and SFC retention measurements by using a different stationary phase to see if it affected the accuracy of prediction and also to see if the model could be extended to other compounds.

The interaction of testosterone with a polar cyanopropyl column was significantly different and the model does not account for this factor. The model is also restricted for solubility predictions for testosterone only as different analytes have different affinity towards a stationary phase and is demonstrated on figures 6.12-6.13. Correction factors will have to be introduced to take into account for the interaction of testosterone with different stationary phases if predictions were to be carried out using the same model. The effect of retention on different statinary phase was established (figure 6.14) by measuring the capacity factor of testosteone at different pressures and temperatures, but at constant density of 0.63 g/ml using a biphenyl column and a cyanopropyl column. The results showed (table 6.6) that testosteone was less retained on the more polar cyanopropyl column. A similar effect was then observed with megestrol acetate (table 6.7, figure 6.15) and pregnenolone (table 6.8, figure 6.16). Unfortunately, due to instrumental breakdown and lack of time no further investigation was possible.

Table 6.6 The capacity factor results obtained for testosterone using two different columns.

Temperature (°C)	k (biphenyl column)	k (cyanopropyl column)
100	1.24	0.44
90	1.49	0.61
80	2.15	0.81
70	2.88	1.13
55	5.50	2.03

Table 6.7 The capacity factor results obtained for megestrol acetate using two different columns.

Temperature (°C)	k (biphenyl column)	k (cyanopropyl column)
100	1.5	0.49
90	1.85	0.67
80	2.51	0.87
70	3.54	1.24
55	6.42	2.24

Table 6.8 The capacity factor results obtained for pregnenolone using two different columns.

Temperature (°C)	k (biphenyl column)	k (cyanopropyl column)
100	1.06	0.25
90	1.30	0.34
80	1.71	0.46
70	2.47	0.64
55	4.26	1.14

Figure 6.12 Retention of three different analytes on biphenyl column

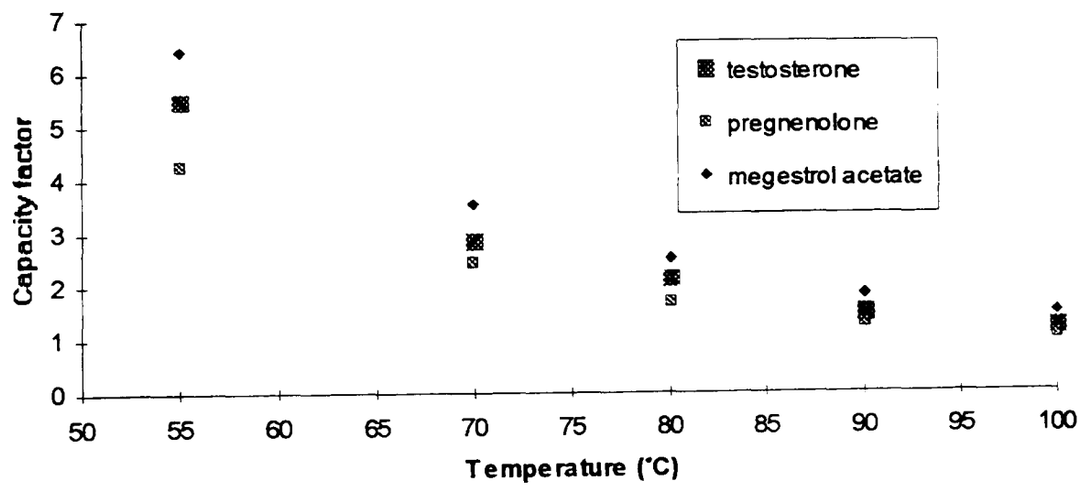


Figure 6.13 Retention of three different analytes on SB cyano propyl column

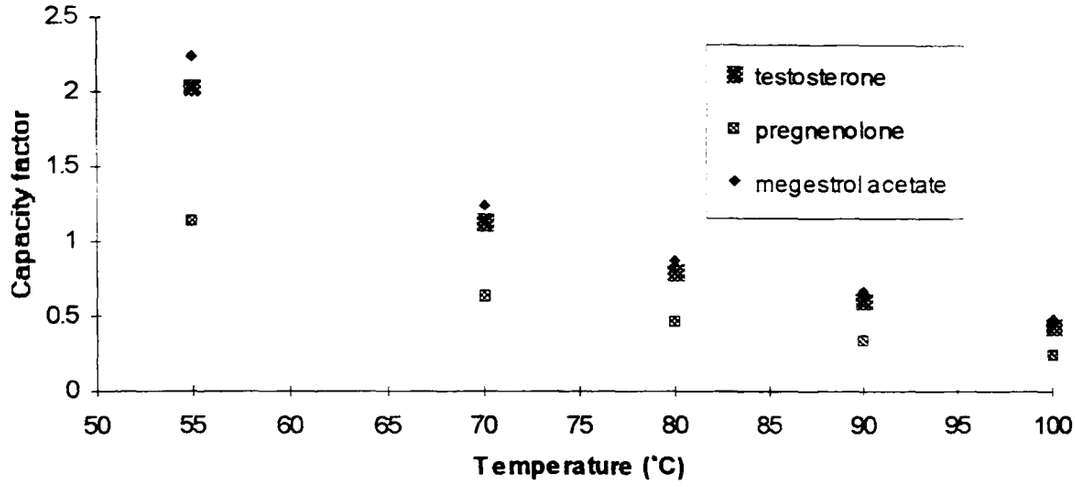


Figure 6.14 Retention of testosterone on two different columns

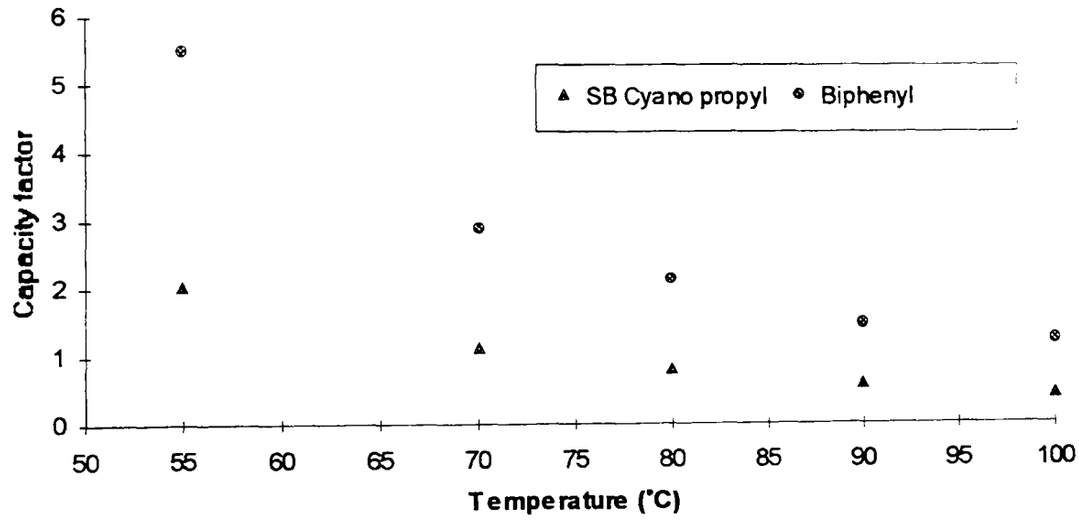


Figure 6.15 Retention of megestrol acetate on two different columns

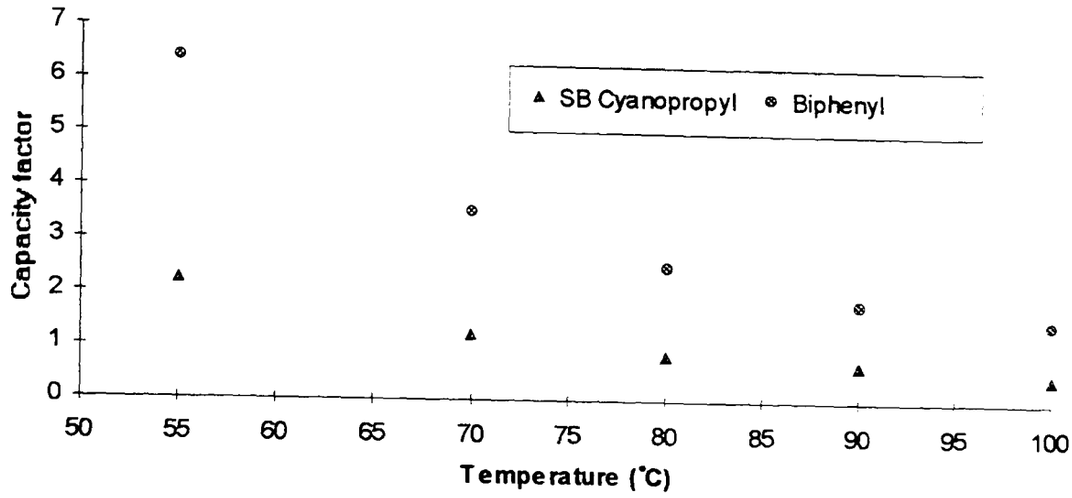
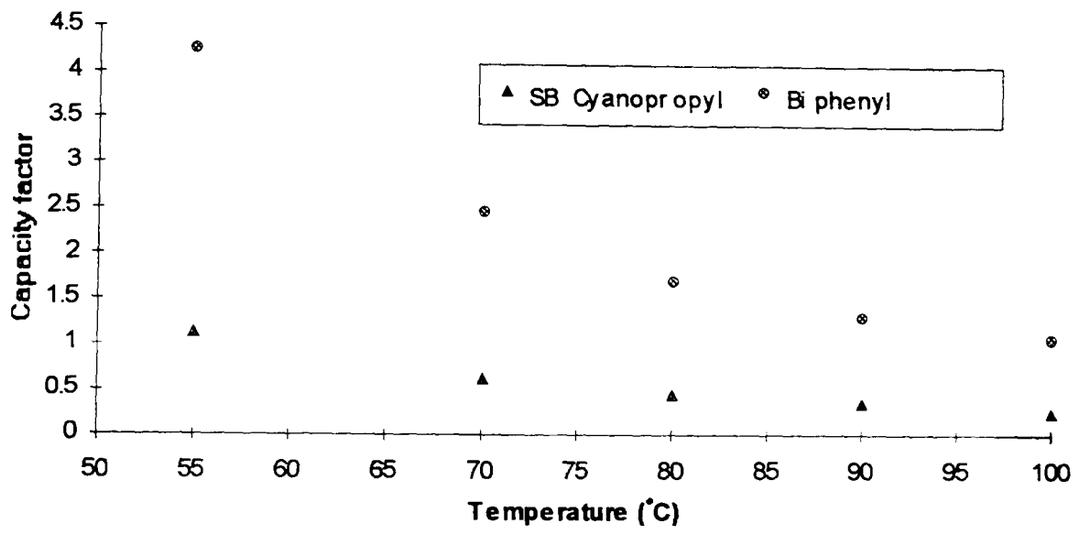


Figure 6.16 Retention of pregnenolone on two different columns



6.7 Conclusion

A solubility model based on capacity factor measurements was presented for the prediction of testosterone solubility in supercritical CO_2. The column utilized for the capacity factor measurements was a biphenyl column and solubility predictions were carried out for four different temperatures for which literature data exists. The solubility predicted appeared to correlate reasonably well with the literature solubility

data based on capacity factor measurements carried out with the biphenyl column only. Changing the column changed retention of testosterone significantly and the model did not hold. It was assumed that the model will not extend to different steroids. It was shown that interactions of different analytes with different stationary phases are significantly different and for the model to hold for different analytes and different stationary phases, correction factors will have to be introduced to take into account of all the interactions. Chapter 7 presents an accurate way of measuring solubility of steroids in supercritical CO_2.

Chapter 7.0 Solubility Determination of Steroids in Supercritical Carbon Dioxide

7.1 Introduction and Aim

The solubility of an analyte in supercritical CO_2 gives an indication of the relative extractibility of the substance as a function of pressure and temperature. The solubility gives the concentration of solute in equilibrium with the pure solute. This part of this thesis reports the solubility data of a series of steroids with a range of polarities using UV analysis following extraction with supercritical CO_2. The objectives of the experiments were to provide increased knowledge on the solubility of steroid hormones in supercritical carbon dioxide, and at the same time, to back up the hypothesis that the solubility of a molecule can be predicted using ClogP and solubility parameter theory.

7.2 Experimental Methods for Obtaining Solubility

There are several experimental methods available to determine solubilities of analytes in supercritical fluid and the methods can be classified in two different ways. The first method relates to the way in which the saturated solution is obtained, which can be static or dynamic, i.e., in a closed cell or in a flow system. The second method is involved with how the solution is analysed. These methods can be grouped into four different categories: gravimetric, spectrometric, chromatographic, and miscellaneous (e.g. titration after extraction). Of the techniques mentioned, the gravimetric method is the most widely used. This method involves the production of a saturated solution by passing the supercritical fluid over the solute in an extraction cell, dropping the pressure to precipitate the solute, and weighing it. The chromatographic method is the second most popular technique. Most chromatographic methods used are modifications of the gravimetric method. For example, in one type of modification, the solute is precipitated as before in a trap, containing a solvent. The solute is then

washed out of the trap, made to volume, and analyzed by any suitable chromatographic method, such as GC, TLC and HPLC. A different type of chromatographic method involves the use of chromatographic retention measurements in SFC to produce qualitative solubility data. The other experimental methods available to determine solubility are attempts to find alternatives, but none of these have yet been generally accepted. Spectroscopic techniques can offer convenience if *in situ* analysis is carried out by making absorption measurements in the UV directly in a high pressure static equilibrium cell, but here the effect of the supercritical fluid on the absorption properties often complicates the procedure.

7.3 Solubilities of Analytes in Supercritical Fluids

The solubility of a solute can be described either as the mole fraction of solute in solution, x, or by the amount (as moles or mass) of solute per volume, expressed in terms of concentration, S, at saturation.[24] The relationship between these quantities is trivial and involves the density of the solution in moles per unit volume. As the solutions are dilute, it is assumed to be the density of the pure fluid. Therefore, S can be expressed in moles per unit volume by the following equation;[24]

$$S = x/V \qquad 7.1$$

where V is the molar volume of the pure fluid.

The solubility of a substance in a supercritical fluid can be explained by two factors, the volatility of the substance and the solvating effect of the supercritical fluid. Figure 7.1 shows the schematic of the solubility of a substance at constant temperature as a function of pressure and in terms of mole fraction.[24]

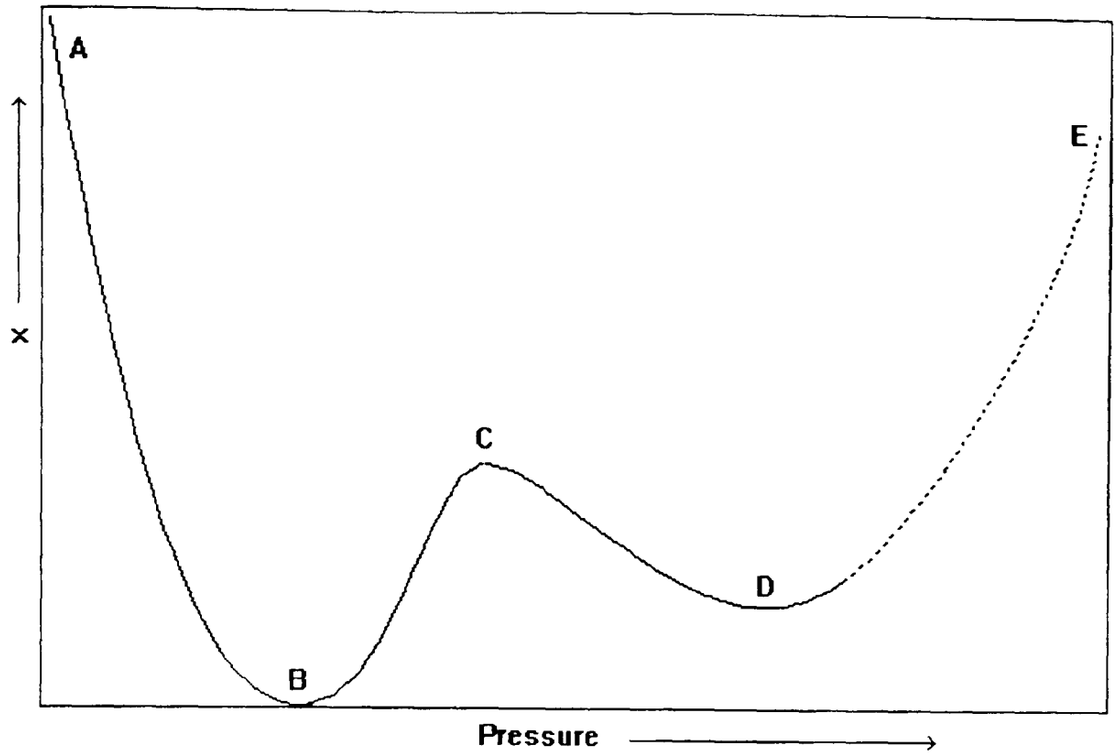


Figure 7.1 Schematic diagram of supercritical fluid solubility against pressure at constant temperature.[24]

At very low pressures (sections A-B), x falls as the solute is diluted by the fluid. Thereafter, in section B-C, a rapid rise in x is noticed at a threshold pressure characteristic of the solute-fluid system, which is a pressure somewhat above the critical pressure of the fluid. This takes place due to the rapid rise in density. The next two features may or may not occur for any particular system, especially if a narrow pressure range is employed. A fall in section C-D may occur because as higher pressure is approached, the repulsive forces may squeeze the solute out of solution. The rise in D-E takes place for moderately volatile solutes, if there is a critical line in the mixture phase at higher pressures.[24]

7.4 Experimental Section

The Jasco SFE system was used in these experiments to measure the solubility of steroids gravimetrically. A sample of pure steroid (10 mg) was mixed with Celite, an inert support, and placed in a sample cell. The sample cell was made of stainless steel (10 cm x 5.0 mm i.d.) with a volume of ~ 2.0 ml. Celite does not exhibit matrix effects and was a suitable surface to investigate the solubility of pure compounds. Extracted analytes were collected in a glass vial placed underneath the BPR outlet. A small volume of solvent (methanol was used in all cases) was placed at the bottom of the collection vial for dissolution of the analyte. Analysis of the extracts was performed using a Shimadzu UV/VIS spectrometer equipped with 1.0 cm path length silica cells for all measurements. Quantification was achieved by diluting all extracted steroids and measuring the absorbance against a five-point linear calibration graph. The steroids investigated (figure 7.2) were testosterone, testosterone-17-propionate, beclomethasone-17,21-dipropionate, betamethasone-17,21-dipropionate, cortisone acetate, hydrocortisone, prednisolone, prednisone and megestrol actetate. Steroids were provided in pure form by Pfizer Central Research, Sandwich , Kent and Glaxo Manufacturing Services, Barnard Castle, Co. Durham.

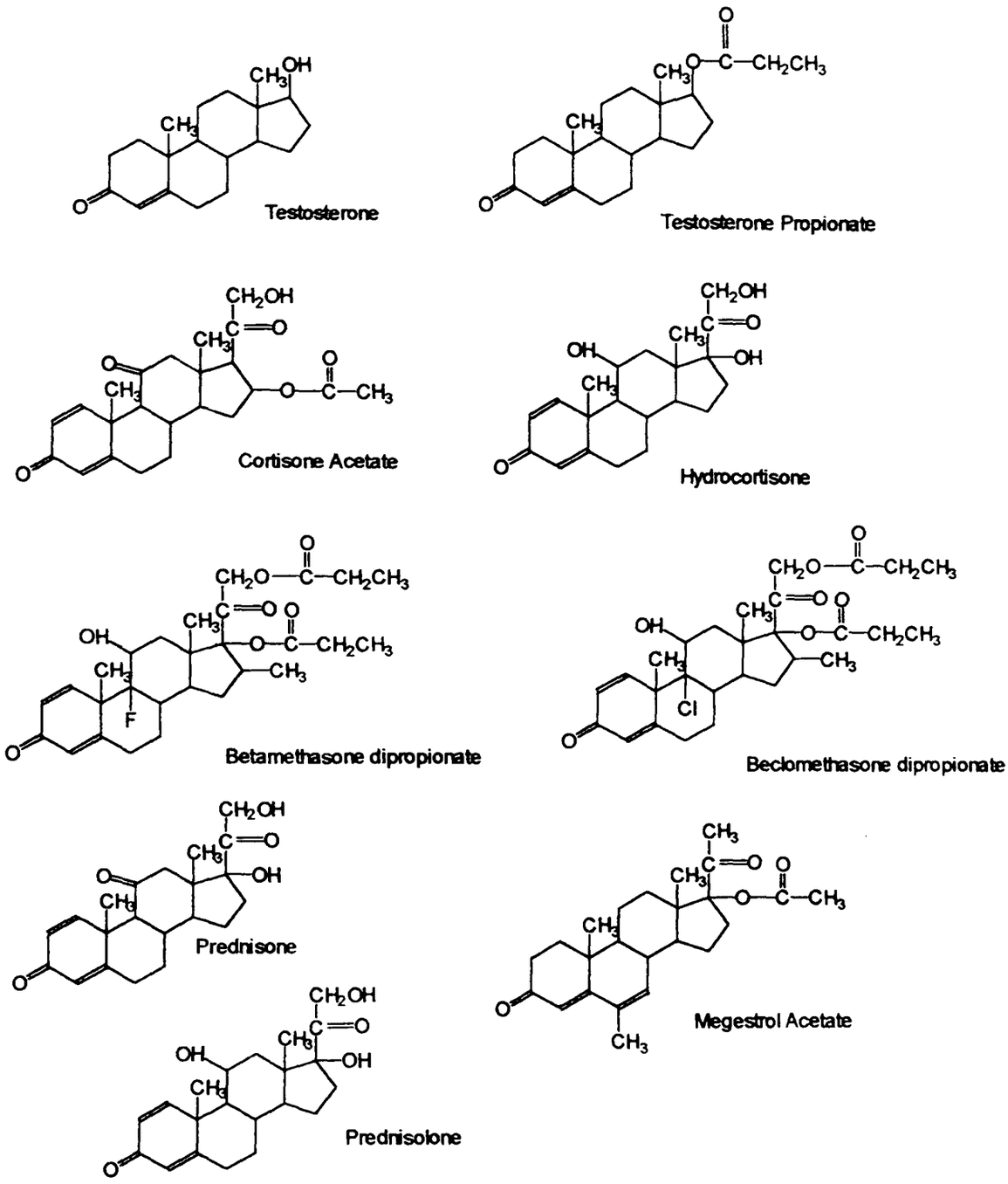


Figure 7.2 Structures of the investigated steroids.

Extraction conditions:

The SFE collection system used required the supercritical solvent to be pumped through the sample cell in dynamic extraction mode. However, all steroid extractions utilised a two minute static extraction period prior to dynamic extraction for 10 minutes at a pump flow rate of 1 ml min^{-1}. The static extraction allowed the supercritical fluid to penetrate the sample increasing the natural partitioning of the analytes into the solvent. The 10 minute extraction time provided enough mass transfer to enable the analytes to be collected in a small amount of solvent at the end of the back pressure regulator. The internal volume of the extraction cell being swept slightly more than 6 full times with supercritical fluid. The mole fraction solubility is dependent on the volume of carbon dioxide used in the extraction and is calculated as follows:

mole fraction extracted =

[moles of analyte extracted]/[moles of analyte extracted + moles of CO_2 used]

for a 10 minute extraction at 1 ml min^{-1} a volume of 10 ml of carbon dioxide is used. Therefore the number of moles of carbon dioxide used is given by

moles of CO_2 =

[volume of CO_2 (ml) x density of liquid CO_2 (g/ml)]/ mwt of CO_2

where the density of liquid CO_2 is 0.93 g ml^{-1}.

An example of the mole fraction solubility calculation for testosterone at temperature 55°C and pressure 149 kg/cm^2 is as follows:

Weight of testosterone extracted = 1.2×10^{-3} g.

Molecular weight of testosterone = 288.41

Mole fraction = $1.2 \times 10^{-3}/288.41 = 4.16 \times 10^{-6}$

Moles of CO_2 = $(10 \times 0.963)/44$

= 0.211

Mole fraction weight extracted = $[4.16 \times 10^{-6}]/[4.16 \times 10^{-6} + 0.211]$

= 1.95×10^{-5}.

7.5 Results and Discussion

Steroid Solubility Studies

In this part of the thesis, the solubility data for a series of steroid hormones, including testosterone, for which literature data exists is reported. A direct comparison is given from the data available from the literature for testosterone and the experimental data accumulated during the course of this work. Thus allowing the robustness of the experimental method to be evaluated.

7.5.1 Initial Studies using Testosterone

Testosterone, like all other steroid hormones shares the same basic four ring cyclepentanoperhydrophenanthrene structure. It is know to be soluble in supecritical CO_2.

Testosterone's solubility was determined by Kosal et al.[186] using apparatus capable of extracting greater quantities of solubilised solid than the Jasco instrumentation used in this case. However, a comparison was undertaken between experimentally measuremented solubility and reported literature solubility by Kosal at 55°C over a range of 10 different pressures. Table 7.1 shows shows that the mole fractions experimentally determined for testosterone are similar to that of the literature values. This is verified by the isotherms of both experimental and literature values.

The establishment of this close correlation for the two sets of results was necessary before other steroid compounds could be extracted using the same method. The fact that the extraction method and the chosen method of analysis provided very similar results to the literature values lead to the conclusion that this was a suitable method of solubility determination. The greatest variance from the literature values was observed at the lower pressures studied. Errors in this region are thought more likely due to the very small solubility shown by testosterone and the other steroids at the resulting density conditions.

7.5.2 Steroid Solubility Studies: Extension to other Steroids

The solubility results for testosterone propionate, betamethasone dipropionate, beclomethasone dipropionate, cortisone acetate, hydrocortisone, prednisolone, prednisone, and megestrol acetate are shown in tables 7.1-7.10 with the solubility isotherms at temperatures and pressures studied in figures 7.3-7.12. The solubilities of all the steroids investigated in this chapter were already predicted using CLogP/solubility parameter theory in chapter 5 and the predictions were as follows:

Steroid	Predicted Solubility in Supercritical CO_2
megestrol acetate	soluble
betamethasone dipropionate	soluble
cortisone acetate	intermediate
beclomethasone dipropionate	soluble
hydrocortisone	insoluble
prednisolone	insoluble
testosterone	soluble
testosterone propionate	soluble
prednisone	insoluble

The qualitative data obtained from chapter 5 identified trends in steroid solubility in supercritical carbon dioxide. Figure 7.13 shows the solubility isotherm of all nine steroids at 55°C which compare agreeably with the experimentally determined values for nine steroids in supercritical carbon dioxide. It was observed that steroid hormones can be solvated in greater amounts as their polarity decreases and the molecule becomes more hydrophobic, for example, steroids such as testosterone propionate and testosterone are more soluble in supercritical carbon dioxide than steroids such as hydrocortisone and prednisone.

Table 7.1 Comparison of Literature and Measured Solubilities for Testosterone at 55°C

Pressure kg cm^{-2}	Literature value at 55°C mole fraction	Measured value at 55°C mole fraction
84.00	0.039E-05	0.009E-05
101.00	0.095E-05	0.027E-05
116.00	0.308E-05	0.752E-05
132.00	0.844E-05	0.998E-05
149.00	1.370E-05	1.951E-05
164.00	2.471E-05	2.350E-05
181.00	4.230E-05	3.011E-05
200.00	4.830E-05	4.791E-05
215.00	6.190E-05	6.251E-05
23100	7.011E-05	7.490E-05

Figure 7.3 Comparison of literature solubility and measured solubility for testosterone at 55°C

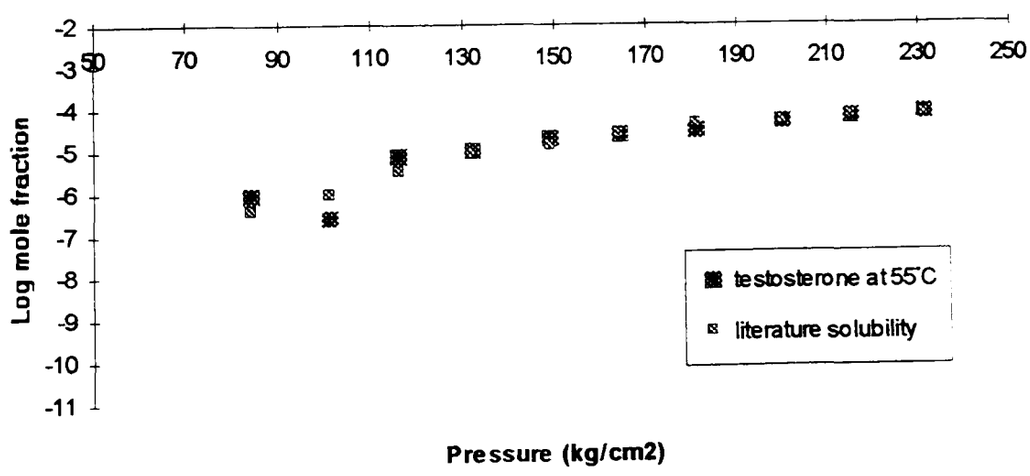


Table 7.2 Experimental results for the extraction of Testosterone at 55°C and 100°C.

Pressure kg cm^{-2}	TESTOSTERONE at 55°C mole fraction	TESTOSTERONE at 100°C mole fraction
231.00	0.009E-05	9.050E-05
215.00	0.027E-05	8.530E-05
200.00	0.752E-05	6.390E-05
181.00	9.981E-05	3.940E-05
164.00	1.951E-05	2.460E-05
149.00	2.350E-05	1.640E-05
132.00	3.011E-05	0.197E-05
116.00	4.791E-05	0.156E-05
101.00	6.251E-05	0.082E-05
84.00	7.490E-05	0.046E-05

Figure 7.4 Plot of log mole fraction vs pressure for testosterone

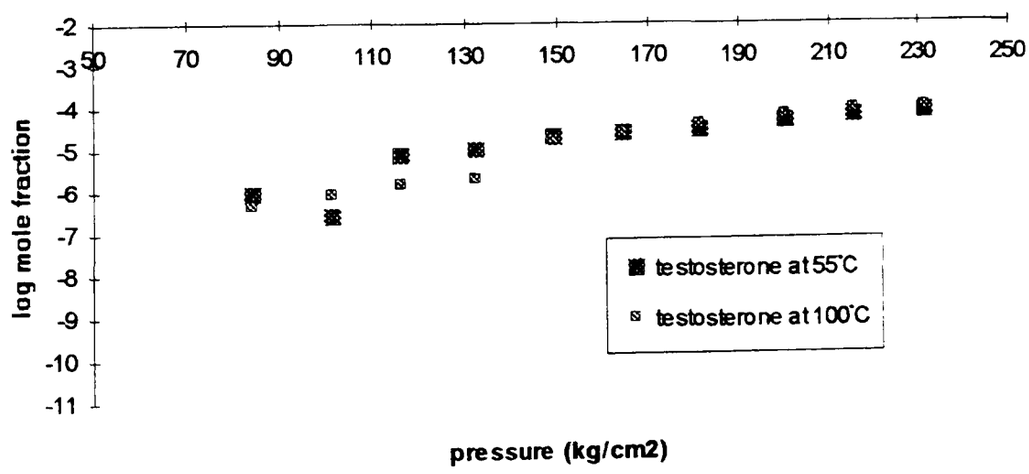


Table 7.3 Experimental results for the extraction of Testosterone Propionate at 55°C and 100°C.

Pressure kg cm^{-2}	TESTOSTERONE PROPIONATE at 55°C mole fraction	TESTOSTERONE PROPIONATE at 100°C mole fraction
231.00	9.100E-05	9.951E-05
215.00	8.250E-05	8.530E-05
200.00	7.580E-05	7.390E-05
181.00	6.350E-05	5.360E-05
164.00	4.130E-05	1.650E-05
149.00	2.620E-05	0.122E-05
132.00	1.650E-05	0.564E-05
116.00	1.310E-05	0.131E-05
101.00	0.701E-05	0.100E-05
84.00	0.131E-05	0.056E-05

Figure 7.5 Plot of log mole fraction vs pressure for testosterone propionate

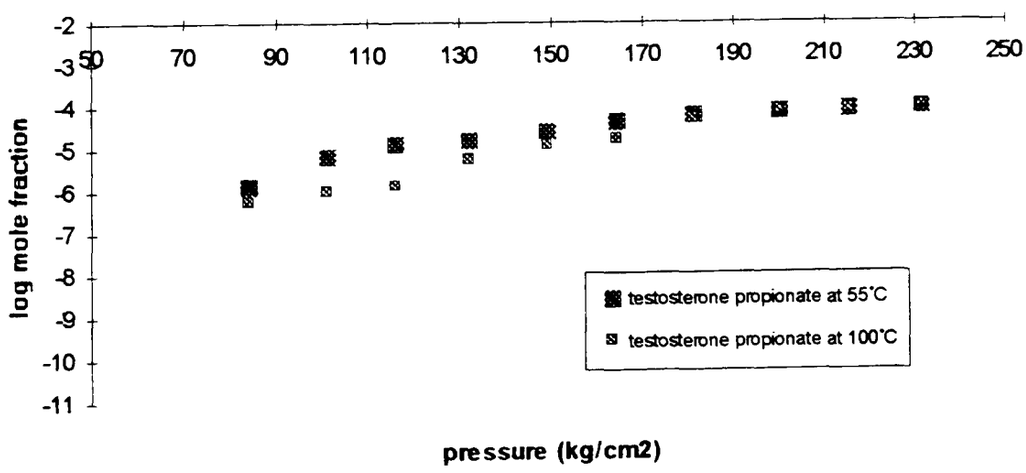


Table 7.4 Experimental results for the extraction Betamethasone Dipropionate at 55°C and 100°C

Pressure kg cm^{-2}	BETAMETHASONE DIPROPIONATE at 55°C mole fraction	BETAMETHASONE DIPROPIONATE at 100°C mole fraction
231.00	7.120E-05	6.190E-05
215.00	5.890E-05	5.550E-05
200.00	2.660E-05	2.120E-05
181.00	1.890E-05	1.190E-05
164.00	1.400E-05	0.899E-05
149.00	0.500E-05	0.311E-05
132.00	0.171E-05	0.0085E-05
116.00	0.021E-05	0.003E-05
101.00	-	-
84.00	-	-

Figure 7.6 Plot of log mole fraction vs pressure for betamethasone dipropionate

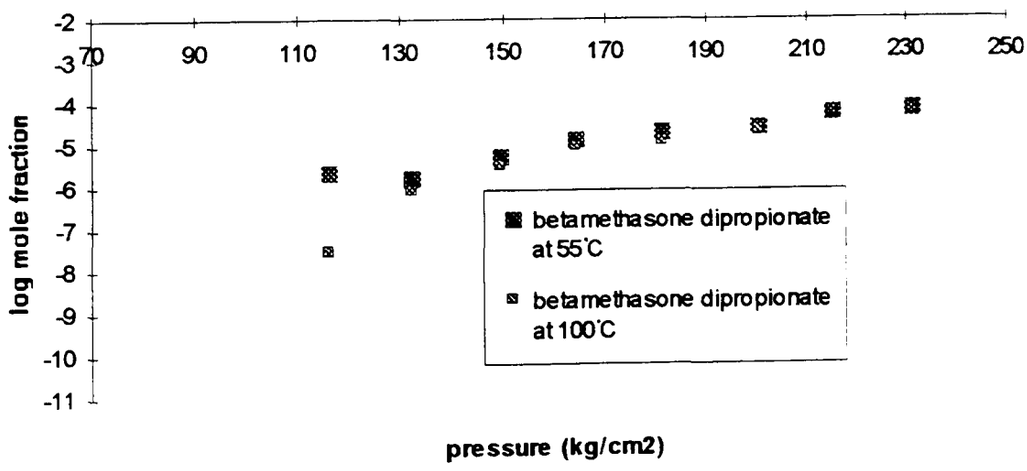


Table 7.5 Experimental results for the extraction of Beclomethasone Dipropionate at 55°C and 100°C

Pressure kg cm^{-2}	BECLOMETHASONE DIPROPIONATE at 55°C mole fraction	BECLOMETHASONE DIPROPIONATE at 100°C mole fraction
231.00	4.880E-05	3.090E-05
215.00	3.410E-05	1.250E-05
200.00	2.990E-05	1.010E-05
181.00	2.590E-05	8.950E-05
164.00	1.100E-05	6.010E-05
149.00	0.911E-05	0.321E-05
132.00	0.471E-05	0.080E-05
116.00	0.233E-05	0.055E-05
101.00	0.055E-05	0.012E-05
84.00	0.019E-05	-

Figure 7.7 Plot of log mole fraction vs pressure for beclomethasone dipropionate

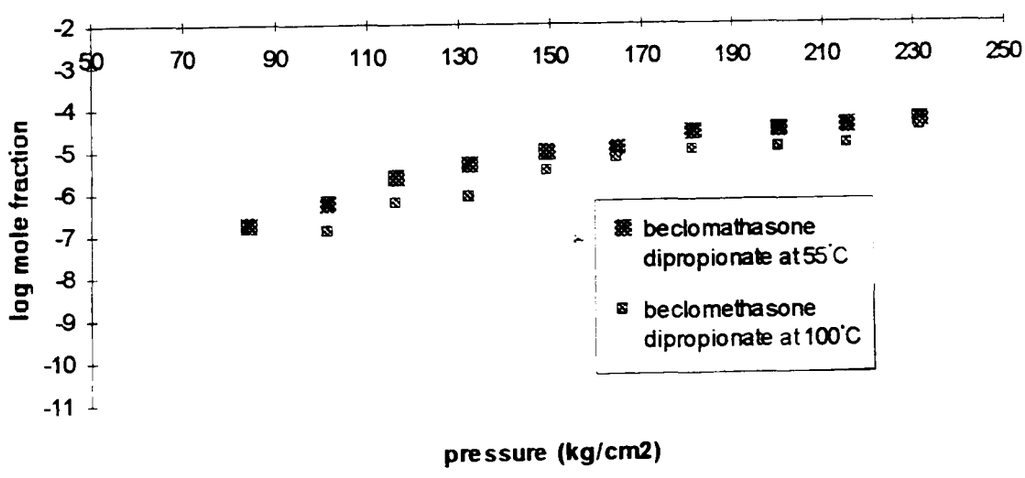


Table 7.6 Experimental results for the extraction of Cortisone Acetate at 55°C and 100°C

Pressure kg cm^{-2}	CORTISONE ACETATE at 55°C mole fraction	CORTISONE ACETATE at 100°C mole fraction
231.00	2.550E-06	2.310E-06
215.00	2.010E-06	1.910E-06
200.00	1.550E-06	1.450E-06
181.00	1.490E-06	1.320E-06
164.00	1.420E-06	1.250E-06
149.00	1.200E-06	1.110E-06
132.00	0.550E-06	0.490E-06
116.00	0.451E-06	0.351E-06
101.00	0.281E-06	0.225E-06
84.00	0.145E-06	0.111E-06

Figure 7.8 Plot of log mole fraction vs pressure for cortisone acetate

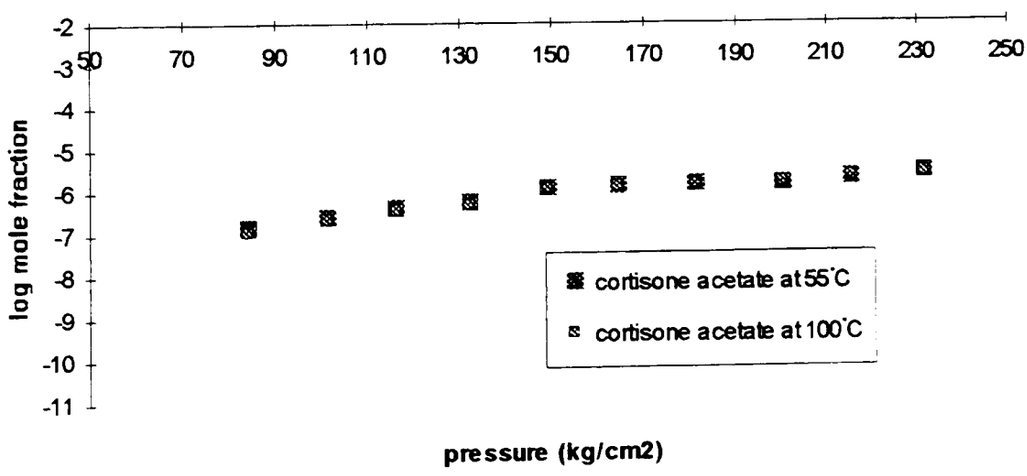


Table 7.7 Experimental results for the extraction of Hydrocortisone at 55°C and 100°C

Pressure kg cm^{-2}	HYDROCORTISONE at 55°C mole fraction	HYDROCORTISONE at 100°C mole fraction
231.00	8.770E-07	7.340E-07
215.00	7.810E-07	6.440E-07
200.00	6.160E-07	5.020E-07
181.00	5.350E-07	4.570E-07
164.00	2.750E-07	1.640E-07
149.00	1.900E-07	1.390E-07
132.00	1.370E-07	0.631E-07
116.00	0.592E-07	0.025E-07
101.00	0.059E-07	-
84.00	0.019E-07	-

Figure 7.9 Plot of log mole fraction vs pressure for hydrocortisone

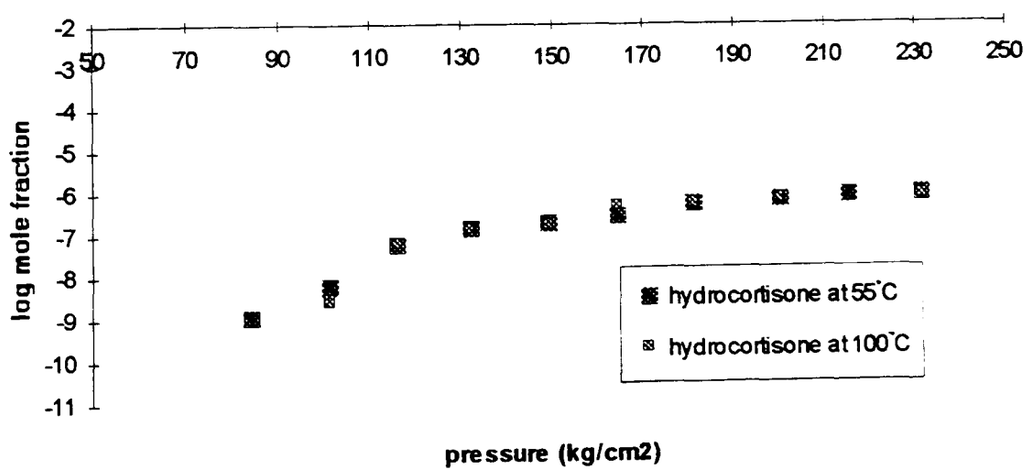


Table 7.8 Experimental results for the extraction of Prednisolone at 55°C and 100°C

Pressure kg cm^{-2}	PREDNISOLONE at 55°C mole fraction	PREDNISOLONE at 100°C mole fraction
231.00	12.900E-08	9.860E-08
215.00	11.800E-08	8.580E-08
200.00	9.720E-08	7.010E-08
181.00	6.550E-08	5.400E-08
164.00	4.590E-08	3.290E-08
149.00	3.890E-08	1.460E-08
132.00	2.650E-08	1.130E-08
116.00	1.110E-08	0.905E-08
101.00	0.355E-08	0.005E-8
84.00	0.112E-08	-

Figure 7.10 Plot of log mole fraction vs pressure for prednisolone

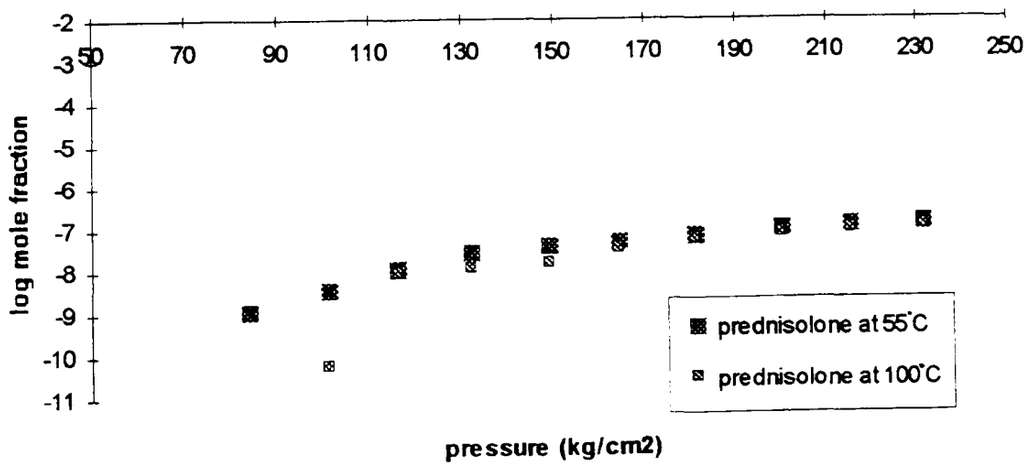


Table 7.9 Experimental results for the extraction of Prednisone at 55°C and 100°C

Pressure kg cm^{-2}	PREDNISONE at 55°C mole fraction	PREDNISONE at 100°C mole fraction
231.00	6.090E-07	5.880E-07
215.00	4.311E-07	3.130E-07
200.00	2.610E-07	1.910E-07
181.00	1.290E-07	1.120E-07
164.00	0.798E-07	0.788E-07
149.00	0.365E-07	0.341E-07
132.00	0.161E-07	0.140E-07
116.00	0.060E-07	0.050E-07
101.00	0.014E-07	0.005E-7
84.00	0.011E-07	-

Figure 7.11 Plot of log mole fraction vs pressure for prednisone

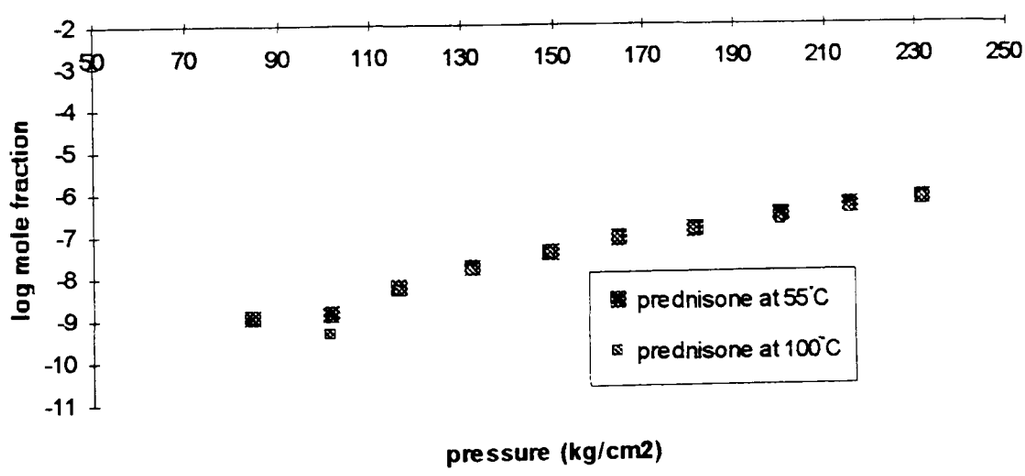


Table 7.10 Experimental results for the extraction of Megestrol Acetate at 55°C and 100°C

Pressure kg cm^{-2}	MEGESTROL ACETATE at 55°C mole fraction	MEGESTROL ACETATE at 100°C mole fraction
231.00	5.310E-05	5.540E-05
215.00	5.170E-05	5.070E-05
200.00	4.930E-05	4.360E-05
181.00	3.820E-05	2.950E-05
164.00	2.340E-05	2.330E-05
149.00	1.360E-05	1.270E-05
132.00	0.716E-05	0.606E-05
116.00	0.232E-05	0.109E-05
101.00	0.109E-05	
84.00	0.064E-05	

Figure 7.12 Plot of log mole fraction vs pressure for megestrol acetate

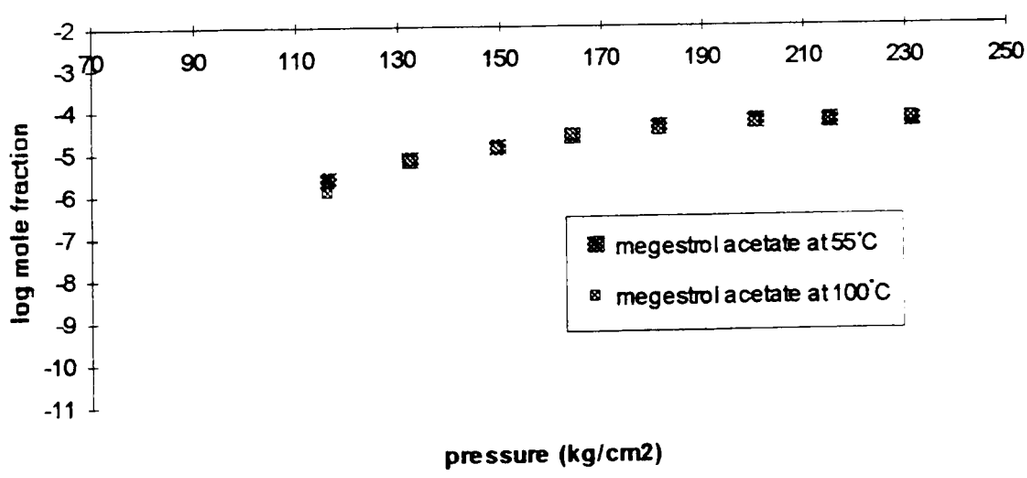
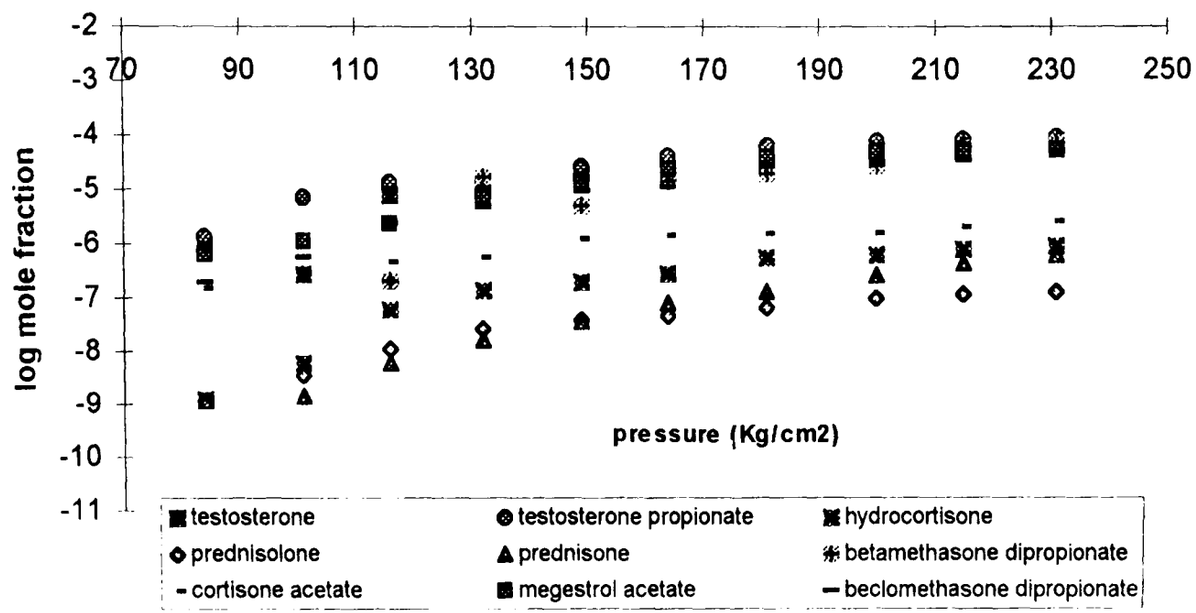


Figure 7.13 Solubility isotherm for nine steroids at 55°C



7.6 Conclusion

This chapter presented solubility results for a series of steroids with ranging polarities in supercritical carbon dioxide. As expected, the solubility of the steroids increased with increasing the density of the supercritical fluid. The experimentally determined values of the steroid solubility in supercritical carbon dioxide obtained in this chapter relate favourably with the predicted steroid solubility in supercritical carbon dioxide based on the ClogP/solubility parameter theory.

Chapter 8.0 The Determination of Fluconazole by SPE/HPLC, SFE/HPLC and SFE/GC from an Animal Feed Matrix

8.1 Introduction and Aims

Sample preparation of pharmaceutical compounds is often more difficult and time-consuming than the actual analysis procedure. Furthermore, extraction of an analyte from a matrix is generally the most time-consuming step of sample preparation and leads to relatively inefficient analyte recoveries. Off-line SFE provides an alternative to traditional Soxhlet or liquid extraction methods. The present method for the isolation of fluconazole from the animal feed involes time consuming liquid extraction/solid phase extraction.[137] The objective of this study was to investigate the use of supercritical fluid CO_2 to extract fluconazole from spiked animal feed matrix. The SFE method is also compared with the established SPE method.

8.2 Optimization Parameters of SFE

There are two basic requirements that must be fulfilled to successfully perform off-line analytical supercritical fluid extraction. Firstly, the extraction parameters must be chosen correctly, given that the analyte is soluble in the extraction fluid. Even at this point, strong matrix interactions may preclude direct extraction of the analyte. Secondly, the collection system used must be efficient to trap all of the analytes during extraction.

For an SFE to be successful, the main operating variables relevant to extraction must be controlled. This is particularly true if extractions are to be carried out at pressures and temperatures near the critical region where a small change in either pressure or temperature has a noticeable difference in the supercritical CO_2 density as demonstrated by the phase diagram in Chapter 1 section 1.2. Designing meaningful

SFE involves considering several variables, other than temperature and pressure, which are important for obtaining a final reproducible extraction. Table 8.1 is a list of possible variables which are of greatest importance for optimizing an extraction with supercritical fluids.

Table 8.1 List of possible variables for SFE.

Supercritical fluid	**Modified supercritical fluid**	**Sample variable**	**Instrumental parameters**	**Sample accumulation**
choice	selection of modifier	size	choice of extraction cells	mode of extraction, e.g. on-line or off-line
pressure	concentration	matrix	extraction cell temperature	choice of collection , e.g. dry collection or solvent collection
temperature		particle size	extraction cell geometry	choice of collection solvent
flow rate		type of analyte	type of restrictor	
			restrictor temperature	

Some of these variables listed will depend on the instrumentation availability, but most of them are common to all types of supercritical fluid extraction. The four main variables for SFE are considered to be the extraction pressure, temperature, time and the flow rate.[187] However, interactions between two, or even several variables are quite possible. An experimental design approach was considered for extraction of fluconazole to account for the possible interactions between the four main operating variables of SFE.

8.3 Experimental design

The term experimental design can be defined as a test or a series of tests in which meaningful changes are made to the input variables of a process or a system so that the reasons for changes in the response may be identified.[188] Experimental design can act as a statistical means of obtaining the greatest amount of information in the fewest number of experiments.

In order to design a successful experimental design technique there are several factors which must be considered.

1. Identifying the factors which may affect the result of an experiment
2. The choice of factors and the levels at which the factors will be studied
3. Designing the experiment so that the effects of uncontrolled factors are minimized
4. Choice of experimental design
5. Using statistical analysis to separate the effects of the various factors involved.

The extraction efficiency for SFE will be found dependent on one or more of the variables listed in table 8.1. However, the relative contribution of each factor will not be equal and the most important factors must be chosen carefully when designing an experiment. Once the relevant factors are identified it is important to keep all the other factors as constant as possible hence reducing the chances of systematic error. Also,

before designing the experiments, an understanding of the final objectives of the experiment is necessary. The reason for this is that there are several possibilities to the design of experiments and it is not possible to find the relevant a approach unless the final objectives are defined. Statistical methods should be used to manipulate the information obtained from experiments so that the results and the conclusions are drawn in an objective manner. Graphical representations are often used for interpretation of results.

8.3.1 Factorial Designs

Factorial designs are an efficient way to study the factors of an experiment where two or more factors can affect the final response of the experiment. Factorial designs can be defined as the exploration of all possible chosen factor levels. The effect of a factor is the change in response produced by a change in the level of factor. This is commonly known as the main effect and it refers to the primary factors of interest in an experiment. When interactions between factors exists, the difference in response between the levels of one factor is not the same at all levels of the other factor. Where factor interaction is possible an experimental design is required which will allow estimation of both the main effects and the interactions. Factorial designs are very efficient and allow the effects of a factor to be estimated at several levels with respect to the other factors. Hence, factorial designs allow for conclusions to be drawn over a range of experimental values.

8.3.1.1 2^k Factorial Designs

These are the most commonly used factorial designs and consider all factors at two levels, i.e. high and low level. The number of treatment combinations, i.e. experiments undertaken is 2^k, where k is the number of variables under investigation. For example, to design an experiment with 2 variables there must be four experiments undertaken for a non-replicated study. Table 8.2 shows the treatment combination matrix for a 2^k design known as the design matrix.

Table 8.2 The treatment combination matrix for a 2^k design.

Treatment combinations/or experiment	**Variable A level**	**Variable B level**
1	high	high
2	high	low
3	low	high
4	low	low

For each treatment of combination (experiment), a response Y (experimental measurement of the dependent variable) is observed. A 2^k design allows estimation of all the main effects and the interaction effects of the following type model

$$Y = B_0 + B_1 x_1 + B_2 x_2 \ldots B_k x_k + B_{12} x_1 x_2 + B_{23} x_2 x_3 \ldots$$

Here B_0 is the intercept, B_i are the coefficients representing the model parameters and x_i are the variables. Note: that this design is linear and does not account for any curvature in the experimental response.

8.3.1.2 Fractional Factorial Design

The main problem with a factorial design is that the number of experiments required for a full factorial design is equal to the power of the number of factors involved in the experiments. This means that for a 2^k design with four variables (k = 4) requires a total of 16 experiments for all b coefficients to be estimated unambiguously. If replicate experiments are required for estimating experimental errors the total number of experiments becomes large.

If information on higher order interactions can be assumed to be negligible, it is possible to omit a significant number of experiments by using a fractional factorial design. Fractional factorial designs allows to neglect certain interactions with very small B coefficients. This sort of decision can be made from process knowledge if an interaction can be assumed to be unlikely. An example of a fractional factorial design is a $2^{(k-1)}$ or a half factorial design. This type of design would require only the half the number of experiments of a full 2^k design. So, when k is considered at 4, the total number of experiments is reduced from 16 to 8. One problem in using a factorial design to determine which factors have a significant effect on the responses that, for factors which are continuous variables, the effect depends on the high and low levels used. If the high and low levels are too close together, the effect of the corresponding factor may be found not significant. On the other hand, if the levels are further apart they may fall on either side of the maximum, and still give a difference in response which is not significant.

8.3.1.3 Response Surface Methodology

The response surface methodology (RSM), is normally dependent on several variables, and the idea is to find the optimum response within a given area of the variable range. The RSM uses statistical and mathematical techniques to model and

analyse data prior to displaying the information graphically. In RSM, the relationship between the response and the independent variables is usually unknown. A model is built which accounts for the observed response. this is generally achieved by using a polynomial equation. Process knowledge can be used to arrange the model either as a low or a high order polynomial. It is also possible to use a model which incorporated a mixture of both higher and lower modelled polynomials and these terms are called mixed models.

8.4 Optimization of Main Operating Extraction Parameters: Pressure, Temperature, % Modifier and Extraction Time

The extraction efficiency for SFE has been assumed to depend on a number of experimental factors e.g. pressure, temperature, time of extraction and flow rate. However, in cases, where the polar analtyes are bound or locked into a matrix, the presence of a modifier is known to be essential for successful extraction. The role of modifier is thought to be two fold. Firstly, the modifier increases the polarity of the extraction fluid and thus competes with the drug for the active site on the matrix. Secondly, the modifier acts to decrease the adsorption of solutes on the surface of the matrix by increasing the swelling of the matrix which in turn increases the likelihood of solute diffusion out of the matrix. Thus, designing an experimental design for analytes should involve consideration of all these variables in order to obtain maximum extraction efficiency.

A fractional factorial design based on response surface methodology was designed for extraction of fluconazole from an animal feed matrix. Four variables were considered, pressure, temperature, time and % modifier. Flow rate was kept at a constant value of 2 ml/min in order to keep the number of experiments down.

8.5 Experimental Section for the Analysis of Fluconazole by SPE/HPLC, SFE/HPLC and GC

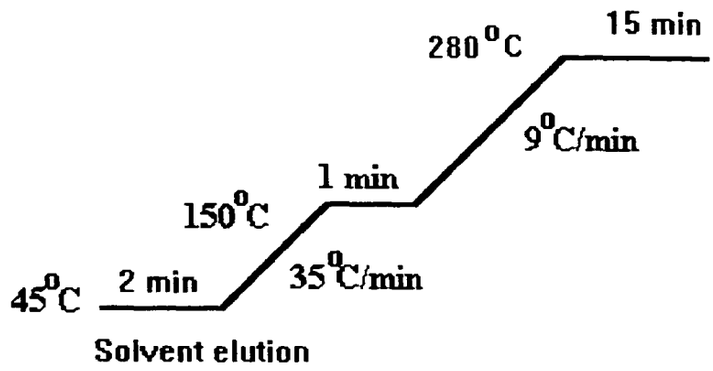
Instrumentation for the Analysis of Fluconazole

The HPLC system consisted of a Gilson model 305 master pump and slave pump, Gilson 805 S monometric module, Gilson 811 B dynamic mixer, Anachem OMRON E5CS column oven, Jasco UV 975 UV-VIS detector, LDC-Milton Roy Integrator. The analysis of the fluconazole SPE extracts of animal feed were performed using a published method by Pfizer.[137] Chromatography was achieved on a 10 cm x 2 mm , 5 μm C_8 Spherisorb analytical column at 35 °C. The mobile phase was 25/75 methanol / water. It was filtered and degassed by vacuum and sonication. The mobile phase flow rate was 0.4 ml min^{-1}. The column eluent was monitored for UV absorption at a wavelength of 210 nm.

The columns investigated for the analysis of SFE fluconazole animal feed extract were a 20 cm x 4.6 mm, 5 μm C_{18} Spherisorb and a 15 cm x 4.6 mm 5 μm CN Spherisorb as well as the 10 cm x 2 mm , 5 μm C_8 Spherisorb.

The GC system used was a MEGA series Carlo Erba system (CU600) with a flame ionisation detector. All samples (2 μl) were injected using on-column injection into a 15 m long x 0.3 mm diameter capillary column coated with DB5 stationary phase of 0.2 μm thickness. Propiophenone was used as the internal standard.

The temperature ramp used for the GC programme was as follows:



The GC-MSD system consisted of a Hewlet Packard 5890 series 2 GC with 5972 MSD. Samples were injected on-column with an Hewlett Packard 7673 autosampler. The GC conditions were the same as described above and the separation used a 25 m long x 0.22 mm diameter capillary column coated with a film of BPX5 stationary phase of 0.25 μm thickness. Single ion monitoring was carried out with ions being monitored at mass 77 and 105 amu for propiophenone and 127 and 224 amu for fluconazole.

Supercritical fluid extraction was undertaken on a Jasco Supercritical fluid extraction system. After the completion of each extraction, the extracts were made up to volume in methanol ready for analysis.

Reagents

Fluconazole reference standard was obtained from Pfizer Central Research (Sandwich, Kent, U.K). For the SPE-HPLC analysis, stock solutions of fluconazole (1 mg ml^{-1}) were prepared in methanol / water (75/25) and stored at 4°C. Working solutions containing 2, 5, 8, 10, 12 and 15 μg ml^{-1} fluconazole were prepared by diluting the stock solution. The correlation coefficient for the peak areas against

concentration was 0.999 (n = 5). While for GC-FID analysis stock solutions of fluconazole were prepared (10 mg ml^{-1}) with an internal standard (250 mg ml^{-1}) and stored at 4 °C. Working standards were prepared by serial dilution over the range, 50 - 400 μg ml^{-1}. An aliquot (20 μl) of internal standard (propiophenone) was added to each of the working standards and the extracts analysed by GC-FID. The calibration plot for GC-FID showed linearity over the chosen range with a correlation coefficient (r) of 0.974 (n = 5). The peak height ratios of sample/standard was used to quantify the extracts. For the GC-MSD analysis, stock solutions of fluconazole (1 mg ml^{-1}) were prepared in methanol / water (75/25) with an internal standard (25 mg ml^{-1}) and stored at 4 °C. Working solutions containing 5, 10, 15, 20 and 25 μg ml^{-1} fluconazole were prepared by diluting the stock solution. An aliquot (20 μl) of internal standard (propiophenone) was added to each of the working standards and the extracts analysed by GC. The correlation coefficient for the peak areas against concentration was 0.999 (n = 5). The peak area ratios of fluconazole and the internal standard were used for quantitation by GC-MSD. The extraction recovery was determined by comparing the peak area ratios of the extracted samples with that of a 100 % standard. Methanol and water of HPLC grade were used throughout. Supercritical fluid grade carbon dioxide (Air Products, Sunderland) of certified purity 99.995% was used for extraction.

Solid Phase Extraction Methodology

A method was adopted from literature published by the manufacturer of fluconazole for the extraction of fluconazole by SPE.[137] A fluconazole standard of concentration 20 μg ml^{-1} in dichloromethane was prepared which corresponded to a spike level of 40 mg drug kg^{-1} of feed. The following procedure was followed for the extraction. A portion of feed (5 g) was accurately weighed in a glass jar and spiked with 10 ml of the fluconazole standard (20 μg ml^{-1} in dichloromethane). The jar was capped, shaken vigorously and the unopened container was then left overnight to allow the

solvent to evaporate. The spiked samples were extracted with 20 ml of dichloromethane at room temperature by stirring with a magnetic stirrer for 1.5 hrs. The slurry mixture was vacuum filtered and the filtrate was quantitatively transferred to two cyano - propyl (500 mg) SPE cartridges (Bond-Elute, Jones Chromatography Ltd., Hengoed, U.K.) of 2.8 ml capacity, connected in series using a manual adapter and preconditioned with dichloromethane. The washings were collected and put through the cartridges again to ensure that all of the fluconazole was trapped onto the chromatographic packing material. Both cartridges were then left to dry under vacuum, the eluent was discarded and fluconazole was eluted with 20 ml of solvent $MeOH/H_2O$ (35/65). The eluent was quantitatively transferred into a 25 ml volumetric flask and made to volume with the appropriate solvent. The procedure was repeated five times and a blank extraction was also prepared. All extracts were analysed by HPLC using with UV-visible detection using a five-point calibration plot.

Supercritical Fluid Extraction Methodology

The extraction efficiency of SFE for the recovery of fluconazole from rodent feed was determined at two levels, 10 g of drug Kg^{-1} of feed and 500 mg Kg^{-1} of feed. The spiked feed samples of known fluconazole content were prepared by adding aliquots of the fluconazole standard in dichloromethane to a 1 g weighed sample of animal feed in a glass jar. These jars were capped, shaken vigorously and the contents were left to dry overnight in an open jar. The spiked sample was quantitatively transferred to a cell and the extraction was carried out to investigate SFE variable dependance. The flow rate of extraction was maintained constant at 2 ml min^{-1} throughout the experimentation. The collection vial contained a few drops of methanol and two cyanopropyl-propyl Bond Elut cartridges were placed in series at the exit to prevent any loss of fluconazole. Following extractions, all extracts were made up to volume in a 25 ml volumetric flask. The SFE extracts obtained at the 500 mg of drug Kg^{-1} of feed level were analysed by GC-MS, while the extracts obtained at the 10 g of drug

Kg^{-1} of feed were analysed by GC with flame ionization detection. All SFE extracts were filtered through a 0.45 μm membrane filter prior to analysis. All experiments were done in duplicate.

8.6 Results and Discussion

8.6.1 SPE-HPLC

The SPE method was utilized to selectively extract fluconazole from the feed prior to reversed phase HPLC and to simultaneously transfer the fluconazole from the hydrophobic extraction solvent to an essentially aqueous phase, compatible with reversed phased chromatography. The animal feed extracts of fluconazole obtained from the SPE were visibly clean and the extracts were analysed by the hplc method suggested by pfizer.[137] The results obtained gave a mean fluconazole recovery of 99.1% (98.1%, 100.7%, 97.9%, 100.2%, 98.7%) with a RSD of 1.3 %. The method described provides a convenient way of determining fluconazole quantitatively. The final solution obtained using this method was both sufficiently concentrated and also free from coextractives making it compatible with HPLC analysis.

Figure 8.1 shows a SPE-HPLC chromatogram of fluconazole in an animal feed extract.

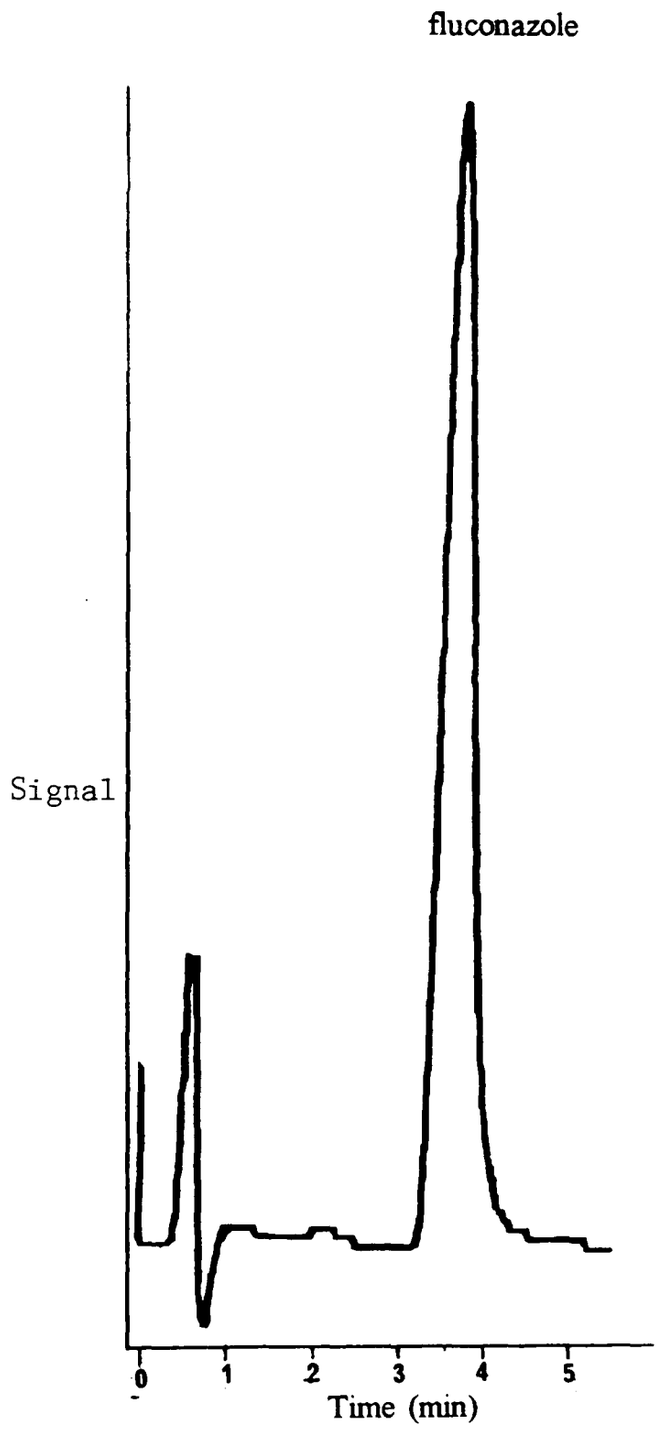


Figure 8.1 A typical SPE-HPLC chromatogram of fluconazole in an animal feed extract using a C_8 column and a mobile phase containing 25/75 methanol/water.

8.6.2 SFE-HPLC of Fluconazole from Animal Feed Matrix

Preliminary Extraction of Fluconazole from Celite matrix

Extraction of fluconazole from a Celite matrix was performed to determine the extraction profile of fluconazole in the absence of any matrix effects from the animal feed. In order to maximise the information available from the minimum number of experimental results the use of an experimental design approach was pursued. The experimental design selected was a fractional factorial 2^{k-1} design. This involved the use of a simple model of the form:

$$Y = b_0 + b_1v_1 + b_2v_2 + b_3v_3 + b_4v_4 \qquad 8.1$$

where, Y is the response (percentage extracted) ; v_1, v_2, v_3 and v_4 are the four main variables selected: pressure, temperature, time of extraction and percentage methanol, respectively; b_1, b_2, b_3 and b_4 are the parametric coefficients; b_0 is the intercept.

The experimental conditions were chosen at two levels, a high level and a low level. The pressure was at 250 kg/cm^2 and 110 kg/cm^2, temperature was at 80°C and 40°C, time of extraction was considered at 20 minutes and 5 minutes and % modifier was considered at 20% and 0%. All extractions were performed in duplicate. All extracts were analysed by HPLC at the same chromatographic method described for the SPE. The results obtained (table 8.3) indicated that fluconazole can be quantitatively extracted from Celite provided that the temperature and the % modifier are maintained at high levels.

Table 8.3: Percentage recovery data for the HPLC analysis of fluconazole from Celite using a fractional factorial design. All experiments were done in duplicate.

Pressure ($kg\ cm^{-2}$)	Temperature (°C)	Time (mins)	% MeOH	% Recovery
250	80	20	20	98.2
250	80	5	0	7.3
250	40	5	20	70.0
110	80	5	20	90.1
110	40	20	20	76.4
110	40	5	0	10.91
110	80	20	0	11.4
250	40	20	0	3.7
170	65	10	10	96.6

Extraction of Fluconazole from the Animal Feed Matrix

The major problem to be overcome in the development of SFE procedures for fluconazole in animal feed is that of obtaining a final sample that is sufficiently free from endogenous materials that fluconazole can be determined quantitatively. The investigation into the extraction of fluconazole from the animal feed began with the extraction of blank animal feed. The study involved extractions of the blank animal feed matrix at 80°C, 250 kg/cm^2, 20 % MeOH for 20 minutes at flow rate of 2 ml/min. This was necessary in order to ensure that no compounds extracted from the matrix itself would interfere with the HPLC analysis. These extractions produced a yellow precipitate in the extract, thus showing that part of the matrix was indeed

mobile and therefore extractable. The analysis of the filtered SFE extracts indicated that the presence of the precipitate in the collection solvent interfered with the existing HPLC method used for the analysis of the SPE extracts of fluconazole.

8.6.3 Method Development for HPLC

A HPLC method development was neccesary to establish a quantitative method for the determination of fluconazole from the animal feed. Blank extractions of animal feed (1 g) were carried out at a pressure of 250 kg/cm^2 , temperature 80°C with 20% methanol for 40 minutes at a flow rate of 2 ml/min and the extracts (25 mls) were spiked with known amounts of fluconazole standard (at spike levels of 100-500 mg of drug/kg of feed). These experimental conditions were chosen based on the results obtained from Celite matrix where the highest recovery was achieved under these set of conditions. Three different approaches were investigated in order to achieve a HPLC method for the quantitation of fluconazole

Initially a HPLC method suggested by Pfizer (the manufacturer of fluconazole) was tried.[137] This was based on a 10 cm x 2 mm C_8 column, UV detection at 210 nm and a binary mobile phase containing 25/75 $MeOH/H_2O$. Unfortunately, co-extractives from the animal feed severely interfered with the fluconazole peak. Systematic HPLC method development was carried out in an attempt to improve the separation of the fluconazole peak.

8.6.3.1 Effect of Mobile Phase Composition

The simplest approach was first tried where the mobile phase composition was varied. The mobile phase was altered to $MeOH/H_2O$ (20/80) and $MeOH/H_2O$ (15/85). This was carried out to investigated if reducing the MeOH concentration increases interaction of the analyte with stationary phase and if the fluconazole peak can be

separated from the rest of the interfering peaks. The result was peak tailing of fluconazole and the fluconazole peak was not resolved (figure 8.2). A C_{18} (2 cm) guard column was also placed before the C_8 column to increase the retention of fluconazole but did not result in any advantage.

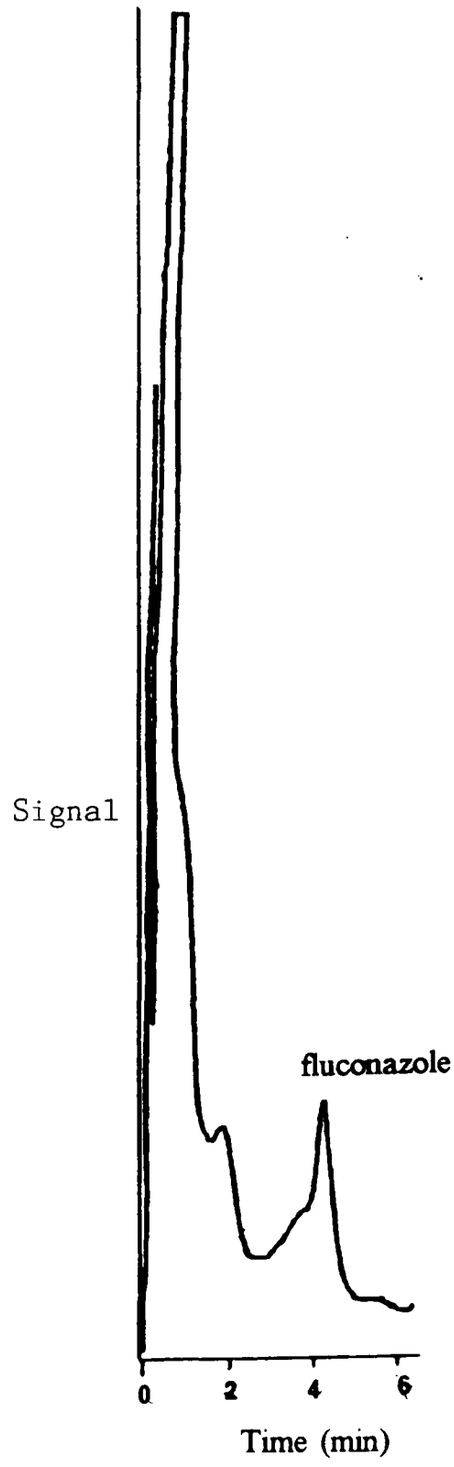


Figure 8.2 Example chromatogram of a fluconazole animal feed extract using a C_8 column and a mobile phase containing 15/85 methanol/water.

8.6.3.2 Effect of Additives

Fluconazole is a weak base. It is known that basic analytes can interact with silanols on the column packing and this can influence resolution and peak shape, e.g cause peak tailing. It was therefore decided to investigate the effect of blocking the silanols by adding a amine modifier to the mobile phase to see if this would significantly affect the peak shape of the fluconazole peak. The same silanol effects that result in band tailing can also be manifested as changes in retention. The addition of varying amounts of amine modifier can often result in changes in band spacing. When basic compounds are observed to tail due to silanol interactions, the an amine modifier such as triethylamine is often added. Triethylamine was introduced in the mobile phase [MeOH/50 mM triethylamine buffer at pH 7.0] and different mobile phase compositions were also tried to obtain higher selectivity and sharper peak but the results were not different (figure 8.3) from what had been found previously.

Next, addition of an ion-pair reagent was tried to influence the separation of the fluconazole from the interfering co-extractives. The retention in ion-pair HPLC can be described as the formation of an ion-pair between a sample ion and the ion- pair agent in the mobile phase, followed by hydrophobic association of the ion-pair with the stationary phase. An ion- pair reagent (5 Mm heptanesulphonic acid) was introduced to the MeOH/50 mM triethylamine mobile phase but baseline resolution of fluconazole (figure 8.4) was still not achieved.

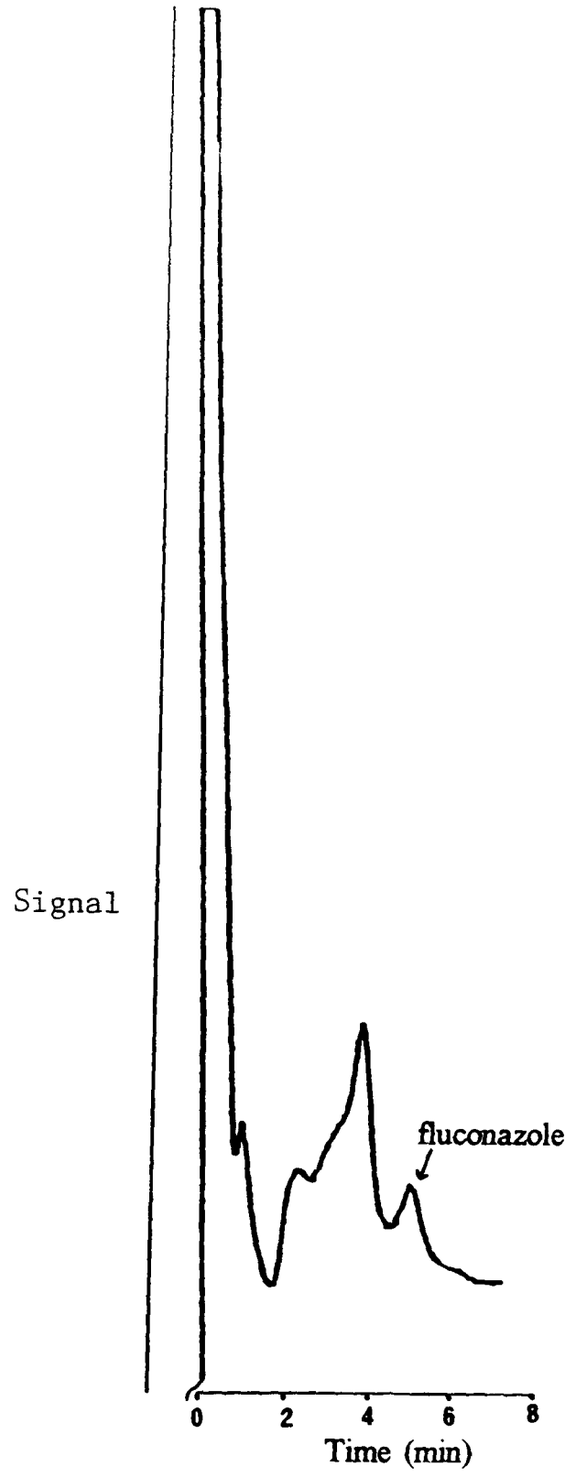


Figure 8.3 Example SFE-HPLC trace of a fluconazole animal feed extract using a C_8 column and a mobile phase containing 85/15 50 mM TEA buffer/methanol.

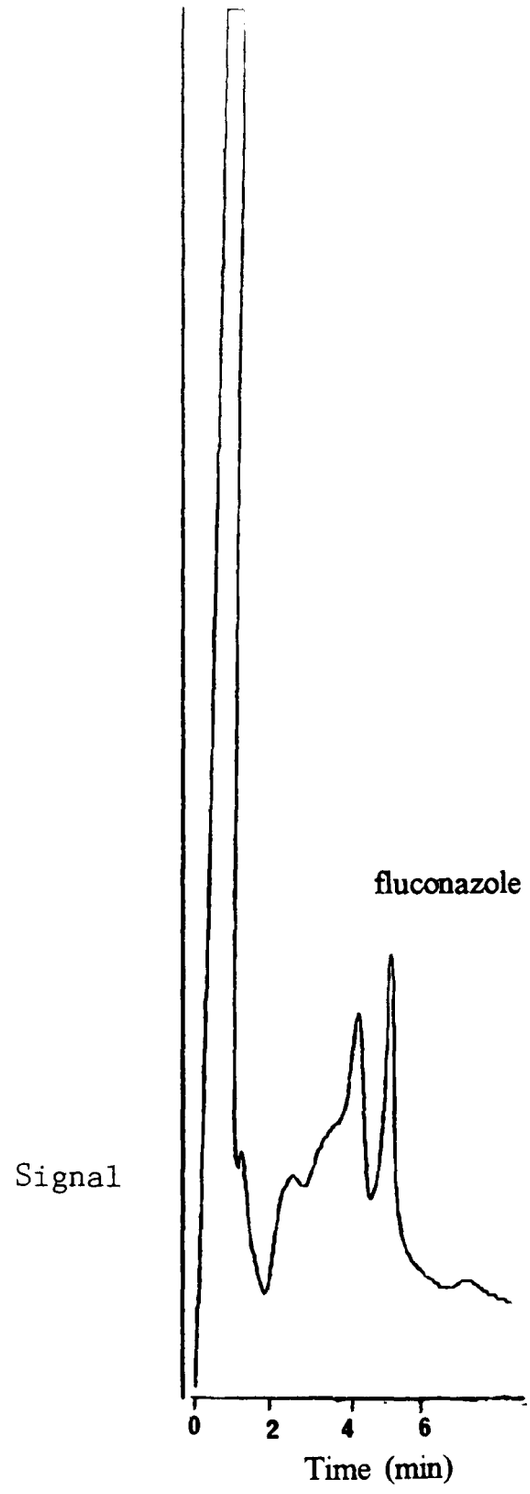


Figure 8.4 Example SFE-HPLC trace of a fluconazole animal feed extract using a C_8 column and a mobile phase containing 15/85 methano/ 50 mM TEA, 5 mM heptanesulphonic acid.

8.6.3.3 Effect of Stationary Phase

Different columns were to be investigated as a change in the nature of the stationary phase generally affect both retention and selectivity. The existing C_8 column was replaced with a C_{18} column (20 cm) which was slightly more retentive than the C_8 column and the flow rate was increased from 0.4 ml/min to 1 ml/min. Different $MeOH/H_2O$ mobile phase combinations were examined and it was possible to delay the retention of fluconazole to 9 minutes so it would not interfere with the coextractives eluting at the baseline. However, the fluconazole peak was broad and could not be separated from an interfering small peak using single wavelength detection (figure 8.5).

MeCN was used instead of MeOH to investigate if it would result in sufficient change in peak spacing, but no noticeable difference in the chromatogram was noted.

Replacing a C_{18} column with a different column, such as phenyl or cyano usually results in a change in band spacing and it was decided to change the C_{18} column for a more polar CN column and as before different mobile phase compositions of $MeOH/H_2O$ were studied as it cannot be assumed that the same mobile phase that was optimized for the C_8 column will also be optimum for the cyano column. The difference in column polarity had the greatest in changing the band spacing as it was found that a mobile phase composition of 15/85 $MeOH/H_2O$, flow rate of 1 ml/min, detection at 210 nm with a 15 cm cyano column gave the best chromatographic separation of fluconazole from other co-extractive peaks (figure 8.6) but still was not considered acceptable below 500 mg of drug/kg of feed.

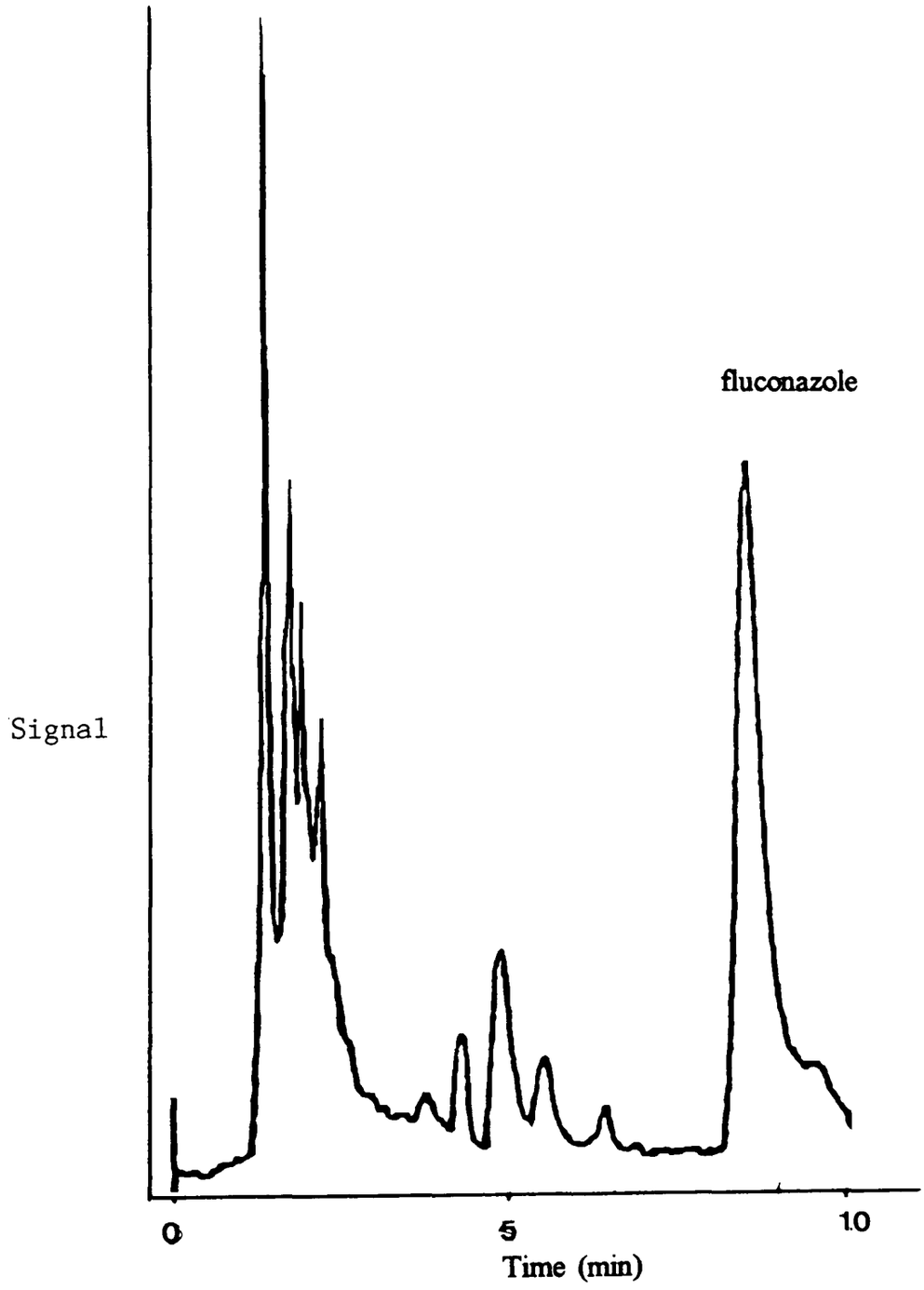


Figure 8.5 A typical SFE-HPLC trace of fluconazole in an animal feed extract using a C_{18} column and a mobile phase containing 20/80 methanol/water.

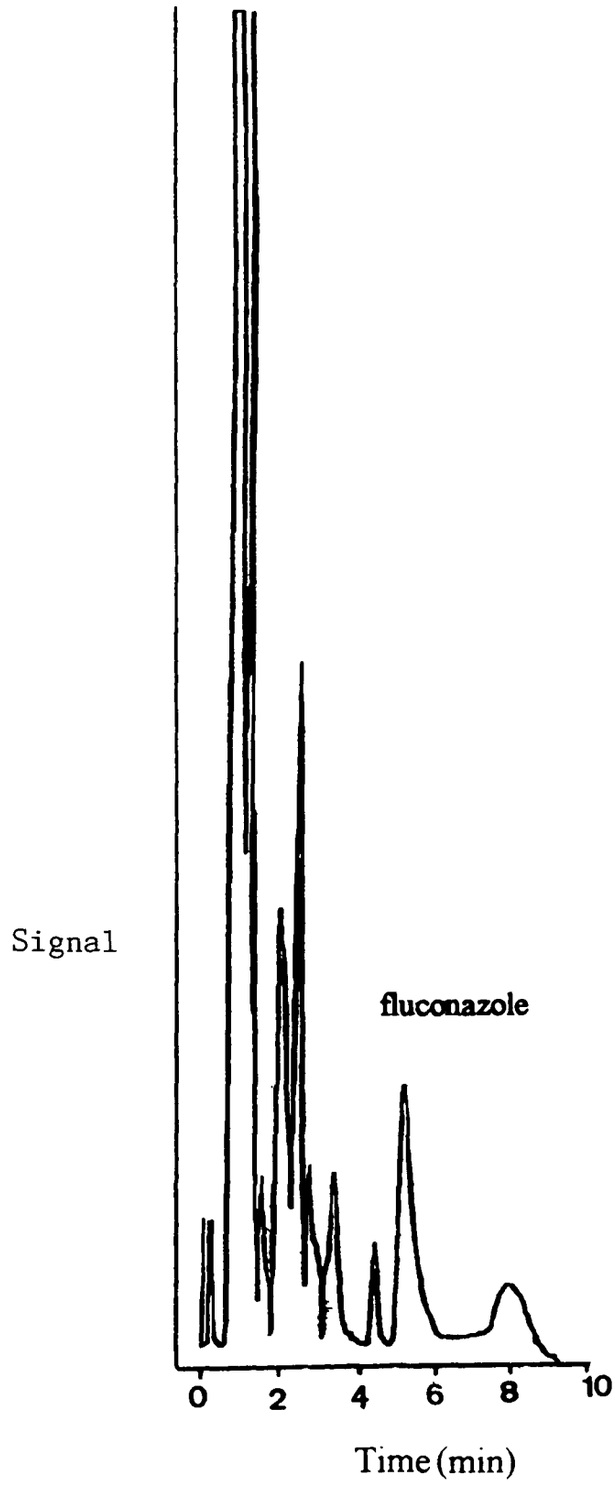


Figure 8.6 Example chromatogram obtained for a SFE-HPLC animal feed extract using a CN column and a mobile phase composition of 15/85 methanol/water.

8.6.3.4 Effet of CO_2 Pre-Extraction as a Clean-up for Animal Feed

Following these investigations it was decided to study if it was possible to extract the coextractives prior to extracting fluconazole. Pre-extractions of interfering compounds was studied with pure CO_2 only (different experimental conditions, i.e different pressure, temperature and extraction time were investigated). It was found from these investigations that CO_2 alone failed to extract any coextractives (figure 8.7) that interfere with the fluconazole peak and introduction of MeOH modifier (20%) in the extraction after CO_2 clean-up extracted the endogenous materials from the animal feed (figure 8.8). Hence problems arise with SFE where addition of MeOH as a modifier is required to aid extraction but at the same time the extracts contain a vast amount of endogenous material from the feed which interferes with the HPLC analysis. The other problem faced with the HPLC analysis of the SFE extracts is that although the samples were filtered prior to injection, the normal column life time was reduced significantly (typically 1-2 weeks).

Because a satisfactory HPLC analysis could not be developed GC analysis was investigated. Immidiately, GC afforded excellent selectivity required for the analysis of fluconazole from the animal feed extract.

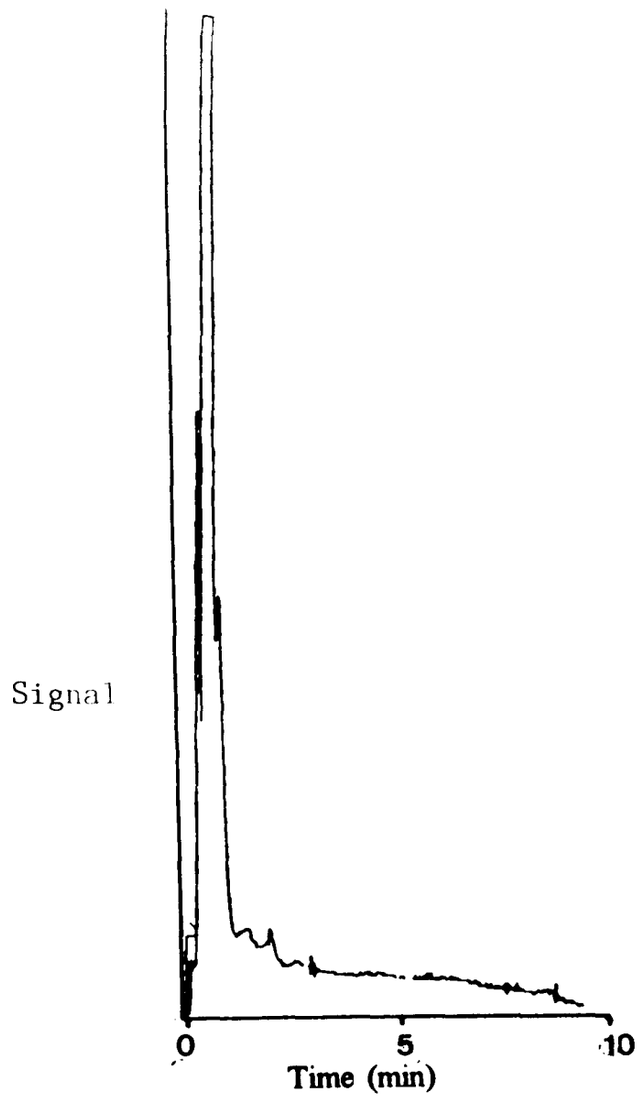


Figure 8.7 Example chromatogram obtained from CO_2 only extraction -1 hour, 80C and 250 Kg/cm2, using a C_8 column and a mobile phase containing 15/85 methanol/water.

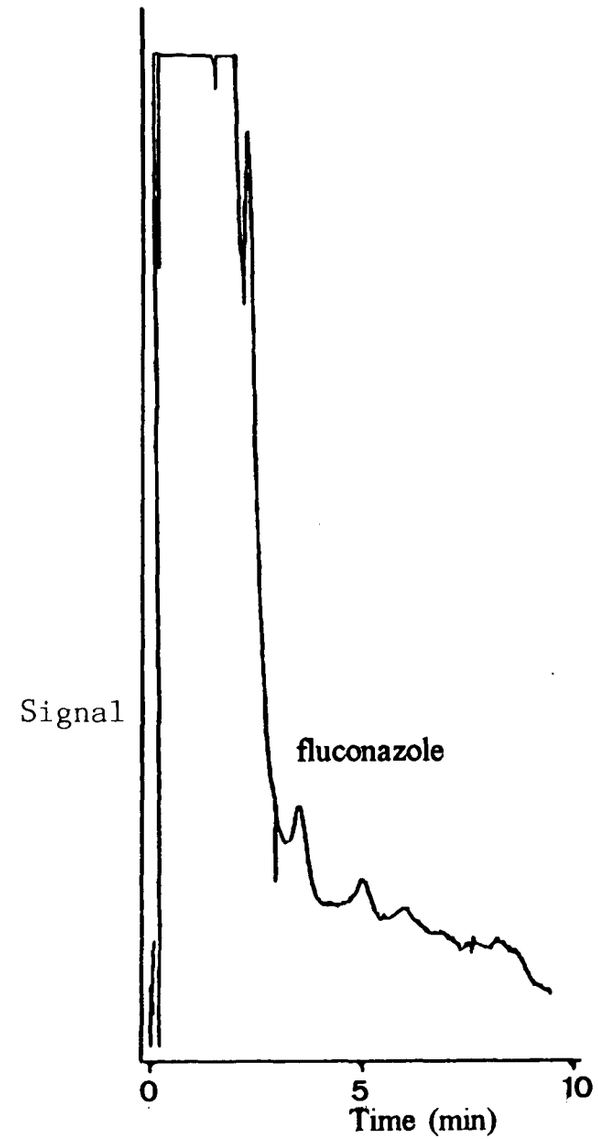


Figure 8.8 Example chromatogram obtained for a SFE fluconazole animal feed extract with 20% methanol for 40 minutes after 1 hour CO_2 only extraction using a C_8 column and a mobile phase of 15/85 methanol/water.

8.7 SFE-GC of Fluconazole from an Animal Feed Matrix

SFE is sufficiently powerful to extract other materials from the matrix which interfere with analyte determination. The nature of the co-extracted material prevented quantitative analysis by HPLC with UV detection. Because of this GC was used for all subsequent analysis. It had been noted previously that fluconazole could be analysed using GC.[190-193] In order to facilitate analysis and detection at high and intermediate spiking levels it was necessary to use GC separation with either flame ionization or mass selective detection. However, in order not to be sensitivity limited (particularly in the case of GC with FID detection) the spiking level of fluconazole was done at the 10 g kg^{-1} (high level). With the GC-FID analysis, baseline resolution of fluconazole, from the endogenous material co-extracted from the feed, was achieved with the conditions described at a retention time of 19 minutes (figure 8.9). An intermediate spiking level of 500 mg kg^{-1} was also prepared and these extracts were analysed by GC with MSD detection. It is apparent however, that the inherent sensitivity of GC-MSD will allow lower spiking levels to be analysed. Figure 8.10 shows the GC-MSD trace of an animal feed extract of fluconazole.

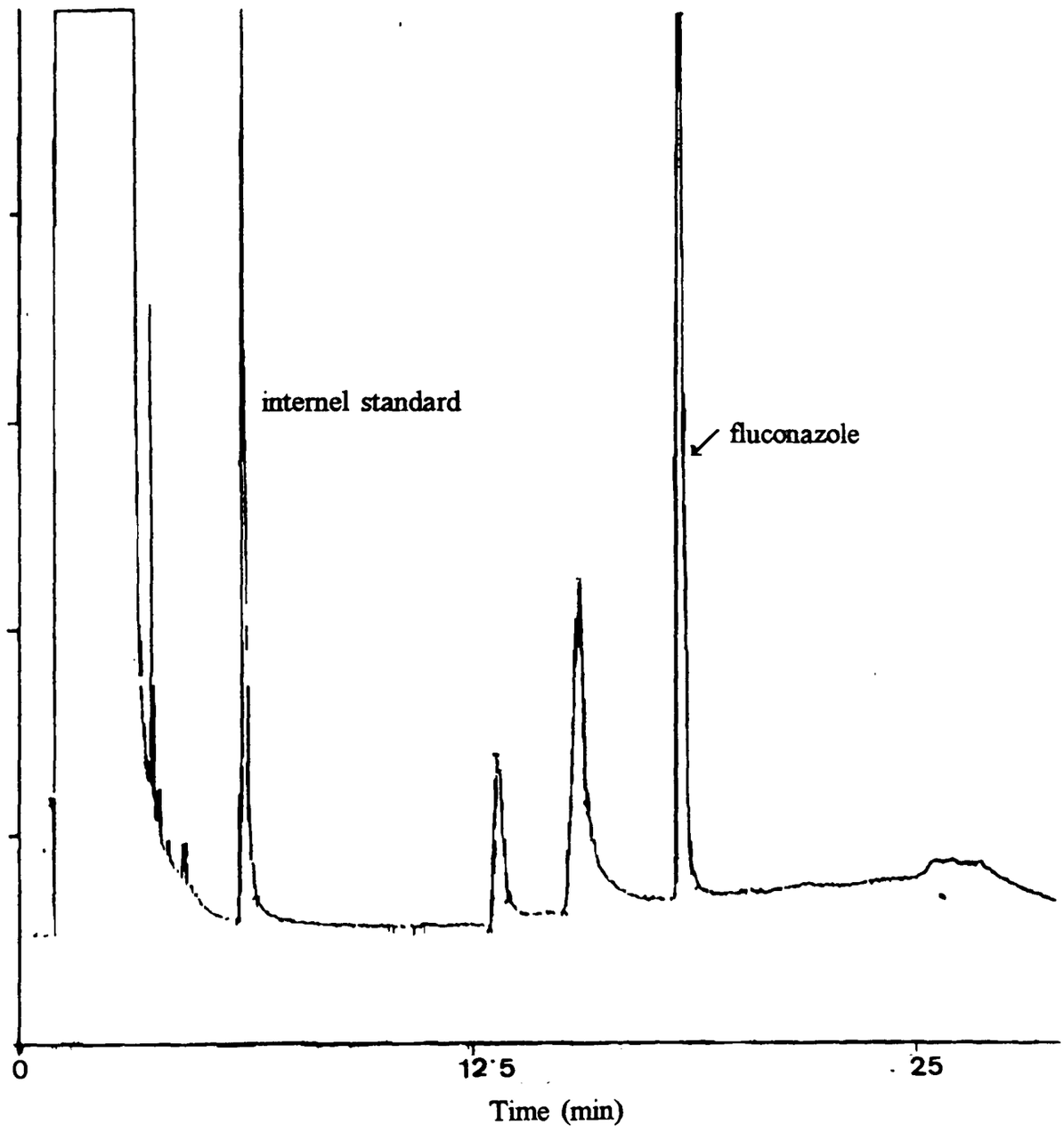


Figure 8.9 A typical SFE-GC-FID trace of a fluconazole animal feed extract.

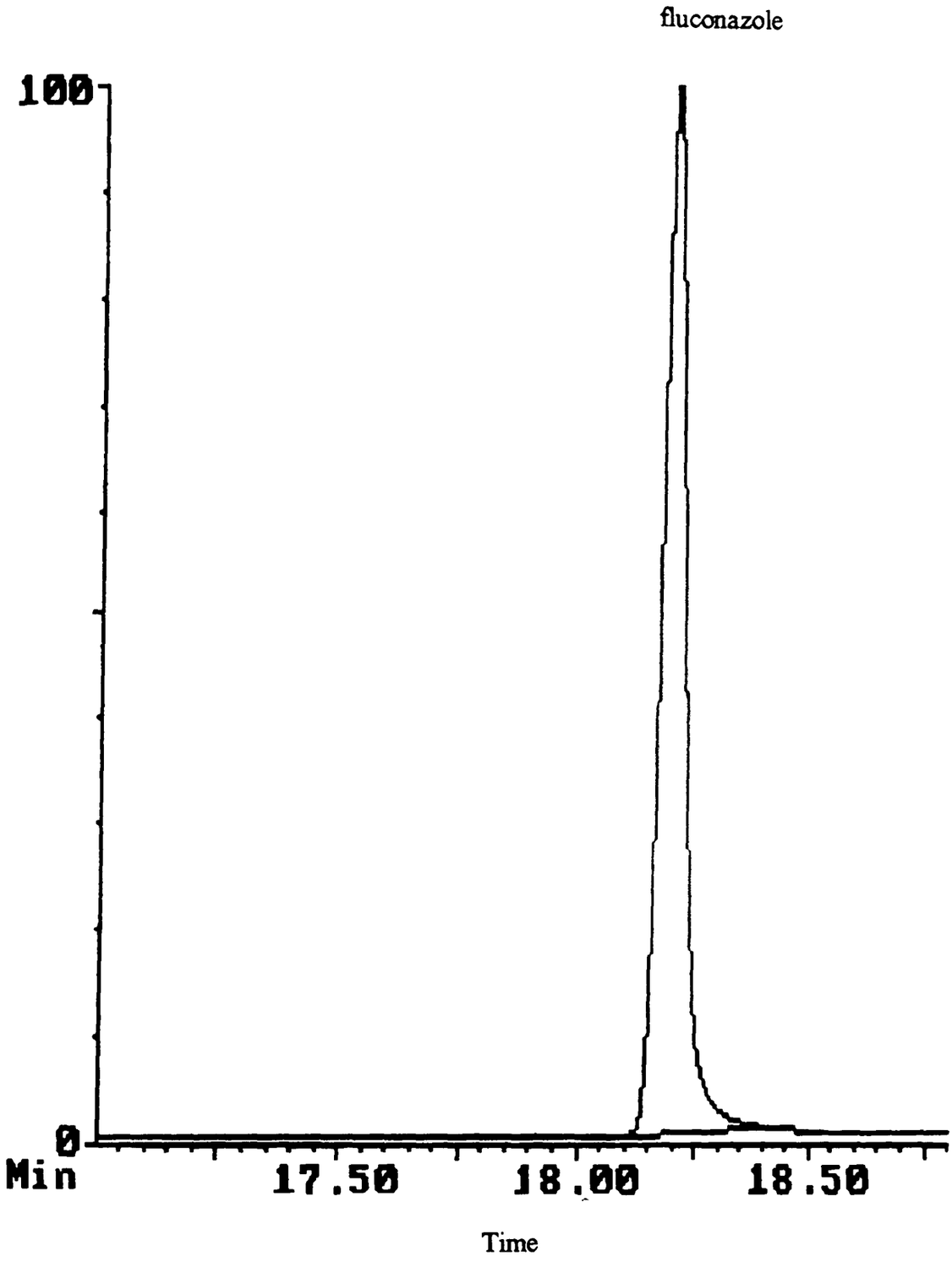


Figure 8.10 A typical SFE-GC-MS trace of a fluconazole animal feed extract

SFE of Fluconazole from Animal Feed using Experimental Design

Spiked fluconazole animal feed was prepared according to the procedure described in the experimental section and a fractional factorial design carried out as discussed for the Celite extractions in the previous section at the two different spike levels. The extraction time was extended, within the design, from 20 mins to 40 mins in order to compensate for any potential matrix effects. The results obtained for both levels of feed are shown in Tables 8.4 and 8.5.

Table 8.4 : Percentage recovery data for the GC-FID analysis of fluconazole (10 g kg^{-1} of feed) from an animal feed using a fractional factorial design. All experiments were done in duplicate.

Pressure ($kg\ cm^{-2}$)	**Temperature (°C)**	**Time (mins)**	**% MeOH**	**% Recovery**
250	80	40	20	87.0
250	80	5	2	76.6
250	40	5	20	79.0
110	80	5	20	90.9
110	40	40	20	80.5
110	40	5	2	16.9
110	80	40	2	22.3
250	40	40	2	27.3
170	65	20	10	97.4

Table 8.5: Percentage recovery data for the GC-MSD analysis of fluconazole (500 mg kg^{-1} of feed) from an animal feed using a fractional factorial design. All experiments were done in duplicate.

Pressure ($kg\ cm^{-2}$)	**Temperature (°C)**	**Time (mins)**	**% MeOH**	**% Recovery**
250	80	40	20	99.6
250	80	5	0	5.3
250	40	5	20	82.7
110	80	5	20	99.7
110	40	40	20	72.5
110	40	5	0	5.1
110	80	40	0	16.8
250	40	40	0	5.8
170	65	20	10	100.3

It should be noted that the percentage methanol was considered at three levels 0, 10 and 20 % in the case of the 500 mg kg^{-1} level whereas at the 10 g kg^{-1} level it was considered at 2, 10 and 20 %. Multilinear regression was used to calculate the coefficients in equations (8.2 and 8.3) . The coefficients are reported in Tables 8.6 and 8.7.

Table 8.6 Coefficients of regression and standard error of linear equation (8.2) for the GC-FID analysis of fluconazole from animal feed (10 g kg^{-1}level).

Variable	Descriptor	Beta	standard error	b	standard error	t(4)	p-level
pressure	v_1	0.2115	0.2605	0.0974	0.1199	0.8120	0.4623
temperature	v_2	0.3166	0.2605	0.5090	0.4188	1.2153	0.2911
time	v_3	-0.1980	0.2605	-0.3647	0.4797	-0.7603	0.4894
%MeOH	v_4	0.7389	0.2605	2.6472	0.9332	2.8366	0.0470

Table 8.7 Coefficients of regression and standard error of linear equation (8.3) for the GC-MSD analysis of Fluconazole from animal feed (500 mg kg^{-1} level).

Variable	Descriptor	Beta	standard error	b	standard error	t(4)	p-level
pressure	v_1	0.0198	0.2460	0.0109	0.1363	0.0893	0.9398
temperature	v_2	0.1817	0.2146	0.4054	0.4788	0.8467	0.4449
time	v_3	-0.0164	0.2137	-0.0419	0.5463	-0.0767	0.9425
%MeOH	v_4	0.9070	0.2139	4.0620	0.9582	4.2394	0.0133

Significance is determined by a t-test with a probability (p) of 0.05 at the 95% confidence level with 4 degrees of freedom. In Tables 8.5 and 8.6, a coefficient with a p-level of less than 0.05 or a t-test of greater than 2.78 will be considered significant. In both cases the results obtained indicate that percentage methanol was the only significant variable for the extraction of fluconazole from the animal feed matrix. The difference in the b coefficients (-0.3647 and -0.0419 for the 10 g kg^{-1} and 500 mg kg^{-1} spiking levels, respectively) reflect the influence that the percentage methanol - time of extraction interaction has on extraction recovery. The consequence of the addition of a small amount of methanol (2 %) as opposed to CO_2 only had a pronounced effect on extraction recovery (Tables 8.4 and 8.5). The model for the 10 g kg^{-1} extraction results is:

Percentage recovery of fluconazole =-4.751 + 0.097 (pressure) + 0.509(temperature)
- -0.365 (time of extraction) + 2.647 (percentage methanol) 8.2

$r = 0.8535$ ($r^2 = 0.7286$) at the 95% confidence interval

Whereas, the model for the 500 mg kg^{-1} results is as follows:

Percentage recovery of fluconazole = -11.813 + 0.010 (pressure) +0.405(temperature)
-0.042 (time of extraction) + 4.062 (percentage methanol) 8.3

$r = 0.9199$ ($r^2 = 0.84620$) at the 95% confidence interval.

Equations 8.2 and 8.3 can be used to predict percentage recovery of fluconazole from an animal feed matrix for any given pressure, temperature, time of extraction and % methanol at the two specified levels.

A typical response surface was generated for fluconazole (figure 8.11) showing the effect of temperature and percentage methanol, as modifier, on extraction recovery.

The percentage extraction efficiency repeatability for the isolation and cleanup of fluconazole from animal feed followed by GC with either FID or MSD detection was determined to be 87.0% (RSD 8.4%) at the 10 g kg^{-1} level and 91.0% (RSD = 13.2%) at the 500 mg kg^{-1} level. In each case the number of replicates was ten. Figure 8.11 shows the response surface obtained from the SFE-GC-FID analysis of fluconazole and figure 8.12 shows the response surface obtained from the SFE-GC-MSD analysis of fluconazole.

Figure 8.11 Percentage Recovery of Fluconazole from animal feed using SFE-GC-FID

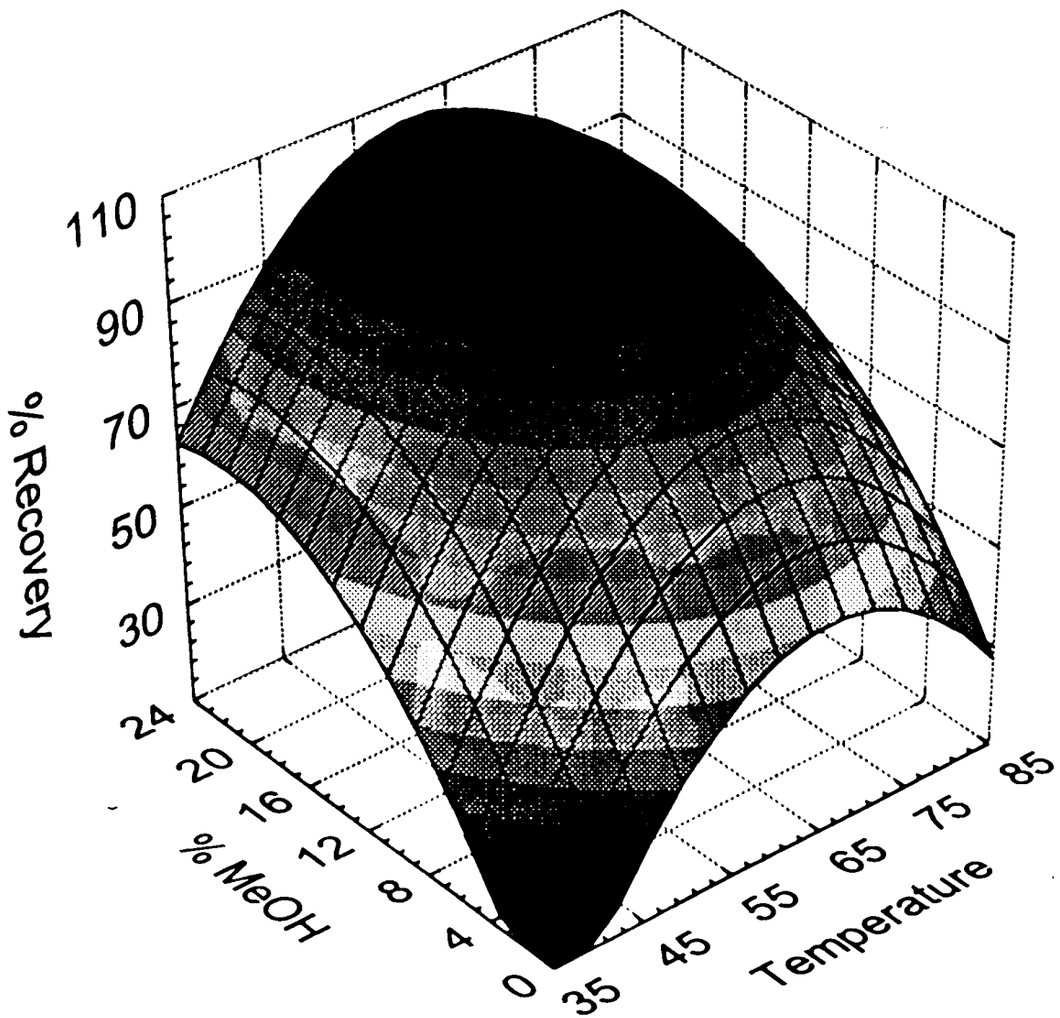
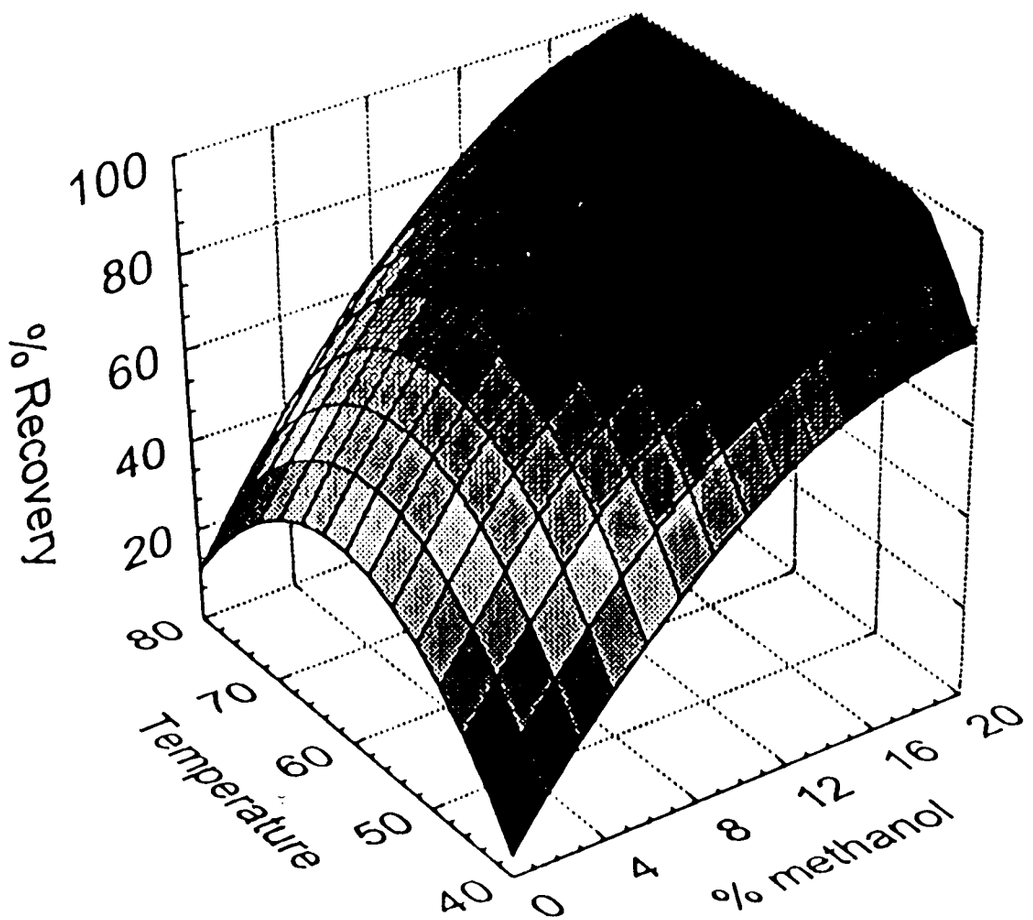


Figure 8.12 Percentage Recovery of Fluconazole from an animal feed using SFE-GC-MS



This study clearly demonstrated the ability of supercritical CO_2 to quantitatively extract fluconazole from an animal feed matrix. The problem lies with the analysis of an SFE extract. The recovery of fluconazole from animal feed was determined, using a fractional factorial design approach, to be dependent upon the addition of modifier in the extraction fluid. The modifier's function is thought to be two fold. Firstly, the modifier increases the polarity of the extraction fluid and thus competes with the drug for the active site on the matrix. Secondly, the modifier acts to decrease the adsorption of solutes on the surface of the matrix by increasing the swelling of the matrix which in turn increases the likelihood of solute diffusion out of the matrix.

8.8 Conclusion

A comparison of SPE with SFE for the isolation and cleanup of fluconazole from animal feed has been made. The inter-dependence of the extraction procedure on the method of analysis was effectively demonstrated. The unselective nature of SFE, when methanol-modified supercritical CO_2 was used, prevented analysis by HPLC with UV detection. Quantitative recovery of fluconazole was possible using GC with either FID or MSD detection at the 10 g kg^{-1} and 500 mg kg^{-1} spike levels, respectively. However, the inherent sensitivity of GC-MSD should allow lower spike levels to be quantified. SPE-HPLC was shown to be an effective method of isolating and analysing fluconazole from animal feed at a low spike level (40 mg kg^{-1}).

Chapter 9.0: The Determination of Antifungals by SFE/Packed SFC from Animal Feed Matrix.

9.1 Introduction and Aims

Supercritical fluid chromatography is complementary to both gas chromatography and high performance liquid chromatography for the analysis of pharmaceuticals, and like HPLC, does not require vapourisation of the sample. The operating conditions of pSFC are mild and so it is able to handle thermally labile drug substances. Antifungals such as fluconazole, tioconazole and UK-47,265 are commonly analysed by HPLC. In Chapter 8, the use of HPLC and GC for the analysis of fluconazole has been discussed. The objective of this study was to evaluate the potential of pSFC using MeOH modified carbon dioxide for the analysis of four antifungals following the SFE of these compounds from an animal feed matrix.

Packed column SFC is most useful in combination with high density mobile phases. In general, the closer the SFC resembles LC, the more similar will be the column technology and the required instrumentation. As is the case with LC, SFC can be used for the routine analysis of samples. By applying specific sample preparation techniques or specific detection methods, complicated separation problems may be reduced to simple chromatograms by using pSFC.

9.2 The Main Optimizing Parameters of pSFC

One of the key features of SFC is its flexibility. Optimisation of a fluid chromatographic separation can be obtained by altering a variety of parameters. The main operating variables (table 9.1) for optimization of a SFC separation are as follows:

Table 9.1 The main operating variables for optimization of a SFC separation.

selection of a stationary phase
selection and/or programming of the mobile phase
optimisation of the analysis temperature
addition of a modifier
eluent composition
flow rate of the mobile phase
pressure

It is apparent from studying this list that in SFC, there are more parameters which can influence separations than in any other chromatographic method. Besides variations of stationary and mobile phases, retention, selectivity, and resolution are easily optimised by adjusting temperature, pressure, and the density of the mobile phase. Mobile phase density is thought to be the key parameter in SFC and most separations are performed using variations in density. Pressure programming can be used as the same way as density programming to elute components of high molecular weight or to shorten analysis times. Temperature is often used as a second parameter to improve resolution in density or pressure programmed SFC analyses. At constant density, analyses are faster at higher temperatures. At constant pressure, short analyses are obtained at temperatures slightly below, or well above, the critical temperature of the eluent. Maximum retention and resolution are found at temperatures somewhat above critical. The solvent strength of the mobile phase can be adjusted either by choosing a suitable eluent or by adding a suitable second eluent component.

Even with so many operating parameters to vary, method development in SFC is easy and does not require much time. Once the choice of stationary and mobile phase is

made which are suitable for the analytical problem, retention as well as selectivity and resolution may be adjusted by varying density, pressure and / or temperature.

9.3 Retention Behaviour in SFC

Under the isobaric conditions in SFC, the mobile phase strength increases with the density of the supercritical fluid. The lower the temperature, the higher the density becomes, and hence the shorter the retention times obtained. Increasing the column temperature therefore results in longer retention times of solutes. However, when the column temperature is much higher, contributions of vapour pressures of solutes become greater and retentions become shorter due to the vapour pressure contribution. Even though solutes are not volatile, the solutes often melt by heat and then are solubilized, resulting in shorter retentions. In this temperature region, contributions of vapour pressure and density change contribute in an opposite way, offering higher selectivity for separation.

Under isothermal condition, the higher the pressure, the higher the density is obtained. Since higher the density, the higher the solvating power becomes resulting in shorter retention times as the pressure goes higher.

9.4 Addition of Modifier Solvent

In SFC, a small amount of an organic solvent is often added to the supercritical fluid. The role of the modifier is thought to be two fold. Firstly, to deactivate active sites of the packing material (typically silanols of silica gel) and secondly to increase the solvating power of the fluid.

While using a silica gel stationary phase in adsorption mode, the solute molecules compete with modifier molecules in adsorption onto the active sites. Therefore, the

retention behaviour is dependent on the degree of affinities of the sample solute and the modifier molecule to the stationary phase. In general, a polar solvent such as alcohol is used for deactivating the stationary phase. Addition of alcohol also increases the solvating power of the mobile phase.

When using a bonded stationary phase in the partition mode, such as ODS-silica, addition of a modifier increases the solvating power of the mobile phase. Accordingly, the partition of the sample solute shifts toward the mobile phase, resulting in shorter retention time.

In both modes, addition of a modifier decreases the retention. Increasing the modifier concentration as a function of time works in a similar manner to gradient elution in HPLC.

9.5 Experimental Section

Reagents

Tioconazole, fluconazole and UK-47,265 were provided in pure form by Pfizer Central Research, Sandwich, Kent. Hexaconazole was provided by Zeneca Agrochemicals, Jeallots Hill, Berkshire. Triethylamine and methanol were obtained from Aldrich Chemicals Ltd. and were HPLC grade. Supercritical fluid grade carbon dioxide (Air Products Ltd., Sunderland) of certified purity 99.995% was used for extraction. The chromatographic column used was a 15 cm x 4.6 mm id stainless-steel column packed with cyano bonded phase (5 μm, Phase Separations Ltd).

The triethylamine was dissolved in the MeOH prior to mixing with the CO_2. The amount of modifier in the CO_2 was regulated by the flow rate of the master pump. Stock solutions were prepared by dissolving appropriate amounts of the drugs into a

50 ml volumetric flask and diluting to volume with MeOH. Working standards were prepared by serial dilution over the appropriate range. Samples were introduced onto the column via a Rheodyne injector fitted with a 10-µl sample loop. The column eluent was monitored for UV absorption at a wavelength of 210 nm. Calibration curves of peak height verses concentration were generated for all the compounds. The percentage recovery was calculated by comparing the peak heights of the extracted samples with those obtained by injections of the 100% standard solutions.

Supercritical Fluid Extraction Methodology

The animal feed was spiked with fluconazole and tioconazole individually whereas hexaconazole and UK-47,265 were spiked jointly for the extraction and analysis of the drugs using the same spiking method described in the previous chapter. The extraction efficiency of SFE for the recovery of fluconazole from the rodent feed was determined at two levels, 10 g of drug/kg of feed and 500 mg of drug/kg of feed. The extraction efficiency of SFE for the recovery of tioconazole from the rodent feed was determined at two levels: 2.5 g of drug/kg of feed and 5 g of drug/kg of feed. The extraction efficiency for the recovery of hexaconazole and the UK-47,265 of fluconazole from the animal feed was also determined at two levels 1 g of UK-47,265/ kg of feed, 1.25 g of hexaconazole/kg of feed and 2.5 g of UK-47,265/kg of feed and 1.875 g of hexaconazole/kg of feed. The spiked feed samples of the known drug content were prepared in the same manner as described in the previous section. The spiked sample was quantitatively transferred to a cell prior to extractions being performed. The collection vial contained a few drops of MeOH and two cyano-propyl Bond Elute cartridges were placed in series at the exit to prevent any loss of the analyte. Following extractions, all extracts were made up to volume in a 25 ml volumetric flask. All SFE extracts were filtered through a 0.45 µm membrane filter for the SFC assay. All experiments were repeated 5 times and triplicate injections of each extracted sample were carried out.

9.6 Results and Discussion

9.6.1 Optimization of SFE

It has already been established in chapter 8 that the efficiency of SFE from animal feed is directly related to the extraction conditions and successful extraction can be achieved provided % methanol was kept high.

The experimental conditions used for the extraction of fluconazole were: temperature 80°C, carbon dioxide + 20% MeOH at 250 kg/cm^2 pressure, flow rate of 2 ml/min and extraction time 40 min.

Experimental conditions for the extraction of tioconazole, hexaconazole and UK-47,265 at both levels were: temperature 60°C, carbon dioxide + 20% MeOH at 250 kg/cm^2 pressure, flow rate of 2 ml/min and extraction time 40 min.

Blank extractions were carried out in duplicate at the optimized extraction conditions. This was necessary in order to ensure that no compounds extracted from the matrix itself would interfere with the pSFC analysis.

9.6.2 Quantitative Analysis of Animal feed Extracts

Optimization of the Operating Chromatographic Parameters for Analysis of the Antifungal Compounds

Because of the more favourable mass transfer kinetics on SFC, owing to the high diffusivity and low viscosity, SFC has the potential of giving better separation performance and shorter analysis time compared to HPLC. The objectives of this study were to evaluate the potential of pSFC with carbon dioxide and polar modifiers as the mobile phases for the analysis of the antifungal compounds. With pure carbon

dioxide, acceptable peak shape was obtained only for hexaconazole. Tioconazole was not eluted with pure carbon dioxide. This can be attributed to interactions between the solutes and active sites, such as residual silanol present on the stationary phase and also to the low polarity of carbon dioxide. Consequently, a mobile phase modifier had to be applied.

9.6.3 Effect of Mobile Phase Composition

As expected, the concentration of the modifier in the mobile phase had the greatest impact on retention of all the compounds compared with any other instrumental parameters tested. Fluconazole was eluted as a broad peak (figure 9.1) from the cyano column when pure carbon dioxide was used as the mobile phase at 60°C and an outlet pressure of 250 kg/cm^2. The flow rate was set at 3 ml/min. A similar effect was observed with the UK-47,265. Tioconazole was not eluted with 100% CO_2. The addition of low concentrations of methanol to the mobile phase dramatically decreased retention time and improved peak shapes for both fluconazole and UK-47,265. To obtain reasonable elution times for fluconazole, a mobile phase containing 8% modifier was required. Similarly, to obtain a reasonable elution time for the UK-47,265, a mobile phase composition containing 12% modifier was required. Hexaconazole was eluted with lower modifier concentration with good peak shapes but a modifier concentration of 12% was also chosen for hexaconazole as it was extracted together with the UK-47,265 from the feed. One area of difficulty uncovered during the analysis of tioconazole was adsorption of the drug by active silanol sites with the column. This was not unexpected as similar silanol interactions are commonly encountered in hplc. A modifier concentration of 14% at 55°C and 272 kg/cm^2 outlet pressure eluted tioconazole from the polar cyano column, but the compound emerged as a severly broad peak (figure 9.2). A further increases in the modifier concentration from 14% to 20% did not improve the peak shape. Consequently the presence of a very polar additive was found to be necessary to

reduce the retention time and to improve peak shape. Different concentrations of additive-methanol mixture were used as the chromatographic mobile phase with the cyano column. The addition of triethylamine in the MeOH (0.25 mls of triethylamine per 50 mls of MeOH) decreased the retention time and improved the peak shape of tioconazole dramatically (see figure 9.6 later). Table 9.2 shows the effect of % MeOH modifier on retention for hexaconazole, fluconazole and UK-47,265.

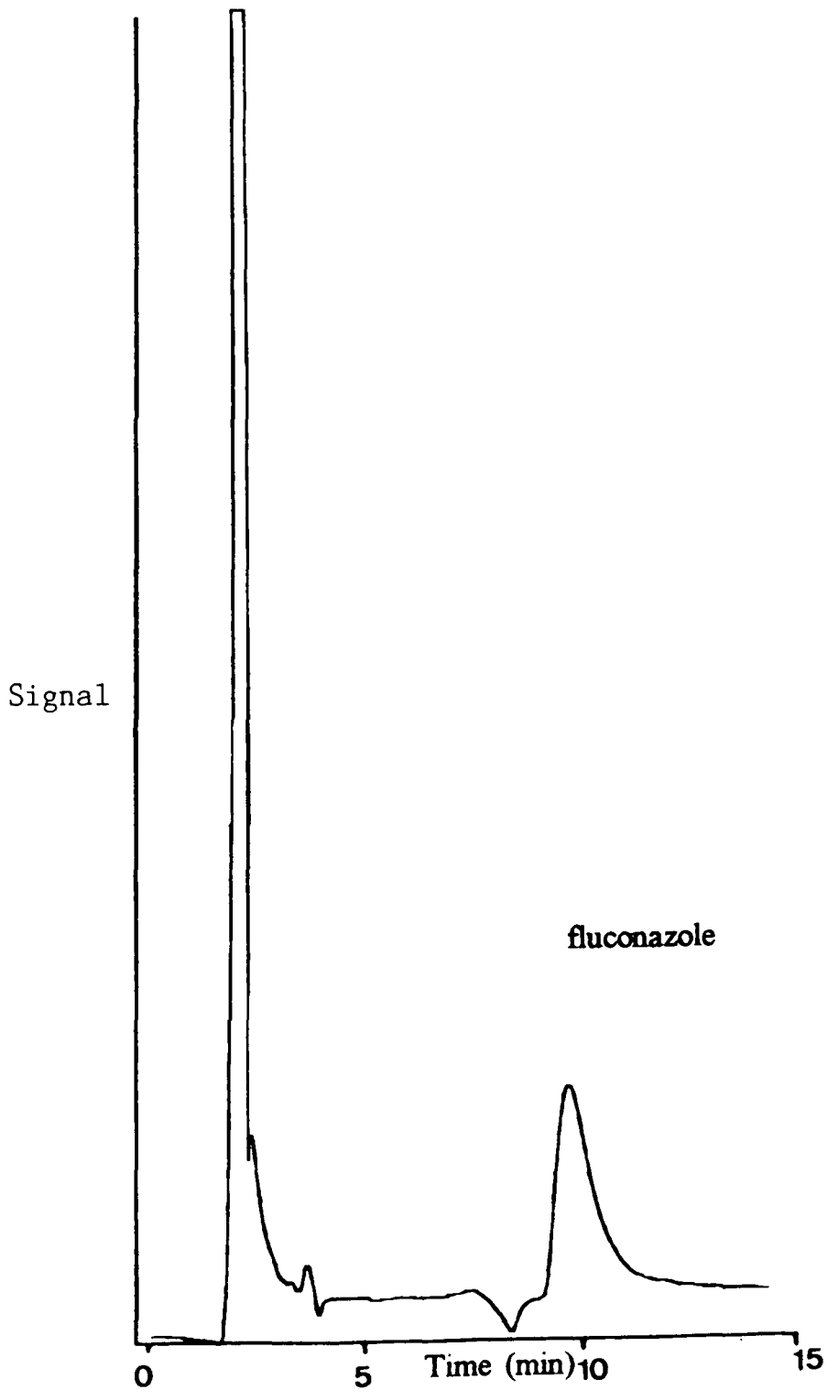


Figure 9.1 Example pSFC chromatogram obtained for an animal feed extract of fluconazole. For chromatographic conditions, see text.

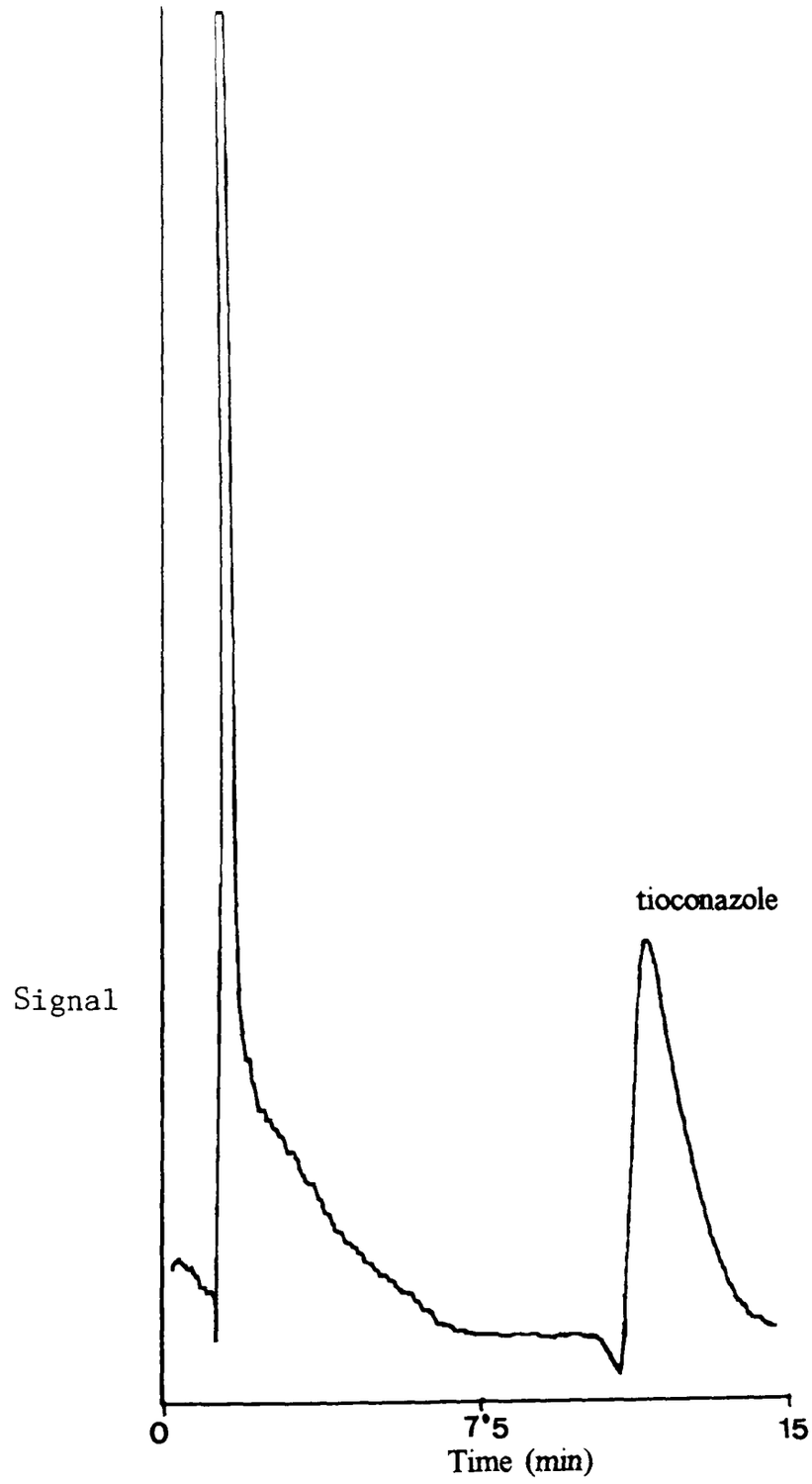


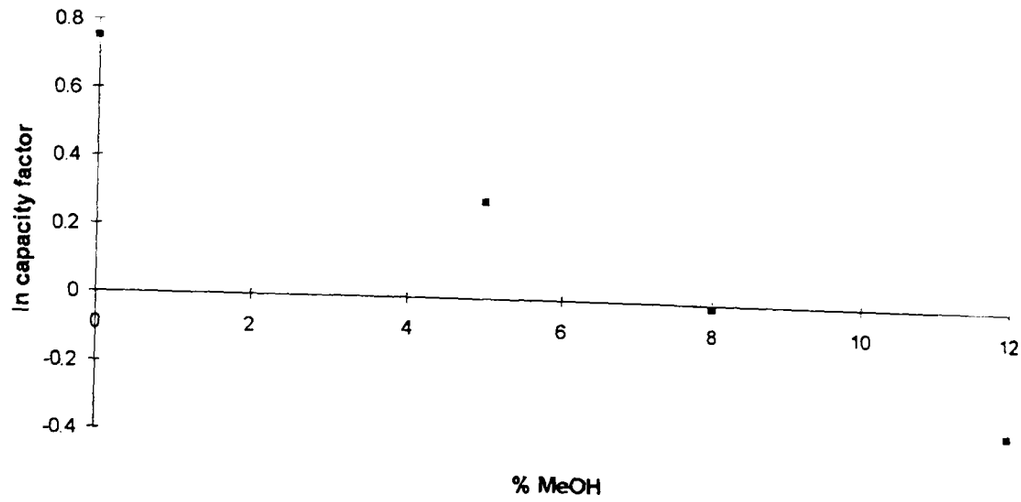
Figure 9.2 Example pSFC chromatogram obtained for an animal feed extract of tioconazole. For chromatographic conditions, see text.

Table 9.2 showing the effect of %MeOH on retention for hexaconazole, fluconazole and UK-47,265.

Compound	Retention time	Capacity factor (k)	%MeOH	Pressure (kg/cm^2)	Flow rate (ml/min)	Temp (°C)
Hexaconazole	1.11	0.59	12	250	3	65
,,	1.39	0.99	8	250	3	65
,,	1.75	1.33	5	250	3	65
,,	3.45	2.13	0	250	3	65
Fluconazole	1.45	1.01	12	292	2	65
,,	3.40	2.47	8	292	2	65
,,	4.98	3.48	5	292	2	65
,,	≈10.5	-	0	292	2	65
UK-47,265	1.85	1.68	12	250	3	65
,,	3.84	2.84	8	250	3	65
,,	5.21	3.70	5	250	3	65
,,	≈11	-	0	250	3	65

A typical effect of addition of a modifier on retention behaviour in SFC is demonstrated by plotting ln capacity factor against % MeOH for hexaconazole (figure 9.3). The graph (r = 0.999) illustrates the fact that addition of a modifier shortens the retention of an analyte in SFC.

Figure 9.3 Example of relationship between ln capacity factor (k) and % modifier for hexaconazole



9.6.4 Effect of Pressure on Retention

The effect of column outlet pressure on retention of all the compounds produced marginally small shifts on retention as compared with the modifier concentration. The operating pressure for the optimization of the compounds from the feed was varied between 250-322 kg/cm^2. Table 9.3 shows an example of the effect of pressure on retention for hexaconzole.

Table 9.3 An example of the effect of pressure on retention for hexaconzole.

Retention time	Capacity factor (k)	Pressure (kg/cm^2)	Temperature (°C)	%MeOH	Flow rate (ml/min)
1.49	1.13	322	65	8	2
1.51	1.16	300	65	8	2
1.54	1.20	272	65	8	2
1.59	1.27	250	65	8	2

9.6.5 Effect of Temperature on Retention

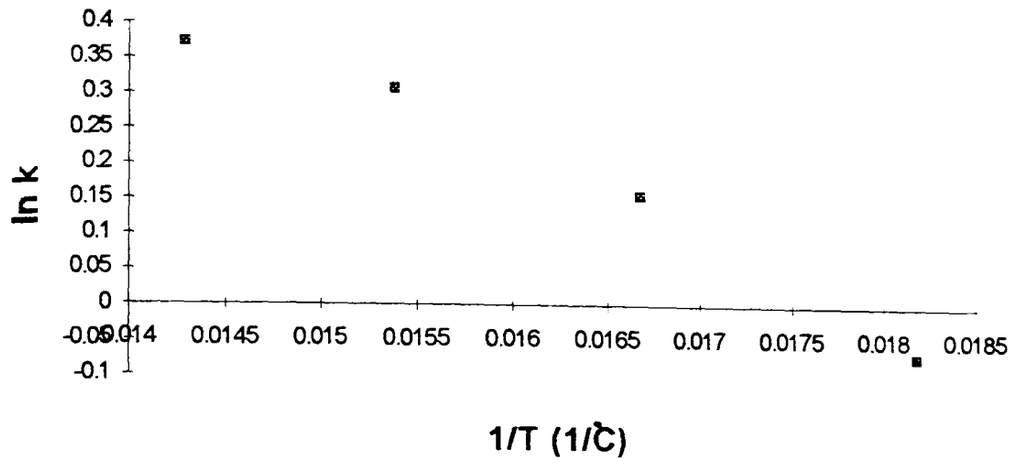
Temperature changes caused changes in retention for all of the compounds. The retention of all of the compounds increased when the temperature was increased. This effect is expected in SFC, as the retention and selectivity depend strongly depend on the density of the fluid and temperature changes are therefore an important factor in the optimization process. The range of temperature studied for the extraction of the compounds from the feed varied between 55°C and 70°C. Table 9.4 shows an example of the effect of temperature on retention for hexaconazole.

Table 9.4 An example of the effect of temperature on retention for hexaconazole.

Retention time	**Capacity factor (k)**	**Pressure (kg/cm^2)**	**Temperature (°C)**	**%MeOH**	**Flow rate (ml/min)**
1.31	0.93	250	55	6	2
1.50	1.17	250	60	6	2
1.65	1.36	250	65	6	2
1.84	1.84	250	70	6	2

A typical effect of temperature on retention is demonstrated by plotting ln capacity factor against 1/temperature for hexaconazole (figure 9.4). The graph illustrates the fact that in SFC , the moblie phase strength increases as the density goes high. The lower the temperature, the higher the density is obtained. Therefore, the relationship between ln k vs T^{-1} becomes a negative slope in isobaric elution and the lower the temperature, the shorter the retention becomes.

Figure 9.4 Relationship between ln capacity factor (ln k) vs 1/temperature



9.7 Analysis of SFE Animal Feed Extracts of Fluconazole, Tioconazole, Hexaconazole and UK-47,265

The optimum chromatographic conditions were first determined with the standards. The blank SFE extracts were then run at the optimum conditions to establish that the co-extracted material from the feed did not interfere with the pSFC analysis at the chosen chromatographic conditions. The chromatogram for the blank feed was free of infering peaks at the retention time of all four compounds, allowing selective quantitation of all four compounds.

The optimum chromatographic conditions established for the analysis of fluconazole at 10 g/kg of feed level from the animal feed were; temperature 55°C, carbon dioxide + 8% MeOH at 292 kg/cm^2 and a flow rate of 2 ml/min. Under these conditions, fluconazole was retained at 3.0 minutes.

The optimum chromatographic conditions established for the analysis of fluconazole at 500 mg/kg of feed level from the animal feed were; temperature 65°C, carbon dioxide + 8% MeOH at 292 kg/cm^2 and a flow rate of 2 ml/min. Under these

conditions, fluconazole was retained at 3.4 minutes. Figure 9.5 shows an example chromatogram is of a blank animal feed eaxtract and a fluconazole animal feed extract.

The optimum chromatographic conditions established for the analysis of tioconazole for both levels of feed level from the animal feed were; temperature 65°C, carbon dioxide + 20% MeOH containing triethylamine (0.25 mls of triethylamine per 50 ml of MeOH) at 250 kg/cm^2 and a flow rate of 3 ml/min. Under these conditions, tioconazole was retained at 1.6 minutes. Figure 9.6 shows an example chromatogram of a blank animal feed extract and a tioconazole animal feed extract

The optimum chromatographic conditions established for the analysis of hexaconazole and UK-47,265 for both levels of feed from the animal feed were; temperature 65°C, carbon dioxide + 12 % MeOH at 250 kg/cm^2 and a flow rate of 3 ml/min. Under these conditions, hexaconazole was retained at 1.1 minutes and the internal standard of fluconazole was retained at 1.85 minutes. Figure 9.7 shows an example chromatogram of a blank animal feed extract and a hexaconazole and UK-47,265 animal feed extract.

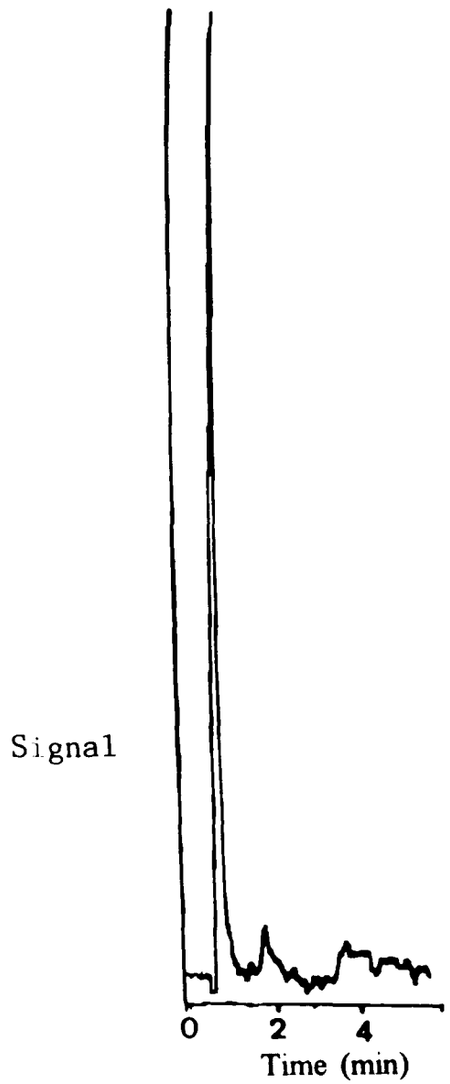


Figure 9.5 a A typical SFE-pSFC trace of a blank animal feed extract

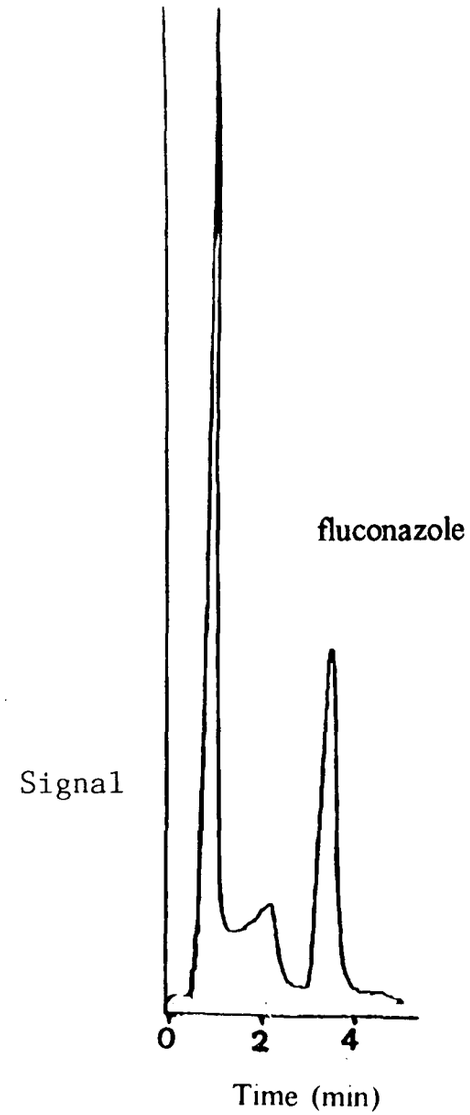


Figure 9.5 bA typical SFE-pSFC trace of a fluconazole animal feed extract at a level of 500 mg/Kg of feed.

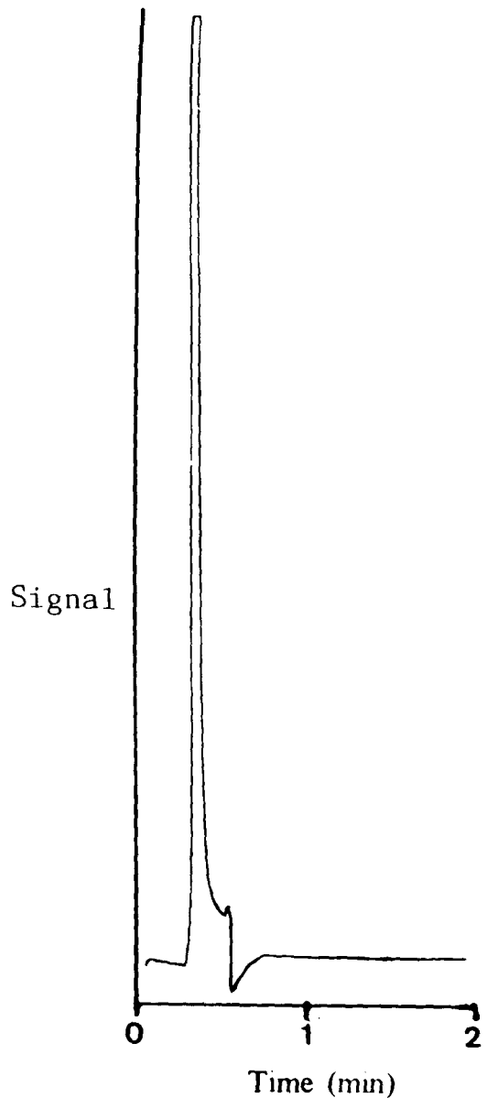


Figure 9.6 a A typical SFE-pSFC trace of a blank animal feed extract

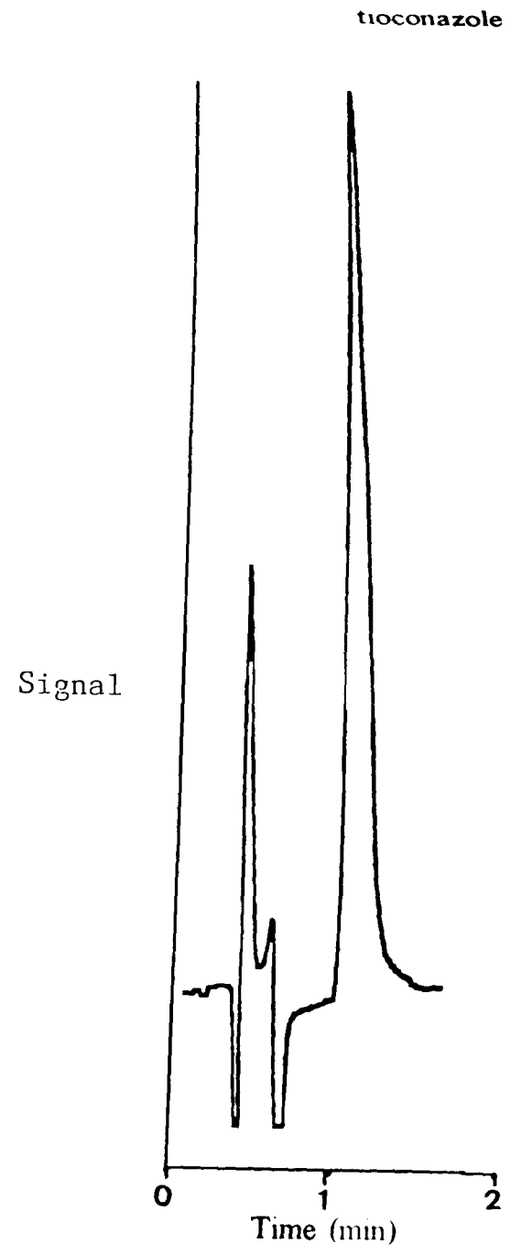


Figure 9.6 b A typical SFE-pSFC trace of a tioconazole animal feed extract at a level of 2 5g/Kg of feed.

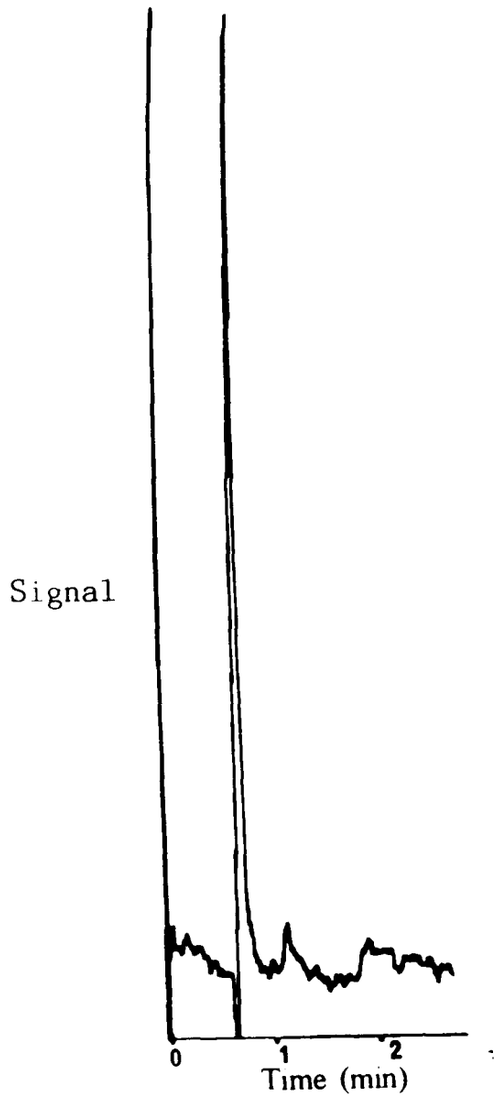


Figure 9.7 a A typical SFE-pSFC trace of a blank animal feed extract.

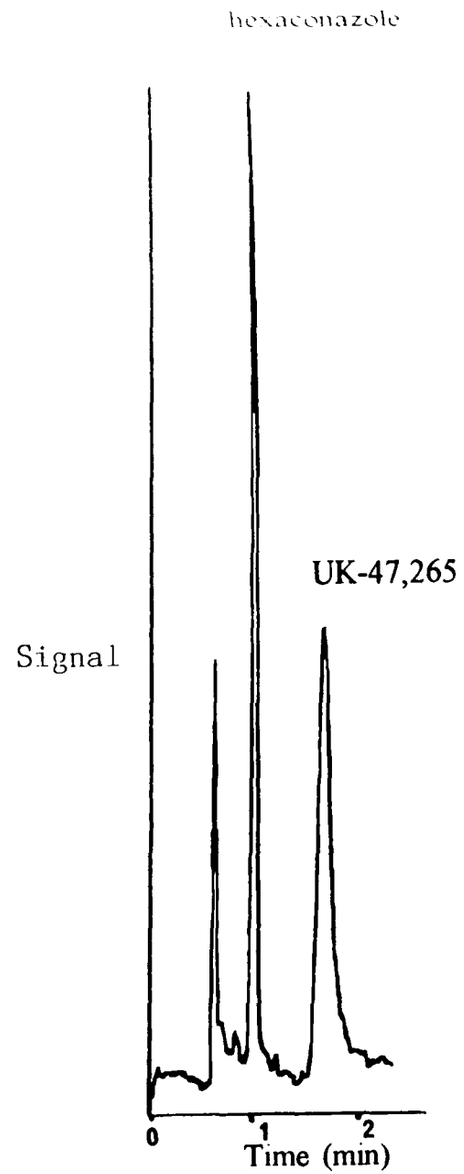


Figure 9.7 b A typical SFE-pSFC trace of a hexaconazole and UK-47,265 extract at a level of 1.25g/K of Hexaconazole and 1g/Kg of UK-47,265.

The results obtained for both levels of feed for all of the compounds are shown in tables 9.5-9.10.

Table 9.5 Percentage recovery data for the pSFC analysis of fluconazole (500 mg/kg of feed) from animal feed .

Extract (n = 3 for each extract)	**% Recovery Mean ± S.D**	**% R.S.D**
1	85.3 ± 0.8	9.4
2	85.6 ± 5.0	5.8
3	84.6 ± 1.3	1.5
4	88.5 ± 8.0	9.0
5	82.6 ± 4.5	5.4

The average recovery obtained for fluconazole at the 500 mg/kg of feed level was 85.32% with an RSD of 2.5%.

Table 9.6 Percentage recovery data for the pSFC analysis of fluconazole (10 g/kg of feed) from animal feed .

Extract (n = 3 for each extract)	**% Recovery Mean ± S.D**	**% R.S.D**
1	96.7 ± 4.1	4.2
2	99.0 ± 0.7	0.7
3	95.8 ± 4.8	5.0
4	96.6 ± 2.6	2.7
5	87.1 ± 3.5	4.0

The average recovery obtained for fluconazole at the 10 g/kg of feed level was 95.6% with an RSD of 5.3%.

Table 9.7 Percentage recovery data for the pSFC analysis of tioconazole (2.5 g/kg of feed) from animal feed .

Extract (n = 3 for each extract)	% Recovery Mean ± S.D	% R.S.D
1	96.6 ± 2.1	2.2
2	98.8 ± 2.1	2.1
3	95.4 ± 3.1	3.2
4	94.1 ± 6.0	6.4
5	88.3 ± 2.8	3.2

The average recovery obtained for tioconazole at the 2.5 g/kg of feed level was 94.6 % with an RSD of 4.2 %.

Table 9.8 Percentage recovery data for the pSFC analysis of tioconazole (5 g/kg of feed) from animal feed .

Extract (n = 3 for each extract)	% Recovery Mean ± S.D	% R.S.D
1	96.8 ± 6.1	6.3
2	96.5 ± 4.2	4.4
3	84.7 ± 3.7	4.4
4	76.7 ± 3.9	5.1
5	90.2 ± 5.5	6.1

The average recovery obtained for tioconazole at the 5 g/kg of feed level was 91.0 % with an RSD of 6.4 %.

Table 9.9 Percentage recovery data for the pSFC analysis of hexaconazole (1.25 g/kg of feed) and (1 g/kg of feed) from animal feed .

Extract (n = 3 for each extract)	% Recovery for Hexaconazole Mean ± S.D	% R.S.D	% Recovery for UK-47,265 Mean ± S.D	% R.S.D
1	90.3 ± 5.8	6.4	80.3 ± 4.3	5.4
2	92.4 ± 2.9	3.1	81.0 ± 5.5	6.8
3	92.1 ± 6.5	7.1	76.9 ± 6.3	8.2
4	86.4 ± 5.1	5.9	77.4 ± 4.8	6.2
5	92.0 ± 2.9	3.2	79.3 ± 5.3	6.7

The average recovery obtained for hexaconazole at 1.25 g/kg of feed and the UK-47,265 at 1 g/ kg of feed level were 90.6 % and 78.9 % with a RSD of 2.6 % and 2.4 % respectively.

Table 9.10 Percentage recovery data for the pSFC analysis of hexaconazole (1.875g/kg of feed) and theUk-47,265 (2.5 g/kg of feed) from animal feed .

Extract (n = 3 for each extract)	% Recovery for Hexaconazole Mean ± S.D	% R.S.D	% Recovery for UK-47,265 Mean ± S.D	% R.S.D
1	97.4 ± 1.5	1.5	93.2 ± 3.1	3.3
2	101.0 ± 3.5	3.5	91.1 ± 4.6	5.0
3	99.4 ± 5.4	5.4	93.2 ± 2.9	3.1
4	92.4 ± 5.0	5.4	91.8 ± 2.2	2.4
5	92.1 ±1.1	1.2	92.2 ± 5.8	6.3

The average recovery obtained for hexaconazole at 1.875 g/kg of feed and the UK-47,265 at 2.5 g/ kg of feed level were 96.5 % and 92.3 % with a RSD of 4.2 % and 1.0 % respectively.

9.8 Discussion

The SFC method developed for the determination of antifungals from animal feed provides an alternative separation selectivity to the existing reverse phase HPLC technique with a shorter analysis time. Good chromatograms were achieved with the cyano bonded column using MeOH-CO_2 based solvent modifiers. For elution of tioconazole, the addition of TEA in the mobile phase was necessary to obtain acceptable chromatography and it is suggested that the TEA acted as a competing base to mask free silanols on the surface of the stationary phase in order to reduce their interactions with tioconazole.

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difficult and not very accurate, even when there are some experimental values. The solubility model proposed for testosterone (Chapter 6), based on the capacity factor measurements can be used as a resonable solubility predictor for testosterone at different temperatures using the biphenyl column. Changing the stationary phase to a cyanopropyl column however changes the interaction of the analyte to the stationary phase significantly and the model is not effective.

The effect of temperature and pressure on the solubilities of steroids in supercritical carbon dioxide were demonstrated in chapter 7. Near the critical pressure of carbon dioxide, solubility increased dramatically as was expected because of a rapid increase in density with increasing pressure at constant temperature.

The objective of the study with antifungals was to examine the extension of supercritical fluid extraction to the extraction of polar drugs. The ultimate aim was to determine antifungals from animal feed using supercritical fluid extraction and supercritical fluid chromatography and thereby demonstrate the versatility of SFE/SFC.

Initial experiments with antifungals involved optimizing the conditions for the extraction of fluconazole, to determine the most significant variables influencing extraction. The information obtained allowed the development of subsequent extractions of fluconazole, tioconazole, hexaconazole and UK-47,265 with supercritical carbon dioxide. The technique is shown to have many parameters that require careful consideration and understanding if it is to be successfully used. Carbon dioxide was ineffective in extracting all four antifungals from the animal feed. The introduction of methanol as a co-solvent into supercritical carbon dioxide modified the polarity and solvating power of the supercritical fluid and extended the range of applications of SFE. A large percentage of the modifier was necessary for the extraction of these polar compounds. The matrix material, animal feed in this case,

associated with the antifungals had pronounced effects on the successful isolation of target analytes in the SFE experiments. An inert support material Celite, was used to study the solubility of fluconazole. The complexity of the extraction process was undoubtly increased by the introduction of the sample matrix. The results from the SFE experiments showed that increasing temperature, time, and % methanol all facilitate greater solubilization of all four antifungals, proving that the efficiency of SFE is directly related to the extraction conditions.

The analysis of fluconazole from SFE proved difficult with HPLC. Although the SFE procedure was found to be simpler and less time consuming compared to the liquid extraction/SPE, however the SFE extracts contained vast amounts of co-extractives which interfered with the HPLC analysis. This was one major disadvantage the SFE method had over the existed method of analysis, specially where the analysis of the drug was required at low levels. After extensive method development, the best RPHPLC separation achieved for fluconazole was at a level of 500 mg of drug/kg of feed. The mobile phase required for separation of fluconazole from animal feed was 15/85, water/methanol (v/v) and was indicative of the fact that NPHPLC is a better choice for the separation of fluconazole in presence of co-extractives and lead us to the use of pSFC as an alternative to NPHPLC.

The GC analysis of fluconazole presented no problems even in the presence of the co-extractives. Although possible derivitisation of fluconazole (a free OH- group in the fluconazole structure) was achievable, was not required for the GC analysis. GC-FID is a good choice of analysis at high levels of fluconazole concentration where the sensitivity of GC-MSD will enable to detect fluconazole concentration at lower levels.

A SFC method was developed for the determination of 4 antifungals in animal feed matrix. The procedure provided an alternative separation selectivity to the existing RPHPLC techniques with much shorter analysis time.

The modification of the mobile phase with polar methanol was necessary to elute the antifungals. The retention in SFC is based on the selective interactions of the analyte with the mobile phase and the stationary phases. The retention characteristics of the antifungals were influenced by several parameters, of which, beside the nature of the mobile and stationary phases and the type of antifungal, the density of the mobile phase, the flow rate and the temperature were the most important. The method development time required was much shorter than the HPLC analysis and the peak shapes obtained for all four compounds were superior than RPHPLC analysis. The necessity to include triethylamine in the mobile phase to obtain acceptable chromatographic results for tioconazole suggests that it is necessary to use a competing base to mask free silanols on the surface of the stationary phase in order to reduce their interactions with the analyte. The levels of the antifungals analysed by pSFC with UV detection were at levels 500 mg to 10 g of the antifungals/kg of feed. The physico-chemical properties of supercritical fluids that provide important chromatographic advantages are important for the transport and gas phase introduction of analyte molecules into the ion source of a mass spectrometer, which could provide a highly sensitive and selective detection pSFC for analysis of very low levels of antifungals.

10.1 Future Recommendations

Future calculations of C logP/solubility parameter should be carried with other classes of pharmaceuticals than steroids to investigate if the relationship could be used as a solubility predictor for other compounds.

In this thesis, the solubility data for a series of steroids is presented. Future work should involve extractions of some of the soluble steroids such as testosterone, by using SFE from their formulation matrices, i.e. from tablets, capsules or ointment formulations. The solubility of pure compounds in supercritical fluids can be

enhanced by the addition of a cosolvent such as methanol and the SFE method may provide a faster solution to sample preparation especially in the analysis of steroids whose formulation require lengthy liquid/liquid extraction.

Far more investigations are required is capacity factor measurements are to be used as an accurate solubility predictor for steroids, such as testosterone. Investigations should include finding an appropriate stationary phase where testosterone capacity factor can be measured accurately for both high densities and low densities. In order to extend the solubility model to more than one stationary phase, it will be necessary to run testosterone at different densities on different stationary phases to calculate the proportionality constant (i.e. interaction of the analyte with different stationary phases) which could then be used as the correction factor to correct between column.

In the pharmaceutical industry, the assay of drug substances from the feed matrices is required at very low levels. Further work on determination of drugs from animal feed should involve quantitation of the drugs such as fluconazole, at lower levels, typically down to a concentration range of 4 mg/kg of feed. Since detector sensitivity was a limiting factor for the analysis of fluconazole by GC, the inherent sensitivity of GC-MSD should be explored to see if fluconazole can be detected at the required low levels. The use of on column ECD should also be considered as a detection method for analysis of fluconazole as this detection method may prove to be sensitive enough to measure drug levels of fluconazole encountered in toxicological studies. Both clean up and pre-concentration step (SPE) will be required if HPLC is chosen for the method of analysis following SFE.

The use of pSFC was shown as the alternative approach to GC and HPLC in this thesis. This technique provides a promising new approach for determining fluconazole, and other antifungals, in animal feed. However, if pSFC is to compete with HPLC, further work is needed in the development of a rigorous analytical

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A comparison between solid phase extraction and supercritical fluid extraction for the determination of fluconazole from animal feed

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Abstract

The application of supercritical fluid extraction with carbon dioxide and modified carbon dioxide for the determination of fluconazole from an animal feed was studied. A fractional factorial design approach was used to examine the significant experimental variables for quantitative extraction of fluconazole. Gas chromatography with either flame ionisation or mass selective detection was used for quantitation of the extracts. The results indicated that modifier (methanol) had the greatest effect on the recovery of fluconazole from the animal feed.

Keywords: Supercritical fluid extraction; Fluconazole; Animal feed; Fractional factorial design; Gas chromatography

1. Introduction

In chronic toxicity studies, administration of drug substances to laboratory animals may involve incorporating the drug into the animals' feed [1]. The determination of the drug substance level in the feed matrices is necessary to monitor dosage levels, verify dose uniformity throughout the feed mix and confirm the drug's stability. Animal feeds are complex mixtures of proteins, lipids, glucicide, cellulose and mineral matter [2] and, if coextracted, the components can interfere in the determination of the analyte of interest. Various means of sample preparation have been investigated to extract drug substances from feed prior to assay. These methods include liquid–solid extraction [3], solid-phase extraction [4], Soxhlet extraction [5] and liquid–liquid extraction [6]. The use of supercritical fluid extraction to determine analytes from animal feed matrices has been investigated only briefly for several pharmaceutical compounds [7–9]. The present method for the isolation of drugs from feed involves time consuming extraction/cleanup steps which also introduce analytical uncertainty and a limited ability to automate such assays. Supercritical fluid extraction offers a simpler isolation procedure with the potential for in situ cleanup of analytes from complex matrices and the possibility of automation. This paper focuses on the use of supercritical fluid extraction for the extraction of fluconazole from animal feed matrix prior to chromatographic determination.

Fluconazole (UK-49588; 2-(2,4-difluoropheny-1,3-bis(1*H*-1,2,3-triazol-1-yl)-2-propanol; Fig. 1) is a triazole compound with potent

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also prepared. All extracts were analysed by HPLC with UV/vis detection using a seven-point calibration plot with a correlation coefficient, r, of 0.9979.

2.4. Supercritical fluid extraction methodology

The extraction efficiency of SFE for the recovery of fluconazole from rodent feed was determined at two levels, 10 g of drug kg^{-1} of feed and 500 mg kg^{-1} of feed. The spiked feed samples of known fluconazole content were prepared by adding aliquots of the fluconazole standard in dichloromethane to a 1 g weighed sample of animal feed in a glass jar. These jars were capped, shaken vigorously and the contents left to dry overnight in an open jar. The spiked sample was quantitatively transferred to a cell and the extraction was carried out, using a fractional factorial design, to investigate SFE variable dependence. The flow-rate of extraction was maintained constant at 2 ml min^{-1} throughout the experiment. The collection vial contained a few drops of methanol and two cyanopropyl Bond Elute cartridges were placed in series at the exit to prevent any loss of fluconazole. Following extractions, all extracts were made up to volume in a 25 ml volumetric flask. The SFE extracts obtained at the 500 mg of drug kg^{-1} of feed level were analysed by GC/MS, while the extracts obtained at the 10 g of drug kg^{-1} of feed were analysed by GC with flame ionisation detection. All experiments were done in duplicate.

3. Results and discussion

Previously reported work [4] on the use of SPE followed by HPLC with UV detection for the isolation and determination of fluconazole from animal feed was done at the 40 mg kg^{-1} level. The methodology was repeated in this study to allow a direct comparison with SFE. The isolation and cleanup offered by SFE proved to be insufficient to eliminate extraneous material which coextracted with methanol-modified supercritical CO_2. The nature of the coextracted material prevented quantitative analysis by HPLC with UV detection. It had been noted previously that fluconazole could be analysed using GC [14–16]. Therefore, quantitative isolation by SFE followed by GC with either FID or MSD has been evaluated. However, in order not to be sensitivity limited (particularly in the case of GC with FID detection) the spiking level of fluconazole was done at the 10 g kg^{-1} (high) level. An intermediate spiking level of 500 mg kg^{-1} was also prepared and extracted using methanol-modified supercritical CO_2. These extracts were analysed by GC with MSD detection. It is apparent, that the inherent sensitivity of GC-MSD will allow lower spiking levels to be analysed.

3.1. SPE-HPLC

The extracts obtained from the SPE were visibly clean and the results obtained gave a mean fluconazole recovery of 99.1% (98.1%, 100.7%, 97.9%, 100.2%, 98.7%). The method described provides a convenient way of determining fluconazole quantitatively. The final solution obtained using this method was both sufficiently concentrated and also free from coextractives to make it compatible with HPLC analysis.

3.2. SFE of fluconazole from animal feed

In order to maximise the information available from the minimum number of experimental results, the use of an experimental design approach was pursued. A fractional design was selected [17]. This involved the use of a simple model of the form

$$Y = b_0 + b_1 v_1 + b_2 v_2 + b_3 v_3 + b_4 v_4 \quad (1)$$

where Y is the response (percentage extracted); v_1, v_2, v_3 and v_4 are the four main variables selected, i.e. pressure, temperature, time of extraction and percentage methanol, respectively; b_1, b_2, b_3 and b_4 are the parametric coefficients; b_0 is the intercept.

Owing to the presence of coextractives from the animal feed matrix, when methanol modifier was present, the use of HPLC with UV detection for the analysis of extracts was impractical. However, it had been reported that fluconazole could be analysed by GC [14–16]. In order to facilitate analysis and detection at high and intermediate spiking levels, it was necessary to use GC separation with either flame ionization or mass selective detection. The calibration plot for GC-FID showed acceptable linearity over the chosen range (50–400 μg ml^{-1}) with a correlation coefficient (r) of 0.974. The peak height of either the sample or standard was used for quantitation. Baseline resolution of fluconazole, from the endogenous

Table 1
Percentage recovery data for the GC-FID analysis of fluconazole (10 g kg^{-1} of feed) from an animal feed using a fractional factorial design. All experiments were done in duplicate

Pressure (kg cm^{-2})	Temperature (°C)	Time (min)	% MeOH	% Recovery
250	80	40	20	87.0
250	80	5	2	76.6
250	40	5	20	79.0
110	80	5	20	90.9
110	40	40	20	80.5
110	40	5	2	16.9
110	80	40	2	22.3
250	40	40	2	27.3
170	65	20	10	97.4

Table 2
Percentage recovery data for the GC-MSD analysis of fluconazole (500 mg kg^{-1} of feed) from an animal feed using a fractional factorial design. All experiments were done in duplicate

Pressure (kg cm^{-2})	Temperature (°C)	Time (min)	% MeOH	% Recovery
250	80	40	20	99.6
250	80	5	0	5.3
250	40	5	20	82.7
110	80	5	20	99.7
110	40	40	20	72.5
110	40	5	0	5.1
110	80	40	0	16.8
250	40	40	0	5.8
170	65	20	10	100.3

material co-extracted from the feed, was achieved under the conditions described at a retention time of 19 min.

Spiked fluconazole animal feed was prepared according to the above procedure and a fractional factorial design carried out at two different concentration levels. The extraction time was extended, within the design, from 20 to 40 min in order to compensate for any potential matrix effects. The results obtained for both levels of feed are shown in Tables 1 and 2. It should be noted that the percentage of methanol was considered at three levels, 0, 10 and 20%, in the case of the 500 mg kg^{-1} level, whereas at the 10 g kg^{-1} level it was considered at 2, 10 and 20%. Multilinear regression was used to calculate the coefficients in Eq. (1) [18]. The coefficients are reported in Tables 3 and 4. Significance is determined by a t-test with a probability (p) of 0.05 at the 95% confidence level with four degrees of freedom. In Tables 3 and 4, a coefficient with a p-level of less than 0.05 or a t-test of greater than 2.78 will be considered significant. In both cases, the results obtained indicate that the percentage of methanol was the only significant variable for the extraction of fluconazole from the animal feed matrix. The difference in the b coefficients (−0.3647 and −0.0419 for the 10 g kg^{-1} and 500 mg kg^{-1} spiking levels, respectively) reflects the influence that the percentage of methanol–time of extraction interaction has on extraction recovery. The consequence of the addition of a small amount of methanol (2%) as opposed to CO_2 only had a pronounced effect on extraction recovery (Tables 1 and 2). The model for the 10 g kg^{-1} extraction results is

$$\begin{aligned}\text{Percentage recovery of fluconazole} &= -4.751 + 0.097(\text{pressure}) \\ &\quad + 0.509(\text{temperature}) \\ &\quad - 0.365(\text{time of extraction}) \\ &\quad + 2.647(\text{percentage methanol})\end{aligned}$$

$r = 0.8535$ ($r^2 = 0.7286$) at the 95% confidence interval.

However, the model for the 500 mg kg^{-1} results is as follows:

Table 3
Coefficients of regression and standard error of linear Eq. (1) for the GC-FID analysis of fluconazole from animal feed ($10\ g\ kg^{-1}$ level)

Variable	Descriptor	Beta	Standard error	b	Standard error	$t(4)$	p-level
Pressure	v_1	0.212	0.261	0.097	0.120	0.812	0.462
Temperature	v_2	0.317	0.261	0.509	0.419	1.215	0.291
Time	v_3	−0.198	0.261	−0.365	0.480	−0.760	0.489
% MeOH	v_4	0.739	0.261	2.647	0.933	2.837	0.047

Table 4
Coefficients of regression and standard error of linear Eq. (1) for the GC-MSD analysis of fluconazole from animal feed ($500\ mg\ kg^{-1}$ level)

Variable	Descriptor	Beta	Standard error	b	Standard error	$t(4)$	p-level
Pressure	v_1	0.020	0.246	0.011	0.136	0.089	0.940
Temperature	v_2	0.182	0.215	0.405	0.479	0.847	0.445
Time	v_3	−0.016	0.214	−0.042	0.546	−0.077	0.943
% MeOH	v_4	0.907	0.214	4.062	0.958	4.239	0.013

Percentage recovery of fluconazole
$= -11.813 + 0.010(\text{pressure})$
$+ 0.405(\text{temperature})$
$- 0.042(\text{time of extraction})$
$+ 4.062(\text{percentage methanol})$

$r = 0.9199$ ($r^2 = 0.8462$) at the 95% confidence interval.

A typical response surface was generated for fluconazole (Fig. 3) showing the effect of temperature and percentage methanol, as modifier, on extraction recovery. The percentage extraction efficiency repeatability for the isolation and cleanup of fluconazole from animal feed followed by GC with either FID or MSD detection was determined to be 87.0% (RSD 8.4%) at the $10\ g\ kg^{-1}$ level and 91.0% (RSD = 13.2%) at the $500\ mg\ kg^{-1}$ level. In each case the number of replicates was ten.

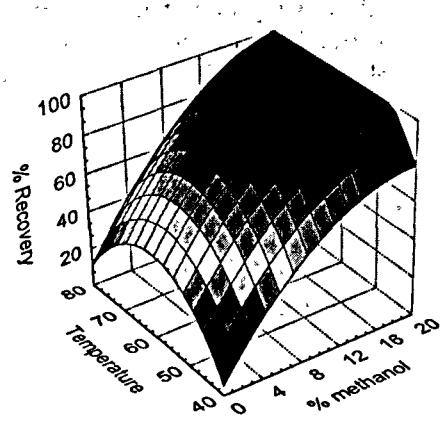

Fig. 3. Response surface for fluconazole showing the effect of temperature and percentage modifier on extraction efficiency.

The recovery of fluconazole from animal feed was determined, using a fractional factorial design approach, to be dependent upon the addition of modifier in the extraction fluid. The modifier's function is thought to be two-fold. Firstly, the modifier increases the polarity of the extraction fluid and thus competes with the drug for the active site on the matrix. Secondly, the modifier acts to decrease the adsorption of solutes onto the surface of the matrix by increasing the swelling of the matrix, which in turn increases the likelihood of solute diffusion out of the matrix.

4. Conclusions

A comparison of SPE with SFE for the isolation and cleanup of fluconazole from animal feed has been made. The inter-dependence of the extraction procedure on the method of analysis and determination was effectively demonstrated. The unselective nature of SFE, when methanol-modified supercritical CO_2 was used, prevented analysis by HPLC with UV detection owing to coextractives. Quantitative recovery of fluconazole was possible using GC with either FID or MSD detection at the $10\,000\ mg\ kg^{-1}$ and $500\ mg\ kg^{-1}$ spike levels, respectively. However, the inherent sensitivity of GC-MSD should allow lower spike levels to be quantified. SPE-HPLC was shown to be an effective method of isolating and analysing

fluconazole from animal feed at a low spike level (40 mg kg^{-1}). In addition, the time taken by the SPE-HPLC method was shorter than that for the SFE-GC method (SPE-HPLC, SPE, ≈20 min; HPLC, 10 min compared to SFE-GC; SFE, 40 min; GC, 25 min).

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Estimation and Determination of Steroid Solubility in Supercritical Carbon Dioxide

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The solubility of ten steroids in supercritical carbon dioxide is reported over a range of temperatures (35–100 °C) and pressures (84–231 kg cm^{-2}). The solubility of testosterone, determined in this work, compares favourably with solubility data previously reported. The use of the solubility parameter and a hydrophobicity term (log *P*), has been shown to provide a qualitative estimate of steroid solubility.

Keywords: Steroid solubility; supercritical carbon dioxide; solubility prediction; solubility parameter; hydrophobicity, log P

Introduction

The development of suitable methods for the extraction of analytes from matrices using supercritical fluids is greatly assisted if some indication of the analyte solubility is available. Various methods for the prediction of analyte solubility have been suggested and include the use of equations of state,[1] chromatographic retention data[2] and solubility parameters.[3] This paper compares the results of a predictive method, based on solubility parameters and the hydrophobicity term (log *P*),[4] with experimentally determined solubility data for ten steroids.

Hydrophobicity in Terms of Octanol–Water Partitioning Behaviour

A commonly applied physico-chemical characteristic employed to determine drug activity in quantitative structure activity relationships (QSAR) is the hydrophobic nature of a drug compound. Hydrophobicity is normally measured using the logarithm of the partition coefficient in octanol–water systems (log *P* or k_{ow}). This group[4] has previously shown, for a group of benzophenone compounds, that the hydrophobic nature of a molecule may be related to its solubility in the supercritical carbon dioxide phase.

Several experimental methods are available to determine log *P* and include the shake-flask method,[5–7] slow stirring,[8] continuous partitioning method[9,10] and isocratic[11–13] and gradient[14,15] HPLC. However, all are time consuming and are prone to excessive experimental error. Alternative approaches are available based on mathematical treatment of the molecular structure. These molecular approaches can be divided into two classes; those which use molecular fragments[12,16] and those based on contributions from individual atoms.[17,18] The former are to be preferred. The use of molecular fragments has been approached from two different perspectives. The first, described as a constructionist approach, was developed by Hansch and Leo[16] whereas the latter method, proposed by Rekker,[12] uses a reductionist approach. In each example, molecular fragments are used to derive a log *P* value. Molecular fragment contributions are available in tabular form for each approach. However, a computer-based approach was adopted in this work [*C* log *P*, Medchem (1989), Daylight Chemical Systems, Pomona College Medical Chemistry Project, Pomona College, Claremont, CA, USA].

Determination of the Solubility Parameter

The prediction of benzophenone solubility[4] relied on the correlation between log *P* and the solubility parameter. There are several methods available for calculating the solubility parameter. These have been evaluated by King[19] who concluded that a 10% variation between different methods is commonplace. Perhaps the simplest method of calculating the solubility parameter is by the group contribution method proposed by Fedors.[20] The main advantage of this method is that it allows the estimation of the solubility parameter from only a knowledge of the molecular structure of the solute. Structurally complex solutes can thus be calculated without prior knowledge of their thermodynamic properties. The Fedors method has been related to solubility in supercritical carbon dioxide by King and Friedrich[3] *via* the reduced solubility parameter proposed by Giddings *et al.*[21] King concluded that a large body of SFE data could be correlated in a quantitative manner to changes in the molecular structure.

The solubility parameter, δ, was described by Hildebrand in terms of thermodynamic properties on the mixing of dilute solutions.[20] For low molecular mass compounds this was found to be related to the energy of vaporization of a substance, ΔE_V, at a given temperature, and the molar volume at that temperature, *V*. The solubility parameter (δ_2) is related to these two physical properties by eqn. (1):

$$\delta_2 = (\Delta E_V/V)^{0.5} \quad (1)$$

By examining the vast amount of molar volume and energy of vaporization data available for liquids, Fedors found that a general system for estimating both ΔE_V and *V* could be tabulated. The approach assumes that the group contributions of individual functional groups on a molecule can be summed to give an over-all estimate of the solubility parameter.

In this paper, experimentally obtained solubility data, determined at different temperatures and over a range of pressures are reported for ten steroids. The experimental results are compared with the Fedors solubility parameter and

the hydrophobic term, log *P*, as a qualitative estimation of steroid solubility in supercritical carbon dioxide.

Experimental

The Jasco SFE–SFC system used in these experiments incorporates a Rheodyne switching valve and the Jasco back-pressure regulator (BPR). A sample of 10 mg of pure steroid mixed with Celite, an inert support, was placed in each sample cell. The sample cells were made of stainless steel (10 cm × 5.0 mm id) with a volume of approximately 2.0 ml. The steroids investigated were: testosterone; testosterone-17-propionate (given as testosterone propionate in Fig. 1); beclomethasone-17,21-dipropionate; betamethasone-17,21-dipropionate; cortisone acetate; betamethasone; hydrocortisone; prednisolone; prednisone; and megestrol acetate (Fig. 1). Steroids were provided in pure form by Glaxo Manufacturing Services, (Barnard Castle, Co. Durham, UK). All solubility experiments were performed in duplicate.

Extraction Conditions

The SFE collection system used required the supercritical solvent to be pumped through the sample cell in dynamic extraction mode. However, all steroid extractions utilized a 2 min static extraction period prior to dynamic extraction for 10 min at a pump flow rate of 1 ml min^{-1}. The static extraction allowed the supercritical fluid to penetrate the sample increasing the natural partitioning of the analytes into the solvent. The 10 min extraction time provided enough mass

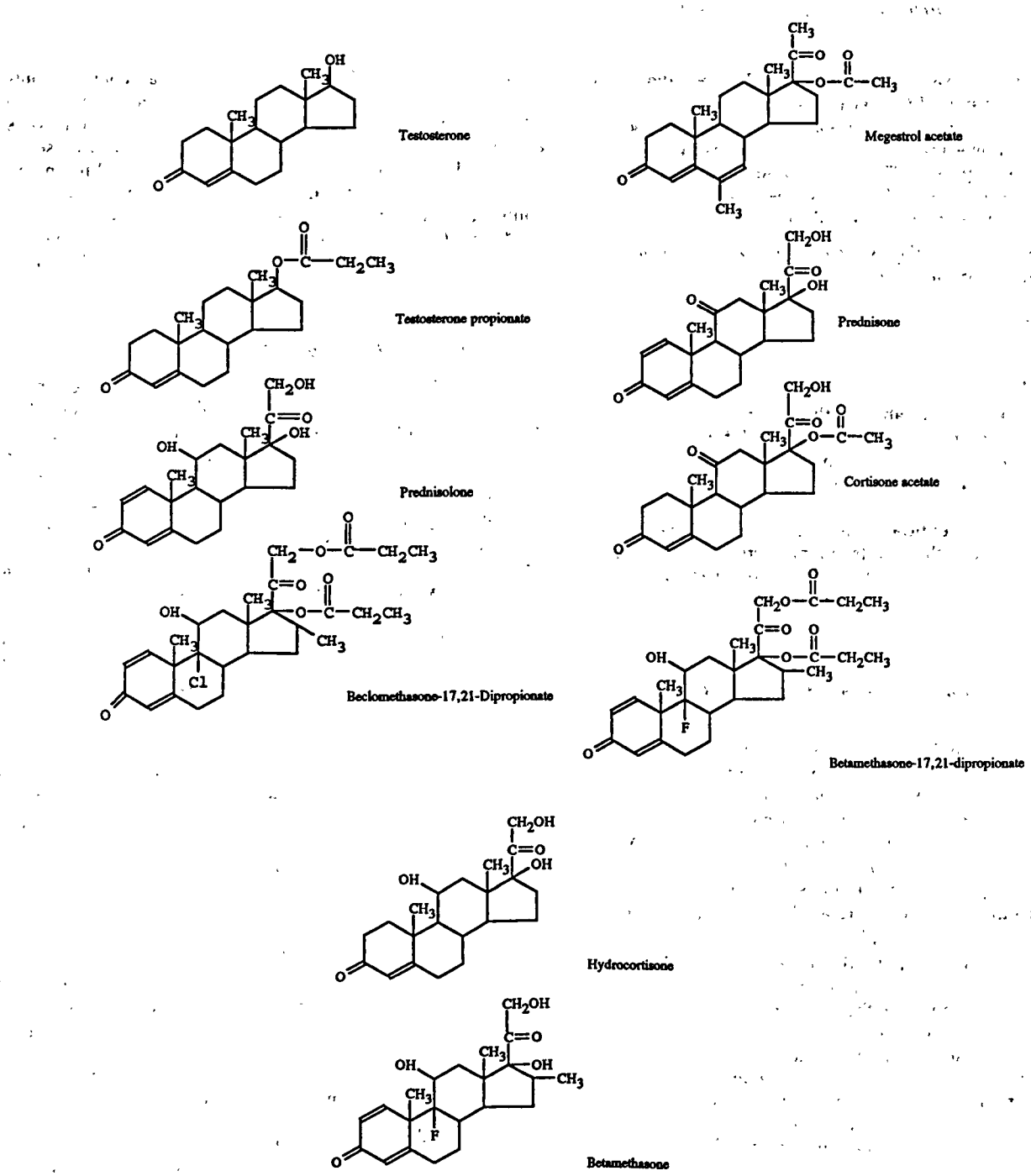


Fig. 1 Structures of the investigated steroids.

transfer to enable the analytes to be collected in a small amount of solvent at the outlet of the BPR, the internal volume of the extraction cell was swept slightly more than six full times with supercritical fluid. The mole fraction solubility is dependent on the volume of carbon dioxide used in the extraction and is calculated as follows:

$$\text{mole fraction solubility} = \frac{\text{moles of analyte extracted}}{\text{moles of analyte extracted} + \text{moles of } CO_2 \text{ used}}$$

For a 10 min extraction at 1 ml min^{-1} a 10 ml volume of carbon dioxide is used. Therefore the number of moles of carbon dioxide used is given by

$$\text{moles of } CO_2 = \frac{\text{volume of } CO_2 \text{ (ml)} \times \text{density of liquid } CO_2 \text{ (g ml}^{-1})}{\text{relative molecular mass of } CO_2 \text{ (g mol}^{-1})}$$

where the density of liquid CO_2 is 0.93 g ml^{-1}. Analysis of the extracts was peformed on a UV/VIS spectrophotometer using 10 mm silica cells for all measurements. Quantification was achieved by diluting all extracted steroids to 10 ml and measuring the absorbance against a four-point linear calibration graph.

Table 1 Comparison of literature and measured solubilities for testosterone at 35 and 55 °C

Pressure/ kg cm^{-2}	Literature values at 35 °C/mole fraction $\times 10^{-7}$	Measured values at 35 °C/mole fraction $\times 10^{-7}$	Literature values at 55 °C/mole fraction $\times 10^{-7}$	Measured values at 55 °C/mole fraction $\times 10^{-7}$
84	22.8	65.7	3.90	9.53
101	73.1	82.2	9.50	28.6
116	119	131	30.8	82.5
132	201	246	84.4	104
149	258	296	137	197
164	316	312	247	230
181	343	329	423	279
200	398	378	483	477
215	450	411	619	690
231	503	526	701	739

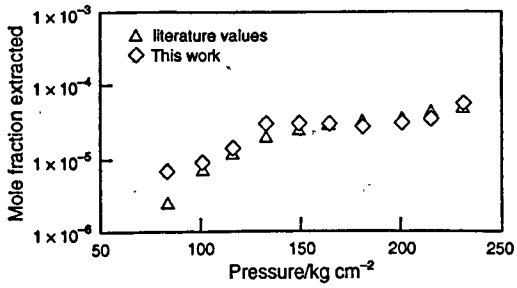


Fig. 2 Solubility isotherm for testosterone at 35 °C.

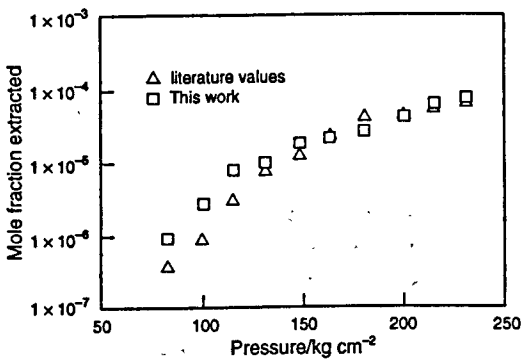


Fig. 3 Solubility isotherm for testosterone at 55 °C.

Table 2 Measured steroid solubility data

Pressure/ kg cm^{-1}	Measured values at 35 °C/mole fraction	Measured values at 55 °C/mole fraction	Measured values at 100 °C/mole fraction
Testosterone—			
84	6.57×10^{-6}	9.53×10^{-7}	4.60×10^{-7}
101	8.22×10^{-6}	2.86×10^{-6}	8.20×10^{-7}
116	1.31×10^{-5}	8.25×10^{-6}	1.56×10^{-6}
132	2.46×10^{-5}	1.04×10^{-5}	1.97×10^{-6}
149	2.96×10^{-5}	1.97×10^{-5}	1.64×10^{-5}
164	3.12×10^{-5}	2.30×10^{-5}	2.46×10^{-5}
181	3.29×10^{-5}	2.79×10^{-5}	3.94×10^{-5}
200	3.78×10^{-5}	4.77×10^{-5}	6.39×10^{-5}
215	4.11×10^{-5}	6.90×10^{-5}	8.53×10^{-5}
231	5.26×10^{-5}	7.39×10^{-5}	9.05×10^{-5}
Testosterone-17-propionate—			
84	ND*	1.31×10^{-6}	5.64×10^{-7}
101	ND	7.01×10^{-6}	9.76×10^{-7}
116	ND	1.31×10^{-5}	1.31×10^{-6}
132	ND	1.65×10^{-5}	5.64×10^{-6}
149	ND	2.62×10^{-5}	1.22×10^{-5}
164	ND	4.13×10^{-5}	1.65×10^{-5}
181	ND	6.35×10^{-5}	5.36×10^{-5}
200	ND	7.58×10^{-5}	7.39×10^{-5}
215	ND	8.25×10^{-5}	8.53×10^{-5}
231	ND	9.10×10^{-5}	9.95×10^{-5}
Beclomethasone-17,21-dipropionate—			
84	1.46×10^{-6}	1.87×10^{-7}	ND
101	4.00×10^{-6}	5.03×10^{-7}	1.20×10^{-7}
116	2.27×10^{-5}	2.30×10^{-6}	5.51×10^{-7}
132	2.53×10^{-5}	4.68×10^{-6}	8.01×10^{-5}
149	2.82×10^{-5}	8.88×10^{-6}	3.21×10^{-5}
164	4.32×10^{-5}	1.09×10^{-5}	6.01×10^{-6}
181	ND	2.62×10^{-5}	8.95×10^{-6}
200	8.00×10^{-5}	3.00×10^{-5}	1.01×10^{-5}
215	8.64×10^{-5}	3.39×10^{-5}	1.25×10^{-5}
231	8.64×10^{-5}	4.90×10^{-5}	3.09×10^{-5}
Betamethasone-17,21-dipropionate—			
84	5.64×10^{-7}	ND	ND
101	2.72×10^{-6}	ND	ND
116	3.48×10^{-6}	2.12×10^{-7}	ND
132	6.39×10^{-6}	1.70×10^{-6}	8.51×10^{-7}
149	2.19×10^{-5}	4.62×10^{-6}	3.11×10^{-6}
164	2.39×10^{-5}	1.38×10^{-5}	8.99×10^{-6}
181	4.66×10^{-5}	1.88×10^{-5}	1.19×10^{-5}
200	5.30×10^{-5}	2.62×10^{-5}	2.12×10^{-5}
215	6.06×10^{-5}	5.84×10^{-5}	5.55×10^{-5}
231	6.72×10^{-5}	7.03×10^{-5}	6.19×10^{-5}
Megestrol acetate—			
84	ND	6.40×10^{-7}	1.09×10^{-6}
101	ND	1.09×10^{-6}	2.84×10^{-6}
116	ND	2.32×10^{-6}	8.06×10^{-6}
132	ND	7.16×10^{-6}	1.37×10^{-5}
149	ND	1.36×10^{-5}	2.33×10^{-5}
164	ND	2.34×10^{-5}	2.95×10^{-5}
181	ND	3.82×10^{-5}	4.36×10^{-5}
200	ND	4.93×10^{-5}	5.07×10^{-5}
215	ND	5.17×10^{-5}	5.54×10^{-5}
231	ND	5.31×10^{-5}	6.61×10^{-5}
Cortisone acetate—			
84	4.83×10^{-7}	1.49×10^{-7}	1.11×10^{-7}
101	9.87×10^{-7}	2.76×10^{-7}	2.25×10^{-7}
116	1.93×10^{-6}	4.49×10^{-7}	3.51×10^{-7}
132	2.18×10^{-6}	5.40×10^{-7}	4.90×10^{-7}

continued —

Table 2—*continued*—

149	2.65×10^{-6}	1.23×10^{-6}	1.11×10^{-6}
164	3.10×10^{-6}	1.42×10^{-6}	1.25×10^{-6}
181	3.09×10^{-6}	1.52×10^{-6}	1.32×10^{-6}
200	3.24×10^{-6}	1.61×10^{-6}	1.45×10^{-6}
215	3.78×10^{-6}	2.08×10^{-6}	1.91×10^{-6}
231	4.14×10^{-6}	2.55×10^{-6}	2.31×10^{-6}
Betamethasone—			
84	1.33×10^{-8}	1.21×10^{-9}	ND
101	3.71×10^{-8}	3.62×10^{-9}	ND
116	7.73×10^{-8}	2.54×10^{-8}	ND
132	1.21×10^{-7}	3.63×10^{-8}	ND
149	1.49×10^{-7}	4.71×10^{-8}	ND
164	2.28×10^{-7}	7.73×10^{-8}	ND
181	2.67×10^{-7}	8.33×10^{-8}	ND
200	3.31×10^{-7}	1.12×10^{-7}	ND
215	3.45×10^{-7}	1.21×10^{-7}	ND
231	3.63×10^{-7}	1.28×10^{-7}	ND
Hydrocortisone—			
84	ND	ND	ND
101	4.58×10^{-8}	4.25×10^{-9}	2.51×10^{-9}
116	9.81×10^{-8}	9.79×10^{-8}	6.31×10^{-8}
132	1.24×10^{-7}	1.63×10^{-7}	1.39×10^{-7}
149	1.48×10^{-7}	2.32×10^{-7}	1.64×10^{-7}
164	2.54×10^{-7}	3.93×10^{-7}	4.57×10^{-7}
181	5.16×10^{-7}	5.68×10^{-7}	5.02×10^{-7}
200	5.75×10^{-7}	6.93×10^{-7}	6.44×10^{-7}
215	8.73×10^{-7}	8.65×10^{-7}	ND
231	9.01×10^{-7}	8.78×10^{-7}	7.34×10^{-7}
Prednisolone—			
84	3.94×10^{-9}	1.22×10^{-9}	ND
101	2.76×10^{-8}	3.75×10^{-9}	ND
116	6.67×10^{-8}	1.15×10^{-8}	9.05×10^{-9}
132	9.34×10^{-8}	2.68×10^{-8}	1.13×10^{-8}
149	1.17×10^{-7}	3.92×10^{-8}	1.46×10^{-8}
164	1.70×10^{-7}	4.66×10^{-8}	3.29×10^{-8}
181	2.55×10^{-7}	6.56×10^{-8}	5.40×10^{-8}
200	3.50×10^{-7}	9.79×10^{-8}	7.01×10^{-8}
215	4.13×10^{-7}	1.22×10^{-7}	8.58×10^{-8}
231	7.28×10^{-7}	1.31×10^{-7}	9.86×10^{-8}
Prednisone—			
84	6.57×10^{-9}	1.22×10^{-9}	ND
101	2.63×10^{-8}	1.35×10^{-9}	ND
116	5.13×10^{-8}	6.28×10^{-9}	5.01×10^{-9}
132	7.89×10^{-8}	1.60×10^{-8}	1.40×10^{-8}
149	1.18×10^{-7}	3.87×10^{-8}	3.41×10^{-8}
164	1.54×10^{-7}	8.00×10^{-8}	7.88×10^{-8}
181	2.88×10^{-7}	1.26×10^{-7}	1.12×10^{-7}
200	4.85×10^{-7}	2.66×10^{-7}	1.91×10^{-7}
215	6.59×10^{-7}	4.27×10^{-7}	3.13×10^{-7}
231	8.93×10^{-7}	6.37×10^{-7}	5.88×10^{-7}

* ND = Not determined.

Table 3 Example calculation of solubility parameter by the Fedors method for testosterone $\delta_2 = (\Delta E_V/V)^{0.5} = 11.16$

Contribution	ΔE_V per fragment	V per fragment	Total ΔE_V for Testosterone	Total V for Testosterone
CH_3^-	1125	33.5	2250	67
CH_2^-	1180	16.1	9440	128.8
CH^-	820	−1.0	3280	−4
HC=	1030	13.5	1030	13.5
C–	350	−19.2	700	−38.4
C=	1030	−5.5	1030	−5.5
C=O	4150	10.8	4150	10.8
OH	7120	10	7120	10
Ring closure 5–6 atoms	250	16	1000	64
Conjugated double bonds	400	−2.2	400	−2.2
Total			30 400	244

Results and Discussion

Steroid Solubility Studies

Initial work focused on a direct comparison between solubility data reported for testosterone[22] and experimental data acquired during the course of this work, thus allowing the robustness of the experimental method to be evaluated. The experiments were designed to provide increased knowledge on the solubility of steroid hormones in supercritical carbon dioxide, which at the same time, allowed realistic selection of conditions for the extraction of steroid hormones from tablets, ointment or creams.

Testosterone has a greater solubility in supercritical CO_2 than most of the nine other steroids studied in this work. Testosterone's solubility in supercritical CO_2 has been determined[22] using apparatus capable of dealing with much greater amounts of solubilized solid than the analytical SFE instrumentation used in this work. However, a comparison was undertaken and the solubility determined at two different temperatures (35 and 55 °C) and over a range of pressures. Table 1 shows that the measured mole fraction solubilities for testosterone are similar to the literature values. This is shown

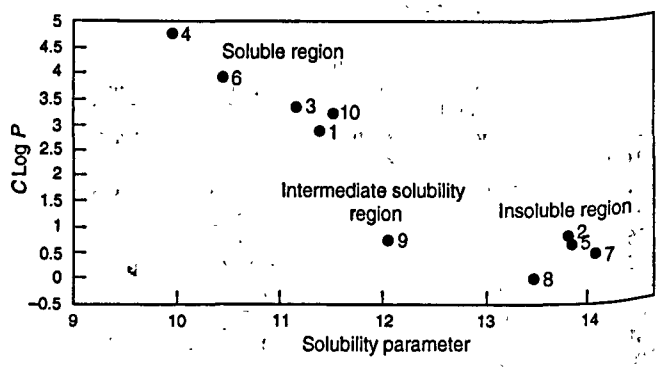
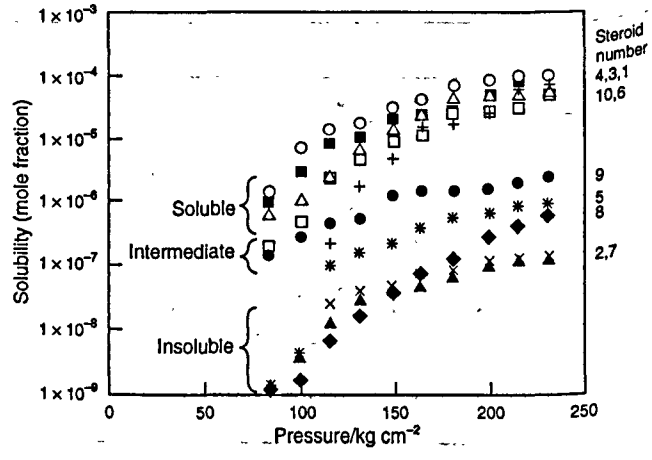


Fig. 4 Prediction of steroid solubility in supercritical carbon dioxide.



■ Testosterone (3)
□ Beclomethasone-17,21-dipropionate (10)
+ Betamethasone-17,21-dipropionate (1)
● Cortisone acetate (9)
× Betamethasone (2)
✱ Hydrocortisone (5)
▲ Prednisolone (7)
◆ Prednisone (8)
△ Megestrol acetate (6)
○ Testosterone-17-propionate (4)

Fig. 5 Solubility of steroids at 55 °C.

Table 4 Prediction of steroid solubility in supercritical CO_2 using solubility parameter and C log P

Steroid	Identifier for Fig. 4	Solubility parameter, δ_2	C log P*	Predicted solubility in supercritical CO_2
Betamethasone-17,21-dipropionate	1	11.42	2.872	Soluble
Betamethasone	2	13.83	0.806	Insoluble
Testosterone	3	11.16	3.349	Soluble
Testosterone-17-propionate	4	9.95	4.784	Soluble
Hydrocortisone	5	13.87	0.658	Insoluble
Megestrol acetate	6	10.44	3.904	Soluble
Prednisolone	7	14.09	0.444	Insoluble
Prednisone	8	13.48	−0.018	Insoluble
Cortisone acetate	9	12.08	0.700	Intermediate
Beclomethasone-17,21-dipropionate	10	11.53	3.212	Soluble

* Taken from C log P, Medchem (1989), Daylight Chemical Systems, Pomona College Medical Chemistry Project, Pomona College, Claremont, CA, USA.

by the isotherms of both experimental and literature values in Figs. 2 and 3. All experimental values were determined at least in duplicate. The experimental variation in the solubility determination was estimated to be $< 3\%$ s_r.

The fact that the extraction method and the chosen method of analysis provided very similar results to the literature values, led to the conclusion that this was a suitable method of solubility determination. The greatest variation from the literature values was observed at the lower pressures studied. Errors in this region are thought more likely because of the very small solubility shown by testosterone and the other steroids at the resulting density conditions and also the smaller experimental scale of this work. The results for testosterone and the other nine steroids are shown in Table 2.

Steroid Solubility Prediction Using C log P and Solubility Parameter

The solubility parameter for each steroid was calculated according to the scheme shown in Table 3. Table 4 summarizes all the calculated solubility parameter data with the computer database values for C log P. A plot of solubility parameter *versus* C log P is shown in Fig. 4. From this it can be deduced that the probable solubility of the selected steroids is likely to be as indicated in Table 4. Obviously, this scheme does not estimate supercritical conditions, such as, temperature and pressure but the trends shown in Fig. 5, a plot of steroid solubility isotherms at 55 °C, indicates a reasonable approximation to solubility in supercritical carbon dioxide.

Conclusions

A prediction of steroid solubility in supercritical carbon dioxide is described, based on the solubility parameter and the hydrophobic term, C log P. The qualitative data identifies trends in steroid solubility which compare favourably with the experimentally determined values for ten steroids in supercritical carbon dioxide over a range of temperatures and pressures.

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(a_1, a_2) define the contribution of each variable X_i to a given principal component, PC_x:

$$PC_x = a_1{*}X_1 + a_2{*}X_2 \ldots \quad (1)$$

A factor score represents the value of an individual sample with respect to a given principal component. The score for sample number n on principal component PC_x is:

$$\text{score} = a_{1x}{*}X_{1n} + a_{2x}{*}X_{2n} \ldots \quad (2)$$

where the a_{ix} are the loadings for PC_x and X_{in} are the values of the variables describing sample number n.

When the scores for factors 1 and 2 were plotted against one another three distinct groups were indicated. Investigation of the associated loadings showed that the most important factors in determining group membership of the samples were (for powder): aluminium, copper, manganese, molybdenum and silicon; and (for wire): aluminium, manganese, molybdenum and silicon.

However, the molybdenum content showed the largest percentage difference between the three groups with group 1 containing 51.5% and 48.6%, group 2 containing 36.1% and 39.3% and group 3 containing 12.4% and 12.1% for powder and wire, respectively. Similar findings have suggested that ores (ferberite and reinite) from the two tungsten-producing areas of the Uganda Protectorate could be characterized according to their manganese[4] and molybdenum[5] content.

Conclusions

The use of a chemometric approach to data interpretation has shown that a significantly smaller number of element assays are required to elucidate the quality of tungsten for electric light filament application. The continued use of chemometrics may alleviate the necessity for high-cost instrumentation for routine analysis.

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Extraction of Ibuprofen by Supercritical Carbon Dioxide

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Supercritical fluids have unique properties which are particularly effective in extraction processes.[1] They exhibit solvation properties similar to those of liquids and have lower viscosities and higher diffusivities which leads to rapid and efficient extraction of analytes. Moreover, the solvent strength of a supercritical fluid[2] increases with increasing density, thus allowing alteration of the extraction selectivity simply by changing the pressure or the temperature. Carbon dioxide is the most common supercritical fluid in use due to its low critical temperature, moderate critical pressure, relative inertness, non-toxicity and ready availability in high purity at low cost.

In the pharmaceutical industry, the use of supercritical fluid extraction for sample preparation is becoming more widespread, owing to its safety and environmental advantages relative to other extraction methods. This paper reports the extraction of the non-steroidal anti-inflammatory drug ibuprofen[3] [racemic 2-(4-isobutylphenyl)propionic acid; Fig. 1] using supercritical carbon dioxide.

Experimental

Instrumentation

An Isco Model 260D pump module and controller (SFX 2-10) was used for extraction. Supercritical fluid chromatography (SFC) grade carbon dioxide (Air Products, Sunderland) was used with a certified purity of 99.995%. A schematic diagram of the apparatus is shown in Fig. 2.

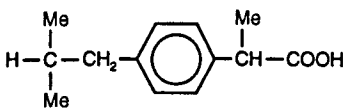

Fig. 1 Structural formula of ibuprofen

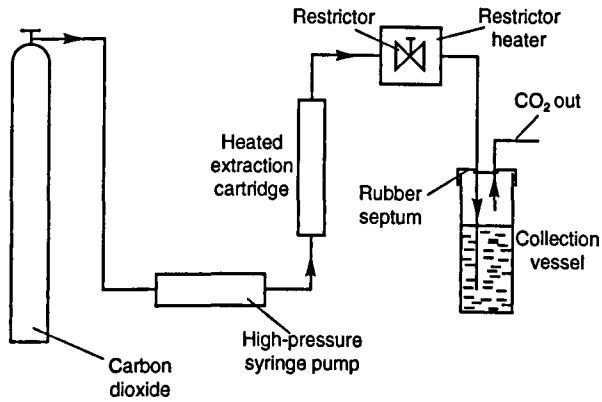

Fig. 2 Schematic diagram of supercritical fluid extractor

Liquid carbon dioxide was pumped using a syringe pump capable of delivering a flow rate of 1 µl min^{-1} to 90 ml min^{-1} at pressures up to 7500 psi.† The extraction vessels consisted of a metal body with upper and lower endcaps fitted with filters. These filters were inserted into the endcaps which then were screwed onto the metal body containing the sample. The extraction chamber was tightly fitted into an aluminium heater block consisting of a stainless steel body capable of withstanding the high pressures (10 000 psi) and temperatures (150°C) used in the extraction processes. The aluminium block acts as a very effective heat-transfer medium between the extraction vessel and chamber for stable temperature control. Depressurization was achieved using a silica fused capillary restrictor (50 µm) into an appropriate collection solvent. The collection solvent was placed in a screw-capped amber vial fitted with a rubber septum. A hypodermic needle pierced the septum to

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† 1 psi = 6.894 76 × 10^3 Pa.

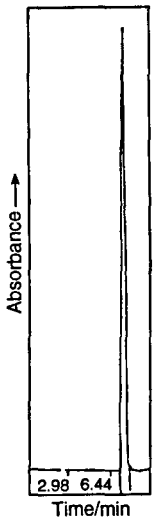

Fig. 3 Typical high-performance liquid chromatography trace for ibuprofen. Column: C_{18}; acetonitrile–phosphate buffer (6 + 4); pH 3.0; flow rate 1 ml min^{-1}; detection at 254 nm

allow carbon dioxide to vent. Blockages were minimized by heating the entire length of the fused silica restrictor to 100 °C.

HPLC Analysis of Ibuprofen

The high-performance liquid chromatography (HPLC) was carried out using an LDC Spectromonitor III with ultraviolet detection (254 nm). Chromatographic peaks were recorded using an LDC/Milton Roy Cl-10 integrator. A 20 µl sample of ibuprofen was injected onto an octadecyl column with acetonitrile–phosphate buffer (6 + 4) at pH 3.0 and at a flow rate of 1 ml min^{-1}. The retention time of ibuprofen was 7 min. Each extract was injected three times and the average peak area was used for calculating the percentage amount extracted. Fig. 3 shows a typical HPLC trace of ibuprofen.

Supercritical Fluid Extraction Procedure

The extraction of ibuprofen involved spiking Celite with 100 µl (50 000 ppm) of ibuprofen standard in methanol. The spiked sample was then quantitatively transferred into the 2.5 ml extraction vessel and dynamic extraction carried out for 10 min with supercritical carbon dioxide, at different temperatures and pressures. After extraction, the collection vessel was rinsed several times with HPLC mobile phase and the final volume adjusted to 10 ml. The experiment was performed in triplicate at each set of extraction conditions. The extracted sample was analysed by HPLC.

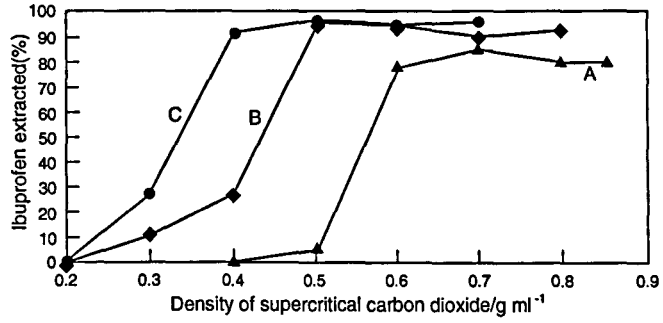

Fig. 4 Solubility isotherm for ibuprofen in supercritical carbon dioxide. A, 50; B, 60; C, 70 °C

Results and Discussion

The primary factors that influence extraction, *i.e.*, density and temperature, were investigated. From the results obtained, a series of isotherms were constructed (Fig. 4) at three temperatures (50, 60 and 70 °C). It is apparent that ibuprofen, although being slightly polar, is soluble in supercritical carbon dioxide at moderately high density and temperature. As Fig. 4 shows, small changes in pressure had dramatic effects on percentage extracted across the whole of the isotherm. The optimum conditions found for the extraction of ibuprofen were a temperature of 70 °C and a density of 0.7 g ml^{-1}.

Conclusion

It is clear that SFE can be used as a sample preparation technique in the pharmaceutical industry. However, it must be noted that the extraction of an analyte in different pharmaceutical matrices does present recovery problems. Future work will involve extraction of ibuprofen from different tablet matrices.

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Predicting Supercritical Fluid Extraction Using Computational Chemistry

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Recent developments in computer technology are leading to dramatic increases in the use of computational chemistry for modelling, optimization and artificial intelligence. All these methods[1,2] have been adapted and applied to supercritical fluid extraction. This paper, however focuses on two particular types of computational chemistry: one in which a neural network is used to predict analyte solubility in supercritical carbon dioxide and another where a molecular modelling program is used to investigate the solute–solvent interactions which occur during supercritical fluid extraction. The software

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