Drug recovery from medicated animal feeds

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Drug Recovery from Medicated Animal Feeds.

by

Ian Michael Barwick

A Doctoral Thesis submitted in partial fulfilment of the requirements for the award of

Doctor of Philosophy
of the Loughborough University of Technology

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Research supervisor: Dr P. Warwick

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Investigations into the poor recovery of sulphadimidine from medicated animal feeds have shown that irreversible drug-feed binding, and not drug degradation, is responsible for the poor recoveries. The experimental work involved the novel use of $^{14}$C-sulphadimidine in analytical studies and in autoradiography of $^{14}$C-sulphadimidine-bound feed. The latter showed that the drug was not bound preferentially to specific feed constituents but was widely distributed on nearly all the feed particles.

Further work on sulphadimidine recovery from feeds demonstrated an inverse relationship between drug recovery and feed moisture content. The role of moisture in the binding mechanism was then considered, and experiments conducted on the adsorption of moisture by feeds showed that the rate controlling mechanism was diffusion.

A hypothesis is presented in which sulphadimidine is partially dissolved by the moisture in the feed and the resulting solution then diffuses into the internal regions of the feed particles via pores and cracks in the constituent particles. The deep penetration of the drug into the feed prevents the drug from being recovered by the extraction solution. Experimental evidence was found to support this hypothesis.

Experimental work also investigated the causes of poor recoveries of sulphadiazine, trimethoprim and dinitolmide.
Acknowledgements

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Chapter 1

INTRODUCTION
CHAPTER 1 - INTRODUCTION

1.1 AGRICULTURE AND THE USE OF MEDICINAL ADDITIVES
Livestock production today is a multi-million pound industry with high productivity due to a variety of reasons. Better stock-breeding programmes, as reported by Bindloss [1], have ensured that genetically improved animals have increased productivity in the areas of either lactation, egg production, or rapid and economical growth. Better nutrition for animals has also played a role in increasing productivity. This has been achieved by research to understand the nutritional requirements of farm animals. This has led to a wide range of feed rations being available to the farmer which enable him to feed his animals a ration containing the optimum levels of nutrition as cheaply as possible.

Coupled with both selective breeding and improved animal feeds, has been the use of medicinal additives in feeds. This has enabled large numbers of animals to be kept in close proximity to each other, a necessity of modern livestock production. The animals are thus kept healthy, and the risk of an outbreak and spread of disease much reduced.

With public opinion beginning to swing towards food produced organically, ie without the use of artificial chemicals or additives, livestock producers have to consider carefully the implications of using medicinal additives in feeds. Provided the strict guidelines for the use of medicinal additives in feeds are adhered to then there should be no danger to either human or animal health. But if drug residues do occur in animal products, then the consumer may be at risk due to the possibility of an allergic reaction being triggered.

The use of medicinal additives in feeds before World War II, to administer drugs to farm animals, was mainly limited to controlling diarrhoea in pigs and outbreaks of internal parasites in chickens as reported by Hanson [2]. Since the herds / flocks were small in comparison to today’s standards and had more room in which to live, problems with outbreaks and spread of diseases were not too serious. However, as demand for animal products was stimulated by the war, new intensive farming methods, enabling high productivity became wide spread. Although these methods did increase productivity, by keeping large numbers of animals in relatively small areas, there was a drawback. This was that if an outbreak of disease did occur, then there usually followed a rapid spread of the disease throughout the other animals.
One area of the animal industry which experienced problems in stepping up production was the poultry industry where outbreaks of a disease called coccidiosis, a disease of the intestinal tract, were extremely common, and extensive research was conducted during the 1940s to develop effective drugs, called coccidiostats, against coccidiosis. As these new drugs were developed, researchers began to notice that feeding sub-therapeutic levels of the drugs resulted in increased rates of growth in the animals.

Over the last fifty years, the use of medical additives in the low level dosing of feeds increased dramatically. To provide some evidence of the scale of medicinal additive inclusion in feeds at the present time, an estimated Anon [3] 33% of all animal feeds produced in the UK contain medicinal additives licensed without the prescription of a veterinary surgeon. The different uses of medicinal additives in animal feeds have been summarised as being:

(i) **Therapeutic** use is where the drugs are administered under the close supervision of a veterinary surgeon as a result of a disease outbreak. High dosage levels are administered over a short period of time, usually a few days. Approximately 5% of animal feeds contain medicinal additives for therapeutic use.

(ii) **Prophylaxis** or disease prevention is the most important use of medicinal additives. Prophylaxis differs from therapeutic use in that the drugs or medicinal additives are added at relatively low levels to the feeds but are administered over a much longer period of time. By including the medicinal additive in the feed the risk of the spread of disease is minimised, if not removed.

(iii) **Growth promotion** is a result of research into prophylactics’ use, since in the absence of any clinical signs of disease, the growth rates of animals fed low levels of medicinal additives were found to improve. It was thought that the animal was developing natural immunity and could therefore utilise more of its energy for growth and weight gain as opposed to using some of its energy to fight off disease. As well as the use of antibiotics, anabolic steroids are used as growth promoters.

As mentioned previously, concern over the use of medicinal additives is increasing, and since 1986 Sweden has banned the use of antibiotic growth promoters. Hardy [4] reported that
previous to the ban, both Carbadox and Olaquindox had been used in the diets of young pigs, which were replaced by Avoparcin and Virginiamycin once the pigs had reached 40 kg live weight. Following the ban, the incidence of post-weaning diarrhoea increased from 15-20% when the growth promoters were used, to 30-50% when the ban was in force. An added complication was that because growth rates were slower, overcrowding occurred which further increased the incidence of the diarrhoea. Hardy continued in his paper by estimating the effect of removing the growth promoters from pig diets in the UK. The absence of growth promoters would naturally decrease the daily gain in weight. To compensate for this, the diets would have to be improved nutritionally; the only drawback is that the cost of improving the diet would be approximately five times the cost of including the growth promoter, and this extra cost would have to be met by increased prices.

This example serves to illustrate that if highly efficient livestock production methods are employed, then medicinal additives are a requirement. But this necessitates that safeguards exist to ensure that both consumer and animal are not subjected to risks by the use of medicinal additives in feeds.

1.2 LEGISLATION AND CONTROL OF MEDICINAL ADDITIVES

The use of medicinal additives in feeds to achieve healthy and productive animals is not without its problems. It has already been stated that, since the animal products or the animals themselves will be consumed by man, there is a danger that any drug residues which remain in the product / animal will produce unfavourable reactions in man.

In the case of medicinal additives used for therapeutic use, no real problems should be encountered since the drugs are administered under the supervision of a veterinary surgeon. He will ensure that the withdrawal period, a designated length of time when the animal is fed non-medicated feed to allow any drug residues in the animal to be removed by normal metabolic processes, before slaughter / consumption is adhered to. Prophylactic use of medicinal additives in feeds is a different situation since the medicated feeds are administered by the farmer, and not by a veterinary surgeon. Drug residues will occur in animal tissues / products if there is misuse of the medicated feeds. Allred and Dunmire [5] reported that two distinct areas are responsible for residues occurring in animal products:
1. On-farm usage
   (i) Failure to comply with the stated withdrawal period
   (ii) Misuse of medicated feeds or drug concentrates
   (iii) Improper cleaning of storage / feed equipment resulting in cross contamination.

2. Manufacturing errors
   (i) mislabelling a medicated feed
   (ii) carry-over of a drug between a medicated and non-medicated feed batch during production.

A specific case of residues occurring in animal tissues is reported by Horwitz[6] in the United States. Sulphadimidine, or as it is known in the US, sulfamethazine, was found in swine tissues at levels greater than the permitted tolerance of 0.1 µgg⁻¹. He reported that in the period of 1974-1981 monitoring of swine tissues revealed that approximately 10% of samples tested contained SDM residues above the permitted limit. This is only one example of such residues occurring.

In the UK, Hardy [4] reported that residues in meat have not been a problem, with the exception of pigs, where approximately 14% of samples tested have contained residues of sulphonamides which exceed the permitted tolerance level. Clearly, it is necessary to counter this problem by implementing strict guidelines on the usage of medicinal additives. In the U.K., Crosby [7] reviewed the strict legislation [8-21] in force, which is supplemented by EC Directives to specifically to address all areas of concern. The main points of the acts governing medicinal additives in animal feeds are summarised below.

   (i) There are two types of medicinal additives defined.

   (ii) Prescription Only Medicines (POM) are medicinal additives that can be included in a feed only on the written direction of a veterinary surgeon. These are for therapeutic use.

   (iii) Permitted Merchant List (PML) medicines are medicinal additives that can be added without restriction, provided that the conditions in the product licence held by the feed manufacturer are adhered to. The compounds in this category are for prophylactic and growth promotion use.
The manufacturer incorporating a medicinal additive into a feed must follow detailed codes of good manufacturing practice which cover such aspects as training of personnel, documentation and quality control. This means that only properly trained and responsible personnel oversee the addition of medicaments to the feed in the mill. Also the manufacturer must declare the nature and amount of the medicinal additive in the feed.

Despite the in-house checks and procedures, it is necessary for regulatory bodies to inspect and test both feeds, for the correct levels of drug inclusion, and animals, to ensure that drug residues are not present. This necessitates that analytical chemists have methods which are accurate and precise. Determinations of drug residues in animal tissues will be problematical because of the low levels of concentrations usually found. Analysis of feeds is not without its problems since, although the drugs are present at greater levels than in animal tissue samples, coloured and reactive components that are co-extracted from the feed will interfere with the determination. For the purpose of this thesis, only the analysis of drugs in feeds will be addressed.

1.3 PROJECT OBJECTIVES

The analysis of animal feeds for medicinal additives has associated problems because the recoveries of certain medicaments are found to decrease with time, and this decrease is more marked in feeds that have been pelleted. For a regulatory analyst, the poor recovery of certain medicaments from feeds is a major problem, since feeds are routinely tested to ensure that they contain the correct drug at the correct inclusion level. Feeds found to contain a drug below the permitted lower level could simply be due to the poor analytical recovery of the medicament from the feed and not due to accidental / deliberate under addition by the feed manufacturer. Conversely, an analytical result which is found to be within the accepted limits could in fact contain more drug than is permissible due to the extraction of the drug from the feed being less than quantitative.

Additionally, there have been cases where a farmer has purchased a medicated feed to prevent the outbreak of a certain disease in his animals only to find that an outbreak has occurred. Litigation has then followed since the farmer believed that the feed manufacturer added insufficient drug, and subsequent analysis of the feed for the farmer has indeed shown that
reduced drug levels are recovered. Clearly this causes a problem for both parties, since the feed manufacturer knows that the required amount of drug was added during manufacture, but the farmer will only accept the result of the analysis, performed some weeks after manufacture, showing that the amount of drug detected is reduced. Clearly, for regulatory purposes, analytical methods for medicinal additives in animal feeds need to be both accurate and precise and to take into account the poor recovery effect.

The work presented in this thesis investigates the problem of poor analytical drug recovery from medicated animal feeds with particular emphasis on feed composition, feed processing conditions and feed storage conditions. The principal drug used in analytical studies was sulphadimidine together with $^{14}$C-labelled sulphadimidine. Sulphadiazine, trimethoprim and dinitolmide have also been used, though to a lesser extent.

1.3.1 The causes of poor analytical recovery

It has been suggested by Crosby [22], that the principal causes of poor analytical recoveries are either degradation of the drug or irreversible binding of the drug to feed constituents. To illustrate the problem of decreasing recovery with time and also the difference in recoveries between pelleted and non-pelleted feeds, Figures 1.1 and 1.2 are presented and show results generated by The Laboratory of The Government Chemist (LGC). In all cases, there appears to be a large initial drop in recovery with time followed by a more gradual decrease. The pelleted feeds at both levels of drug addition show poorer recoveries than the non-pelleted feeds.
Figure 1.1

Recovery of SDM from Meal and Pelleted Feed (100 mg/kg)

Figure 1.2

Recovery of SDM from Meal and Pelleted Feed (200 mg/kg)
1.4 Animal Feeds and Their Constituents

To provide some background to the problem of drug recovery from animal feeds, it is necessary to know something of the composition of animal feeds.

Animal feeds have developed over the years from being simple mixtures of constituents or straights to complex mixtures which ensure that clearly defined levels of nutrition are available for the animal. With the large variety of animal rations on the market today, the feed industry is anything but a small concern with mills operating 24 hours a day. Wilson [23] reported on the developments in the composition of animal feeds where originally the majority of raw materials used were waste products from the flour milling and oil extraction industries. Gradually, as intensive farming methods developed, the best quality raw materials were being used in animal feeds, with many competing directly with the supplies for human food.

Analysis methods for the determination of medicinal additives in feeds usually rely upon the extraction of the drug from the feed with a solvent. The complex and differing nature of animal feeds provides for a very difficult matrix from which to extract the drug. The various constituents used in a particular feed will vary according to the time of year and the availability of constituents. Despite these variations, it is possible to draw up a list of commonly included constituents [24] which are shown in Table 1.1. The list is by no means extensive, but serves to illustrate the variety of ingredients used. The main constituents are then described below.

1.4.1 Cereals

The carbohydrate requirement is usually met from the use of cereals which are high in starch and are a source of energy. The major factor in deciding which cereals are used and in which proportions is cost. Differences in cereals grains are ascertained by examining whether or not the grains separate from their fibrous coatings during the threshing process. Maize, wheat and triticale all produce grains separated from their fibrous coatings and are called 'naked' grains. In contrast, oats and barley are both termed clothed grains in that after threshing, they are still enclosed by two fibrous layers. This means that oats and barley contain more fibre and less starch than maize, wheat or triticale.
Table 1.1

Commonly Included Ingredients [24]

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Maximum Inclusion Level (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cereals</strong></td>
<td></td>
</tr>
<tr>
<td>Barley meal</td>
<td>55</td>
</tr>
<tr>
<td>Maize meal</td>
<td>45</td>
</tr>
<tr>
<td>Wheatfeed</td>
<td>25</td>
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<td>Oat meal</td>
<td>15</td>
</tr>
<tr>
<td>Wheat meal</td>
<td>60</td>
</tr>
<tr>
<td><strong>Vegetable Proteins</strong></td>
<td></td>
</tr>
<tr>
<td>Peas</td>
<td>15</td>
</tr>
<tr>
<td>Beans</td>
<td>15</td>
</tr>
<tr>
<td>Soya Bean meal extract</td>
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<td>Rapeseed meal extract</td>
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<tr>
<td><strong>Animal Proteins</strong></td>
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<td>Fishmeal</td>
<td>open</td>
</tr>
<tr>
<td>Meat and Bone meal</td>
<td>20</td>
</tr>
<tr>
<td>Feather meal</td>
<td>5</td>
</tr>
<tr>
<td>Blood meal</td>
<td>5</td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td></td>
</tr>
<tr>
<td>Biscuit meal</td>
<td>5</td>
</tr>
<tr>
<td>Brewer's grains</td>
<td>15</td>
</tr>
<tr>
<td>Malt culms</td>
<td>15</td>
</tr>
<tr>
<td>Maize gluten meal</td>
<td>15</td>
</tr>
<tr>
<td>Molasses</td>
<td>10</td>
</tr>
<tr>
<td>Rice bran</td>
<td>5</td>
</tr>
</tbody>
</table>
1.4.1.1 Wheat
Wheat is grown in most temperate zones in the world and can be broadly classified into three groups:

(i) soft wheat - milled to flour for cakes
(ii) hard wheat - milled to flour for bread making
(iii) durum wheat - hard wheat used mainly for pasta

The type of wheat generally used in feeds is soft wheat, with both spring and winter varieties being used. Wheat quality is not only dependent on the moisture and protein content, but also on the physical appearance of the grain, and the contamination with weed seeds. As regards the storage of wheat, it should be dried to below 16% moisture to prevent mould formation, fermentation and sprouting.

1.4.1.2 Barley
Barley is a crop cultivated widely throughout the world as a result of its short growing period and its hardiness. As with wheat, both spring and winter varieties are used for feed barley. In the UK, the majority of barley is used for malting. Barley used for malting should have a low protein content. The barley that fails the malting standards is used in animal feeds, and should be below 16% moisture and free from impurities such as wheat seeds, chaff and stones. Barley with a moisture content of between 16-18% may be used to produce rolled barley which can be used in coarse mixes.

1.4.1.3 Maize
Maize is mainly cultivated in the United States and Southern Europe. The actual maize grains grow attached to a central stem, often called the cob. The maize harvesting is timed to occur just before it is completely ripe to reduce losses. Although maize is low in protein (protein quality being lower than that of wheat) because of its high oil content, the energy value of maize is higher than that of other cereals.

1.4.1.4 Wheatfeed
Wheatfeed is a by product of flour milling and mainly consists of very fine bran and bran powder mixed with the mill tailings which are mainly starch and proteinaceous material. Wheat feed is known by a variety of different names: wheatings, thirds, pollards, midds, sharps
and bran. The majority of wheatfeed is home produced, although some is imported and the latter can be contaminated with fungus.

1.4.1.5 Malt culms

In the malting process of barley, the grains are allowed to germinate until the sprouts have formed to approximately an inch in length. The barley is then dried, and the sprouts, which are known as malt sprouts or malt culms, are removed by screening.

1.4.1.6 Molasses and Molaferm

Molasses or Molaferm are both by-products from cane or beet sugar manufacture. Molasses is the liquor from the refining process from which no more sugar can crystallise out. Molaferm is a blend of molasses and condensed molasses solubles. Various types of molaferm are found with Molaferm 20 being a blend of 80% molasses and 20% condensed molasses solubles. The materials are used because the sugar content from both aids palatability and is a source of energy.

1.4.2 Vegetable proteins

These sources are either obtained from whole seeds such as beans and peas, or from the products remaining after the crushing of certain seeds and fruits to remove their oil. Oil can be removed by two methods. The first, expelling, is where the seed is cracked and then the oil is expelled by hydraulic or screw presses. This method is not particularly efficient since approximately 5-12% of the oil is left in the raw material. The second method, extraction, uses an organic solvent to remove the oil and is more efficient than the expeller method. Following extraction, the organic solvent is removed by heat, and the remaining material has a residual oil content of approximately 3%.

1.4.2.1 Peas and beans

Both peas and beans are grown in temperate regions of the world and are used with a maximum moisture level of 16%. With peas they can be broadly classified into four variety groups: white pea, marrowfat, red and large blue. The first two varieties are mainly used in this country. Beans can be divided into two groups depending on the colour of their flowers.
1.4.2.2 Soya beans and extracted soya bean meal
Whole soya bean meals have an oil and protein content of approximately 20 % and 38 % respectively. Present in whole soya beans is a factor called trypsin inhibitor, which inhibits the digestive enzyme trypsin. This factor can be destroyed, and the resultant product is referred to as full fat soya which is mainly used in pig and poultry feeds. Ruminants can be fed whole unprocessed soya bean meal because the trypsin inhibitor enzyme is degraded in the rumen. Whole soya is processed to remove the oil for human consumption. The hulls, which constitute the fibre, are removed from the bean prior to oil extraction. After processing, the hulls can be blended with the extracted soya. Soya 50 (50 % crude protein), commonly called Hypro, has no hulls added following oil extraction. Other extracted bean products such as Soya 44 (44 % crude protein) are a blend of Hypro and hulls.

1.4.3 Animal proteins

1.4.3.1 Fishmeal
Fishmeal is prepared from the heads, bones and adhering flesh and whole fish failing to meet human standards, but not the entrails. These are then cooked and dried. The protein content ranges from 60-75 %. Fishmeal is known under a variety of different names which indicate either the type of fish used to prepare the meal or the country of origin.

1.4.3.2 Meat and bone meal
This is manufactured from pieces of meat and carcass with a high proportion of bone. The bone meal is selected to result in a finished product with 40-55 % protein. Products from different rendering plants may have different characteristics and specifications, but the main differences depend on whether the oil is removed by solvent extraction or whether it is mechanically removed.

1.4.4 Fats
Fats can be of vegetable or animal origin and are composed of glycerol and individual fatty acids. These fatty acids can vary in chain length (2-28) and also in the degree of saturation. Hard fats are saturated while the soft oils are unsaturated. The fats of vegetable origin are normally liquid at room temperature and unsaturated (oil), whilst those of animal origin are normally solid at room temperature and saturated (fat) in nature.
1.5 FEED PROCESSING

An overview to feed processing is shown in Figure 1.3 based on the production facilities at Paul’s Agriculture Lindum Mill, Shepshed, Loughborough, Leicestershire, and also Dalgety’s Mill at South Wigston, Leicestershire, which is typical of the modern mill operation.

1.5.1 Raw materials intake

The first stage consists of raw material intake whereby materials are delivered, tested and, if they are within the specifications, stored in large holding bins. Delivery of raw materials carries on around the clock to meet demand. Dalgety Agriculture’s Mill has 53 raw material bins on site to enable production of 29 different finished products with output being some 2000 tons of finished feed per week. This illustrates the size of the operation together with the range of products that can be produced. All operations are computer controlled to ensure that a specific raw material is correctly sent to the appropriate holding bin.

1.5.2 Feed processing

The computer is set to produce a certain ration and the ingredients are then selected from the corresponding bin and brought to the blending bin via pipelines. In the blending stage / size reduction stage, two different practices are commonly employed:

(i) Pre-reduction is where each constituent is ground separately then mixed, but because each constituent is a different particle size, achieving a uniform mix is fairly difficult and compression more difficult.

(ii) Post-reduction is where the constituents are first roughly blended then reduced to a uniform particle size. This results in easier mixing and hence easier compression.

In the mixing stage, the constituents are mixed in 2.5 tonne batches for approximately 2 minutes. It is during this stage that the vitamins, minerals and premixes are added so that they do not undergo the size reduction step which would damage them.

After all the solids have been mixed the liquids (molasses, propionic acid) are added, and following mixing, a meal results. From here, the feed is in the form of a meal and either goes to storage or passes on to the pelleting stage.
**Feed Processing**

- Raw materials intake
- Blending / Sieving / Grinding
- Mixing
- Meal
  - Meal storage bins
  - Meal for pelleting
    - Conditioning
    - Pelleting
      - Cooling and Sieving
      - Fats coating
      - Bulk storage bins
1.5.3 The pelleting process
Efficient food production demands that feeds meet the precise nutritional standards required, are in a form most suitable for handling and transportation, and finally, are in a form which is appetising to the animal. Pelleting feeds fulfils all these requirements with approximately 75% of all UK feeds supplied in pellet form [23]. The main benefits of pelleting are summarised in Figure 1.4 [24].

The pelleting process consists of a conditioning stage when steam is injected into the feed to increase the moisture content. Dairy feeds also have molasses injected. The moisture content is thus increased from 12% to 18% where both steam and molasses have been injected, and from 12% to 15% where only steam is injected. The temperature of the feed in the conditioner, or ripener as it is also called, is between 75-80 °C, although, in some pelleting presses, temperatures in excess of 100 °C are employed. The feed is then forced through a circular die of the required size, then cut to length. Obviously, the temperature the feed reaches depends upon the size of the pellets being produced, with smaller pellets experiencing higher temperatures than the larger pellets. The temperature aimed for pellets leaving the press is approximately 80 °C.

Once the pellets have been cooled, sieving is used to remove small particles and undersize pellets. Finally, the fats coating stage increases the durability of the pellets and also reduces the amount of dust. After passing through quality control, the pellets can either be bagged or stored in bulk for transportation.
The advantages of pelleting [24]

**Effects of Pelleting**

- Increased bulk density
- Increased feed intake
- Improved digestibility
- Prevents 'de-mixing' of ingredients

**Consequences for compounder/farmer**

- Facilitates bulk transport and storage
- Reduces transport costs
- Improved flow and metering characteristics
- Reduces waste on farm
- Improved efficiency of food production
- Reduces bacteriae e.g. Salmonella
- Stock cannot 'select' ingredients
- Allows drug additions without risk of inaccurate dosage
- Easier formulation changes without rejection

- More cost efficient feed production therefore increased profitability for compounder
- Improved nutritional quality of ration and increased profitability for farmer

More cost efficient feed production therefore increased profitability for compounder

Improved nutritional quality of ration and increased profitability for farmer
1.6 THE SULPHONAMIDES

1.6.1 History of the sulphonamides

The sulphonamides are an important group of antibacterial compounds with the general structure as shown in Figure 1.5.

![General Formula for a Sulphonamide](image)

Although the sulphonamide, sulphanilamide, was synthesised in 1908 by Gelmo as part of an azo dye development programme, its antibacterial activity was not discovered until 25 years later when Domagk experimented with the treatment of infection of streptococci with a compound called sulfamidochrysoidine. Indeed this discovery was quite by accident because it was not until further investigation of results that it was shown that in vivo, reduction of sulfamidochrysoidine by the enzyme azo reductase, reduces the azo linkage to yield sulphanilamide as the active compound of the two products formed. This is shown in Figure 1.6.

![In vivo release of sulphanilamide from sulfamidochrysoidine](image)
By 1940, the key biological features concerning the mode of action of the sulphonamides were understood when Woods [25] demonstrated that p-aminobenzoic acid (PABA), shown in Figure 1.7, prevents the bacteriostatic action of sulphanilamide.

![Figure 1.7: p-aminobenzoic acid and sulphanilamide](image)

Fildes [26] postulated that PABA was an essential metabolite that was responsible for one or more enzymatic processes involved in bacterial growth. Because of the similarities between the sulphonamides and PABA, it was suggested that the sulphonamides may act by blocking the enzyme system or systems in which PABA is involved and on which many bacteria depend for normal growth and development. Further studies confirmed that the inhibition between sulphonamides and PABA is competitive.

### 1.6.2 Chemical Properties

Sulphonamides are white, crystalline powders which are usually only sparingly soluble in water. As they are weak acids, they form salts with bases, and these salts are more soluble than the free sulphonamides. Sulphonamides, which are the amides of sulphonic acids, can be classified by the number of groups on the nitrogen as either unsubstituted, monosubstituted or disubstituted. Unsubstituted and monosubstituted sulphonamides are weakly acidic whereas disulphonamides are much stronger acids. They are prepared by a variety of different processes, the main two being shown in Figure 1.8.
1. Acetanilide (I) is treated with chlorosulphonic acid to give the sulphonyl chloride (II) which is reacted with the appropriate amine to give required intermediate product (III) which then undergoes acidic/alkaline hydrolysis to give the desired sulphonamide (IV)

2. Reaction of p-nitrobenzenesulphonylchloride (I) with appropriate amine gives an intermediate (II) which if reduced leads directly to the desired sulphonamide (III).

1.6.2.1 Structure and Activity

In the course of research conducted into sulphonamides, attempts have been made to correlate the chemotherapeutic activity of sulphonamides [27] with various chemical and physical parameters such as pKa, protein binding and electronic charge distribution.

1.6.2.2 Electronic charge distribution

The structure of the PABA molecule is a benzene ring with an amino group, para to which is an ionic group containing two very negative oxygen atoms. With the sulphonamides, the group which resembles the CO₂ ion in PABA is the SO₂ group. Comparing the oxygen-oxygen distances in both cases shows that in PABA the distance is 23 nm and in the sulphonamides it is 24 nm. Also the general geometry of the sulphonamides is very similar to PABA as is shown in Figure 1.9.
Measurements of the base constants of all the amino groups of the sulphonamides have shown that they are all of the same order of magnitude. This implies that it is the differences in the SO₂ groups of the sulphonamides compared to the CO₂ ion that can account for the different bacteriostatic activities of the compounds. With dipole-moment studies, the SO₂ group has been shown to be a relatively negative group, but the CO₂ group is more negative since it carries a negative charge. It was proposed by Bell and Roblin [28] that the more negative the SO₂ group of the sulphanilamide type compound, the greater the bacteriostatic activity of the compound. They then proceeded to evaluate the relative negative character of the SO₂ group in terms of their acid dissociation constants and relate this to their bacteriostatic activity.

1.6.2.3 pKa

Bell and Roblin postulated that the antibacterial activity of a sulphonamide is related to pKa. The theory was that the maximum antibacterial activity occurred when pKa was equal to pH and was based upon the assumption by researchers at that time that sulphonamides penetrated the cell wall as undissociated molecules but acted in ionised forms.
The equation linking pKa to pH is:

$$pK_a = pH + \log \frac{[HA]}{[A^-]}$$

Thus, when the sulphonamide is 50% dissociated, $[HA] = [A^-]$ and so $[HA]/[A^-] = 1$ and $\log 1 = 0$ which then gives:

$$pK_a = pH$$

By considering the N-substituted derivatives, they were able to investigate the effect the R group had upon the acid constants. For the R group to be acid strengthening, it must be electronegative and attract electrons from the adjacent amide nitrogen so the hydrogen can leave as a proton in solution, leaving the anion. They published their results in graphical form of Activity against E.coli at pH 7 against pKa of the sulphonamide, over a range of pKa values between 3 and 11. This is shown in Figure 1.10.

**Figure 1.10**

*Drug activity as a function of pKa for a series of sulphonamides[28]*
Interpretation of the graph is that a decrease in pKa over the range from 11 to 7 results in an increasing proportion of ionised sulphonamide; the ion is more active than the neutral species, so activity increases over this range. From pKa 5 to 3, the sulphonamide is essentially totally ionised, but, instead of the activity levelling out, it decreases. This decrease is due to the fact that pKa is now a measure of electron withdrawal by R away from the SO₂N group. This shows that the essential property of the sulphonamide is not simply ionisation but the charge that exists on the oxygen atoms of the SO₂ group. The one drawback of their theory was that it does not apply to all series of these drugs since the pKa of many sulphonamides lie outside the 6.0-7.5 range that would give approximately 50% dissociation.

The study by Bell and Roblin [28] successfully accounted for the enhanced bacteriostatic efficiency of heterocyclic sulphonamides whose pKa values are near 7. Their theory also explained why some sulphonamides such as sulfaguanidine and dapsone, which cannot ionise at all as acids, are so potent. With these types of compounds, a large negative charge can accumulate on the SO₂ group by resonance delocalization. Although Bell and Roblin put forward these theories, one criticism is that their work was based on analogues rather than experimental data concerning the physico-chemical properties of the SO₂ group. Nevertheless their theory provided a major stimulus to subsequent researchers in this field.

1.6.2.4 Protein Binding
Sulphonamides are known to bind to plasma proteins and suggested protein binding sites are the basic centres of histidine, arginine and lysine. Agren et al [29] have investigated physico-chemical factors influencing the binding of sulphonamides to human albumin. They reported that the degree of binding increased remarkably near the pKa of the drug. A sulphonamide with a low pKa was reported as having a comparatively high degree of binding compared to a drug with a high pKa. They concluded that the uncharged species is hardly bound. The groups involved in sulphonamides in the binding are alkyl, alkoxy, alkylthio, halo, aryl or heteroaryl at N1. The main forces involved in the Sulphonamide-protein interaction are hydrophobic bonding and ionic attraction.
Newbould and Kilpatrick [30] investigated the causes of two new sulphonamides, sulphaphenylpyrazole and sulphamethoxypyridazine, to be found in blood at persistently high levels after a single daily dose. They concluded that the new sulphonamides tested were bound to plasma-proteins to a greater degree than other older sulphonamides, and thus had less bacterial activity. Additionally, Klotz and Walker [31] investigated the binding of some sulphonamides by bovine serum albumin and in particular the formation of complexes formed between sulphonamides and crystallised serum albumin. Calculation of binding energies were made which were related to the structure of the sulphonamides used in the study.

1.6.3 Sulphadimidine

Sulphadimidine [4-amino-N-(4,6-dimethyl-2-pyrimidinyl)benzenesulphonamide, sulfamethazine, SDM] is also known by a variety of other names: sulfamethazine, sulfadimerazine, sulfadimethylpyrimidine as well as many trade names, and is a widely used antimicrobial drug. Its structure is shown in Figure 1.11. The usual withdrawal period is 10 days prior to slaughter.

Figure 1.11
Structure of Sulphadimidine

The large range of products [32] containing SDM either on its own or in combination with other drugs such as chlortetracycline, penicillin or tylosin phosphate, is shown in Table 1.2.
<table>
<thead>
<tr>
<th>Product</th>
<th>Active compounds</th>
<th>Dosing level*</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyfac HS</td>
<td>SDM</td>
<td>73.2</td>
<td>Cyanamid</td>
</tr>
<tr>
<td></td>
<td>Chlortetracycline</td>
<td>73.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Penicillin</td>
<td>36.6</td>
<td></td>
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<tr>
<td>Micro-Bio:</td>
<td>SDM Premix 10%</td>
<td>100</td>
<td>Microbiologicals</td>
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<tr>
<td>SDM Pure</td>
<td>SDM</td>
<td>1000</td>
<td></td>
</tr>
<tr>
<td>Microfac HP</td>
<td>SDM</td>
<td>81.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chlortetracycline</td>
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</tr>
<tr>
<td></td>
<td>Penicillin</td>
<td>40.95</td>
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</tr>
<tr>
<td>Pharmshure</td>
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<td>100</td>
<td>PW</td>
</tr>
<tr>
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<td>Peter Hand</td>
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<td>SDM</td>
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<td></td>
</tr>
<tr>
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<tr>
<td>Tylasul Premix</td>
<td>SDM</td>
<td>20</td>
<td>Elanco</td>
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<td></td>
<td>Tylosin Phosphate</td>
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</tr>
<tr>
<td>Tylasul Premix 100</td>
<td>SDM</td>
<td>100</td>
<td>Elanco</td>
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<tr>
<td></td>
<td>Trimethoprim</td>
<td>25</td>
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</tr>
</tbody>
</table>

*Dosing level expressed in g of drug per 1000g of feed.*
The properties of SDM are as would be expected of a sulphonamide [33], with it being a white to yellowish powder, and odourless or near odourless. On exposure to light, the powder may darken. Its solubility in water and in alcohol is only slight, while in acetone, mineral acids and solutions of alkali hydroxides and carbonates it is reported as being soluble.

1.6.3.1 Pharmacokinetics

Once administered, SDM is rapidly absorbed from the gastrointestinal tract with approximately 60-80 % bound to plasma albumin. It is highly acetylated with 30-40 % in the blood being in the acetyl derivative. Within two days of administration, 50 % is excreted, with 33 % of this in the acetyl derivative form.

SDM is a short acting sulphonamide with a half-life dependent on the rate of acetylation. In rapid acetylators, the half-life is 1.5-4 hours while in fast acetylators it is 5.5-8.8 hours. Studies on the metabolism of SDM [27,34,35] have shown the major metabolites of SDM to be as shown in Figure 1.12.
Figure 1.12

Major metabolites of SDM

Protein bound SDM

\[ \overset{4\text{NH}_2}{\text{SO}_2} \overset{1\text{NH}}{\text{CH}_3} \overset{\text{CH}_3}{\text{N}} \overset{\text{CH}_3}{\text{N}} \]

\[ \overset{\text{NHCOCH}_3}{\text{SO}_2} \overset{\text{NH}}{\text{CH}_3} \overset{\text{CH}_3}{\text{N}} \overset{\text{CH}_3}{\text{N}} \]

Sulphadimidine

Acetysulphadimidine

Conjugation at N^4 and N^1

\[ \overset{\text{NH}_2}{\text{SO}_2} \overset{\text{NH}}{\text{CH}_3} \overset{\text{CH}_3}{\text{N}} \overset{\text{CH}_3}{\text{N}} \]

Glucuronic acid

\[ \overset{\text{NH}_2}{\text{SO}_2} \overset{\text{NH}}{\text{CH}_3} \overset{\text{CH}_3}{\text{N}} \overset{\text{CH}_3}{\text{N}} \]

Sulphuric acid

5-Hydroxy-4,6-dimethyl 2-sulphanilamidopyrimidine

4-Hydroxymethyl,6-methyl-2-sulphonamidopyrimidine
1.7 REVIEW OF ANALYSIS METHODS

A thorough review of analytical methods for sulphonamides in both tissues and feed up until 1981 has been made by Horwitz [6 and 36]. It is useful to review in detail those methods for the determination of SDM in feeds that have only been briefly mentioned in the review by Horwitz as well as methods published after 1981. Horwitz reported that the colorimetric based Bratton-Marshall method lacked specificity in sulphonamide analysis. He further reported that although the chromatographic-based methods were more specific, the methods gave low drug recoveries and reproducibilities.

A major criticism of nearly all the analytical methods reviewed is that in the method validation studies, no allowance is made for the fact that a regulatory chemist often receives feed samples that are several days or even weeks old. This creates problems since SDM recoveries from feeds are inversely proportional to the length of time SDM has been in contact with the feed. A method which quantitatively extracts SDM from freshly spiked samples in a method validation study may not be satisfactory in extracting SDM from aged feed samples.

Allred and Dunmire [5] reported on a high performance liquid chromatography (HPLC) method for determining SDM at low levels in non-medicated feeds. Acetonitrile extraction of SDM was followed by a concentration, then clean-up step using a column of C18/Porasil B and AX/Corasil. The C18/Porasil B retained the non-polar compounds and the AX/Corasil retained the polar interferents eluting before SDM on the analytical column. Results indicated that 89-112 % recoveries were obtained with no differences being reported for the level of fortification.

Cieri [37] reported an HPLC method for the determination of SDM, or sulfathiazole. SDM is extracted with 50 % methanol, which is then filtered and injected in to a liquid chromatograph. Recoveries from synthetic samples were reported as ranging from 100.2-103.6 % for the dosing levels used. Commercially produced samples gave recoveries of 77.8-82.9 %.

Holder et al [38] found that when SDM had been in contact with the feed for a few hours, quantitative recovery was obtained when the SDM had been added in the form of a premix, but not when added as either the dry chemical, or dissolved in an organic solvent then added. They
also reported that the recovery of SDM varied inversely proportionally to the time the drug was in contact with the feed; good recoveries being obtained with a variety of solvents provided the extraction was performed within 15 minutes of spiking. After a contact time of 96 hours recoveries of only 70% were obtained.

Stringham et al [39] described a liquid chromatographic method involving post-column derivatisation for the determination of sulfamethazine and sulfathiazole in feeds and feed premixes. The drugs are extracted with 0.15N hydrochloric acid in 25% methanol, then diluted, clarified and injected onto a reversed-phase C18 column with the use of a post-column derivatising agent, dimethylaminobenzaldehyde. Recoveries for sulfamethazine and sulfathiazole are reported as being 100.6 and 96.3% for respectively. The advantage in using post-column derivatisation was reported that the co-extracted compounds that interfere with UV detection during chromatography do not need to be removed if post-column derivatisation forms a coloured compound with the drugs. The detection is then simplified due to the fact that the co-extracted interferents will not absorb in the visible region.

Munns and Roybal [40] developed a gas-liquid chromatographic method for the determination of SDM in swine feed whereby SDM was extracted with ammoniated acetone and separated from co-extractants on a Sephadex LH-20 column. The eluate is then methylated, evaporated to dryness and dissolved in a solvent before injection onto an OV-25 GLC column. Recoveries of SDM from feeds ranged from 87-104%. It was found that the length of time SDM was exposed to the feed material affected the recovery with a 10 minute extraction giving higher recoveries than a 30 minute extraction.

McGary [41] reported a method for determining SDM and Carbadox in animal feeds by paired ion HPLC. Chloroform-methanol extraction of the drugs is followed by vacuum filtration through Celite. Clean-up using liquid-liquid extraction with aqueous sodium chloride – sodium hydroxide is followed by recovery from monobasic potassium phosphate with chloroform. Evaporation of solvent followed by dissolution in mobile phase allowed injection onto the HPLC column. Recoveries were reported as being 104-106% for SDM and 103% for carbadox.
Schwarz [42] investigated the effect of particle size on SDM extraction and reported that the finer the particles produced during grinding, the longer it took to extract the SDM quantitatively when the ground feed was mechanically shaken with the solvent. Further investigation showed that quantitative recovery could be obtained regardless of the degree of fineness of the feed, merely by allowing the feedstuff to be wetted by the solvent and left for longer than 3 hours. It was also investigated whether there was any difference in the way the SDM was incorporated into the feed; either by adding the SDM directly or by first mixing it with a carrier. No differences were observed between the two methods of addition, which contradicts the findings of Holder et al. Finally, the diminished recovery of ground feeds stored in polyethylene bags and exposed to sunlight was reported. The magnitude of the loss varied considerably from 50 % SDM loss in 3 days to 12 % loss in 5 weeks. He recommended that the feeds are stored in a dark and cool place when not in use.

Smallidge et al [43] described a method for the determination of SDM and sulfathiazole in swine feeds at the residue level using reversed-phase chromatography and post-column derivatisation. The extraction was performed with HCl and diethylamine followed by centrifugation and filtering. Injection onto a C18 reverse-phase column with post-column derivatisation using dimethylaminobenzaldehyde allowed quantitation of the two drugs. An internal standard of sulfamerazine was used because it differed from SDM by only one methyl group, but criticism of the use of an internal standard must be made since it would be incorrect to use it to quantify extraction efficiency from the feed of SDM. This would be because SDM and sulfamerazine would have been in contact with the feed for different lengths of time and so would be expected to give different recoveries. Absolute recoveries of the drugs were reported as being 44-80 %.

Houglum et al [44] employed a liquid chromatographic method for the determination of SDM in feeds with extraction into 50 % methanol solution and injection onto a C18 column. Recoveries are reported as being on average 97 %.

Blanchflower and Rice [45] reported that the concentration of SDM found in pelleted feeds especially was dependent on the solvent system used for the extraction. When an acetone-ammonia solvent system was used as the extractant, SDM levels appeared to decrease when the feed samples were finely ground using a laboratory mill. They also comment on the fact
that the published methods for SDM (extraction or homogenisation with a solvent) have shown good recovery figures, but they have usually been spiked after milling and no further process takes place before analysis. In commercial feed samples, the SDM is added before the pelleting process, and these methods give poor extractions on these feed samples. They concluded by saying that, during the pelleting process the feed is subjected to a combination of heat, pressure and moisture which appears to bind the SDM to the feed matrix and makes its extraction more difficult.

Poor recoveries have been noted in both tissue samples and feed samples which were thought to be due to binding effects. As a possible remedy, enzyme digestion using ficin and pepsin was proposed to release the bound sulphonamides. Extending this to feeds, Merwin [46] did not find any differences in the digestions of stored feed with and without the use of ficin.

The problem of protein binding of sulphonamides in tissue samples by plasma proteins was addressed by Bevill et al [47] who successfully used phenylbutazone to displace the sulphonamides from the binding sites, and resulted in a four-fold increase in the measured level of sulfamethazine.

1.7.1 Current analysis method
The current analysis method by Conway [48] used for sulphadimidine in feeds comprises extraction of the drug with aqueous acetonitrile. The clean-up stage that separates sulphadimidine from the co-extractives is achieved using a cation-exchange cartridge. Sulphadimidine is eluted from the cation-exchange column using the mobile phase used in the HPLC stage. HPLC analysis of the eluted solution using a C18 reversed-phase column resolves the SDM peak well, but problems with the method involve the poor recoveries of feeds that have been stored for some time, with recoveries being only 70% in some cases.

Conway reported the results of analysing feeds spiked with SDM either in the form of a premix or in the form of an SDM solution. The major findings were that for a feed spiked with the SDM premix, using pure acetonitrile as the extraction solution and analysing 24 hours after spiking, recoveries of ~96% were obtained. When the analysis method was used to test a feed spiked with SDM in solution, only a 41% recovery was obtained, although when the feed was analysed within 1 hour of spiking with the solution, the recovery was 100%. Further investigations on the composition of the extraction solution showed that a 70:30 v/v mixture
of acetonitrile:water extracted 83% of the total SDM 134 days after storage compared to only 46% extraction using 100% acetonitrile 138 days after spiking. The presence of the water in the extraction solution is thought to assist in the solubilisation of the protein-bound SDM, and that the acetonitrile precipitates the protein.

The Analytical Methods Committee [49] considered current methods of analysis for SDM in feeds. The method supplied by the manufacturer of the drug was initially examined but was found to be unsuitable, since it gave only 90% recoveries from freshly spiked feeds and poorer recoveries from older feed samples. An amendment then followed which comprised of a chloroform extraction clean-up stage. A collaborative study showed that in all but one case, recoveries of SDM from pellets were lower than from a meal. The clean-up stage was then amended to the cation-exchange column procedure described by Conway. Collaborative studies again showed that pelleted feeds gave poorer recoveries than from the same feeds before pelleting. There was also some indication of a drop in recoveries with storage.
Chapter 2

RECOVERY OF SDM FROM FEEDS
CHAPTER 2 - RECOVERY OF SULPHADIMIDINE FROM FEEDS

The experimental work described in the following chapters investigates the causes of poor analytical recovery of medicinal additives from feeds. The majority of work centred on SDM, but sulphadiazine, trimethoprim and dinitolmide were also considered. A feature of the work was to use radiolabelled medicinal additives in studies.

The experimental work was divided into 4 sections.

(i) Validation of the analysis method and determination of the experimental error of the method. This work was performed to ensure that the SDM not detected on the HPLC system was not lost in the analytical stages.

(ii) Development of a dosing protocol to introduce the drug into the feed. This was necessary because it was essential to know precisely how much drug had been added to the sample in order to calculate the recovery of the drug after extraction.

(iii) Investigations into the recovery of SDM from both complete feeds and from individual constituents of the feed looking, in particular, at factors that affect SDM recovery.

(iv) Investigation of experimental findings from (iii) using radiolabelled SDM. This work is described in Chapter 4.
2.1 ANALYTICAL METHOD FOR SDM

2.1.1 Experimental
The analysis method used throughout this paper is that described by Conway [48] and later modified by the AMC [49]. A schematic view of the analysis method is shown in Figure 2.1, in which SDM is extracted from the feed with aqueous acetonitrile followed by cation-exchange clean-up. The resulting solution is analysed using a reversed-phase HPLC system with uv detection. The only modification to the method was that a column processor was used to enable simultaneous processing of several solid-phase extraction columns. A validation exercise was performed to investigate whether processing columns by positive pressure, as specified by Conway, produced different results for the extraction of SDM as opposed to the use of a vacuum system. No significant differences were found between samples processed by the two different methods. The column processor was therefore used in all subsequent studies.

![Figure 2.1](image)

**Figure 2.1**
Stages in Analysis

- Ground to pass through Immsieve
- Extraction with 70% aqueous MeCN
- Filtration through glass fibre filter paper
- Pump
- Diode array detector
- HPLC Analysis
- Clean-up using cation-exchange column
Reagents: As specified by Conway [48]
Self-indicating silica gel: (Fisons, Loughborough, Leicestershire, UK)
Swine Feed: (a) PigBreed 16 Meal (Pauls Agriculture Ltd., Shepshed, Leicestershire, UK).
(b) UltraFinisherMeal (Dalgety Agriculture Ltd., South Wigston, Leicestershire, UK).

Apparatus:
HPLC System: Philips PU 4021 Multi Channel Detector.
Philips PU 4100 Liquid Chromatograph fitted with 20 \( \mu l \) injection loop.
Philips P 3202 Computer with PU 6003 Diode Array Software and P6000 Integration Software.

HPLC Columns: Apex ODS, 5 mm, 250 m x 4.6 mm i.d. (Fisons).
Apex ODS, 5 mm, 50 m x 4.6 mm i.d. guard column (Fisons).
Sample Tubes: Falcon 50 ml Polypropylene Disposable Sample Tubes (Northern Media, Nottingham, UK).

Column processor:
Baker spe-10 Column Processor (J.T.Baker, Hayes, Middlesex, UK).

2.1.2 Validation of Analytical Method

2.1.2.1 Loading and recovery of SDM from the cation-exchange column
To demonstrate that SDM was not being lost in the clean-up stage of the analysis, the following experiment was performed.

SDM (93.9 mg) was weighed into a 100 ml volumetric flask and diluted to the mark with extraction solution. After placing this in a sonic bath for 10 minutes, to ensure complete
dissolution, the solution was mixed thoroughly. A 10 ml aliquot was taken from this solution, pipetted into a 100 ml volumetric flask and diluted to the mark with extraction solution to give the working standard solution.

After conditioning the column, 10 ml of the working standard solution was added to glacial acetic acid (5 ml) to give the load solution. This was then added to the column and the method described by Conway [48] followed.

The load solution, after passing through the column, was collected as was the solution used to elute the SDM. Both solutions were analysed by HPLC. The chromatogram of the load solution after passing through the cation-exchange column is shown in Figure 2.2 and the chromatogram of the solution used to elute SDM is shown in Figure 2.3. By analysis of the working standard solution both before and after clean-up, it was possible to determine whether quantitative loading and recovery of SDM had occurred.

When the SDM was eluted from the column, the experimental SDM concentration in the eluate solution was found to be 37.8 ug ml\(^{-1}\) compared to a theoretical concentration of 37.6 ug ml\(^{-1}\). This demonstrated that, within experimental error, complete recovery of the SDM from the column was obtained.

The effectiveness of the clean-up stage on feed samples is demonstrated with a feed extract shown before and after the clean-up stage in Figures 2.4 & 2.5 respectively. Figure 2.4 of the feed extract shows that the SDM peak, which elutes just before 6 minutes, is not completely resolved from interfering coextractives. After the clean-up stage, baseline resolution is achieved by removal of the interfering coextractives, as is shown in Figure 2.5.

The clean-up stage of the analytical method had been demonstrated to be both effective in improving resolution of the SDM peak by removing interferents and also quantitative in that no SDM was lost in the process.
Figure 2.2
Chromatogram of load solution
after passing through cation-
exchange column

Figure 2.3
Chromatogram of solution used
to elute SDM from cation-
exchange column
Figure 2.4
Chromatogram of extraction solution before cation-exchange clean-up

Figure 2.5
Chromatogram of extraction solution after cation-exchange clean-up
2.1.2.2 Short-term precision of method

The precision of the method was evaluated over one day by analysis of both working standard solutions of SDM and feed sample extracts that had undergone the clean-up process. Five 10 g samples of a batch of feed were taken and analysed as described by Conway [48]. The results of the analyses are shown in Table 2.1.

Table 2.1
Analysis of Feeds – Short Term Precision Study

<table>
<thead>
<tr>
<th>Sample</th>
<th>SDM Found (μg g⁻¹)</th>
<th>RSD for Triplicate Injections (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>38</td>
<td>1.3</td>
</tr>
<tr>
<td>2</td>
<td>36</td>
<td>2.3</td>
</tr>
<tr>
<td>3</td>
<td>36</td>
<td>2.6</td>
</tr>
<tr>
<td>4</td>
<td>36</td>
<td>0.6</td>
</tr>
<tr>
<td>5</td>
<td>39</td>
<td>2.0</td>
</tr>
</tbody>
</table>

The samples taken showed good precision in terms of SDM content since levels found ranged from 36-39 μg g⁻¹ which gave a relative standard deviation of 4.25 %. This indicated that in the samples taken for analysis, SDM was uniformly distributed throughout the feed and that sampling did not affect the results significantly.
2.1.2.3 Short-term precision of SDM working standard solutions
Addressing the precision obtained for the analysis of the SDM working standard solution, eight injections of the SDM solution were chromatographed in two batches of four and the results are shown in Table 2.2. The RSD for the eight injections was only 0.8 % indicating a high level of precision.

Table 2.2
Short-term Precision of SDM Working Standard Solution
Injection onto HPLC Column

<table>
<thead>
<tr>
<th>Injection No.</th>
<th>SDM Peak Area (units²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>32.202</td>
</tr>
<tr>
<td>2</td>
<td>32.060</td>
</tr>
<tr>
<td>3</td>
<td>32.118</td>
</tr>
<tr>
<td>4</td>
<td>32.203</td>
</tr>
<tr>
<td>5</td>
<td>32.798</td>
</tr>
<tr>
<td>6</td>
<td>32.685</td>
</tr>
<tr>
<td>7</td>
<td>32.477</td>
</tr>
<tr>
<td>8</td>
<td>32.347</td>
</tr>
</tbody>
</table>

2.1.2.4 Long-term precision of SDM Working Standard Solutions
To investigate the precision of the SDM working standard solutions used in the quantitation of the extracted SDM solutions, the results from the injections of different concentration working standard solutions were analysed. Eleven different concentrations of SDM working standard solutions ranging from 1.16 - 25.76 µg ml⁻¹ were injected over a period of 2 years. The procedure followed was to inject the working standard solutions in triplicate and take the average integrated SDM peak area for use in the calculations. In total 60 batches of triplicate injections were made and in order to show the variation in the results, it was
decided to divide the average integrated SDM peak area by the concentration of the injected SDM working standard solution to obtain a ratio (Area / Concentration). The arithmetic mean of the 60 ratios was 2.96 with a RSD of 3.0% which was good considering that the analyses were made over a period of two years using two different HPLC columns. A plot of SDM peak area against SDM working standard solution concentration is shown in Figure 2.6. The correlation coefficient of the line of 0.996 indicated that Beer’s law was obeyed over the concentration range investigated.

![Figure 2.6](image)

**Correlation Coefficient = 0.996**

2.1.2.5 Estimation of errors

In order to estimate the errors associated with the analysis method, each stage in the analytical procedure was considered. The stages in both sample preparation and SDM working standard solution preparation are shown respectively in Figures 2.7 and 2.8, together with the associated error of each stage if applicable. Thus, in weighing out 10 g of feed, the error in the measurement is 0.0002 g. The errors in the use of the volumetric glassware are as stated by the manufacturers.
Figure 2.7
Errors associated with sample preparation

<table>
<thead>
<tr>
<th>Analysis stage</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weighing out feed (10g)</td>
<td>0.0002 / 10</td>
</tr>
<tr>
<td>Addition of 100 ml of extraction solution by pipette</td>
<td>0.12 / 100</td>
</tr>
<tr>
<td>Filtering</td>
<td>-</td>
</tr>
<tr>
<td>Removal of 10 ml aliquot by pipette</td>
<td>0.04 / 10</td>
</tr>
<tr>
<td>Addition of 5 ml glacial acetic acid</td>
<td>-</td>
</tr>
<tr>
<td>Addition of sample to clean-up column</td>
<td>-</td>
</tr>
<tr>
<td>Quantitative recovery of SDM in 25 ml of mobile phase</td>
<td>0.04 / 25</td>
</tr>
<tr>
<td>Filtering through 0.45 μm filter</td>
<td>-</td>
</tr>
<tr>
<td>Injection of 20 μl of solution onto HPLC column via injection loop</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 2.8
Errors associated with preparation of SDM working standard solution

<table>
<thead>
<tr>
<th>Analysis stage</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weighing 100 mg into volumetric flask</td>
<td>0.0002 / 0.10</td>
</tr>
<tr>
<td>Dilution to mark with methanol</td>
<td>0.1 / 100</td>
</tr>
<tr>
<td>Take 1 ml aliquot</td>
<td>0.02 / 1</td>
</tr>
<tr>
<td>Dilute to 100 ml in volumetric flask</td>
<td>0.1 / 100</td>
</tr>
<tr>
<td>Filter through 0.45 μm filter</td>
<td>-</td>
</tr>
<tr>
<td>Injection onto of 20 μl of solution onto HPLC column via injection loop</td>
<td>-</td>
</tr>
</tbody>
</table>
By combining all the error terms that arose due to the various sample manipulation stages, the error interval in the determination of SDM in animal feeds could be determined. Thus if \( x \) is the error associated with the measurement of \( X \), then

\[
\left( \frac{x}{X} \right)^2 = \left( \frac{0.004}{25} \right)^2 + \left( \frac{0.12}{100} \right)^2 + \left( \frac{0.04}{10} \right)^2 \\
+ \left( \frac{0.04}{25} \right)^2 + \left( \frac{0.0002}{0.1000} \right)^2 + \left( \frac{0.1}{100} \right)^2 + \left( \frac{0.02}{10} \right)^2 + \left( \frac{0.1}{100} \right)^2
\]

\[ x = 0.0207 \]

Therefore, if \( X = 55 \), \( x = \pm 1.16 \) which means that in the analysis of a feed containing SDM at a concentration of 100 \( \mu g \) g\(^{-1}\), then the error in the measurement is \( \pm 2.1 \% \)\( -\pm 2 \% \). For studies in which SDM has been added in the laboratory, the error is increased to \( \pm 4 \% \).

2.1.3 Discussion of method validation experiments

In the validation of the analytical method for SDM as described by Conway [48], it was demonstrated that the clean-up step enabled the separation of SDM from interfering coextractives with quantitative retention and then elution from the clean-up column. The precision of the method in the analysis of feed samples containing SDM was demonstrated, as was the precision associated with the SDM working standard solution. The estimation of errors showed that the theoretical error interval in the analysis of commercial feed samples was \( \sim \pm 2 \% \). Samples of feed in which the SDM was added in the laboratory gave an error interval of \( \sim \pm 4 \% \), with the additional 2\% being due to the error in the preparation of the SDM dosing solution and subsequent addition to the feed samples.

As was stated previously, errors due to mixing inefficiency resulting in an uneven distribution of drug throughout the feed, and sampling errors have been neglected. This was because it was assumed that the industrial mixing process is efficient in producing a homogeneous feed containing the drug and that an unbiased sampling procedure had been followed. The method was found to give both precise and accurate results for the samples tested.
2.2 DEVELOPMENT OF A DOSING METHOD
An inherent problem with any drug dosing method is firstly knowing the amount of drug added, and secondly, how well distributed the drug is in the sample. On the industrial scale, high efficiency mixers ensure that, on the whole, the drug is well distributed. On the laboratory scale, the problem arises in trying to obtain a uniform mix of drug and sample, especially with constituents with a wide variation in particle sizes. This ultimately leads to problems since, if a sub-sample is taken from the bulk, then the result will be influenced to a larger degree by the efficiency of the mixing and not by the decomposition /binding phenomena under investigation. It was, therefore, necessary to develop a small-scale dosing procedure for use in analytical studies so that samples can be dosed accurately with little or no errors due to sampling which would enable quantitation of non-recovered SDM.

2.2.1 Experimental
Mixer: Pascal No. 2 Conical Tumbler Mixer, (Pascal Engineering Company Ltd, Crawley, W. Sussex).
Chemicals: Calcium carbonate SLR, (Fisons, Loughborough, Leicestershire, UK).

2.2.2 Preparation of premix
2.2.2.1 Procedure
A SDM premix was prepared by adding SDM (25.1 g) to calcium carbonate (225.0 g) in a sample bottle and mixing for 6 hours using a conical tumbler mixer. The theoretical concentration of the premix was 10.0% by weight. Samples were then removed from the bottle from various locations. To allow for the increased concentrations of SDM present in the premix compared to a feed, 0.1g samples were used in the analysis instead of 10g specified by Conway. Additionally, 1 ml of the filtered extraction solution was taken for clean-up after addition of 9 ml of fresh extraction solution. These two modifications to the method ensured that the amounts of SDM being injected onto the HPLC column were the same as those when analysing feeds containing 100 µg g⁻¹ of SDM.
2.2.2.2 Results

Results from the analyses of these samples are shown in Table 2.3.

Table 2.3
Preparation of SDM Premix

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>SDM Content (µg g⁻¹)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Added</td>
<td>Found</td>
</tr>
<tr>
<td>1</td>
<td>10.0</td>
<td>7.9</td>
</tr>
<tr>
<td>2</td>
<td>10.0</td>
<td>7.6</td>
</tr>
<tr>
<td>3</td>
<td>10.0</td>
<td>7.7</td>
</tr>
<tr>
<td>4</td>
<td>10.0</td>
<td>6.8</td>
</tr>
<tr>
<td>5</td>
<td>10.0</td>
<td>9.7</td>
</tr>
</tbody>
</table>

The results show 68-97 % recovery of the drug which is probably due to the inefficiency of the mixing and inaccuracy of sampling. It is interesting to note that if the mixing is so inefficient, then it would be expected that, in some samples the SDM content would be greater than the theoretical content. In all cases, the SDM contents for the samples are less than the theoretical content. This might imply that there is some interaction occurring between SDM and the calcium carbonate. Alternatively, the fact that only 0.1 g of premix was used in the analysis could account for the poor distribution.
2.2.3 Dosing of wheat constituent with SDM premix

2.2.3.1 Procedure
The SDM premix prepared in 2.2.2.1 (0.3963 g) was added to Wheat (401.5 g) which had been ground for 30 seconds in a coffee grinder then passed through a 1 mm sieve. This was then mixed for 6 hours using a conical tumbler mixer. The theoretical SDM concentration was 99 μg g⁻¹.

2.2.3.2 Results
The results for analysis in duplicate after 24 hours contact time are shown in Table 2.4, together with results from the investigations reported in 2.2.4, 2.2.5 and 2.2.6.

Table 2.4
Results Summary for Dosing Experiment

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Sample</th>
<th>Dosing Method</th>
<th>Mixing Time (mins)</th>
<th>Stressing Time (mins)</th>
<th>Contact Time (hours)</th>
<th>SDM Content (μg g⁻¹) Added</th>
<th>SDM Content (μg g⁻¹) Found</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>A</td>
<td>Dry addition</td>
<td>360</td>
<td>-</td>
<td>24</td>
<td>99</td>
<td>103</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>99</td>
<td>106</td>
<td>107</td>
</tr>
<tr>
<td>Beans</td>
<td>A</td>
<td>solution</td>
<td>60</td>
<td>-</td>
<td>&lt;2</td>
<td>100</td>
<td>89</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td></td>
<td>60</td>
<td></td>
<td></td>
<td>100</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>HiPro Soya</td>
<td>A1</td>
<td>solution</td>
<td>5</td>
<td>-</td>
<td>&lt;2</td>
<td>94</td>
<td>92</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>A2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>96</td>
<td>92</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>B1s</td>
<td>solution</td>
<td>5</td>
<td>5</td>
<td>&lt;2</td>
<td>92</td>
<td>87</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>B2s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>96</td>
<td>94</td>
<td>98</td>
</tr>
<tr>
<td>Bone Meal</td>
<td>MB1</td>
<td>solution</td>
<td>5</td>
<td>-</td>
<td>24</td>
<td>93</td>
<td>95</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>MB2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>95</td>
<td>101</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>MB1s</td>
<td>solution</td>
<td>5</td>
<td>5</td>
<td>24</td>
<td>93</td>
<td>91</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>MB2s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>93</td>
<td>85</td>
<td>91</td>
</tr>
</tbody>
</table>

2.2.4 Dosing of beans with a solution of SDM in methanol (MeOH)

2.2.4.1 Procedure
SDM (0.1158 g) was weighed into a 100 ml volumetric flask and dissolved in MeOH, then diluted to the mark to give an SDM solution of concentration 1.158 mg ml⁻¹. A quantity of beans (314.8 g), which had been ground for 30 seconds in the grinder, was then dosed with 27.1 ml of the SDM solution to give a dosing level of 100 mg kg⁻¹. The sample was then mixed
on the conical tumbler mixer for 1 hour. After mixing, the MeOH was removed under gentle vacuum for 50 minutes.

2.2.4.2 Results
Duplicate analysis of the samples, as shown in Table 2.4, produced large variations in the values for SDM content which would indicate that the mixing had not been uniform. This could be explained by the fact that not all the dry material was ‘wetted’ by the dosing solution. The only disadvantage in dosing in this way is that it does not mimic the industrial method of addition. However, it was hoped that removing the MeOH after addition would allow the same interactions to occur as in a dry addition process.

2.2.5 Dosing of individual samples of HiPro Soya (HPS) with SDM in MeOH solution
Further work in the development of the dosing protocol was to investigate the use of a solution of SDM in methanol to dose small feed samples. It was considered best to dose the same quantity of feed as used in the analysis stage ie 10g, with a solution of SDM in methanol. It was hoped that, by adding the drug to the quantity of feed used in the extraction stage and then adding the extraction solution directly to the feed container then errors caused by uneven distribution of the drug in the feed and also errors caused by sampling would be removed.

2.2.5.1 Procedure
Four 250 ml conical flasks labelled A1, A2, B1s and B2s had 10g of HPS, which had been passed through a 1 mm sieve, accurately weighed into each. A dosing solution of SDM in MeOH of concentration 1.053 mg ml\(^{-1}\) was prepared, and 1 ml of this solution was added to each of the conical flasks in turn. The flasks were stoppered and shaken for 5 minutes to aid mixing. Flasks B1s and B2s were then stressed in a domestic pressure cooker for 5 minutes. The rationale of using the pressure cooker is explained in section 2.6.

2.2.5.2 Results
Extraction and HPLC analysis gave the results that are displayed in Table 2.4 and indicated that good recoveries were obtained.
2.2.6 Dosing of individual samples of Meat and Bone meal (MB) with SDM in MeOH solution

2.2.6.1 Procedure
Four 10g portions of MB were accurately weighed into plastic bottles labelled MB1, MB2, MB1s and MB2s. All samples were dosed with 1 ml of a freshly prepared solution of SDM in MeOH of concentration 0.951 mg ml\(^{-1}\). The sample bottles were then capped and shaken for 5 minutes to aid mixing. Samples MB1s and MB2s were then stressed for 5 minutes. The stressing process is described in detail in section 2.6.

2.2.6.2 Results
Extraction and HPLC analysis gave the results that are displayed in Table 2.4 and show that the stressed samples gave slightly higher recoveries than the non-stressed samples.

2.2.7 Discussion of dosing experiments results
A dosing method involving adding SDM in the form of a premix, and SDM in calcium carbonate, to large quantities of feed materials was not found to be suitable for use in analytical studies due to the inhomogeneity of the final mixture. Samples taken from the mixture would then have been dependent upon the distribution of the drug premix throughout the feed and would have masked any interaction effects that were to be studied. Additionally, errors would have been associated with the distribution of SDM in the premix which further complicated the method. Similarly, dosing large quantities of feed materials with a solution of SDM in methanol introduces distribution problems due to the fact that not all portions of the feed are wetted by the dosing solution.

In the method of dosing small portions of feed with a premix of SDM, although errors due to distribution of the premix in the feed sample are negligible because the whole sample is used in the analysis, errors are still present due to the distribution of SDM in the premix. This becomes all the more important because only a small quantity of premix is used to dose the individual samples.

The method chosen finally was dosing small feed samples with a solution of SDM in methanol. It was felt that, although this did not mimic the industrial method of drug
addition, the advantages in that, an exact amount of drug could be added to each sample, far outweighed the disadvantages. Thus, the size of feed portions dosed were the same size as those used in the analysis stage. This allowed addition of the drug dosing solution to the sample container, mixing of the solution and feed, and evaporation of the methanol followed by addition of the extraction solution directly to the sample container. This eliminated sampling errors.

2.3 ANALYSIS OF FEED SAMPLES
The problem of progressively poorer recoveries with time can be seen in the results generated for the analyses of samples at LGC and also subsequent analyses of the same samples at Loughborough University of Technology (LUT). A results summary for these batches, analysed previously by LGC, are shown in Table 2.5. There appeared to be a uniform decrease in the SDM recoveries found by LUT compared to those found by LGC, with this shown in Figure 2.9. Calculations show that this decrease is between 20-22% in all cases. At first sight, this was thought to have been due to a systematic error occurring, but on closer inspection of the results for the analyses, no evidence could be found for such a gross error. It was proposed that because all the feed samples had been stored in the same type of plastic containers, filled to the same level, and stored in the laboratory together, all the feeds had experienced similar effects. The fact that SDM recoveries decrease on storage, might be used to explain that if the storage conditions of a series of feed batches are identical then similar decreases in SDM recoveries should result. The effect of storage conditions on SDM recoveries is addressed in Chapter 3 in detail.
### Table 2.5

**Analysis of Feeds**

<table>
<thead>
<tr>
<th>Feed Sample</th>
<th>2/8/90</th>
<th>11/9/90</th>
<th>4/12/90</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M</td>
<td>72</td>
<td>56</td>
<td>45</td>
</tr>
<tr>
<td>2M</td>
<td>80</td>
<td>51</td>
<td>56</td>
</tr>
<tr>
<td>3M</td>
<td>61</td>
<td>70</td>
<td>55</td>
</tr>
<tr>
<td>4M</td>
<td>71</td>
<td>62</td>
<td>49</td>
</tr>
<tr>
<td>5M</td>
<td>63</td>
<td>64</td>
<td>51</td>
</tr>
</tbody>
</table>

### Figure 2.9

**Analysis of Feed Samples 1M-5M at time intervals**

![Graph showing SDM concentration over time for different feed samples.](image-url)
2.4 RECOVERY OF SDM FROM FEED STRAIGHTS (I)

To simplify the recovery problem, it was decided to investigate the recovery of SDM from individual feed components. Consultation with a feed mill led to samples being supplied of the following constituents or straights: soya hulls, rice bran, maize gluten, barley, cottonseed, salseed, oatfeed, choc mix, wheatfeed and rapeseed. The SDM premix used in the study had been prepared by LGC, and shown to be well mixed. This was therefore used to dose the constituents to mimic the industrial drug addition process.

2.4.1 Experimental

Thirteen feed constituents (250 g) were weighed into individual 1 l amber wide-necked glass storage bottles. To each was added 16.11 g of a 10 % SDM in calcium carbonate premix prepared by LGC to give a dosing level of 52 μg g⁻¹. All bottles were then sealed and sent to LGC where each was mixed for 24 hours on a high efficiency ‘Turbula’ mixer then sent back to LUT. It was hoped that, by use of the high-efficiency mixer, good distributions of SDM throughout the constituents could be achieved. To investigate the mixing efficiency, random samples were taken from a selection of the bottles and analysed.

2.4.2 Results

The results of the analysis are shown in Table 2.6 and indicate that, in spite of the efficiency of the mixer, discrepancies occurred between the theoretical dosing level and the experimentally found level. It was, therefore, decided to dose small samples with a solution of SDM in MeOH in order to reduce errors.
<table>
<thead>
<tr>
<th>Sample No.</th>
<th>SDM Content ($\mu$g g$^{-1}$)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Added</td>
<td>Found</td>
</tr>
<tr>
<td>Beans</td>
<td>52</td>
<td>44</td>
</tr>
<tr>
<td>Barley</td>
<td>52</td>
<td>46</td>
</tr>
<tr>
<td>Oat Feed</td>
<td>52</td>
<td>50</td>
</tr>
<tr>
<td>Rape Seed</td>
<td>52</td>
<td>34</td>
</tr>
<tr>
<td>Pig Feed</td>
<td>52</td>
<td>41</td>
</tr>
</tbody>
</table>

2.5 RECOVERY OF SDM FROM FEED STRAIGHTS (II)

The previous recovery study investigating the recovery of SDM from feed straights did not give satisfactory results when many of the constituents were analysed to determine the efficiency of the mixing process. To improve on this, small-scale dosing of samples were performed, and, in view of the large number of samples that needed to be prepared, dosing and analysis in the same container could not be achieved using the 250 ml conical flasks used previously.

2.5.1 Experimental

Several different containers were evaluated as to their suitability, with the final choice being 50 ml plastic sample tubes supplied by Northern Media which were capable of withstanding temperatures of up to 140 °C, necessary for use in stressing studies. The only problem was that because of the 50 ml capacity, sample analysis size had to be scaled down. It was found that 2 g of constituent was a suitable amount for each tube, and in order to keep the same ratio of constituent and extraction solution volume as used in the method developed by
Conway, 20 ml of extraction solution was used. It was also decided to increase the dosing level to 500 μg g⁻¹, so that although only 20% of the normal quantity of feed was used, the same level of SDM would be injected onto the HPLC column. Addition of the SDM solution was performed by means of a 1000 μl glass syringe.

The constituents chosen were Wheatfeed (WF), HiPro Soya (HPS) and Rapeseed (R) and the typical nutritional characteristics are summarised in Table 2.7. The HPS and R are high in protein while the WF is high in starch.

Table 2.7

Nutritional information on selected constituents [50]

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Starch (g/kg)</th>
<th>Sugar (g/kg)</th>
<th>Crude Protein (g/kg)</th>
<th>Oil* (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheatfeed</td>
<td>300</td>
<td>90</td>
<td>170</td>
<td>45</td>
</tr>
<tr>
<td>HiPro Soya</td>
<td>10</td>
<td>120</td>
<td>520</td>
<td>10</td>
</tr>
<tr>
<td>Rapeseed</td>
<td>60</td>
<td>100</td>
<td>420</td>
<td>25</td>
</tr>
</tbody>
</table>

* Oil determined by petroleum ether extraction

Two grams of constituent were accurately weighed into separate sample tubes to which was added 500 μl of a 2.1 μg ml⁻¹ SDM in MeOH solution. Each sample tube was capped, then mixed for 10 seconds using a vortex mixer to ensure sufficient interaction of the solution with the constituent. Half the samples were then stressed for 10 minutes (caps removed), and then the stressed and non-stressed samples were left for 1 hour in a fume hood to allow the MeOH to evaporate. Removal of the MeOH under vacuum was not possible due to the large numbers of samples.
SDM recoveries were performed at 0 months, 1 month, 2 months and 3 months. At each time point, duplicate samples of stressed and non-stressed constituents were analysed together with two constituent blanks, one of which had been stressed. The blanks served as an indication of any change in the composition of the constituent since various components from the constituent were extracted with the extraction solution. The Diode Array Software permitted all wavelengths between 200 and 400 nm to be monitored.

2.5.2 Results

Results for the analyses of the Initial, One, Two and Three month time points are shown in Figures 2.10-2.12 as graphs of recovery versus time for each constituent.

![Figure 2.10](image-url)

**Figure 2.10**

Recovery of SDM from HiPro Soya (HPS)

SDM Recovery (%)

Time / months

0 1 2 3 4

0 80 90 100

stressed

non-stressed
Figure 2.11
Recovery of SDM from Wheatfeed (WF)

Figure 2.12
Recovery of SDM from Rapeseed (R)
The HPS samples gave some interesting results. The samples that had been stressed would have given a fairly constant decrease in SDM recoveries had it not been for the result obtained at 1 month, which was very low compared to the results at 2 and 3 months. It could only be thought that the result at 1 month was erroneous, since the same dosing solution and syringe had been used to dose all the samples, and subsequent samples gave much improved SDM recoveries.

The HPS samples that had not been stressed gave an approximate 10% decrease in SDM recovery at 1 month as did the stressed HPS sample. Both stressed and non-stressed samples gave two similar SDM recoveries at 2 and 3 months. No explanation can be offered for the behaviour of the stressed and non-stressed samples.

The stressed Wheatfeed sample gave a steady decrease in SDM recovery over the period of the study, and recoveries were always greater than the corresponding non-stressed samples, except at the initial time point. The non-stressed Wheatfeed samples gave a sharp initial drop in SDM recovery from the initial to the 1 month time point, but then showed a more steady decrease.

The stressed Rapeseed samples all showed a fairly uniform decrease in SDM recoveries with the line being almost linear. Similarities were noted to the Wheatfeed samples in that, except in all but the initial and 3 month samples, the stressed samples gave higher SDM recoveries than the corresponding non-stressed samples.

2.5.3 Discussion
It is interesting to note that the constituents with the highest protein contents, Rapeseed and HiPro Soya, did not produce the biggest decreases in SDM recoveries. This was found in the non-stressed wheatfeed samples, but not in the stressed wheatfeed samples. The wheatfeed samples contained the highest starch levels of the three constituents.

Initial suggestions that SDM might be binding to proteins [48], because of its known high affinity for plasma proteins in vivo, should be questioned.
2.6 THE PELLETEDING PROCESS

The pelleting process subjects the feed to high pressures, temperatures and mechanical stresses. Both the moisture content and the temperature of the feed are increased during the conditioning stage of pellet production. Since pelleted feeds have a higher moisture content than non-pelleted feeds, due to the conditioning stage, the effect of increasing both the moisture content and the temperature of a feed on SDM recovery was investigated. The studies performed previously with Wheatfeed, HiPro Soya and Rapeseed all gave results that could not be explained. It was hoped that, by performing additional experiments on the effect of conditioning feed samples, some sense could be made out of the results of the previous studies.

In order to mimic the conditioning process used in pellet manufacture, a domestic pressure cooker was used because of its similar operating conditions. The conditioning process has steam injected at pressures of between 15-45 psi and the product attains temperatures of 70 °C and above. The pressure cooker has a nominal operating pressure of 103 kPa (15 psi) and in initial tests, the feeds were measured at >75 °C after 5 minutes stressing time, indicating that it did mimic the industrial conditioning process in certain aspects. The pressure cooker used was a Prestige Pressure Cooker Model No. 6189 TT.

2.6.1 Effect of pressure and temperature on SDM recovery (I)

2.6.1.1 Experimental

A study was then initiated using a feed previously dosed with a SDM premix. Six 10 g samples were taken from the bottle with two being analysed immediately for SDM. Two were stressed in the pressure cooker for 10 minutes, then analysed. The final two samples were analysed after first extracting the SDM using a Soxhlet apparatus in which the solvent was kept recycling for 7 hours.

2.6.1.2 Results

The results are shown in Table 2.8 and appear to indicate that the stressing in the pressure cooker reduces the recovery of SDM from the feed compared to the non-stressed samples, but some concern was raised over the results for the two unstressed samples since there was quite a large difference between the two results compared to the two values for SDM content.
of the stressed samples. Also the Soxhlet extraction performed on the remaining two feed samples did not give any improvement in recoveries compared to the normally used extraction with cold extraction solution for 1 hour.

Table 2.8

Effect of stressing on SDM recovery from feeds

<table>
<thead>
<tr>
<th>Sample</th>
<th>SDM Content (µg g⁻¹)</th>
<th>Recovery (± 4 %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Added</td>
<td>Found</td>
</tr>
<tr>
<td>1</td>
<td>52</td>
<td>38</td>
</tr>
<tr>
<td>2</td>
<td>52</td>
<td>46</td>
</tr>
<tr>
<td>3*</td>
<td>52</td>
<td>32</td>
</tr>
<tr>
<td>4*</td>
<td>52</td>
<td>31</td>
</tr>
<tr>
<td>5*</td>
<td>52</td>
<td>35</td>
</tr>
<tr>
<td>6*</td>
<td>52</td>
<td>37</td>
</tr>
</tbody>
</table>

* stressed for 10 minutes then analysed

* analysed after Soxhlet extraction

2.6.1.3 Discussion

In light of the results of this experiment that stressing appeared to reduce the SDM extracted, further investigations into the stressing process were planned.

Addressing the question of the effect of stressing on both SDM and the feed led to two hypotheses. Firstly, with the increased temperature, pressure and moisture the SDM was degrading. The second was that instead of the conditions during stressing degrading SDM, they were increasing the interaction between the feed and the drug resulting in increased binding.
2.6.2 Effect of pressure and steam on the recovery of SDM (II)
Conway [48] suggested possible SDM-Protein binding to explain the poor recoveries obtained.

2.6.2.1 Experimental
To examine this further, a protein feed constituent, Hi Pro Soya [HPS], was obtained from Dalgety Agriculture Ltd., South Wigston, and 20 g samples were prepared as follows:
1. HPS control (not dosed with SDM and not stressed)
2. HPS control stressed for 5 minutes
3. HPS dosed with premix (97 µg g⁻¹) and not stressed
4. HPS dosed with premix (107 µg g⁻¹) and stressed for 5 minutes

2.6.2.1 Results
The results of the analyses are shown in Table 2.9.

Table 2.9
Effect of stressing on SDM recovery from HPS

<table>
<thead>
<tr>
<th>Sample</th>
<th>SDM Content (µg g⁻¹)</th>
<th>Recovery (± 4 %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Added</td>
<td>Found</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>97</td>
<td>96</td>
</tr>
<tr>
<td>4</td>
<td>107</td>
<td>98</td>
</tr>
</tbody>
</table>
2.6.2.3 Discussion

In sample 3, the recovery of the SDM of 99% was greater than the stressed sample, 4, which was 92%. Although the stressing had produced a slight decrease in the recovery of SDM when the samples were analysed immediately after stressing, it was thought that analysis of the stressed samples with time would show larger differences. This would agree with the results of SDM recoveries shown in Figures 1.1 & 1.2 where initially the difference between pelleted and non-pelleted feeds was negligible but then increased with time.

2.6.3 Effect of stressing SDM

2.6.3.1 Preliminary study

The effect of stressing on SDM was investigated to determine whether the process of stressing caused degradation in the SDM molecule. Three 0.1g samples of SDM were stressed in a pressure cooker for 5, 10 and 15 minutes respectively. After removal from the pressure cooker, the respective samples were dissolved in methanol and analysed using a Fourier Transform Infra-Red spectrometer (FTIR) to detect any changes in structure that might have been induced by the stressing process. Figure 2.13 shows the FTIR spectra for the three solutions indicating that all three are identical. Figure 2.14 compares the sample stressed for fifteen minutes with a reference standard of SDM and shows that there is no difference in the spectra between the two.
Figure 2.13
FT-IR spectra of SDM stressed for 5, 10 and 15 minutes

sdmstrs5: SDM 5mins stressing
sdmstr10: SDM 10mins stressing
sdmstr15: SDM 15mins stressing
Figure 2.14
FT-IR spectra of SDM stressed for 15 minutes and a reference standard of SDM

sdmref: SDM reference
sdmstr15: SDM 15mins stressing
2.6.3.2 HPLC study

The degradation products of SDM are reported by Zajac [51] and are shown in Figure 2.15.

Figure 2.15

Degradation Products of SDM

Extending the previous study, SDM (0.1004g) was stressed for 10 minutes then dissolved in acetonitrile, transferred to a 100 ml volumetric flask and made up to the mark. A 1 ml aliquot was then taken from this solution and diluted to 100 ml in a volumetric flask using HPLC mobile phase to give a solution of concentration 10.04 µg ml⁻¹. Triplicate injections of this solution were made on the HPLC with the run time increased from 8 to 15 minutes. A representative chromatogram is shown in Figure 2.16 and it can be seen that no degradation products can be seen.
Figure 2.16
Chromatogram of stressed SDM
Solutions of 2-amino-4,6-dimethylpyrimidine (2ADMP) and sulphanilamide (SPM) were prepared of concentrations 0.508 and 0.36 mg ml$^{-1}$ respectively. These were then injected and the chromatograms obtained are shown in Figures 2.17 and 2.18. The retention time for 2ADMP was 3.9 minutes with a broad peak. For SPM, the retention time was 2.5 minutes with a sharp peak being obtained. Sulphanilic acid and 2-hydroxy-4,6-dimethylpyrimidine are also shown in Figures 2.19 and 2.20. All but 2,4-dimethyl-6-hydroxypyrimidine produced a peak which demonstrated that had SDM undergone degradation then it would have been observed.

2.6.4 Discussion of SDM stressing experiments

Subjecting SDM to elevated temperatures, pressures and moisture levels did not produce any degradation products. This fits in with the known stability of SDM since when administered to an animal, a large proportion of SDM is excreted as the acetylated form of SDM, ie it has not undergone degradation. Relating the fact that SDM does not undergo degradation when stressed to SDM recovery differences between pelleted and non-pelleted feeds necessitates an alternative explanation.
Figure 2.17
Chromatogram of 2-amino-4, 6-dimethyl pyrimidine reference standard
Figure 2.18
Chromatogram of Sulphanilamide reference standard
Figure 2.19
Chromatogram of sulphanilic acid reference standard
Figure 2.20
Chromatogram of 2,4-dimethyl-6-hydroxypyrimidine
2.7 ADSORPTION OF SDM ON FEED

As degradation of SDM was not observed, irreversible binding of SDM to feed constituents was investigated. It was thought unlikely that SDM was binding to an individual component because of results from studies in which individual feed constituents were dosed with SDM showed that poor drug recoveries were obtained from a range of constituents. Irreversible binding of SDM to feed constituents requires that suitable binding sites are present in the feed. Experiments were designed where the amount of SDM added to uniform amounts of feed were varied to see whether there was a limited number of binding sites in the feed samples. If this were the case, then the results would be expected to show increased SDM recoveries from samples that had the largest amounts of SDM added. This would be due to the fraction of SDM not bound being greater than in a sample where less SDM was added.

2.7.1 Adsorption of SDM on feed - preliminary study

2.7.1.1 Experimental

To investigate the possibility of sorption phenomena occurring, it was decided to vary the amount of SDM added to the feed samples. An initial study was performed whereby 2 g samples of feed were dosed with a solution of SDM in methanol to give the following SDM contents: 23, 113, 226, 565, 1129 μg g⁻¹. All the samples were stored at room temperature and wrapped in foil to protect them from light. Extraction of SDM and analysis was performed after 50 days contact time between SDM and the feed.

2.7.1.2 Results

The results are shown in Table 2.10 with the corresponding plot shown in Figure 2.21. The computer-fitted straight line with a correlation coefficient of 0.998 indicates that there is a relationship between the amount of drug added and SDM recovery. The line does not pass through the origin, and this may be due to the limit of detection (LOD) of the method being ~10 μg g⁻¹ introducing a bias. The LOD would normally be lower because the sensitivity of a single wavelength uv detector is greater than a diode array detector. The LOD was found to be the lowest concentration of SDM, in a freshly spiked feed, that was capable of producing a chromatographic peak that could be identified by taking its uv spectrum.
Table 2.10
AIS – Preliminary

<table>
<thead>
<tr>
<th>SDM Concentration (ug g⁻¹)</th>
<th>SDM Recovery (± 4 %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Added</td>
<td>Found</td>
</tr>
<tr>
<td>11</td>
<td>-</td>
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<td>275</td>
<td>152</td>
</tr>
<tr>
<td>547</td>
<td>330</td>
</tr>
</tbody>
</table>

Figure 2.21
Adsorption of SDM on feed - Preliminary study

\[ y = -12.832 + 0.62096x \quad \text{R}^2 = 0.998 \]
2.7.2 Adsorption of SDM on feed - long-term study

In view of the results obtained in the preliminary study, it was decided to repeat the study with an increased number of samples to increase the number of time points.

2.7.2.1 Experimental

Sample tubes labelled A 1-16, B 1-16 and C 1-16 containing feed (2g) were prepared and the previously described dosing protocol followed using known dilutions of a solution of SDM in methanol. The dosing levels ranged from 11-500 µg g⁻¹. Samples labelled 15 and 16 were controls and were dosed with only 0.5 ml of methanol. Analyses were then performed at 7, 28 and 42 days on all samples.

2.7.2.2 Results

The results of the study are shown in Tables 2.11-2.13 and the graphs of SDM recovered against SDM added are shown in Figure 2.22.

Table 2.11
Adsorption Isotherm Study TP1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Contact Time (days)</th>
<th>SDM Concentration (µg g⁻¹) Added</th>
<th>SDM Concentration (µg g⁻¹) Found</th>
<th>Percentage SDM Recovery (± 4%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>7</td>
<td>12</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A2</td>
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</table>
Table 2.12
Adsorption Isotherm Study TP2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Contact Time (days)</th>
<th>SDM Concentration (ug g⁻¹) Added</th>
<th>SDM Concentration (ug g⁻¹) Found</th>
<th>Percentage SDM Recovery (± 4 %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>28</td>
<td>12</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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Table 2.13
Adsorption Isotherm Study TP3

<table>
<thead>
<tr>
<th>Sample</th>
<th>Contact Time (days)</th>
<th>SDM Concentration (ug g⁻¹) Added</th>
<th>SDM Concentration (ug g⁻¹) Found</th>
<th>Percentage SDM Recovery (± 4 %)</th>
</tr>
</thead>
<tbody>
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<td>C1</td>
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<tr>
<td>C16</td>
<td>42</td>
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<td>-</td>
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</tr>
</tbody>
</table>
The three time points all gave plots approaching straight lines indicating that the relationship between added SDM and recovered SDM that was seen in the preliminary study, was also seen in this study. The change in slope at 7 to 28 days was far greater than the change in going from 28 to 42 days. Thus it would appear that the greatest changes occur in the first few weeks of drug-feed contact. Interpretation of the changes in slopes could be explained by the system approaching some kind of equilibrium.

2.7.3 Adsorption isotherm study using aqueous SDM dosing solution
Some concern was raised with the results of the adsorption isotherm studies since the system was composed of SDM, feed, water and methanol, and, methanol was thought perhaps to alter the system.

2.7.3.1 Experimental
In order to simplify the system, it was proposed that a solution of SDM in water, as opposed to methanol, should be prepared and used to dose feed samples to different levels. The only problem with this was that, owing to the reduced solubility of SDM in water, compared to methanol, only a relatively dilute solution could be prepared. This meant that the maximum dosing level achievable using the undiluted aqueous SDM solution was $\sim 170 \, \mu g \, g^{-1}$. Therefore a pilot study was initiated dosing feed samples at 2 different levels; $\sim 86 \, \mu g \, g^{-1}$ and $\sim 171 \, \mu g \, g^{-1}$. The drug-feed contact time was 87 days when extraction and analysis took place.
2.7.3.2 Results
The results are displayed in Table 2.14. The SDM recoveries were similar between the two dosing levels, although it was thought that the SDM recoveries obtained were low when compared to other feed samples of similar age.

Table 2.14

Adsorption Study using aqueous SDM dosing solution

<table>
<thead>
<tr>
<th>Sample</th>
<th>SDM Concentration (µg g⁻¹)</th>
<th>Percentage SDM Recovery (± 4 %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Added</td>
<td>Found</td>
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<tr>
<td>1</td>
<td>86</td>
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<td>4</td>
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<td>21</td>
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<td>6*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7*</td>
<td>-</td>
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</tr>
</tbody>
</table>

*Control samples dosed with 1 ml water

2.7.4 Discussion of SDM adsorption studies
The results from the studies where methanol was used to prepare a dosing solution of SDM showed the expected relationship, if it is assumed that there are a limited number of binding sites in the feed. Results from the experiment where a solution of SDM in water was used to dose the feed samples did not show any differences in SDM recoveries between the two dosing levels used, but the dosing range was not as large as in the studies using SDM in methanol solutions. Although the SDM in water dosing solution study did not produce the expected results, the recoveries obtained were thought to be lower than would have been obtained using SDM in methanol.
2.8 EFFECT OF INCREASING SPECIFIC SURFACE AREA ON
SDM RECOVERY

2.8.1 Experimental
With the results from the adsorption studies giving the expected results for binding, it was
decided to investigate whether the binding of SDM to the feed constituents could be
increased, ie the recovery of SDM decreased, by increasing the specific surface area of the
feed. The effect of increasing the specific surface area of the feed would be to increase the
number of sites available to SDM. The only drawback in the experimental design concerned
the possibility that, by reducing the particle size of the feed, the process of grinding could
modify the binding sites detrimentally, and thus the expected effect of increased binding of
SDM to the feed constituents would not be seen.

After careful consideration, the effect of grinding on the chemistry of the binding sites was
ignored. This was justified because the the grinding process took place in a domestic coffee
grinder which was not particularly harsh on the particles during size reduction. Thus it was
felt that the chemistry of the binding sites would be unchanged.

The effect of increasing the surface area of a feed, by reducing the particle size in a grinder,
on SDM recovery was investigated. A quantity of feed\(^b\) (~10g) was ground for one minute
using a domestic coffee grinder. The feed was then sized using sieving and a Malvern 2600c
series laser particle sizer to give the particle size distribution of the feed which is shown in
Figure 2.23. The effect of the grinding process on the particle size distribution of the feed
can be clearly seen. Three 2g portions of the ground feed were accurately weighed into
sample tubes (labelled 1-3). Two grams of control feed were weighed into three other
sample tubes (labelled 4-6). Each sample was dosed with a solution of SDM in methanol
to give SDM levels of 662-695 \(\mu g \, g^{-1}\). The samples were then stored in a polystyrene box
and left for 18 days to allow interaction between the SDM and the feed.

2.8.2 Results
Analysis of the samples gave the results shown in Table 2.15.
2.8.3 Discussion

The results indicated that the increase in specific surface area did not produce any decrease in SDM recoveries. It could be deduced from the results, that the binding of SDM was not exclusively confined to the particles' surfaces.

Table 2.15
Effect of increasing surface area on SDM recovery

<table>
<thead>
<tr>
<th>Sample</th>
<th>SDM Concentration (μg g⁻¹) Added ±2% Found</th>
<th>Percentage SDM Recovery (± 4 %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>695</td>
<td>557</td>
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<tr>
<td>2</td>
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</tr>
<tr>
<td>6</td>
<td>679</td>
<td>529</td>
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</tbody>
</table>
2.9 EFFECT OF SOLVENT PRETREATMENT OF FEEDS ON SDM RECOVERY

2.9.1 Background
The report by the Analytical Methods Committee [49] contained details of an experiment in which a quantity of fishmeal was extracted with an organic solvent to remove the fat. The recovery of SDM from the feed treated in this way was compared to the same feed that had been untreated. Additionally, the oil used to remove the fat was distilled off and was medicated with SDM, as were samples of both oleic acid and stearic acid, which were chosen to represent typical unsaturated and saturated fatty acids. After allowing the mixtures and SDM to have the same contact times, the recovery of SDM from the samples was determined. It was reported that recoveries from the fishmeal, the fishmeal oil and the oleic acid fell, but the recoveries from the defatted fishmeal or the stearic acid did not.

2.9.2 Effect of hexane pretreatment of feed samples on SDM recovery
To investigate whether removing the fat from a feed would result in higher SDM recoveries compared to a feed that had not been defatted, 100 g of feed was weighed into a wide-necked 250 ml conical flask. To this was added 500 ml of hexane, and the mixture was agitated for 5 minutes. The flask was then wrapped in foil to protect it from light and left overnight. The following day, the hexane was decanted off and the feed allowed to air-dry. Duplicate samples of the defatted feed and untreated feed were weighed into sample tubes and a solution of SDM in methanol added to each to give SDM concentrations of 508-512 µg/g of feed. The samples were left for 36 days to allow interaction then were extracted. The SDM recoveries from both sets of samples were similar, averaging at 41% of the total drug added. Explanation of the similarity of the results from the defatted and control feed, as in the Analytical Methods Committee [49] study, was thought to have been due to the fact that the total oil content of the feed was 5%, but the oil content of the fishmeal used in the Analytical Methods Committee [49] study was 10%.

2.9.3 Partition of SDM between soya oil and water
The increased recoveries from defatted fishmeal samples was investigated further by examining whether there was a distribution of SDM between an aqueous phase and a soya bean oil phase. A saturated SDM solution was prepared in water and filtered through a 0.45
μm filter to give a concentration of 335 μg ml⁻¹. 50 ml of the SDM solution was pipetted into a 250 ml conical flask and 50 ml of soya oil added. The flask was gently swirled, then wrapped in aluminium foil to protect from light. Samples from the aqueous phase were withdrawn after 3 and 90 days. The concentration at 3 days was found to be 335 μg ml⁻¹ and at 90 days 213 μg ml⁻¹. Observation at 90 days showed that SDM had come out of solution and was resident at the phase boundary. Intermediate time points between 3 and 90 days were not possible due to the diode array detector being repaired. From the results it could be concluded that SDM had little or no solubility in the oil phase and hence the distribution between the oil and water was entirely concerned with the solubility of SDM in the water. This was undertaken to gain an understanding of the structure of the feed.

2.10 MICROSCOPIC EXAMINATION OF FEEDS AND FEED STRAIGHTS
Examination of a feed that had been pelleted, using Scanning Electron Microscopy (SEM), is shown in Figures 2.24 - 2.27. The low magnification samples shown in Figures 2.24 and 2.25 show the smooth nature of the surface of the pellet. Following grinding, Figures 2.26 and 2.27 show the internal structure of the pellets as being porous with a large range of discreet structures being visible.

Examination of the following constituents: wheat, fish meal, rice bran, barley, HPS, calcium carbonate, full fat soya, malt culms, meat and bone meal, and wheatfeed followed.

2.10.1 Wheat
The two SEM micrographs, shown in Figures 2.28a and 2.28b, are of wheat. The first micrograph, Figure 2.28a, is at x35 magnification and shows a typical particle. The second micrograph, Figure 2.28b, is of the surface of the particle at x500 magnification showing starch grains and the porous nature of the particle. Additional scanning of the samples showed what appeared to be two types of particles present: fibrous particles which are probably derived from the husk; and the particles shown in the micrographs.

2.10.2 Fishmeal
Figures 2.29a and 2.29b are of two SEM micrographs of a fishmeal sample. The first micrograph, Figure 2.29a, is at x20 magnification and shows the heterogeneous nature of
the constituent. The second, Figure 2.29b, is an enlargement of the first micrograph, being magnified to x200. The differences in shading of the particles is due to uneven coating with gold in the sputtering process of sample preparation. Overall the heterogeneous nature of the particle shapes and sizes can be seen with the fishmeal showing evidence of being a fairly porous material due to spaces in between particles.

2.10.3 Rice bran
Rice bran is shown in Figures 2.30a and 2.30b. The first micrograph, Figure 2.30a, at x30 magnification and shows the range of particle sizes and shapes. The particles appear to be extremely heterogeneous. The second micrograph, Figure 2.30b, is a close-up of a particle shown in the first micrograph at x89 magnification. It shows the fairly scaly nature of the particle surface. Again, as was seen with fishmeal, the material is extremely heterogeneous.

2.10.4 Barley
Figures 2.31a and Figure 2.31b show two micrographs of a barley sample. The first micrograph, Figure 2.31a, at x40 magnification shows that there are maybe only two components: husk and starch material. A particle from the first micrograph is enlarged at x400 magnification, Figure 2.31b, and shows that the surface is composed of small starch granules tightly packed together.

2.10.5 HPS
Examination of the HPS showed that it is composed of fairly similar particles. The first micrograph in Figure 2.32a shows a particle at x50 magnification in which a hole / fold is evident in the centre of the particle. Further magnification, x250 magnification, Figure 2.32b, shows that there are both large and small cracks.

2.10.6 Calcium carbonate
Figure 2.33a shows a micrograph of calcium carbonate at x350 magnification which is in the calcite form, being rhombic, and is the most stable of the three forms. The larger structures are agglomerates of small particles. Figure 2.33b clearly shows the rhombic structures at x2000 magnification.
2.10.7 Full-fat soya
On first inspection of the sample, it was apparent that it was heterogeneous with a wide range of particle size distributions. There appeared to be large particles with very small granules attached. In Figure 2.34a, the first micrograph at x12 magnification shows the wide range of particle sizes. The second micrograph, Figure 2.34b, shows an individual particle magnified x60.

2.10.8 Malt culms
As with full-fat soya, malt culms appeared to be heterogeneous with a wide range of particle sizes and shapes. Malt culms are the dried rootlets from malting barley, and the various components can be seen in the first micrograph, Figure 2.35a, which is at x12 magnification. A single particle from the field of view is shown in the second micrograph at x75, Figure 2.35b, and the smooth surface, with lots of small particle debris attached to it can be seen.

2.10.9 Meat and Bone meal
The meat and bone meal sample appeared to be heterogeneous and is shown in Figure 2.36a, at x15 magnification. Closer examination of one of the particles is shown in Figure 2.36b at x250 magnification and revealed the complex morphology of the surface, with numerous cracks and pores.

2.10.10 Wheatfeed
The range of particle sizes and shapes is shown in Figure 2.37a which is at x19 magnification. Closer examination of an individual particle, 2.37b, at x150 magnification, shows the porous nature of the particle.

2.10.11 Discussion
The SEM examination of the feed constituents showed that all except the calcium carbonate were heterogeneous and exhibited some degree of porosity.
Figure 2.24
SEM Micrograph of pellet [x17]

Figure 2.25
SEM Micrograph of pellet [x100]
Figure 2.26
SEM Micrograph of pellet fragment after grinding [x250]

Figure 2.27
SEM Micrograph of pellet fragment after grinding [x500]
Figure 2.28a
SEM Micrograph of wheat particle [x35]

Figure 2.28b
SEM Micrograph of wheat particle [x500]
Figure 2.29a
SEM Micrograph of fishmeal [x20]

Figure 2.29b
SEM Micrograph of fishmeal [x200]
Figure 2.30a
SEM Micrograph of Ricebran
[x30]

Figure 2.30b
SEM Micrograph of Ricebran
[x89]
Figure 2.31a
SEM Micrograph of Barley
[x40]

Figure 2.31b
SEM Micrograph of Barley
[x400]
Figure 2.32a
SEM Micrograph of Hi Pro Soya (HPS) particle [x50]

Figure 2.32b
SEM Micrograph of Hi Pro Soya (HPS) particle [x250]
Figure 2.33a
SEM Micrograph of calcium carbonate [x350]

Figure 2.33b
SEM Micrograph of calcium carbonate [x2000]
Figure 2.34a
SEM Micrograph of full-fat soya [x12]

Figure 2.34b
SEM Micrograph of full-fat soya [x60]
Figure 2.35a
SEM Micrograph of malt culms
[x12]

Figure 2.35b
SEM Micrograph of malt culms
[x75]
Figure 2.36a
SEM Micrograph of meat and bone meal
[x15]

Figure 2.36b
SEM Micrograph of meat and bone meal
[x250]
Figure 2.37a
SEM Micrograph of wheatfeed 
[x15]

Figure 2.37b
SEM Micrograph of wheatfeed 
[x250]
2.11 CONCLUSIONS

The work performed in this chapter has shown that:

1. The analytical method used for SDM is both accurate and precise.

2. The best method of dosing samples with drug was the addition of a methanol solution of the drug. Although this method was not the same as that used in industry, the method did allow accurate addition of SDM to samples.

3. Recovery studies from individual feed constituents showed that poor recovery was obtained in all cases, with recoveries decreasing with increasing drug-constituent contact time.

4. The effect of stressing on SDM recovery from feed constituents was similar to the non-stressed samples when the experiments were performed on samples immediately after the stressing process. This agrees with the results shown in Figures 1.1 and 1.2.

5. No evidence of SDM degradation was found when samples of SDM were stressed in a pressure cooker to simulate the conditioning process.

6. In view of the fact that no degradation products of SDM were seen in the course of experimental studies, drug-feed binding was considered the cause of poor SDM recoveries.

7. Experiments investigating the effect of drug dosing level on SDM recovery showed that, the more drug added, the higher the recovery which implies that in each sample there is factor limiting the amount of SDM that can be bound. Such a factor could be a fixed number of suitable binding sites for SDM.

8. Increasing the specific surface area of a feed, by reducing the particle size, did not produce any differences in SDM recoveries compared to a sample of feed that was not reduced in particle size. It could be suggested that the possible binding phenomenon
occurring is not confined exclusively to the surfaces of the particles. If this had been the case, then the samples with increased specific surface area would have been expected to have shown reduced SDM recoveries. Alternatively, if there was an excess of sorption sites on the surface of the particles, size reduction would not necessarily effect increased surface sorption.

9. The hexane pretreatment of feed samples prior to dosing with SDM, did not result in any differences in drug recovery when compared to untreated samples of feed. This differs from the results in the AMC study [49] where solvent extraction of the oils and fats from fishmeal resulted in those samples showing lower recoveries in SDM than the untreated samples.

10. The study investigating the distribution of SDM between a saturated solution of SDM in water and an equal volume of soya bean oil, did not show any significant reduction in concentration of the SDM in the aqueous phase in the initial period of the study. It would have been expected that if a distribution between the two phases occurred, then it would proceed at a rapid rate initially.

11. SEM examination of feed constituents showed that all exhibited a degree of porosity.
Chapter 3

THE INFLUENCE OF MOISTURE ON SDM RECOVERY
CHAPTER 3 - THE INFLUENCE OF MOISTURE ON THE RECOVERY OF SDM FROM FEEDS

3.1 BACKGROUND
Experimental work in Chapter 2 showed that degradation products of SDM were not observed in studies where SDM was subjected to elevated temperatures, moisture levels and pressures in a pressure cooker. This, therefore, ruled out the possibility of SDM being degraded in the pelleting process, which was suggested in Chapter 1 to account for the fact that pelleted feeds give lower recoveries than the same feed before pelleting. Also in Chapter 2, evidence of binding was seen in the adsorption study whereby, as the amount of SDM added to a feed sample was increased, the recovery of SDM increased. This further strengthened the idea of drug-feed binding being the cause of poor recoveries.

Another interesting result from Chapter 2 was the evidence to suggest that storage conditions had some effect on the recovery of SDM from feed samples. The samples analysed in Section 2.3, all showed very similar decreases in recoveries with time which was attributed to identical storage conditions. Clearly, from the results obtained, the recovery problem increases with the time the drug is in contact with the feed, and is affected by the conditions of storage, ie temperature and humidity.

The question of factors affecting the recovery of SDM from feeds was considered carefully. One of the differences between pelleted and non-pelleted feeds is the moisture content, with pelleted feeds having the higher moisture content, due to the conditioning stage in pellet production. A hypothesis was put forward that the increased moisture content in pelleted feeds accounted for the lower drug recoveries, compared to non-pelleted feeds.

Before investigating this hypothesis experimentally, the effect of conditioning and pelleting on biological materials was addressed by reference to literature in this field. Although the effects of conditioning and pelleting of compound animal feeds has not been researched thoroughly, several papers addressed the effects on individual feed constituents.

Studies on soya bean products have shown that extensive structural changes occur during extrusion with Cummings et al [52] showing that extruded soya bean meal took on the
appearance of a fibre-like structure after extrusion. Maurice et al [53] reported that the extruded product became more spongy in texture as the temperature, at which extrusion was carried out, increased.

Stanley and deMan [54] reported results concerning the extrusion of products which contain mixtures of soy and corn, and again found that the extrudates had a spongy appearance. Cegla et al [55] investigated extrusion effects on cotton seed flour and found that the products were highly puffed and expanded structures. Taranto et al [56] studied the microstructure of soy and cottonseed flours texturised by extrusion and non-extrusion processing (performed by a hand-operated press). Similar surface morphologies were reported for both methods. Scanning electron microscopy and transmission electron microscopy showed that neither of the two processes was especially efficient in producing a disruption of the cells and fusion of the cellular contents, but still was enough to produce some texturisation. The authors also suggested that the extrusion process did not necessarily produce texturisation and the combined effect of pressure, heat and time may have even partly destroyed any texturisation that had occurred.

The work reviewed illustrates that the conditioning and pelleting stages in individual constituents result in complex changes. These are particularly induced by the presence of increased moisture levels. Extending these findings to the effect of conditioning and pelleting of feeds, which are mixtures of biological materials, would indicate that very complex changes could occur. Experiments were designed to investigate the effect of feed moisture content on SDM recoveries.

3.2 EXPERIMENTAL WORK

3.2.1 Effect of storage humidity on SDM recovery (I)

3.2.1.1 Experimental

The apparatus and chemicals used were as specified in Chapter 2. A simple experiment was planned to modify the moisture content of a feed already dosed with SDM. This was achieved by placing the feed samples either in a high humidity, water-saturated environment or in a low humidity, dry environment. Two grams of a non-medicated feed were accurately
weighed into 40 sample tubes (labelled 1-40). A solution of SDM in methanol (0.5 ml) was added to each sample tube to give an average dosing level of 300 μg g⁻¹. The tubes were then capped and mixed for 10 seconds, using a whirl mixer to allow interaction between the dosing solution and the feed. After mixing, the sample tubes were left uncapped in a fume hood for 2 hours to allow the methanol to evaporate.

Tubes 1-20 were then placed (uncapped) in an airtight box containing 500 g of self-indicating silica gel to absorb moisture from the feed. Samples 21-40 were placed (uncapped) in another air-tight box containing 500 ml of HPLC grade water to provide a water saturated environment to increase the feed moisture content. Both air-tight boxes were placed inside polystyrene insulation containers, which were then placed in an air conditioned room maintained at 20 ±3 °C. After 11, 25 and 35 days in their respective environments, 4 samples were removed from each box. Two samples were dried to constant mass in an oven maintained at 105 °C to determine the moisture content. SDM was extracted from the remaining two samples.

3.2.1.2 Results
The results, shown in Table 3.1, are displayed graphically in Figures 3.1 and 3.2. With both the samples stored in high humidity and in low humidity, an inverse relationship between feed moisture content and SDM recovery can be seen.
Table 3.1
Humidity Effects on SDM Recovery

<table>
<thead>
<tr>
<th>Contact Time (d)</th>
<th>Moisture (%)</th>
<th>SDM Recovery (%)</th>
<th>Moisture (%)</th>
<th>SDM Recovery (%)</th>
</tr>
</thead>
<tbody>
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<td>5</td>
<td>24</td>
<td>32</td>
<td>6</td>
</tr>
</tbody>
</table>

Figure 3.1
SDM recovery from low moisture content feed samples
Additionally, the samples which had been dried to constant mass were then analysed to determine the SDM recovery after the drying process. These results are shown in Table 3.2. It is interesting to note that the longer the period of oven drying of the wet samples, the better the SDM recovery. Conversely, low moisture content samples were adversely affected by prolonged oven drying, showing a decrease with increasing drying time.

Table 3.2
Humidity Effects on SDM Recovery After Drying*

<table>
<thead>
<tr>
<th>Contact Time (d)</th>
<th>Drying Time (d)</th>
<th>Percentage SDM Recovery (±4%)</th>
<th>Dry</th>
<th>Wet</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>7</td>
<td>66</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>5</td>
<td>54</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>11</td>
<td>48</td>
<td>91</td>
<td></td>
</tr>
<tr>
<td>52</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

(* Drying at 105°C for 5-11 days)
3.2.1.3 Discussion

The results from the first part of the study appear to agree with the hypothesis that higher moisture contents in pelleted feeds cause lower SDM recoveries than from lower moisture, non-pelleted feeds.

The results from the second part of the study, looking into the effect of oven drying feed samples on SDM recoveries, appear anomalous. In the previous part of the study, it had been observed that the lower the moisture content of the feed samples, the higher the SDM recovery. In the oven drying study, five out of six of the results showed the reverse of this trend with all the low moisture content samples showing a reduction in SDM recoveries as the samples were oven dried. In the case of the high moisture content samples, the recoveries after 11 days contact time, followed by 7 days oven drying, and the samples after 25 days contact time and 5 days drying time, both showed a reduction in SDM recoveries despite the fact that the moisture levels were reduced. An explanation of this will be offered later.

3.2.2 Effect of modifying feed moisture content (before dosing) on SDM recovery

Since the results from 3.2.1 had shown that an inverse relationship existed between the feed moisture content and the recovery of SDM with time, when the moisture content was modified after SDM dosing, the effect of modifying the feed moisture content before dosing was investigated.

3.2.2.1 Experimental

Two grams of non-medicated feed were accurately weighed into 60 sample tubes (labelled 1-60). Samples 1-20 and 21-40 were placed in air-tight boxes, containing silica gel and water respectively. Samples 41-60 were placed in an empty air-tight box to serve as a control. All three boxes were placed inside polystyrene insulation containers as before for 6 days. Representative samples were taken from each environment after 6 days and each sample dried for 6 hours at 105 °C. The drying time of 6 hours was used since this was the time used in the determination of moisture in samples in industry. The feed moisture content had been previously measured as 12% w/w before the study began, and it was found that the moisture content in samples 1-20 had decreased to 5% w/w; samples 21-40 had increased to 18% w/w and samples 41-60 remained unchanged. The samples were then removed from their respective environments and dosed with a solution of SDM in methanol.
(0.5 ml) and the procedure described in section 3.2.1.1 followed. After removal from the fume hood, the sample tubes were capped, wrapped in foil and stored in the air conditioned room, maintained as stated previously. Four samples were then removed from each batch after 0, 7, 28, 42 and 55 days drug-feed contact time, and two samples analysed for SDM with the results displayed in Figure 3.3. The remaining two samples were oven dried at 105 °C for 6 hours, left for a further 7 days, then analysed for SDM content. The results are displayed in Figure 3.4.

Figure 3.3
SDM recovery as a function of feed moisture content

Figure 3.4
SDM recovery from feed samples after oven drying
3.2.2.2 Results

In both parts of the study, the low moisture content feed, 6% w/w, showed the slowest and most gradual decrease in SDM recoveries with time, followed by the control feed at 12% w/w moisture content. The high moisture content feed, 18%, showed the most rapid decrease in SDM recovery with time. The 18% and 6% w/w feed samples were artificially engineered to be vastly different from the normal feed moisture content of 12%. It appeared that the recoveries from the first part of the study were levelling off. This might be indicative of the process causing poor recoveries approaching equilibrium.

It was interesting to note that the effect of decreasing the moisture content of the samples by oven drying for 6 hours, and then allowing a further 7 days contact time between the feed and the drug, did not result in any higher recoveries being obtained. Indeed, if the effect of drying is ignored and the additional contact time considered, then superimposing the results of the second study on the graphs of results from the first study shows that almost identical decreases in recoveries are obtained. This is probably a result of the samples picking up moisture from the headspace of the stoppered tube.

3.2.2.3 Discussion

The study clearly showed that a kinetic relationship existed between feed moisture content and SDM recovery with the higher the feed moisture content, the faster the decrease in SDM recoveries with time.

3.2.3 Effect of storage humidity on SDM recovery (II)

It was decided to repeat the experiment looking at the effect of storage conditions on SDM recovery with improvements to the experimental design. The first modification was to increase the number of different humidities used in the study. The second modification was to maintain these humidities accurately to prevent wide fluctuations of humidity with temperature. The third, and final modification, was to increase the total number of time points used in the study, paying particular attention to the period immediately after dosing. To ensure the relative humidities in the environments remained as constant as possible, saturated salt solutions, as reviewed by Young [57], were used. Saturated solutions of sodium chloride, which provided a relative humidity of 75%, and magnesium chloride, which provided a relative humidity of 33%, were chosen to be used alongside the silica gel (0% relative humidity) and an ambient humidity environment.
3.2.3.1 Experimental

Two gram portions of feed were weighed into 112 sample tubes. Addition and mixing of the SDM in methanol dosing solution was as described previously. Sample tubes, labelled 1-28, were placed in an air-tight box which was the ambient humidity environment and was to serve as the control. Sample tubes 29-56 were placed in an air-tight box containing a saturated solution of sodium chloride (75% relative humidity). Sample tubes 57-84 were placed in an air-tight box containing silica gel (0% relative humidity). Finally, sample tubes 84-112 were placed in an air-tight box containing a saturated solution of magnesium chloride (33% relative humidity). Samples were removed at 3 day intervals, with two samples being used for moisture determinations and two samples being analysed for SDM recoveries.

3.2.3.2 Results

The results, illustrated in Figure 3.5, show that with the samples stored in 0% relative humidity, there was little or no change in SDM recoveries over the course of the study. In the case of the samples stored in the 33% relative humidity environment, the results at 6, 9 and 12 days contact time are not shown. This was because these samples had fallen into the solution of magnesium chloride. Despite this, interpolation showed the overall trend in SDM recoveries remained fairly constant.

![Figure 3.5](image)

**Figure 3.5**

SDM recovery as a function of storage humidity

- 0 %
- 33 %
- 75 %
- Ambient
With both the samples stored in the ambient relative humidity and the 75% relative humidity environments, SDM recoveries decreased steadily over the period of the study. Both sets of samples agreed quite closely in recoveries. This was to have been expected if the relative humidity of the ambient relative humidity was taken as being approximately 70%.

3.2.3.3 Discussion
The kinetic relationship between feed moisture content and SDM recovery was again shown by the data. It was interesting to observe that the different moisture contents resulted in fairly constant ratios of SDM recoveries. In the AMC paper [49], a study was performed on the recovery of SDM from wheat meal, soya meal and fishmeal both before and after pelleting. The results from two laboratories are summarised in Table 3.3 and show that the ratios between the recoveries from the pelleted and non-pelleted samples are fairly constant, at 0.7-0.8. If it is assumed that the moisture contents of the meal samples is 11% and that of the pelleted samples is 15%, then the ratio of pelleted moisture to meal moisture is 0.73.

Table 3.3
Recovery of SDM from feed constituents - AMC [50]

<table>
<thead>
<tr>
<th>Sample</th>
<th>Laboratory A</th>
<th>Laboratory B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat meal</td>
<td>112</td>
<td>110</td>
</tr>
<tr>
<td>Wheat pellets</td>
<td>80 0.71</td>
<td>84 0.76</td>
</tr>
<tr>
<td>Soya meal</td>
<td>80 0.78</td>
<td>95 0.80</td>
</tr>
<tr>
<td>Soya pellets</td>
<td>62 0.78</td>
<td>76 0.80</td>
</tr>
<tr>
<td>Fish meal</td>
<td>75 0.72</td>
<td>82 0.71</td>
</tr>
<tr>
<td>Fish pellets</td>
<td>54</td>
<td>58</td>
</tr>
</tbody>
</table>

Figure shown outside bracket is = \( \frac{\text{SDM recovery from pellets}}{\text{SDM recovery from meal}} \)
Since we have shown that the recovery of SDM, R, is inversely related to the moisture content, M, of the feed, i.e., \( R \propto 1/M \) then:

\[
\frac{R_p}{R_m} = \frac{M_m}{M_p}
\]

This could be taken as direct evidence that the conditioning process, which increases the moisture content of the feed prior to pelleting, results in lower recoveries than from non-pelleted feeds.

### 3.3 Stability of SDM in Aqueous Solution

#### 3.3.1 Experimental

In view of the fact that increased feed moisture contents resulted in greater decreases in SDM recoveries, compared to feeds of lower moisture contents, it was decided to investigate whether hydrolysis of SDM was occurring. Hydrolysis could explain the experimental results obtained from the moisture studies.

Previous work reported by Holder et al. [38] on the stability of SDM in deionised water at concentrations of 50 and 500 \( \mu g \) ml\(^{-1}\) indicated that, over the 16 day period of the study, there was no significant change in SDM concentration in the two dilutions of SDM, both in samples exposed to light and in samples protected from light. To investigate the stability of SDM over a longer period of time, the following study was performed.

SDM (65.7 mg) was weighed into a 100 ml volumetric flask to which was added 80 ml of HPLC grade water. The flask was stoppered, shaken thoroughly then placed in an ultrasonic bath for 2 minutes to aid dissolution. On removal from the ultrasonic bath, the solution was made up to the mark, shaken then filtered through a 0.45 \( \mu m \) filter. Aliquots of the filtered solution were then transferred to two 30 ml glass storage bottles labelled
1 and 2. Bottle 1 was wrapped in aluminium foil to protect it from light, while bottle 2 was left exposed to light. Samples of the solution were taken from each bottle after 0, 1, 8 and 62 days for analysis by HPLC to determine SDM concentration and whether any degradation products were present.

3.3.2 Results
The results are presented in Table 3.4 and show that SDM was stable in aqueous solution over the period of the study.

Table 3.4

<table>
<thead>
<tr>
<th>Sample</th>
<th>Average SDM Peak Area at Time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>SDM*</td>
<td>1042.8</td>
</tr>
<tr>
<td>SDM+</td>
<td>1071.3</td>
</tr>
</tbody>
</table>

*Stored protected from light  
+Stored exposed to light

3.3.3 Discussion
As the stability of SDM in water was demonstrated, it appeared that the explanation of irreversible binding between SDM and feed constituents, which was put forward in Chapter 2, was further strengthened.
3.4 REVIEW OF EXPERIMENTAL FINDINGS

In order to elucidate a mechanism for the poor recovery of SDM from feeds, it is useful to summarise briefly the findings of the experimental work performed from the preceding studies.

(i) Experimental studies investigating SDM recovery from both complete feeds and individual constituents have shown that recoveries decrease the longer SDM is in contact with the feed. It can be inferred that, if irreversible binding is occurring, then it is not to a single feed constituent. As regards the mechanism for binding, either several different, yet specific, mechanisms exist or, there is one general mechanism which accounts for all the binding. Also the fact that recoveries decrease with contact time indicates that the process accounting for the poor recoveries is a function of time.

(ii) SDM recoveries were found to be directly related to the amount of SDM added to the feed.

(iii) Increased feed moisture levels were found to decrease SDM recoveries compared to feed samples with a lower moisture content. This could be explained by the increased moisture allowing greater interaction between the feed and drug compared to a lower moisture content feed.

After considering the experimental findings, it would appear that irreversible binding, and not degradation of SDM, is the cause of the recovery problem. The fact that the feed moisture content affects SDM recoveries was chosen for further investigation.

3.4.1 The role of moisture in SDM recovery

The role of moisture in the recovery of SDM from animal feed was considered. Conway [48] reported that extraction solutions composed entirely of acetonitrile could only quantitatively extract SDM from samples, if the time between dosing and extraction was less than 1 hour. If 24 hours were allowed to elapse between dosing and extraction, then recoveries were reported as decreasing to only 41% of the total amount of drug added. Improvement in SDM recovery was achieved by using an extraction solution composed of acetonitrile: water (70:30 v/v). Recoveries using the aqueous acetonitrile extraction solution were reported as
97% after 24 hours contact time. The only disadvantage in including water in the extraction solution is that components which interfere in the chromatography were also extracted.

Additionally, in the paper by Cody et al [58], the effect of 5% water in the extraction solution on the recovery of a range of drugs was reported. The results are shown in Table 3.5. It was suggested that the improvement in drug recoveries from certain feeds, especially those that were aged, was due to the penetration of the water into the feed matrix which improved extraction efficiency.

Table 3.5

Effect of water in extraction solution - Cody et al [58]

<table>
<thead>
<tr>
<th>Drug</th>
<th>Recovery (%)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MeCN</td>
<td>MeCN + 5% water</td>
<td></td>
</tr>
<tr>
<td>Amprolim</td>
<td>43</td>
<td>89</td>
<td></td>
</tr>
<tr>
<td>Sulphaquinoxaline</td>
<td>51</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>Halofuginone</td>
<td>-10</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Ethopabate</td>
<td>-10</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>difficult to detect</td>
<td>35</td>
<td></td>
</tr>
</tbody>
</table>

There appears to be an anomaly in the role of water since work by both Conway [48] and Cody [58] reported that quantitative recoveries can only be achieved from aged feed samples using an acetonitrile:water extraction solution. Experimentally, it has been found that the higher the feed moisture content, the poorer the SDM recovery. Clearly, there are two distinct processes involving water. The first concerns when SDM is added to the feed. If excess moisture is present, either before or after the drug is added, then it is apparent that greater interaction between the drug and the feed constituents is possible, than if the feed contained less moisture.
The second process concerns the extraction of SDM. In this case, quantitative extraction of the drug, after 24 hours contact time, can only be achieved with water present in the extraction solution. The water must help in either solubilising SDM or helping in allowing the extraction solution to penetrate the feed matrix. Although SDM is reported as being only slightly soluble in water, the study investigating the stability of an aqueous SDM solution showed that solubilities approaching 400\mu g g^{-1} could be attained. Using this figure for the solubility of SDM in water, theoretical calculations were then performed. It was assumed that a 10 g portion of feed, containing 100 \mu g g^{-1} of SDM, was extracted with 100 ml of 70\% aqueous acetonitrile as the extraction solution. Theoretically, it is possible for all the SDM present in the feed to be dissolved in the water fraction of the extraction solution.

If the feed moisture is assumed to be 12 \% w/w, then it is also theoretically possible for \sim 50 \mu g of SDM to be dissolved in the moisture contained in the feed. The fact that water could be a vehicle for the extraction of SDM from feeds, also suggests that water is a vehicle for the interaction of SDM with the feed constituents. It has already been seen that increased moisture contents in feeds result in accelerated decreases in recoveries with time, compared to a lower moisture content feed. The question to be addressed is how water allows increased binding of SDM to feed constituents.

As was stated previously, the fact that poor SDM recoveries were obtained from a range of different feed constituents suggests that the binding is not specific. Since this appears to be the case, then the irreversibility of the binding implies that either several specific binding mechanisms exist, or one general mechanism is responsible. A possible general binding mechanism could involve water. This can be supported by the following experimental results.

(i) Theoretical calculations involving moisture have shown that the moisture present in a feed can dissolve some of the SDM present.

(ii) An inverse relationship between feed moisture content and SDM recovery has been observed.
(iii) The rate of decrease in SDM recoveries with time in high moisture content samples is greater than in lower moisture content samples.

(iv) In the adsorption of SDM on feed, the more SDM added to the samples, the higher the SDM recovery. An explanation of this could be that the more SDM is present in the feed sample, the greater the non-dissolved fraction available for extraction.

(v) If the increased moisture content allows greater dissolution of SDM, then the resulting solution must somehow interact with the feed matrix to prevent SDM from being extracted by the extraction solution. Considering the porosity of the feed constituents, which was observed in Chapter 2, the SDM solutions could diffuse into the pores of the constituents, and prevent the SDM from being extracted by the extraction solution.

(vi) Lower drug recoveries in pelleted feeds could be due to the increased feed moisture levels compared to non-pelleted feeds.

The above hypothesis of drug dissolution in feed moisture and diffusion into feed particle pores is an explanation for the experimental results. But it was decided to consider the interaction of water with a feed before pursuing this hypothesis further.

3.5 THEORETICAL CONSIDERATION OF MOISTURE IN FEEDS

To gain a better understanding of the role of water in the recovery of drugs from feeds, the interaction of water with feeds was considered. Although no specific work was found concerning the interaction of water with feeds, several researchers had considered the adsorption of water by individual biological materials.

3.5.1 Moisture theory - mathematical treatment

A mathematical treatment of the absorption of moisture by biological materials was made in a paper by Young and Nelson [59] and further developed by York [60] and also Zografi et al [61]. It was proposed that there were three different locations for water: monolayer adsorbed moisture, normally condensed moisture and absorbed moisture. These are shown in Figure 3.6a.
The model presented by Young and Nelson was that when a dry material is exposed to moisture, water adsorbs first to surfaces to form a monomolecular layer. This monomolecular layer is subject to both binding forces and diffusional forces. The diffusional forces cause gradual moisture transfer into the material, i.e., absorption. Reduction in the vapour pressure results in water molecules at the surfaces being removed first, before diffusional forces pull moisture out of the interior of the material. Young and Nelson reported that each of the types of water present during water adsorption could be described mathematically.

Figure 3.6a

Moisture in Materials

- Monolayer Adsorbed Moisture
- Normally Condensed Moisture
- Absorbed Moisture
3.5.1.1 Monomolecular layer water (monolayer adsorbed moisture)
At equilibrium, the rate of condensation of moisture on the surface is equal to the rate of evaporation from the surface. Kinetic theory allows an equation to be derived for the rate at which water condenses on a surface to form a monomolecular layer

\[
M = \frac{P}{(2\pi mK T)^{1/2}} (1-\theta) \tag{1}
\]

where
- \(M\) = number of molecules condensed per unit surface area of the adsorbent per unit of time.
- \(P\) = vapour pressure of water
- \(m\) = mass of a water molecule
- \(K\) = Boltzmann’s constant
- \(T\) = absolute temperature
- \(\theta\) = fraction of the surface already covered by a layer of bound moisture

An assumption made in the equation is that the vapour molecules collide with the uncovered portion of the surface and are condensed and held by forces greater than those exerted on normally condensed molecules.

If further molecules collide with the previously covered monomolecular layer, a second layer of molecules is formed. It is assumed that this layer prevents the first layer evaporating.

Boltzmann’s law gives the rate of evaporation of the molecules from the bound moisture layer as:

\[
V = K_0 e^{-q/kT} (\theta - \phi) \tag{2}
\]
where \( v \) = number of molecules evaporated per unit surface area of adsorbent per unit time

\( K_0 \) = evaporation coefficient

\( q_l \) = heat of adsorption of the molecules bound to the surface

\( T \) = absolute temperature

\( \varnothing \) = fraction of surface covered by two or more layers of molecules

From (1) and (2) gives:

\[
\varnothing = \frac{P(2\pi nKT)^{1/2} + K_0 \varnothing \exp(-q_l/KT)}{K_0 \exp(q_l/KT) + P(2\pi nKT)^{-1/2}}
\] (3)

Assuming that in the second and higher layers of molecules, the condensation occurs with a normal heat of condensation, \( q_L \), then:

\[
\theta = \frac{P\theta}{K_0 \exp(-q_L/KT)(2\pi nKT)^{1/2}}
\] (4)

At a vapour pressure \( P_o \), the saturation vapour pressure, the evaporation and condensation from a water surface will occur at equal rates

\[
P_o = K_0(2\pi nKT)^{1/2} \exp(-q_L/KT)
\] (5)

Substituting (4) into (5) gives

\[
\theta = \frac{\varnothing}{\varnothing + (1-\varnothing) \exp((-q_l-q_L)/KT)}
\] (6)

where \( \varnothing \) = relative humidity.
If $E = \exp \left( \frac{(-q_1-q_L)}{KT} \right)$, then

$$\theta = \frac{\rh}{\rh + (1-\rh)E}$$

This equation relates the fraction of the surface covered by a unimolecular layer of water to the relative humidity of the environment.

3.5.1.2 Multimolecular layer of water (normally condensed moisture)

By combining (4) and (5), it can be shown that:

$$\varnothing = \rh \theta$$

Let $\alpha = \text{the total amount of normally condensed moisture on the surface (measured in molecular layers)}$.

Differentiation of (7) allows for the fact that when additional moisture condenses, some will land on a surface covered by bound molecules and some will land on layers of normally condensed molecules.

The resulting equation is:

$$\alpha = \frac{E \rh}{E-(E-1)\rh} + \frac{E^2}{(E-1)} \ln \frac{E-(E-1)\rh}{E-(E-1)(1-\rh)}$$

The total amount of adsorbed moisture can then be obtained by addition of equations (6) and (8).
3.5.1.3 Absorbed moisture

It is assumed that a volume of water, \( V \), can enter into the cells so that it cannot be considered to be adsorbed moisture. It is also assumed that the volume of water is uniformly distributed. The first layer of water molecules is bound to the surface, and this is prevented from moving into the cell, but a normally condensed layer of molecules is accumulated on the surface of the cell with binding forces insufficient to prevent moisture movement into the cell. Consequently, for an initially dry material, the amount of absorbed water held by the material would be proportional to the area covered by one layer of normally condensed water molecules.

\[ Y_s = V \varnothing \]  

\( Y_s \) = volume of absorbed moisture during sorption process  
\( V \) = volume of absorbed moisture at saturation  
\( \varnothing \) = fraction of surface covered by one or more layers of normally condensed moisture.

If the material is saturated, and subjected to a drying environment, the absorbed moisture cannot move out of the material until the layer of bound molecules is removed.

\[ Y_d = V \theta \]

where

\( Y_d \) = volume of absorbed moisture during a desorption process.

If a material is initially dry, and is allowed to reach equilibrium with maximum relative humidity, \( r_{h_{\text{max}}} \), the amount of absorbed moisture held during a desorption process will then be proportional to the area covered by a bound layer of molecules multiplied by the maximum relative humidity that was reached:

\[ Y_d = V \theta r_{h_{\text{max}}} \]
3.5.1.4 Total moisture content

By addition of the 3 types of moisture, the total amount of moisture can be calculated.

If \( W_s \) = total weight of moisture held by the material during a sorption process then:

\[
W_s = PV_m (\theta+\alpha) + PV\phi \tag{10}
\]

Similarly if \( W_d \) = total weight of moisture held by the material during a desorption process then:

\[
W_d = PV_m (\theta+\alpha) + PV\phi_{rh_{max}} \tag{11}
\]

where

- \( P \) = density of water
- \( V_m \) = volume of moisture in unimolecular layer
- \( rh_{max} \) = maximum relative humidity to which material has been subjected

The moisture content is then obtained by dividing the dry weight of the biological material:

\[
M_s = \frac{\sigma V_m}{W} (\theta+\alpha) + \frac{\sigma V\phi}{W} \tag{12}
\]

\[
M_d = \frac{\sigma V_m}{V} (\theta+\alpha) \frac{\sigma V_{rh_{max}}}{W} \tag{13}
\]

putting \( A = \frac{\sigma V_m}{W} \) and \( B = \frac{\sigma V}{W} \)

simplifies the above equations to:

\[
M_s = A(\theta+\alpha) + B\phi \tag{14}
\]

\[
M_d = A(\theta+\alpha) + B\phi_{rh_{max}} \tag{15}
\]
Values for A, B and E for some materials have been reported in the literature by York [61] and Zografi et al. [61], but these are mostly individual materials and not complex mixtures as in feeds. Calculations using the Young and Nelson equations, described previously, for the adsorption of moisture by feeds were not possible due to the difficulty in assuming values for A, B and E for feeds. Overall, the theoretical understanding of moisture adsorption in a biological material is well defined.

The influence of water activity on the quality of extruded products was addressed in a paper by Schwarzer [62]. Water activity, as described by Adams [63], can be thought of as the relative escaping tendency of water from a sample compared to pure water, and is numerically equal to the relative humidity of air which is in equilibrium with the material. Schwarzer reported that the water activity of a product had an influence on the spoilage of the product by mould development and autoxidation, and described how there were three types of water present in the product. The first type of water was associated with proteins, salts and sugars, but was not able to be utilised by micro-organisms. The second type was bound by molecular attractions (Van der Waals), with the third type bound by capillary attractions. The latter two can be utilised by the micro-organisms, and mould development is a significant problem at higher water activities. The 3 types of water described by Schwarzer can be equated with those described in the model by Young and Nelson [59]. Figure 3.6b shows the sorption isotherm illustrating the relationship between moisture content and water activity, and also showing the type of water present. From the origin up to A, the water present is associated strongly with high molecular weight components. From A to B, the water can be described as biologically active, in an absorbed form; from B onwards, the water is in liquid form in capillaries.

Schwarzer also reported that the water activity, $A_w$, increases with moisture content up to about 12%, after which point, it does not vary greatly with moisture content. Additional factors reported to affect the water activity were moisture content, temperature and physical structure. An explanation of this is that at 12 % moisture, a complete monolayer is formed. If the definition of Adams is used concerning water activity, and it is assumed that below 12 % moisture there is an incomplete monolayer, then there will be distinct regions or patches of monolayer coverage. Water molecules in these regions will not be restricted in their translational movement by forces of attraction exerted by the other water molecules.
to as large a degree as water molecules in a complete monolayer. This results in the water activity of an incomplete monolayer being higher than in a complete monolayer.

An interesting point made by Schwarzer concerned the physical structure of a material. He stated that materials with an open or diffuse structure tended to have a higher water activity because the water was not bound so tightly. Conversely, materials with denser structures tended to have lower water activities at the same moisture levels. Additionally, he described the extrusion process as being useful in making the feed nutrients more available to the animal and producing high quality feeds, but mentioned that the extrusion process altered the physical structure of many of the raw materials. Cell walls of cereals were reported as being disrupted, which was accompanied by gelatinisation of the starches present. The effect of these modifications on the physical structure of the raw materials was to lower their water-holding capacity. This agrees with the findings of the researchers [53-57].

Taking the sorption isotherm in Figure 3.6b [64] and modifying it allows a better explanation for pelleted feeds giving lower drug recoveries than non-pelleted feeds. Figure 3.6c [64] shows the moisture sorption isotherm for an extruded feed and a non-extruded feed. The non-extruded feed has a lower water activity at the same moisture contents as the extruded material due to the physical structures of the raw materials being more dense.

The findings reported in the paper by Schwarzer [62] necessitate a modification to the hypothesis presented earlier to account for the poor recovery of SDM from animal feeds. It has been suggested that the drug recoveries from pelleted feeds are lower than from non-pelleted feeds because the former have higher moisture contents. This has been seen in the experimental results reported earlier. This may not be completely the case because, although pelleted feeds have higher moisture contents than non-pelleted feeds, the fact that the pellet processing increases the water activity of the pellets could imply that the assumed differences in feed moisture content need not be as great as originally thought. The increased water activity in the pellets would allow more SDM to be dissolved and, therefore, diffuse into the pores of the feed particles than a non-pelleted feed of similar moisture content.
The papers by Schwarzer [62] and Young and Nelson [59] provide both a theoretical and practical consideration of moisture in feeds and lend weight to the hypothesis that, in pelleted feeds, the increased moisture content and physically-modified structure of the raw materials, resulting in higher water activity, allow increased amounts of SDM to dissolve.

Figure 3.6b

Relationship between moisture content, water activity and water type present in extruded feed [64]

Figure 3.6c

Relationship between moisture content, water activity and water type present in extruded feed and non-extruded feed [64]
3.5.2 Sorption Experiments

If a food material is placed in an environment which is at constant temperature and humidity, given sufficient time, an equilibrium will be reached, and the final moisture content attained can be called the equilibrium moisture content. A typical adsorption isotherm [64] is shown in Figure 3.7 and illustrates the sigmoid shape. Fitting in with the moisture theory equations presented earlier is the fact that the sorption isotherm can be divided into three regions which correspond to the different types of water being present in the food material.

![Typical Sorption Isotherm](image)

Measurement of adsorption and desorption isotherms for a variety of materials has been performed by several researchers [65-71]. Hysteresis, which is separation of the adsorption and desorption isotherms due to absorption of moisture internally, is evident in the majority of materials investigated.
Mathematical treatment of adsorption and desorption data has led to a large number of equations to represent the adsorption process. These are discussed in detail in several papers [68, 71, 73].

The paper on water activity and storage stability by Rockland [65] is a particularly good review of the subject, since it summarises the developments in the theories describing moisture adsorption and desorption in foods. Initially, it was popular to use the equation described by Henderson [74]:

\[ \ln (1 - rh) = -k T M^n \]

where \( rh \) represents relative humidity, \( M \) is total moisture content, and \( n \) and \( k \) are constants. This equation was used, although discrepancies between experimentally obtained data and theoretical data were apparent.

Developments by Rockland in 1957 led to an adapted form of the Henderson Equation being used:

\[ \log \log \left( \frac{1}{1-rh} \right) = n \log M + K \]

This had the advantages that the constants \( n \) and \( K \) could be evaluated more easily than in the previous equation. Use of the equation to treat experimental data did not produce the expected straight line except in special cases. Generally, it was found that the experimental points were better fitted when three straight lines were drawn, and this method was called the local isotherm treatment.

Use of the local isotherm approach to build up a composite adsorption isotherm gave agreement between the theoretical and the experimental data. Links between the three lines drawn to fit the data and the three known types of water present in the material were then made.
This local isotherm treatment of data was then disputed by Iglesias and Chirife [73]. It was shown that, although the local isotherm approach was extremely attractive due to its link with the three types of water present in materials, it was not valid in a large number of cases because the region corresponding to the capillary water occurred at water activities that were too low for capillary water to be present. The conclusions made in the paper were that although the local isotherm approach proposed by Rockland did describe moisture adsorption in a broad sense, it did not permit 'unequivocal definition of the physical state of the water in food'.

The equations described by Henderson and Rockland all rely upon data obtained using a range of different relative humidities to give adsorption and desorption isotherms. The work planned to investigate the adsorption and sorption of moisture by feeds would only involve two relative humidities, 100% and 0%. Although this simplified the experimental procedure, it excluded the use of any of the equations described earlier, since adsorption / desorption isotherms would not be obtained. An advantage in this method of studying moisture adsorption / desorption was that it would allow kinetic studies to be performed and measurements for both adsorption and desorption of moisture on feeds were made as described below.

3.6 ADSORPTION OF MOISTURE BY FEEDS

It was hoped that by investigating the adsorption and desorption of moisture by feeds a better understanding of how water affected the recovery of SDM from feeds could be gained. To investigate the adsorption and desorption of water by feeds, experiments were performed using a Robal Microbalance (Cl Electronics, Salisbury, Wilts, UK.) connected to a microcomputer to record the increase / decrease in mass due to water adsorption / desorption. The apparatus is shown in Figure 3.8.
Figure 3.8

Apparatus for the measurement of water adsorption and desorption in feed

Sample

Connected to thermostatted water bath

Temperature controlled cabinet

Microbalance

Computer

Printer
3.6.1 Effect of temperature on the rate of moisture adsorption

3.6.1.1 Experimental
Feed samples (~100 mg) were placed in the sample pan in the temperature controlled apparatus and exposed to a water-saturated atmosphere. The water-saturated environment was achieved by using 30 ml of distilled water in which a piece of cotton wool had been immersed. The cotton wool increased the surface area available for evaporation of the water thus, ensuring a saturated atmosphere. The change in mass was recorded by a computer at time intervals. To investigate the effect of temperature on the adsorption of water by the feed samples, the temperature of the cabinet was set at 27, 31 and 40 °C, and the increases in mass recorded.

3.6.1.2 Results
The results of this study are shown in Figure 3.9a, and indicate that the greater the temperature of the cabinet housing the apparatus, the faster the rate of moisture adsorption.

Calculations were made involving the Arrhenius equation:

\[ k = Ae^{-E_a/RT} \]  \hspace{1cm} (16)

where

- \( k \) = rate of reaction
- \( A \) = frequency factor
- \( E_a \) = activation energy
- \( R \) = gas constant
- \( T \) = absolute temperature

Equation (16) can be rewritten to give:

\[ \ln k = \ln A - \frac{E_a}{RT} \]  \hspace{1cm} (17)
At each temperature used in the adsorption rate study, the initial rate of moisture adsorption was calculated by measuring the gradient of the tangent. The gradients then represented the values of k at each of the temperatures, and plotting ln k vs 1/T would normally be expected to give a straight line of gradient = -E / R and an intercept on the y-axis of ln A. The graph obtained is shown in Figure 3.9b and showed that the reaction rate was not linear with temperature, but was better fitted by two straight lines.

Clearly, there is a change in mechanism to account for such widely differing values of both \( E_a \) and A. Plotting the data using the parabolic law (see section 3.6.2.2) showed that all three temperatures fitted the parabolic or 1-d diffusion equation. These plots, together with the plot of the parabolic law for the sample of feed that was oven dried, are shown in Figure 3.9c. The moisture adsorption by the feed that had been oven dried did not follow the parabolic law. Individual plots of the parabolic law for moisture adsorption at 27, 31 and 40 °C are shown in Figures 3.9d-f respectively. An individual plot of the parabolic law for the adsorption of feed previously oven dried is shown in Figure 3.9g.

The Arrhenius plot, shown in Figure 3.9b, can be accounted for by reference to Figures 3.9d-f. All plots show a straight line portion, preceded by an initiation period. In Figure 3.9d, adsorption at 27 °C, the initiation period is larger than at the other two temperatures. Clearly, when the initial adsorption rates were calculated, the rate calculated for the data at 27 °C was not on the linear portion of the parabolic law plot. This meant that the rate actually measured was not the rate of moisture adsorption controlled by a 1-d diffusion mechanism as was measured at the other temperatures. Oven drying the sample prior to moisture adsorption had clearly altered the adsorption mechanism, since it was not observed to follow the parabolic law or 1-d diffusion equation.

When the overall adsorption rates were calculated, simply by dividing the final percentage mass increase by the time at which it occurred, an Arrhenius plot of the adsorption rates obtained gave a straight line, as in shown in Figure 3.10. Calculation of the activation energy, \( E_a \), gave a value of 27 kJ mol\(^{-1}\) and a pre exponential factor of 38. The activation energy is approximately twice the value for the strength of a hydrogen bond ~15 kJ mol\(^{-1}\) as reported by Selley [75]. The significance of the low value of the pre exponential factor can be made by reference to a paper by Cordes [76]. Cordes summarised pre exponential factors of both gas-phase and solid-phase reactions. The solid phase reactions have lower
pre exponential factors and can be attributed to the product having greatly reduced entropy. In the case of the adsorption of water, gaseous water condenses upon a surface of a feed particle to condense as liquid water. The large entropy possessed by the water molecule in the gaseous state is reduced when it condenses upon the surface. The pre exponential factor is further reduced due to the translational movement of the water molecule on the surface being restricted.

![Fig 3.9a](image)

**Moisture adsorption rate as a function of temperature**

![Fig 3.9b](image)

**Arrhenius plot of moisture adsorption rates**

129
Fig 3.9c
Moisture adsorption rates as a function of temperature

Fig 3.9d
Parabolic law plot for the adsorption of moisture by feed at 27 °C
Fig 3.9e
Parabolic law plot for the adsorption of moisture by feed at 31 C

Fig 3.9f
Parabolic law plot for the adsorption of moisture by feed at 40 C
Fig 3.9g
Parabolic plot for the adsorption of moisture by feed (previously oven dried) at 31 C

Fig 3.10
Arrhenius plot of moisture adsorption rates
3.6.2 Effect of pre treating feed with organic solvent on adsorption and desorption of moisture.

3.6.2.1 Experimental

Conway [48] reported that using acetonitrile alone to extract SDM from a feed sample that had been spiked 24 hours previously did not result in quantitative extraction of the SDM. In order to investigate the effect that acetonitrile had upon the ability of the feed to adsorb water, a feed sample that had been soaked in acetonitrile for 28 days followed by filtering and air-drying was used on the microbalance. Additionally, samples that had been pre treated in methanol were also used in the study. Both the acetonitrile treated feed and the methanol treated feed were compared to a feed sample that had not been treated with organic solvents to determine whether solvent pre treatment of the feeds altered the adsorption and desorption characteristics of the feed.

Samples were allowed to adsorb moisture until they had seemingly reached a plateau then the water in the apparatus was exchanged for self-indicating silica gel to remove the adsorbed moisture and give a desorption curve.

A complication of the experimental procedure was that the apparatus had originally been designed to monitor the adsorption of moisture by polymers over a very short period of time in the order of minutes. In the adsorption and desorption of moisture on feeds, the time over which the measurements were made was in the order of days. Modification of the computer program allowed data acquisition every 30 seconds. The data were then stored onto disk. The data on the disks had to be transferred via a computer link and interface to a PC which allowed data processing.

The data constituting the adsorption and desorption isotherms are not continuous because only every fifth data file was transferred from the micro computer to the PC. This was because of the spreadsheet and graphical software could not handle all the data points. Despite this fact, every data file or collection of points on the isotherms are composed of 90 data points. This means that there are approximately 1500 data points on each adsorption or desorption plot.
3.6.2.2 Results
Figures 3.11 - 3.16 show the rate of moisture uptake / loss by the feed samples measured as change in mass with time. Figures 3.11 and 3.12 show respectively the adsorption and desorption of water from a feed that was untreated. Figures 3.13 and 3.14 show respectively the adsorption and desorption of water from a feed that was pre treated with acetonitrile before use in the experiment. The adsorption and desorption of moisture from a sample of feed that was pre treated with methanol before use in the experiment are shown in Figures 3.15 and 3.16 respectively.

Figures 3.11 and 3.12 illustrate the adsorption and desorption of moisture by untreated feed, respectively. Figure 3.11 shows the adsorption of moisture, while Figure 3.12 shows the desorption. The mass of the feed is measured in mg, and the time is measured in seconds. The data points are plotted on a graph with time on the x-axis and mass on the y-axis.

Figures 3.13 and 3.14 depict the adsorption and desorption of moisture by feed that was pre treated with acetonitrile. Figure 3.13 shows the adsorption, while Figure 3.14 shows the desorption. The mass of the feed is measured in mg, and the time is measured in seconds. The data points are plotted on a graph with time on the x-axis and mass on the y-axis.

Figures 3.15 and 3.16 display the adsorption and desorption of moisture by feed that was pre treated with methanol. Figure 3.15 shows the adsorption, while Figure 3.16 shows the desorption. The mass of the feed is measured in mg, and the time is measured in seconds. The data points are plotted on a graph with time on the x-axis and mass on the y-axis.
Figure 3.13
Adsorption of moisture by MeCN treated feed

Figure 3.14
Desorption of moisture from MeCN treated feed
Figure 3.15
Adsorption of moisture by MeOH treated feed

Figure 3.16
Desorption of moisture from MeOH treated feed
All the adsorption studies did not proceed to complete saturation, despite having mass increases of between 25-30%. It was found that prolonged exposure of the feed samples to the saturated humidity, resulting in moisture adsorption in excess of 30% w/w, produced microbial growth on the samples. To prevent this from occurring, it was decided to discontinue exposure to the water vapour after the sample had gained between 25-30% moisture w/w.

To analyse the data obtained, the mass loss/gain was converted to the parameter, $\alpha$, which is the extent of reaction, and has a value of between 0 and 1. Thus, the maximum mass increase or decrease obtained in the experiments was equated with $\alpha = 1$ and subsequent values of $\alpha$ calculated. Use of $\alpha$ allowed normalisation of the y axis which corrected for the fact that the slight differences in masses were used in the experiments and was the convention for use in kinetic equations.

Consultation with a paper by Sharp [77] provided several simple equations with which to test the data. Only the 1-diffusion or parabolic law model was found to fit some of the data. It was decided to investigate additional kinetic models, as well as the 1-d diffusion, and these were:

(i) **Zero order**: $\alpha = k$

   If the adsorption of moisture is considered, and if the mechanism follows zero order kinetics, then both the concentration gradient and the surface area of interface remains constant.

(ii) **1-dimensional diffusion or parabolic law**: $\alpha^2 = kt$

   With 1-dimensional diffusion, as the extent of the process increases, the rate decreases. This situation is found in some porous systems where there is some shape to the pores.

(iii) **First order**: $\alpha = kt$

   The rate is proportional to the concentration gradient of the system.
The adsorption and desorption data of moisture from the untreated feed, the acetonitrile treated feed and the methanol treated feed were plotted using the three equations mentioned above.

For the untreated feed, Figures 3.17-3.19 plot the adsorption data using the zero, first and parabolic equation respectively. Figures 3.20-3.22 plot the desorption data using the zero, first and parabolic equation respectively.

For the acetonitrile treated feed, Figures 3.23-3.25 plot the adsorption data using the zero, first and parabolic equation respectively. Figures 3.26-3.28 plot the desorption data using the zero, first and parabolic equation respectively.

For the methanol treated feed, Figures 3.29-3.31 plot the adsorption data using the zero, first and parabolic equation respectively. Figures 3.32-3.34 plot the desorption data using the zero, first and parabolic equation respectively.
Figure 3.18
Adsorption of moisture by untreated feed - First order plot

Figure 3.19a
Adsorption of moisture by untreated feed - Parabolic plot

Figure 3.19b
Adsorption of moisture by untreated feed - Parabolic plot modified

\[ y = 6.395 \times 10^{-2} + 1.085 \times 10^{-5} x \]
\[ R^2 = 0.999 \]
Figure 3.20
Desorption of moisture from untreated feed - Zero-order plot

Figure 3.21
Desorption of moisture from untreated feed - First-order plot

Figure 3.22
Desorption of moisture from untreated feed - Parabolic plot
Figure 3.23
Adsorption of moisture by MeCN treated feed - Zero order plot

Figure 3.24
Adsorption of moisture by MeCN treated feed - First order plot

Figure 3.25
Adsorption of moisture by MeCN treated feed - Parabolic plot
Figure 3.26
Desorption of moisture from MeCN treated feed - Zero order plot

Figure 3.27
Desorption of moisture from MeCN treated feed - First order plot

Figure 3.28
Desorption of moisture from MeCN treated feed - Parabolic plot
Figure 3.29
Adorption of moisture by MeOH treated feed - Zero order plot

Figure 3.30
Adorption of moisture by MeOH treated feed - First order plot

Figure 3.31
Adorption of moisture by MeOH treated feed - Parabolic plot
Figure 3.32  
Desorption of moisture from MeOH treated feed - Zero order plot

Figure 3.33  
Desorption of moisture from MeOH treated feed - First order plot

Figure 3.34  
Desorption of moisture from MeOH treated feed - Parabolic plot
Additional mathematical treatment was performed using the Johnson-Mehl equation \[78\] and described further by Burke \[79\]:

\[
\alpha = 1 - \exp(-kt)^n
\]

which can be rearranged to give:

\[
\log \log \left( \frac{1}{1-\alpha} \right) = n \cdot \log t
\]

where \( t = \text{time} \)

\( \alpha = \text{extent of reaction} \)

\( n = \text{time exponent} \)

Plotting the rearranged equation of \( \log \log \left( \frac{1}{1-\alpha} \right) \) against \( \log t \) allows \( n \) to be calculated from the gradient. The value of \( n \) is indicative of the type of diffusion process occurring. The Johnson-Mehl equation was used to plot the data from the adsorption and desorption of moisture on untreated feed, MeCN treated feed and MeOH treated feed.

3.6.2.3 Graphical Interpretation

Figure 3.35 shows the adsorption of moisture on untreated feed with the linear portion indicated. If this linear region is then replotted and regression analysis performed, as is shown in Figure 3.36, then a straight line is obtained with a gradient of 0.82. According to Burke \[79\], values of \( n \) which are between 2/3 and 3 occur when “diffusion and the interface process are of comparable rate and neither is completely controlling.”
In the context of the adsorption of moisture by the feed sample, when the sample is first exposed to moisture, the surfaces of the particles are gradually covered with water molecules. Once all the available surfaces have been filled, then the rate of diffusion into the material is the same as the rate of coverage of the surfaces. Hence a straight line is obtained in this region. After a certain point, the diffusion rate starts limiting the overall rate of moisture adsorption, and the gradient decreases. But this increases again due to new internal volumes becoming available.
In the case of the MeCN treated feed, the adsorption data seem to fit a curve with only a relatively short linear portion as can be seen in Figure 3.37. Replotting the linear portion, in Figure 3.38, enables calculation of the gradient which is 0.7, indicating that surface processes and diffusion proceed at similar rates.

The MeOH treated feed, when adsorbing moisture, gives a plot of the data shown in Figure 3.39, which could represent an initial stage of adsorption followed by a second layer of adsorption. The small linear portion is replotted in Figure 3.40 to give a gradient of 0.48, indicative of a diffusion process.

By examining the graphs of the zero, first order, parabolic law and Johnson-Mehl equations used to plot the adsorption data, it was possible to determine limits of $\alpha$ between which the data gave a straight line.

For the adsorption data, this is presented in Table 3.6 and shows that, in the case of the MeCN and MeOH treated feed samples, the initial rate mechanism obeys the parabolic law. This parabolic law region in MeCN feed is between $\alpha$ values of 0 and 0.57, and in the MeOH feed between 0 and 0.63. The data fit zero order kinetics at values of $\alpha$ between 0.58 and 0.87 for MeCN treated feed, and 0.79 and 1.00 for MeOH treated feed.
Figure 3.38
Adsorption of moisture on MeCN treated feed - linear portion of Johnson-Mehl plot

\[ y = -3.7941 + 0.7091x \quad R^2 = 1.000 \]

Figure 3.39
Adsorption of moisture on MeOH treated feed - Johnson-Mehl plot

\[ y = -2.7848 + 0.4833x \quad R^2 = 1.000 \]

Figure 3.40
Adsorption of moisture on MeOH treated feed - linear portion of Johnson-Mehl plot

\[ y = -3.7848 + 0.48335x \quad R^2 = 1.000 \]
Table 3.6
Adsorption of moisture on feeds – Microbalance study summary

<table>
<thead>
<tr>
<th>Feed</th>
<th>Limits of $\alpha$ for linear ranges in named equations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Johnson-Mehl</td>
</tr>
<tr>
<td>Untreated (AR)</td>
<td>0.01 - 0.31</td>
</tr>
<tr>
<td>MeCN</td>
<td>0.40 - 0.58</td>
</tr>
<tr>
<td>MeOH</td>
<td>0.11 - 0.40</td>
</tr>
</tbody>
</table>

The desorption of moisture from untreated feed again gave a substantial linear portion as in shown in Figure 3.41. Replotting this linear portion in Figure 3.42 gave a gradient of 1, which, according to Burke, is indicative of diffusion controlled growth in one dimension only, or 1-d diffusion.

Desorption of moisture from MeCN treated feeds produces a linear portion to the graph, shown in Figure 3.43 which is larger than in the adsorption stage. Replotting the linear portion in Figure 3.44 gives a gradient of 1.0. Again, diffusion controlled growth in one dimension only, or 1-d diffusion, is indicated by this value.

Moisture desorption from the MeOH treated sample gave a plot which was similar to the adsorption and desorption plots obtained for the untreated feed samples. Figure 3.45 shows a fairly linear portion which decreases in gradient later, then becomes almost vertical at the end. As in the other desorption samples, the gradient, shown in Figure 3.46, is 1.06.

This agrees with the model proposed by Young and Nelson [59] in section 3.5.1 that once the molecules at the surfaces have been removed, water diffuses out from the interior.
Figure 3.41
Desorption of moisture from untreated feed - Johnson-Mehl plot

Figure 3.42
Desorption of moisture from untreated feed - linear portion of Johnson-Mehl plot

Figure 3.43
Desorption of moisture from MeCN treated feed - Johnson-Mehl plot
Figure 3.44
Desorption of moisture on MeCN treated feed - linear portion of Johnson-Mehl plot

\[ y = -4.4979 + 1.0415x \quad R^2 = 0.999 \]

Figure 3.45
Desorption of moisture from MeOH treated feed - Johnson-Mehl plot

Figure 3.46
Desorption of moisture from MeOH treated feed - linear portion of Johnson-Mehl plot

\[ y = -4.6509 + 1.0640x \quad R^2 = 1.000 \]
3.6.2.4 Discussion
The data from the adsorption and desorption of moisture on feeds has shown that the
treatment of the feed with organic solvents does affect the moisture adsorption characteristics
of the feed.

In the adsorption of moisture by the untreated feed, the experimental data obtained followed
the parabolic law or 1-d diffusion mechanism equation after an initiation period. With both
the MeCN and MeOH treated feeds, the initial region of the graph appeared to follow the
1-d diffusion reaction mechanism equation, but after a period of ~60000 seconds did not
follow this mechanism. This indicates that the solvents have somehow modified the feed.

The 1d-diffusion or parabolic law describe the situation where, in adsorption, a reservoir
of moisture is condensing onto a surface at a certain rate. As progressively more moisture
condenses upon the surface, the layer of condensed moisture increases in thickness which
has the effect of decreasing the condensation rate. This model assumes that there is a plane
surface for the water to condense on.

Use of the Johnson-Mehl equation to treat the data has shown that although the organic
solvents have affected the adsorption of moisture by the samples, which result in different
mechanisms occurring, the desorption of moisture from the samples is similar. This is
because large portions of the desorption plots yield straight lines with gradients of 1. It could
be inferred that the organic solvents have, in some way, modified the surface chemistry
of the particles.

3.6.3 Desorption of water from feed samples at 105 °C
Experiments performed at room temperature allowed mechanisms for moisture adsorption
and desorption to be determined. It was hoped that performing similar mathematical
treatment of the data obtained from drying a feed at 105 °C on a thermobalance would also
allow a kinetic model to be suggested.

3.6.3.1 Experimental
A thermobalance was used to monitor the loss in mass when feed samples were heated at
105 °C. Again, as well as using feed that had not been pretreated with organic solvent,
pretreated samples as in 3.5.2 were used. Additionally, hexane treated feed was used in the studies.

3.6.3.2 Results
The plots of mass loss against time for the untreated, MeCN treated (2 samples), Hexane treated and MeOH treated samples are shown respectively in Figures 3.47 - 3.51. The only difference between the two MeCN samples (MeCN (I) and MeCN (II)) was that the second, MeCN(II), had been stored for ~12 hours in a sample tube before use in the experiment.

Figure 3.47
Desorption of moisture from untreated feed sample on thermobalance

Figure 3.48
Desorption of moisture from MeCN(I) treated feed on thermobalance
Figure 3.49
Desorption of moisture from MeCN(II) treated feed on thermobalance

Figure 3.50
Desorption of moisture from EtOH treated feed on thermobalance

Figure 3.51
Desorption of moisture from MeOH treated feed on thermobalance
With the untreated feed, Figure 3.47 shows that there are three regions. Region I is associated with the greatest loss of moisture. Region II is a steady stepped decrease after a period of a long plateau. Region III is similarly characterised by a plateau followed by a stepped decrease. The three regions correspond to the three types of water as described by Schwarzer [62].

The MeCN(I) sample desorption plot appears different from MeCN(II). If it is assumed that in MeCN(I), the sample has been modified by the acetonitrile, but on storage has picked up moisture from the storage container to constitute sample MeCN(II), then this would explain why the desorption plot for MeCN(II) resembles the desorption plot of the untreated feed.

The hexane treated samples appears to have three regions, whereas the methanol treated sample only appears to have two regions.

Mathematical treatment of the individual regions was performed. In each region, $\alpha$ was calculated and plotted using zero, first order and parabolic equations as previously. This procedure was performed for all samples and the results are summarised in Table 3.7 where an "X" indicates no linear portion to the plot and a "v" indicates a linear portion to the plot.

<table>
<thead>
<tr>
<th>Feed</th>
<th>Region</th>
<th>Straight line plot obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Zero order</td>
</tr>
<tr>
<td>Untreated (AR)</td>
<td>I</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>X</td>
</tr>
<tr>
<td>MeCN (I)</td>
<td>I</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>X</td>
</tr>
<tr>
<td>MeCN (II)</td>
<td>I</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>X</td>
</tr>
<tr>
<td>Hexane</td>
<td>I</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>v</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>v</td>
</tr>
<tr>
<td>Methanol</td>
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<td>X</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>X</td>
</tr>
</tbody>
</table>
3.6.3.3 Discussion
Although no simple equation was found to fit the desorption data for the samples dried at 105 °C, this fact does indicate that oven drying does cause a different mechanism for the removal of water compared to samples dried over silica gel. This is an important finding since it can partly explain the results in section 3.1.2 where oven-drying samples previously stored over silica gel, which gave quantitative SDM recoveries, gave progressively poorer SDM recoveries with increased oven-drying times.

3.6.4 Effect of water on α-soy protein and wheat starch
Consideration had been given to the adsorption of moisture by feeds. The model by Young and Nelson [59] assumes that absorption also occurs. The hypothesis presented suggested that solutions of SDM formed diffused into the pores of the feed particles, thereby preventing the extraction solution from extracting the drug. It was decided to investigate whether absorption could also take place by some feed constituents which would also prevent the extraction solution from extracting the drug.

3.6.4.1 Procedure
The effect of water on the swelling properties of α-soy protein and wheat starch was investigated by measuring the particle size distribution of separate solutions each using a Malvern 3600c series laser particle sizer. Each solution was prepared by adding sufficient amounts of each sample to the sample cell to ensure that the instrumental concentration parameter was in the optimum range. The solution was then pumped into the measurement cell and the particle size measured every few minutes to note if an increase in size occurred due to swelling. It was not possible to extend the period over which the measurements were taken due to the time constraints of instrument usage.

3.6.4.2 Results
The results from the studies are shown in Figures 3.52 and 3.53. Both the starch and the α-protein samples showed very slight increases in size during the course of the experiment and therefore absorbed moisture. If the results are extrapolated to several hours or days immersion time, then it is possible to deduce that absorption does occur.
3.6.4.3 Discussion

The slight swelling of the starch and protein indicated that increased moisture could affect the physical structures of these raw materials. It had been hoped to investigate the effect of swelling at elevated temperatures, but this was not possible with the laser particle sizer.

![Particle size analysis of starch granules in water](image1)

**Figure 3.52**  
Particle size analysis of starch granules in water

![Particle size analysis of alpha-protein soy particles in water](image2)

**Figure 3.53**  
Particle size analysis of alpha-protein soy particles in water
3.7 DISCUSSION AND CONCLUSIONS

The role of moisture in the recovery of SDM from feeds has been discussed. The hypothesis presented is that feed moisture dissolves some of the drug to form solutions which can diffuse into the pores of the feed particles. The recovery of the drug will depend on:

(i) the length of time the drug is in contact with the feed
(ii) the moisture content of the feed, which is related to the storage conditions
(iii) the solubility of the drug in water.

The sorption of water by flour, starch and gluten has been studied by Bushuk and Winkler [80] and also by Babbit [81] who reported that diffusion was the main rate controlling factor in the adsorption process. This agrees with the experimental work on the adsorption of water by feeds which was found to follow 1-d diffusion kinetics. Diffusion is described in detail by Lewis [82] with particular reference to solid diffusion. He reported that solid diffusion was a more complicated process to consider than either gas or liquid diffusion because, although the material may appear to be diffusing through the solid, it could in fact be diffusing through liquid contained in the solid. Alternatively, if the solid exhibited a degree of porosity, then the material could diffuse through the gas phase in the pores.

Solid diffusion is differentiated by Lewis into structure insensitive and structure sensitive types. The former is when the solute is dissolved to form a homogeneous solid solution which is then followed by diffusion, due to concentration gradients existing in the solution. When a material is leached, the leachable solids are held within a framework of insoluble solids called the marc, and also the occluded solution. The diffusion process occurs primarily within the occluded solution, but the framework of insoluble solids, the marc, restricts the diffusion process. In the leaching process, it is therefore necessary to allow the solid to take up the solvent to dissolve the external solute which then allows diffusion to proceed. This particular case of solid diffusion appears to fit in with our experimental findings and the hypothesis presented. The case for solid diffusion is strengthened by the results for the pre exponential factor calculated in section 3.6.1.2 which indicated a solid-phase reaction.
Additional work by Bushuk and Winkler [83], on the relative sorptive capacities of wheat flour for organic vapours, showed the following trend: water > methanol > ethanol > carbon tetrachloride. Adsorption rates were reported to fit reasonably well with first order diffusion equations, and the size of the hysteresis loops on adsorption and desorption was found to be directly related to the size of the adsorbate molecule which was explained by the fact that there was some physical trapping of the sorbate molecules within the adsorbent. This also helps in explaining why water is needed in the extraction solution to extract SDM quantitatively. Assuming that the aqueous solution of drug in the feed diffuses into the interior of the feed particles via pores, then the relative sorptive capacity trend reported by Bushuk and Winkler, if extended to feeds, would imply that the organic sorbates, which were larger than water, would be adsorbed to a lesser degree than water. If this sorptive capacity is equated to penetration of the extraction solvent into the feed matrix, then the extraction solution composed entirely of an organic solvent, e.g., acetonitrile, would not penetrate as deeply into the feed matrix as an acetonitrile-water extraction solution. This would mean that in aged feeds, where the water-drug solution had diffused deep into the feed matrix, the extraction solution composed entirely of acetonitrile would not be able to extract the drug as well as an acetonitrile-water extraction solution, since it would not penetrate deep enough into the feed matrix.

Bomben et al [84], Menting et al [85] and Chandrasekaran and King [86] reported that there was a substantial drop in diffusivity when the solute concentration was increased. Therefore, extending this to the situation in feeds, the lower the feed moisture content, the higher the solute concentration and the slower the diffusion. In the case of high moisture content feeds, the concentration of SDM in the water in the feed will be lower, therefore diffusion will occur at a greater rate.

The experiment investigating the effect of temperature on the rate of moisture adsorption in feeds can be explained by the diffusion rate being greater at high temperatures than at lower temperatures. This in turn can be explained by the Stokes-Einstein equation, Atkins [87].
\[ D = \frac{kT}{6\pi \eta a} \]

where
- \( T \) = temperature
- \( \eta \) = viscosity
- \( a \) = effective hydrodynamic radius of the spherical particle
- \( k \) = constant

The increase in temperature is accompanied by a decrease in viscosity, which results in the diffusivity increasing.

The structure insensitive solid diffusion model described by Lewis [64] also ties in with the need for water to be present in the extraction solutions in order to improve drug recoveries. If the drug has diffused into the pores in aqueous solution, then the further it diffuses into the pores, the less it is available to the extraction solution. Water is then necessary in the extraction solutions to dissolve the external SDM and initiate diffusion from the interior of the particles. This could also explain the oven drying results from section 3.2.1, since in the dry samples, there was insufficient water in the feed to allow the diffusion of SDM out of the pores. This, in itself, implies a surface film effect whereby, in gentle drying, e.g. over silica gel, the moisture on the surface of the feed particles is gently evaporated, pulling out the moisture in the internal pores to replace it. In oven drying, the rate of evaporation of moisture from the surface will be so rapid that moisture from the interior will have insufficient time to diffuse out to the surfaces, and there will be a discontinuous surface layer of water. Extending this to the extraction solution, the water is necessary to complete the surface film layer and allow the diffusion process to occur.

In summary, the work performed in this Chapter has shown that:

1. SDM recovery is inversely related to the moisture content of the feed.
2. The rate at which SDM recovery decreases with time is directly related to the moisture content.
3. The stability of SDM in a saturated aqueous solution has been demonstrated. This excludes SDM hydrolysis as being responsible for reduced SDM recoveries from high moisture content feeds.
4. Experimental results indicated that binding between SDM and feed constituents was occurring, and the reduced recoveries from pelleted feeds was thought to be due to both the increased moisture levels experienced by the feed and the physical modification of the feed constituents.

5. A paper by Schwarzer provided evidence that extrusion resulted in both higher feed moisture contents and water activity compared to a non-extruded feed. This was due to the less dense structure in the extruded product reducing the water holding capacity of the feed. Additionally, Schwarzer reported that, at extruded product moisture contents of 12%, water was present in liquid form. This would agree with the hypothesis that SDM is dissolved in the moisture in the feed, but only the biologically active water could take part in the dissolution process, so the higher the moisture content, the more SDM could be dissolved, resulting in reduced recoveries.

6. Moisture adsorption experiments have shown that in untreated feed, the mechanism is 1-dimensional diffusion, but pretreatment of the feed samples with organic solvents affects the mechanism so that although they follow 1-d diffusion initially, different mechanisms then take over. This has relevance to the requirement of water in the extraction solution to extract SDM quantitatively in aged samples. Clearly, solvent affects the mechanism of moisture adsorption, and also appears to affect the mechanism of moisture desorption, although it is not so pronounced as in adsorption. The role of water would appear to reduce the effect of the organic solvent on the mechanism of moisture adsorption/desorption.

7. The mechanism of moisture desorption from feed samples at 105 °C is not the same as when water is removed over silica gel. A mathematical equation to elucidate the desorption mechanism was not found, but the fact that it was different to the silica gel desorption mechanism could explain the results in Section 3.2.1 where oven drying the low humidity samples produced reduced SDM recoveries, and a similar trend was seen in the two high moisture content samples that were oven dried. Clearly, if moisture adsorption at room temperature follows a 1-dimensional diffusion mechanism in untreated feed, and a similar mechanism in the desorption stage, but does not follow
this mechanism during desorption at 105 °C, then the reduced SDM recoveries can be accounted for by deviation from the 1-dimensional diffusion.

8. Addressing the question of oven drying samples further, an explanation can be made by reference to film theory. If it is assumed that in a feed of 12 % moisture, water is present in all three types, then when the sample is heated in an oven, the bulk water will be removed first. After this is removed, the monolayer water will be removed at such a rate that there is not a complete film of water left over the particle surfaces. This results in the water trapped in pores not being able to evaporate due to the lack of a complete film preventing the 'wick-out' effect. Thus, SDM that has diffused deep into the pore structure of the particles in an aqueous solution, is trapped at the pore tips because there is insufficient water for SDM to allow its diffusion out of the pore. A more elegant summary of this situation is that the diffusion coefficient of SDM out of the pores is slower than that of water.

9. Moisture adsorption by both starch and α-protein showed that slight increases in particle sizes occurred although the experiments were only performed over approximately 15 minutes. This indicated that the same effect could be seen in pelleted feeds, although much quicker, due to increased temperatures and pressures.
Chapter 4

THE USE OF $^{14}C$-LABELLED SDM
CHAPTER 4 - THE USE OF RADIOLABELLED COMPOUNDS

4.1 RADIOLABELLED COMPOUNDS

The use of radiolabelled medicinal additives was an important part of the work performed to investigate the poor recovery of medicinal additives from feeds. The rationale behind their use was that the activity of the radiolabelled compound in the extraction solution, following extraction, could be monitored, and compared to the drug recovery calculated by the HPLC method. A concern raised in the experimental work was that, if SDM was undergoing degradation when it was in contact with the feed, and gave rise to products that were not retained on the HPLC column, this would not have been observed. Work performed in earlier chapters had not provided any evidence of SDM degradation occurring, but it was necessary to show unequivocally that this was the case. Although the main degradation products of SDM had been chromatographed, and with the exception of 2,4-dimethyl-6-hydroxypyrimidine, had been retained on the column, there was still a possibility that unidentified products, although at low concentrations, could elute with the solvent front. The use of radiolabelled medicinal additives would allow any degradation products that were not retained on the column to be detected, if a fraction collector was used to collect small fractions of the mobile phase after passing through the HPLC column, and if the latter were counted on a liquid scintillation counter. Additionally, it was proposed to perform autoradiographic studies using feed samples that had been dosed with the radiolabelled medicinal additive, then extracted with the extraction solution. In this way, it was hoped that any residual activity on the feed particles would be visualised using the autoradiographic film. This would permit the distribution of the residual drug on the feed particles to be determined.

The use of radiolabelled medicinal additives in studies would also allow a check to be made on the analytical method to ensure that none of the drug was lost during the clean-up stages.
4.2 $^{14}$C-LABELLED SDM

SDM was the first medicinal additive to be considered for radiolabelling. It was decided that SDM was to be labelled with 14-carbon ($^{14}$C) since the half-life of 5730 years was long enough to ignore its radioactive decay over the period of the study. It was also considered suitable for labelling SDM because it would be isotopic labelling (using a radionuclide of an atom already in the molecule) and would behave as the non-labelled SDM.

There was initial difficulty in finding a commercial supplier of $^{14}$C-SDM, without the great expense of having it made by custom synthesis. In this respect, it was decided that $^{14}$C-SDM would be synthesised at Loughborough.

It was necessary to devise a synthesis incorporating a commercially available $^{14}$-C compound which met the following criteria:

(i) The labelled precursor must be sufficiently stable for use in the synthesis, ie it should not undergo appreciable self-decomposition.

(ii) The labelled precursor chosen should be able to be incorporated into a synthetic pathway easily.

(iii) The synthetic pathway used should contain the minimum number of steps to maximise both the chemical yield and the specific activity of SDM.

4.2.1 Methods of Synthesis

Reviewing the literature for the synthesis methods for SDM showed that there were two distinct approaches employed. The first approach involved cyclisation reactions which were described by Rose and Swan [88], Korolkovas [89] and in The Pharmaceutical Manufacturing Encyclopaedia [90]. The second approach involves a condensation reaction between 2-amino-4,6-dimethylpyrimidine and p-acetamidobenzenesulphonyl chloride followed by hydrolysis of the acetyl group and was described by Caldwell et al [91]. The cyclisation reaction is used in the industrial preparation of SDM, but the condensation reaction was more compatible with small-scale laboratory preparation and was, therefore, chosen for further investigation.
4.2.2 Choice of suitably labelled precursor

Having decided upon the last two reaction steps to synthesise SDM, it was necessary to address the problem of getting labelled material suitable for incorporation into the last two steps. Considerations were to find a labelled precursor readily available commercially and with sufficient specific activity which could easily be converted to the p-acetamidobenzenesulphonyl chloride. [U-\(^{14}\)C] nitrobenzene (uniformly labelled with \(^{14}\)C in the benzene ring) was chosen to fulfill the requirements since it was readily available and could be easily converted to the sulphonyl chloride as indicated in Figure 4.1.

The conversion of nitrobenzene to p-acetamidobenzenesulphonyl chloride would be achieved by firstly converting nitrobenzene to aniline, which would be converted to acetanilide, which would, in turn, be converted to the p-acetamidobenzenesulphonyl chloride.
4.2.3 Sulphadimidine Preparation

It was decided to perform the synthesis using unlabelled nitrobenzene (cold synthesis) to investigate the success and yield of the reaction before attempting the synthesis using labelled nitrobenzene. This would permit any difficulties in the synthesis to be overcome and optimisation of experimental conditions to ensure maximum yields.

4.2.3.1 Experimental

Apparatus:
Perkin-Elmer 1600 Series FT-IR spectrometer. Varian EM360A nmr spectrometer.

Chemicals:
The following chemicals: nitrobenzene AR, tin metal (granulated), hydrochloric acid, sodium hydroxide, sodium chloride, petroleum ether, potassium hydroxide pellets, sodium acetate AR, chlorosulphonic acid, and pyridine were all obtained from Fisons Ltd, Loughborough, Leicestershire, UK. 2-amino-4,6-dimethylpyrimidine was obtained from Aldrich Chemical Co., Poole, Dorset, UK. Charcoal was obtained from Fluka Chemicals Ltd, Gillingham, Dorset, UK.

4.2.3.2 Step I: nitrobenzene to aniline (Vogel [92])
Nitrobenzene (26 ml) together with granulated tin (60 g) were introduced into a 1 l round-bottomed flask fitted with a reflux condenser. Concentrated hydrochloric acid (135 ml) was measured out, and 10 ml were then added slowly through the top of the condenser to the flask with gentle swirling. The remainder of the acid was added over a 30 minute period in small portions with continued agitation. After the acid had been added, the flask was heated on a steam bath for 30 minutes with occasional swirling after which time the flask was cooled giving a brown coloured solution. Water (50 ml) was added to the reaction mixture and then water (100 ml) in which had been dissolved sodium hydroxide (75 g). The condenser was removed and the flask arranged for steam distillation, with steam passed through the hot liquid until the distillate lost its milky appearance, and a further 100 ml of distillate collected. The distillate was then saturated with sodium chloride (38 g) and extracted twice with ether (50 ml). The ether washings were combined and dried over 10 g of potassium hydroxide pellets. The ether phase was transferred to a distilling flask and the ether removed by heating on a steam bath.
After the ether had been removed, the distillation was continued using a Bunsen burner and when the temperature reached 180 °C the distillation was interrupted and the water condenser replaced by an air condenser and the distillation continued to collect the product distilling over a 10 degree range. Aniline (16 g) was obtained giving a yield of 69 %. The FT-IR Spectra of the product and aniline reference are shown in Figure 4.2 and indicated that the product was aniline and that the purity was very good which was due to the distillation step.

4.2.3.3 Step II: aniline to acetanilide (Vogel [93])

Concentrated hydrochloric acid (18 ml) was added carefully to water (500 ml) in a 1 l conical flask. To the solution was added aniline (16 g). A solution of sodium acetate trihydrate (32.6 g) in water (100 ml) was prepared. The solution containing the dissolved aniline was warmed to 50 °C and acetic anhydride (24 ml) added, with swirling to aid dissolution. The solution of sodium acetate was then added in one portion, and the reaction mixture cooled in an ice bath with stirring to aid crystallisation of the product. The acetanilide was collected by suction filtration and was washed with small quantities of cold water, then the product dried in a desiccator to give a fine, white, crystalline solid (11 g) with a yield of 50 %. FT-IR spectra of both the product and a reference standard of acetanilide are shown in Figure 4.3. Again, good correlation between the product and reference spectra confirmed that acetanilide had been produced.
Figure 4.2
FT-IR spectra of product from Step I and aniline reference standard.
Figure 4.3
FT-IR spectra of product from Step II and acetanilide reference standard

acetref: acetanilide ref
acetprod: acetanilide prod
4.2.3.4 Step III: acetanilide to p-acetamidobenzenesulphonyl chloride (Vogel [94])

Chlorosulphonic acid (31 ml) was added to a dry 250 ml conical flask and then cooled to 10-15 °C in a water bath containing ice. Dry acetanilide (11 g) was added to the chlorosulphonic acid in small portions with thorough mixing. The temperature was not allowed to rise above 20 °C. Following addition of all the acetanilide, the reaction mixture was allowed to warm up to room temperature and was heated on a steam bath for 20 minutes to complete the reaction. The reaction mixture was cooled to room temperature using a water bath containing ice.

Crushed ice (330 g) together with water (55 ml) was placed in a large beaker in a fume cupboard. The reaction mixture was slowly poured into the beaker, with care taken to avoid splattering the chlorosulphonic acid. After addition was complete, the flask was rinsed with a little cold water and transferred to the large beaker. The crude precipitate that formed in the beaker was p-acetamidobenzenesulphonyl chloride and was collected by suction filtration and washed with cold water, then pressed dry to give 34 g of damp product. The next step was carried out immediately.

4.2.3.5 Step IV: p-acetamidobenzenesulphonyl chloride to p-acetamido-N-(4,6-dimethyl-2-pyrimidinyl)benzene sulphonamide (Caldwell et al [91])

2-amino-4,6-dimethylpyrimidine (0.49 mole) was suspended in dry pyridine (125 ml) and p-acetamidobenzene sulphonyl chloride (0.51) added, ensuring that the temperature did not rise above 55 °C, forming a yellow coloured solution. The reaction mixture was then heated on a steam bath for 1 hour, after which time the solution had turned a golden colour. A solution of sodium hydroxide (22 g) in water (110 ml) was added slowly, and the heating continued for a short time. The pyridine was removed by distillation under reduced pressure over a period of 70 minutes, with water being added periodically to maintain volume. The product was collected by filtration and crystallised from glacial acetic acid to give a yellowish white powder of mass 12 g.

4.2.3.6 Step V: p-acetamido-N-(4,6-dimethyl-2-pyrimidinyl)benzene sulphonamide to 4-amino-N-(4,6-dimethyl-2-pyrimidinyl) benzene sulphonamide (Caldwell et al [91])

The p-acetamido-N-[4,6-dimethyl-2-pyrimidinyl] benzene sulphonamide (12 g) was placed in a 500 ml round-bottomed flask and sodium hydroxide (1M, 88 ml) added. A reflux condenser was attached to the flask, and anti-bumping granules were added to the solution.
and heated gently under reflux for 3 hours. The reaction mixture was allowed to cool to room temperature, decolourised using charcoal, and then filtered to remove the charcoal. The filtrate was then acidified to pH6 with hydrochloric acid (0.5M, 40 ml). The solution was left overnight, after which time a white precipitate had formed. Filtration of the product and drying in a desiccator gave a product (0.9 g). The melting point was recorded at >360 °C but was difficult to determine accurately since the compound underwent charring. It appeared that the compound produced was the acid salt.

4.2.3.7 Amendment to method

After refluxing with sodium hydroxide and allowing it to stand overnight, a white gelatinous precipitate was observed to have formed. Neutralisation with hydrochloric acid (1M) to pH 5.9 resulted in the precipitate dissolving, permitting filtration to remove the anti-bumping granules. A portion of the solution (100 ml) was left unfiltered and placed in a separating funnel, to which was added dichloromethane (50 ml). The mixture was shaken, after which time, the lower dichloromethane layer was run off. This was repeated twice more. During the extraction process, the aqueous phase continued to have a yellow colouration and the dichloromethane phase was observed to have small particles present. This appeared to indicate that the solvent extraction using dichloromethane had been only partially successful. The three dichloromethane fractions were combined and placed in a 500 ml round-bottomed flask and evaporated to dryness over a period of 15 minutes. A white crystalline product was produced and was dried in a vacuum desiccator. The melting point was measured as 157-160 °C which indicated that it was not sulphadimidine, but in fact 2-amino-4,6-dimethylpyrimidine. This indicated that the reaction between the benzenesulphonyl chloride and the 2-amino-4,6-dimethylpyrimidine had not been successful.

4.2.4 Repeat of synthesis

As the production of acetanilide starting from nitrobenzene had been successful, it was decided to use commercially obtained acetanilide for use in the later stages of the synthesis. This was done to allow optimisation of the stages involving the addition of p-acetamidobenzenesulphonyl chloride to 2-amino-4,6-dimethylpyrimidine which appeared to be the step at which problems were occurring.

Acetanilide, supplied by Sigma Chemical Co., was converted to the sulphonyl chloride as
described previously to give a very pale pink/white solid. Reaction of the sulphonyl chloride with 2-amino-4,6-dimethylpyrimidine gave a crystalline solid, after allowing the solution to stand overnight. The solution was divided into two 35 ml portions and one portion was poured into ice-water (140 ml), then neutralised to pH 7 using sodium hydroxide (0.5 M). Excess hydrochloric acid (2 M) was then added to give pH 3.8 and the solution cooled in an ice bath. No product was found to have crystallised.

The remaining portion (35 ml) was left for several days, during which time a large amount of golden-coloured crystals were produced. The crystals were filtered off and recrystallised from the minimum amount of glacial acetic acid. The crystallised product was orange-coloured and was dried overnight in a desiccator. The $^1$H NMR spectra of the product was then taken and compared to the spectra of both sulphadimidine and 2-amino-4,6-dimethylpyrimidine. These are shown in Figures 4.4-4.6 respectively. It was apparent that the isolated product was, in fact, 2-amino-4,6-dimethylpyrimidine, since good correlation of the nmr spectra of the product and the 2-amino-4,6-dimethylpyrimidine was obtained. Clearly addition of 2-amino-4,6-dimethylpyrimidine to p-acetamidobenzene sulphonyl chloride had not been successful. This was considered unusual considering the known reactivity of the sulphonyl chloride.

The synthesis of $^{14}$C-SDM was then reviewed carefully, and it became clear that considerably more time would have to be spent on the synthesis than was first envisaged. This created problems since the primary function of the research work was to investigate the causes of poor analytical recovery of SDM from feeds using $^{14}$C-SDM, and not to spend large amounts of time synthesising it. Fresh efforts were made to find a supplier of $^{14}$C-SDM. Fortunately, Sigma Chemical Co. had a quantity of $^{14}$C-SDM which had been left over from a custom synthesis. It was available at reasonable cost, and therefore, it was purchased to enable analytical work to proceed.
Figure 4.4

$^1$H-nmr spectrum of product after reaction of p-acetamidobenzene sulphonyl chloride and 2-amino-4,6-dimethylpyrimidine
Figure 4.5

$^1$H-nmr spectrum of sulphadimidine reference
Figure 4.6

$^1$H-nmr of 2-amino-4,6-dimethylpyrimidine
4.3 USE OF $^{14}$C-SDM IN ANALYTICAL STUDIES

4.3.1 Chemicals
Sulphadimidine-phenyl-ring UL-$^{14}$C was supplied by Sigma Chemical Co, St Louis, USA. The activity was uniformly distributed in the phenyl ring of the molecule and had a specific activity of 8.2 mCi mmol$^{-1}$. The purity was >98 %.

4.3.2 Preparation of $^{14}$C SDM standard solution
$^{14}$C SDM (10 MBq) and carrier SDM (21.5 mg) were transferred into 25 ml volumetric flask and dissolved in methanol and made up to the mark. This was the labelled SDM dosing solution and was of specific activity of 401 kBq ml$^{-1}$ and SDM concentration of 0.96 mg ml$^{-1}$.

4.3.3 Radiochemical Purity of $^{14}$C SDM
$^{14}$C SDM dosing solution (100 µl) was diluted to 10 ml using HPLC mobile phase to give an SDM concentration of 9.6 µg ml$^{-1}$. Aliquots of this solution (20 µl) were then injected into the HPLC column. The column eluate was collected by means of a fraction collector set to collect a fraction for 0.33 minutes which corresponded to a volume of mobile phase of 0.5 ml. After each run, 4.4 ml Ecoscint A was added to each vial which was then stoppered, shaken and counted for 5 minutes on a liquid scintillation counter. The activity values were quench-corrected and background-subtracted. The UV chromatogram and activity profile are shown in Figures 4.7a and 4.7b respectively, and indicated that no radioactive compounds apart from $^{14}$C SDM were present in the $^{14}$C SDM dosing solution. The slightly longer retention time for the SDM peak in the activity profile, compared to the chromatogram, is due to the fraction collector being situated after diode array detector, which introduced a slight time delay.
Figure 4.7a

Chromatogram of $^{14}$C-SDM

Figure 4.7b

Activity profile of $^{14}$C-SDM
4.3.4 Analytical Recovery of SDM from pig feed

Two grams of commercially obtained pig feed were accurately weighed into 6 sample tubes (1-6). To each was added 0.5 ml of the labelled SDM dosing solution to give SDM dosing levels of 232-237 \( \mu \text{g g}^{-1} \). The tubes were then capped and mixed using a vortex mixer, for 10 seconds to allow interaction between the dosing solution and the feed. The samples were then left uncapped in a fume hood to allow the methanol to evaporate. Tubes 1 and 2 were then extracted after removal from the fume hood which corresponded to a drug-feed contact time of 1 hour. Tubes 3 and 4 were extracted after a contact time of 7 days, while 5 and 6 were extracted after 16 days. SDM recoveries were measured by HPLC using uv detection as well as radiochemical detection by fraction collection and liquid scintillation counting. The results are shown in Table 4.1.

To ensure that loss of activity did not occur during the extraction, clean-up and chromatographic stages of the analysis, experimental specific activity of the processing solutions were

<table>
<thead>
<tr>
<th>Sample</th>
<th>Contact Time (days)</th>
<th>Percentage SDM Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HPLC (±4%)</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>94</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>73</td>
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<tr>
<td>4</td>
<td>7</td>
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<td>5</td>
<td>16</td>
<td>60</td>
</tr>
<tr>
<td>6</td>
<td>16</td>
<td>55</td>
</tr>
</tbody>
</table>
compared to theoretical specific activities which enabled a mass balance study to be carried out. Thus, all the activity extracted could be monitored to ensure that no losses occurred during the subsequent processing stages. The results are shown in Table 4.2.

The recoveries showed that there was a steady decrease as the time of contact between the drug and feed increased. Both measurements made using HPLC with UV detection and Liquid Scintillation Counting showed this trend with good agreement.

Table 4.2
Analytical method mass balance

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contact Time (days)</td>
<td>0</td>
<td>7</td>
<td>16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Analytical Stage (± 5%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentagea extraction efficiency</td>
<td>92</td>
<td>88</td>
<td>85</td>
<td>79</td>
<td>68</td>
<td>69</td>
</tr>
<tr>
<td>Percentageb retention of SDM on cation-exchange column</td>
<td>97</td>
<td>96</td>
<td>96</td>
<td>96</td>
<td>90</td>
<td>88</td>
</tr>
<tr>
<td>Percentagec activity removed by washing column with water</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Percentagec activity removed by washing column with methanol</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Percentaged recovery of SDM from column</td>
<td>98</td>
<td>96</td>
<td>81</td>
<td>86</td>
<td>88</td>
<td>83</td>
</tr>
</tbody>
</table>

Extraction Efficiency a is the amount of activity found in the extraction solution as a percentage of the total amount of activity added to feed.

Retention b is the percentage of the extracted activity retained on the cation-exchange column calculated by measuring the activity of the load solution after passing through the column.

Wash c is the percentage of activity retained on the cation-exchange column removed by washing with either water or methanol.

Elution d is the percentage of the retained activity on the cation-exchange column that was removed by elution with 25ml of HPLC mobile phase.
4.4. AUTORADIOGRAPHY STUDIES

4.4.1 Introduction

Autoradiography is defined as the visualisation of the distribution of radiation from a specimen which absorbs and scatters its own activity. Autoradiography is an old technique whose origins date to before the study of radioactivity itself and relies upon placing a sample in close proximity to a nuclear emulsion or solid-state detector which enables the intrinsic radiation pattern to be recorded.

The first recorded evidence of autoradiography being performed was in 1867 when Niepce de St. Victor [95] reported that both silver chloride and silver bromide were blackened on exposure to uranium salts. Becquerel, in 1896 [96], continued this work by observing that such blackening occurred through sheets of black paper in the absence of sunlight. Work by the Curies, in 1898 [95], further developed this work and led to the discovery of radioactivity through their experiments in isolating radium sulphate from pitchblende (an oxide of uranium also containing radium, thorium, cerium and lead). The application of this theory enabled autoradiography to be understood more fully, and, in 1908 an autoradiogram of a frog that had been put in a container with some radium was published. The experiment demonstrated that the frog had taken up radium through its skin which had reacted with the film emulsion to produce an image.

The next developments in autoradiography occurred in the 1940s as a result of the advances in nuclear physics. The construction of the atomic bomb and controlled nuclear fission led to the availability of a new range of man-made radionuclides, and coupled to this was the development of new photographic emulsions.

The 1950s saw the development of liquid emulsions which enabled slides to be dipped into molten solutions of the emulsions, and then progressed to allow stripping film techniques which involved stripping the emulsion off glass slides and placing it against the sample. As autoradiography continued in its use, resolution improved. Nuclear emulsions today are extremely versatile in allowing a wide range of charged particles and ionising radiations to be recorded and also for the wide range of magnifications at which they may be viewed.
As well as emulsions for recording the distribution of radioactivity in a sample, solid-state detectors are also used. Materials used include thin metal foils and layers of plastics such as cellulose nitrate, PVC and acetylbutyrate among others. The man-made plastics have found widest use, since they are generally easier to use because they do not need the use of a darkroom and also because there is greater specificity for the type of radiation to be recorded.

4.4.2. Principles of detection

Nuclear emulsions are essentially photographic emulsions which are specialised to give sensitivity to charged particles and ionising radiation. The emulsion consists of a dispersion of silver halide crystals in a matrix of gelatin. The role of the matrix is to act as a supporting medium which isolates the crystals and allows each to develop independently, without initiating a reaction in its neighbours.

The crystals are mainly silver bromide and can vary in size from 0.07-0.40 μm and are in a very narrow band according to the use of the particular emulsion. Once exposed to ionising radiation or light photons, the bromide ions liberate electrons which become mobile in the conductivity band and leave the electron deficient bromide atoms in the lattice. The transfer of an electron from a bromide atom to a neighbouring bromide ion results in movement of a positive hole through the crystal. The electron travels until it encounters an interstitial silver ion at the point of lattice defect and forms an unstable metallic silver atom. This site becomes an electron trap and forms a latent image centre consisting of several silver atoms.

Modern autoradiographic films have been developed providing a wide range of products specifically suited to the isotope being detected.

4.4.3 Experimental studies

4.4.3.1 Reagents

The autoradiography film used was Hyperfilm-β max supplied by Amersham International, Amersham, Bucks, UK. The developer used was Agfa G 150 and the fixer was Agfa G 350 both supplied by Agfa-Gevaert, Belgium.
4.4.3.2 Procedure
To investigate the distribution of the residual SDM, autoradiography studies were initiated. The autoradiographic procedure is shown in Figure 4.8. After the feed solid was filtered off from the extraction solution, the filter paper and the solid were carefully dried, and adhesive tape strips were then used to remove small portions of the feed from the filter paper which were then fixed onto microscope slides. This method was found to be much easier than trying to embed the feed solid in wax and taking sections using a microtome.

Once the slides had the feed particles affixed to them, they were placed in contact with the autoradiography film and left to expose. Preliminary studies showed that the exposure time necessary to produce sharp images was ~7 days. After this exposure time, the film was placed in the developing tank for 4 minutes followed by the fixing tank for 8 minutes. The film was then rinsed for 12 minutes. Quantitation of the amount of activity associated with a particular sample due to the blackening of the film was not possible, due to the uneven thicknesses of the particles on the slides. Sample 3 required a total of 41 tape transfers to be made onto slides and all were then exposed to the autoradiographic film for 4 days, after which time they were developed. The same procedure was also followed for sample 5. The microscope slides were then photographed with enlargement together with the autoradiography films. Correlation between the residual $^{14}$C SDM and the feed particles was then possible.

4.4.3.3 Results
Photographs of both slides and autoradiographic films are shown in Figures 4.9 and 4.10. The activity was found to be generally throughout all the feed samples which indicates that the binding is non-specific.

4.4.3.4 Discussion
Residual $^{14}$C SDM was observed to be generally distributed throughout the feed. There was no evidence of the $^{14}$C SDM binding to a specific constituent since the microscopic separation of similar feed particles followed by exposure to autoradiographic film showed that activity was associated with all the particles. The difficulty in performing this study was in identifying the feed particles. To overcome this problem, a study was planned in which individual feed components were separately dosed with $^{14}$C SDM.
Figure 4.8
Autoradiography Procedure

Feed on filter paper → Particles removed with clear adhesive tape → Tape affixed to slide

Possible to correlate 14C SDM distribution with feed particles → Film developed → Slide covered with autoradiographic film and exposed for several days
Figure 4.9

$^{14}\text{C-SDM}$ Recovery from feeds
Sample 3
Figure 4.10

$^{14}$C-SDM Recovery from feeds
Sample 5
4.4.4 Study dosing individual feed constituents with $^{14}$C-labelled SDM

To confirm that binding was non-specific, it was decided to dose feed constituents individually with the $^{14}$C-SDM solution and investigate whether, after extraction, residual activity was found on the samples which would confirm the non-specific nature of the binding.

4.4.4.1 Procedure

Individual feed constituents (wheat, barley, meat and bone meal, Hi Pro Soya, rice bran, wheat feed, malt culms, fish meal and full fat soya) were obtained from a local mill. Calcium carbonate was also chosen, since it is used in some laboratories to prepare a SDM premix. Eight samples of each constituent (0.1g) were accurately weighed into scintillation vial inserts, to which were added 20 µl of the $^{14}$C SDM. After addition of the solution, all samples were capped and mixed, using a whirl mixer for 10 seconds to allow interaction between the feed and SDM, then uncapped and left in a fume hood for 1 hour to allow the methanol to evaporate. Duplicate samples from each constituent batch were then extracted, using 70% aqueous acetonitrile, by shaking mechanically for 1 hour, after which time the samples were centrifuged at 6000g for 1 minute to separate the extraction solution from the solid. Aliquots of the extraction solutions (20 µl) were then taken and added to scintillation vials containing 4.2 ml of Ecoscint. Following mixing, all samples were counted in the $^{14}$C channel of the liquid scintillation counter, and a subtraction for background was made. Calculation of the extraction efficiency could be made by dividing the experimentally found specific activity by the theoretical specific activity. The extraction process was repeated after 7, 14 and 25 days contact time between SDM and the feed. The $^{14}$C SDM recoveries are shown in Table 4.3.

As in the previous study, samples extracted after 7 days contact time were then prepared for autoradiography, and subsequent exposure of the slides to the film for 10 days produced sharp images which can be seen in Figure 4.11.

4.4.4.2 Results

The autoradiographs obtained showed that in all samples, except with calcium carbonate, residual activity was found on the constituent particles after extraction with the extraction solvent. Quantitation of the amount of residual $^{14}$C SDM on the constituent particles was difficult because the samples were not of uniform thickness. This resulted in particles that were large producing darker images simply because they were in closer contact with the film.
**Table 4.3**

$^{14}$C-SDM recovery from constituents

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Contact Time/days</th>
<th>0</th>
<th>7</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td></td>
<td>85</td>
<td>76</td>
<td>75</td>
</tr>
<tr>
<td>Fishmeal</td>
<td></td>
<td>83</td>
<td>80</td>
<td>89</td>
</tr>
<tr>
<td>Ricebran</td>
<td></td>
<td>87</td>
<td>80</td>
<td>75</td>
</tr>
<tr>
<td>Barley</td>
<td></td>
<td>93</td>
<td>87</td>
<td>72</td>
</tr>
<tr>
<td>HiPro Soya</td>
<td></td>
<td>97</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Calcium Carbonate</td>
<td></td>
<td>91</td>
<td>95</td>
<td>92</td>
</tr>
<tr>
<td>Full Fat Soya</td>
<td></td>
<td>90</td>
<td>90</td>
<td>73</td>
</tr>
<tr>
<td>Malt Culms</td>
<td></td>
<td>94</td>
<td>79</td>
<td>69</td>
</tr>
<tr>
<td>Meat and Bone Meal</td>
<td></td>
<td>96</td>
<td>91</td>
<td>71</td>
</tr>
<tr>
<td>Wheatfeed</td>
<td></td>
<td>90</td>
<td>73</td>
<td>59</td>
</tr>
</tbody>
</table>
Figure 4.11

$^{14}$C-SDM Recovery from constituents
Although quantitation was not possible, it was still appropriate to make observations on the results obtained. The cereals (wheat, ricebran, barley, malt culms and wheatfeed) all appeared to produce darker images than the other constituents. Both Hi Pro Soya and Full fat soya, despite having large particle sizes, produced only feint images.

4.4.4.3 Discussion

It was obvious that the non-extracted activity was still associated with the feed solid, and subsequent exposure of the feed solid to autoradiographic film confirmed this. Clearly, SDM was binding to the feed components as was suggested by Blanchflower and Rice [45]. The distribution of the labelled SDM on the feed particles was determined by direct comparison of the autoradiography film with the feed particles affixed to the slides. No distinct distribution pattern was seen, and the activity appeared to be generally distributed on nearly all the feed particles. The subsequent recovery study using individual feed components further showed the general nature of the SDM association with the feed particles.

The mechanism, by which the SDM is bound to the feed particles, must now be considered carefully, since Conway [48] suggested that the SDM is protein bound. This must be questioned because the distribution of the labelled drug is so general. Clearly, with so general a distribution pattern being evident, there are either several different binding mechanisms, which would account for the drug being bound to a large number of different particles, or there is a general mechanism. The latter could involve water as was suggested in Chapter 3.

4.5 CONCLUSIONS

The use of the $^{14}$C-labelled SDM has made it possible to rule out SDM degradation as the cause because careful monitoring of the extraction solution showed that all were of lower specific activity than were expected.

From the work performed in this chapter, it has been shown that:

1. Drug recovery studies from feeds showed that incomplete recoveries of $^{14}$CSDM occurred.

2. Recoveries were found to decrease with time.
3. The non-extracted $^{14}$C-SDM was residual on the feed particles.

4. Autoradiography studies on the feed particles after extraction showed that the residual $^{14}$C-SDM activity was generally distributed throughout the feed which indicated general binding.

5. Drug recovery studies from individual feed constituents also showed that incomplete recoveries of $^{14}$C-SDM were obtained.

6. Autoradiography studies on the individual constituents after dosing showed that the non-extracted $^{14}$C-SDM activity was found on all the constituents' particles.

7. No degradation products of $^{14}$C-SDM were found.

8. The incomplete recovery of SDM from feed samples can be attributed to irreversible binding of the drug to feed constituents.
Chapter 5

SULPHADIAZINE AND TRIMETHOPRIM
CHAPTER 5 - SULPHADIAZINE AND TRIMETHOPRIM

5.1 INTRODUCTION
The problem of poor recoveries of drugs from feeds is not confined to SDM, but is a widespread problem. To extend the investigation into the causes, other drugs which are used as medicinal additives in feeds and also give recovery problems, were considered.

Sulphadiazine (SDZ) is another sulphonamide widely used as a medicinal additive, usually in conjunction with Trimethoprim (TMP). The structures of SDZ and TMP are shown in Figure 5.1. The SDZ / TMP premix is used to treat diseases which are caused by bacteria sensitive to potentiated sulphonamide preparations.

Figure 5.1
Structures of Sulphadiazine and Trimethoprim

Sulphadiazine

Trimethoprim
5.1.1 Chemical Properties

5.1.1.1 Sulphadiazine

The chemical properties of SDZ are very similar to sulphadimidine, with it being practically insoluble in water, ether or chloroform, and very slightly soluble in alcohol and in acetone. SDZ is soluble in mineral acids, and in solutions of alkali hydroxides and carbonates. The pKa of SDZ is 6.5 measured at 25°C. It is readily absorbed after oral administration, and, like SDM, is acetylated with up to 15% in the body being present as the inactive N\textsuperscript{4}-acetyl derivative. Twenty four hours after dosing, 50% of the dose is excreted in the urine of which 40% is the N\textsuperscript{4}-acetyl derivative and 50% is unchanged drug. It is a white, yellowish-white or pinkish-white crystals or powder which slowly darkens on exposure to light.

5.1.1.2 Trimethoprim

TMP is white or yellowish-white crystals or a crystalline powder, whose melting point is 199-202°C and pKa is 7.2. Unlike both SDM and SDZ, it is soluble in water (1 in 2500). It is also reported as being soluble in ethanol, chloroform, and methanol in order of increasing solubility. Following oral administration, TMP is readily absorbed and undergoes several metabolic reactions. These include: oxidation of the methylene group to a hydroxymethyl group; N-oxidation, O-demethylation, hydroxylation, and conjugation with glucoronic acid or sulphate. The majority of the drug is excreted unchanged, but the metabolites are conjugated.

5.1.2 Background to experimental work

Consultation with a firm producing a premix containing both drugs, which is used by manufacturers of medicated feeds, revealed that problems had arisen due to poor recoveries of the drugs in a similar fashion to those with SDM.

In a specific case, a consignment of feed was delivered to a farm and fed to the animals, who nevertheless subsequently developed the disease the medicated feed was supposed to prevent. The farmer attributed the disease outbreak to an insufficient level of medication in the feed. Subsequent analysis of the feed by an independent analyst, on behalf of the farmer, showed that only 70% of the stated drug content was found. Complaints by the farmer to the feed manufacturer were redirected to the premix manufacturer for supplying the premix with what
appeared to be below the stated content of drug. The premix manufacturer then had to demonstrate that the product he supplied to the feed manufacturer did contain the stated amount of drug. By the close liaison with the premix manufacturer and a feed mill, it was possible to initiate studies investigating the recovery of both SDZ and TMP on the industrial scale, which are described in detail later.

5.2 DEVELOPMENT OF ANALYTICAL METHOD FOR SDZ AND TMP

In order to investigate the recovery problem, initial work concerned the development of a suitable analytical method for SDZ and TMP in animal feeds. Ideally, it was hoped to develop a simultaneous extraction method, but if this were not possible, then separate extraction of SDZ and TMP would also be acceptable.

The various considerations for a method were:

(i) Selection of a suitable mobile phase
(ii) Selection of suitable extraction solution
(iii) Optimisation of the analytical method.

5.2.1 Experimental

5.2.1.1 Apparatus

As specified in Chapter 2.

5.2.1.2 Chemicals

Reagents as specified by Conway [48]. Premix containing sulphadiazine and trimethoprim was supplied by Cheminex Laboratories Ltd, Kettering, Northants, UK. Sulphadiazine and Trimethoprim supplied by Cheminex.

5.2.2 Mobile Phase Selection

A review of HPLC systems [97-106] employed for the separate analysis of TMP and SDZ showed that generally reversed phase systems were the most common. Several of the reversed phase methods reviewed for the simultaneous determination of TMP with sulphonamides enabled two observations to be made in connection with the TMP peak. Firstly, the TMP eluted late in the chromatogram, and secondly, the TMP exhibited significant broadening. In
several cases an organic modifier was used to reduce the retention time of the TMP.

In one method described by Torel et al [105], a 20 x 0.47 cm Spherisorb ODS Hichrom 5 μm column was used together with a mobile phase of composition acetonitrile: 0.05 N sulphuric acid (22:78 v/v) and a flow rate of 1 ml min⁻¹. The mobile phase specified by Torel et al was used with a 25 cm Apex ODS column, which had been used for the determination of SDM in feeds according to the method described by Conway [48], with injections of a standard solution of TMP. The chromatogram of a TMP standard solution injected onto the column can be seen in Figure 5.2. TMP eluted at ~12 minutes with considerable broadening and tailing of the peak. Additionally, the absorbance was only very low with an unstable baseline. Since the chromatography obtained using this method was less satisfactory than by using the method described by Conway [48], it was decided to investigate the use of the mobile phase described by Conway. Experimental work was then undertaken using standard solutions of SDZ and TMP using the HPLC system described by Conway.

5.2.2.1 Injection of working standard solution of SDZ onto HPLC Column
SDZ (25.7 mg) was weighed into a 100 ml volumetric flask and diluted to the mark with methanol. Sonication of the flask for 5 minutes ensured complete dissolution of the SDZ to give a SDZ stock standard solution of concentration 0.257 mg ml⁻¹. A 1 ml aliquot of this solution was pipetted into a 100 ml volumetric flask and diluted to the mark using HPLC mobile phase which gave a SDZ working standard solution of concentration 2.57 μg ml⁻¹. A representative chromatogram obtained from triplicate injections of this solution is shown in Figure 5.3. It can be seen that there is a single peak eluting at 3.55 minutes. The uv Spectrum of this peak was compared to the uv Spectrum of the working standard solution obtained using a Philips 8700 uv Spectrophotometer. Good correlation between the two spectra confirmed the chromatogram peak identity was SDZ.

Standard solutions of SDZ were prepared with the following concentrations: 11.83, 15.76 and 31.52 μg ml⁻¹ and injected to investigate the linearity of the detector response. The results are shown in Table 5.1 and the resulting correlation coefficient of 0.9998 indicated that the detector response was linear with SDZ concentration over the range investigated.
Figure 5.2
Chromatogram of TMP standard using MeCN/H$_2$SO$_4$ as mobile phase
Figure 5.3
Chromatogram of SDZ working standard solution
Table 5.1

Calibration Graph of SDZ concentration vs. SDZ Peak Area

<table>
<thead>
<tr>
<th>SDZ Concentration /μg ml⁻¹</th>
<th>Area under SDZ peak / units²</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.83</td>
<td>37.473 37.473</td>
</tr>
<tr>
<td>15.76</td>
<td>49.860 48.760 48.860</td>
</tr>
<tr>
<td></td>
<td>48.700 48.700 48.720</td>
</tr>
<tr>
<td>31.52</td>
<td>98.425 98.265</td>
</tr>
</tbody>
</table>

5.2.2.2 Injection of working standard solution of TMP onto HPLC column

TMP (11.7 mg) was weighed into a 100 ml volumetric flask and dissolved in MeOH and diluted to the mark to give a stock standard solution of concentration of 0.488 mg ml⁻¹. A 2 ml aliquot of the stock standard solution was then pipetted into a 100 ml volumetric flask and diluted to the mark with HPLC mobile phase to give a working standard solution concentration of 37.44 μg ml⁻¹. The TMP was then injected several times and gave peaks at 6.3 minutes which showed some tailing. A representative chromatogram is shown in Figure 5.4. A uv spectrum was taken of the peak eluting at 6.3 minutes and compared to the uv spectrum of the TMP standard solution obtained using a Philips 8700 uv Spectrophotometer. Good correlation between the two spectra indicated the peak eluting at 6.3 minutes was TMP.

One feature of the peaks was that only relatively weak absorbances were recorded in spite of the concentration of the working standard solution used. The diode array detector allowed several different wavelengths to be tried but none gave improvements in absorbance.
Figure 5.4
Chromatogram of TMP
working standard solution
5.2.2.3 Loading and Recovery of SDZ from the Cation-Exchange Column

It was decided to take the analysis of SDZ a stage further to investigate whether the clean-up method specified by Conway [48] would also be suitable for SDZ. To investigate this, SDZ (59.4 mg) was weighed into a 100 ml volumetric flask and diluted to the mark with extraction solution. After 5 minutes sonication to ensure complete dissolution, the solution was mixed thoroughly. A 10 ml aliquot was taken, pipetted into another 100 ml volumetric and diluted to the mark with extraction solution. The SDZ concentration in the load solution was 59.4 μg ml⁻¹. After conditioning the column, 10 ml of the load solution was added to the column, according to the method. The load solution was collected after passing through the column, as was the solution used to elute the SDZ. These were then chromatographed and analysed. Injection of the load solution after passing through the cation-exchange column (CEC) gave the chromatogram shown in Figure 5.5. The chromatogram from injection of the elution solution is shown in Figure 5.6. The results are shown in Table 5.2 and indicate that, within experimental error, quantitative recovery of SDZ from the CEC was obtained.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration of SDZ solution / μg ml⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Theoretical</td>
</tr>
<tr>
<td>A</td>
<td>23.76</td>
</tr>
<tr>
<td>B</td>
<td>23.76</td>
</tr>
</tbody>
</table>

5.2.3 Investigation into extraction of TMP - extraction solution selection

To determine whether the acetonitrile-water extraction solution was capable of extracting TMP from a feed, it was decided to perform an extraction on a feed containing both SDZ and TMP. The clean-up stage would be omitted, to simplify the experiment, but the extraction solution would be diluted to the same concentration that would be obtained if the clean-up stage
Figure 5.5
Chromatogram of load solution after passing through cation-exchange column

Figure 5.6
Chromatogram of solution used to elute SDZ from column
had been performed. This then allowed the efficiency of the extraction solution for TMP to be determined.

A feed containing SDZ and TMP (supplied by Cheminex Laboratories Ltd.) was extracted with the acetonitrile-water extraction solution. The feed extract (10 ml) was diluted to 25 ml in a volumetric flask with HPLC mobile phase, to provide similar concentration levels as would be found in samples that had undergone clean-up. This solution was then filtered through a 0.45 μm filter and injected onto the HPLC column. A representative chromatogram is shown in Figure 5.7 and illustrates that although SDZ is present in the extraction solution, no TMP was detected.

The same feed extract solution was then spiked with a TMP standard solution, and the effect of this is shown in Figure 5.8. This implies that the TMP was not being extracted from the feed by the aqueous acetonitrile extraction solution which successfully extracted SDZ. The fact that TMP was not extracted from the feed matrix by the extraction solution suggests that the interaction between the TMP and the feed matrix is greater than compared to that of SDZ.

5.2.4 TMP extraction solution studies

The aim was to extract the SDZ and TMP either simultaneously or by separate extractions. To this end, different solvents were tried with varying degrees of polarity. In all extraction experiments, either 10 g or 2 g of feed were used with either 100 ml or 20 ml of extraction solution respectively. After mechanically shaking the feed with the extraction solution for 1 hour, the mixture was filtered through Whatman GF/A filter paper and 10 ml of the filtrate diluted to 25 ml with HPLC mobile phase. The solutions were filtered through 0.45 μm filters before injection onto the HPLC column. Quantitation of SDZ was achieved by integration of the area under the peak corresponding to SDZ. With TMP, because of variable integration results, peak heights were measured manually.

In order to evaluate whether TMP had been extracted with the various extraction solutions, the PU6003 Software, coupled to the Diode Array Detector was used. This enabled a comparison between the uv spectrum of the chromatographic region where TMP should elute and a TMP reference spectrum. If no match occurred, then TMP was not extracted. This procedure was adopted because, in several chromatograms, small baseline perturbations coincided with the
Figure 5.7
Chromatogram of diluted feed extract showing presence of SDZ and absence of TMP

Figure 5.8
Chromatogram of diluted feed extract (as in Figure 5.7) with TMP spike
region where TMP should elute. There were several occasions where small peaks eluting around the 6 minute region appeared to be TMP, but on taking the uv spectrum, they did not correspond to the TMP reference spectrum. The results for the following extraction solutions used are summarised in Table 5.3.

5.2.4.1 Acidified Methanol
Phosphoric acid (85%, 6 g) was made up volumetrically to 1 l with methanol. The chromatograms obtained from the injection of the extraction solutions are shown in Figure 5.9. SDZ was identified in the chromatogram, but TMP was not observed.

5.2.4.2 Methanol / Acetonitrile
Acetonitrile: methanol (50:50 v/v) was used, and the chromatogram obtained is shown in Figure 5.10. Again SDZ was present, but TMP was not observed.

5.2.4.3 DMF
On filtering the solutions through the 0.45 μm filters, it was noted that the filters were prone to blocking. No TMP was detected, but SDZ was detected. The chromatogram obtained is shown in Figure 5.11.

5.2.4.4 Alkaline DMF
Ammonia (0.88), 3 ml was diluted to 1 l with DMF. Again, when filtering, the 0.45 μm filters became easily blocked. The chromatogram is shown in Figure 5.12 and shows SDZ, but not the presence of TMP.

5.2.4.5 Ethyl Acetate
Ethyl acetate was used. Since ethyl acetate was not miscible with the HPLC mobile phase, 10 ml of the filtered extract was taken down to dryness on a rotary evaporator and then taken up in 25 ml of mobile phase with sonication. Chromatographic analysis of the extraction solution, shown in Figure 5.13, showed that a very ‘clean’ chromatogram was obtained i.e. no co-extractives from the feed were present. As previously, SDZ was detected, but TMP was not.
5.2.4.6 Soxhlet Extraction
Soxhlet apparatus was used with 200 ml of DMF, and the solution was left circulating for 4.5 hours, after which time a very dark coloured solution was obtained. Injection of the solution showed that SDZ was extracted, but not TMP, as can be seen in Figure 5.14.

5.2.4.7 Acetic Acid
Extraction using 0.1M acetic acid produced the chromatogram shown in Figure 5.15. Neither SDZ nor TMP was detected.

5.2.4.8 Hexane
With the use of hexane as the extraction solvent, neither SDZ nor TMP was extracted, as can be seen in Figure 5.16.

5.2.4.9 DMF with Homogenisation
10g of feed was weighed in to a 250 ml plastic bottle, and 100 ml DMF was added. Homogenisation was then tried using a Gallenkamp Homogeniser, with 45 minutes at setting 10 followed by 30 minutes at setting 7. Injection of the filtered extract and chromatographic analysis afforded the chromatogram shown in Figure 5.17. SDZ was detected, but no TMP.

5.2.4.10 Discussion
The results showed that with SDZ extraction, all the extraction solutions tried, with the exception of hexane and acetic acid, extracted SDZ with recoveries between 64-95 %. With TMP extraction, none of the extraction solutions tried were successful. It was evident that the difficulty of the method development would be the extraction of TMP.

5.2.5 Dosing and Recovery with DMF
With all the extraction solutions tried in the previous section, only SDZ has been detected. The feed used in the analytical studies were all of the order of 3 months old. It was decided to dose a blank feed with a solution of TMP in methanol to investigate whether recovery of TMP could be achieved using a freshly dosed feed. A methanol solution of TMP was used in preference to a premix of TMP because the dosing level could be more accurately known with a solution.
A blank feed (10 g) was weighed into two 250 ml conical flasks, labelled A and B. To each was added 1 ml of 0.825 mg ml\(^{-1}\) of TMP in methanol. The flasks were stoppered and shaken for 60 seconds, and to A was added 100 ml of DMF/NH\(_3\), as prepared in 4. To B was added 100 ml of DMF. The extraction procedure was then followed, and the chromatograms obtained from injection of the solutions are shown in Figures 5.18 a and b. The results are shown in Table 5.4.

<table>
<thead>
<tr>
<th>Extraction Solution</th>
<th>SDZ Concentration (μg g(^{-1}))</th>
<th>Percentage Recovery (±4%)</th>
<th>TMP Concentration (μg g(^{-1}))</th>
<th>Percentage Recovery (±4%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Added</td>
<td>Found</td>
<td></td>
<td>Added</td>
</tr>
<tr>
<td>H(^+)/MeOH</td>
<td>400-600</td>
<td>569</td>
<td>95</td>
<td>80-120</td>
</tr>
<tr>
<td>H(^+)/MeOH</td>
<td>400-600</td>
<td>558</td>
<td>93</td>
<td>80-120</td>
</tr>
<tr>
<td>MeOH/MeCN(_2)</td>
<td>400-600</td>
<td>557</td>
<td>93</td>
<td>80-120</td>
</tr>
<tr>
<td>DMF</td>
<td>250</td>
<td>159</td>
<td>64</td>
<td>50</td>
</tr>
<tr>
<td>DMF</td>
<td>250</td>
<td>182</td>
<td>73</td>
<td>50</td>
</tr>
<tr>
<td>DMF/NH(_3)</td>
<td>250</td>
<td>167</td>
<td>67</td>
<td>50</td>
</tr>
<tr>
<td>DMF/NH(_3)</td>
<td>250</td>
<td>177</td>
<td>71</td>
<td>50</td>
</tr>
<tr>
<td>Ethyl Acetate</td>
<td>250</td>
<td>168</td>
<td>66</td>
<td>50</td>
</tr>
<tr>
<td>Hexane</td>
<td>250</td>
<td>-</td>
<td>-</td>
<td>50</td>
</tr>
<tr>
<td>Acetic acid (0.1M)</td>
<td>250</td>
<td>-</td>
<td>-</td>
<td>50</td>
</tr>
</tbody>
</table>
Figure 5.9
Chromatogram of feed extract using MeOH/H⁺ as extraction solution

Figure 5.10
Chromatogram of feed extract using MeOH/MeCN as extraction solution

Figure 5.11
Chromatogram of feed extract using DMF as extraction solution
Figure 5.12
Chromatogram of feed extract using alkaline DMF as extraction solution

Figure 5.13
Chromatogram of feed extract using ethyl acetate as extraction solution

Figure 5.14
Chromatogram of feed extract using DMF with soxhlet extraction as extraction solution
Figure 5.15
Chromatogram of feed extract using acetic acid on extraction solution

Figure 5.16
Chromatogram of feed extract using Hexane as extraction solution

Figure 5.17
Chromatogram of feed extract using DMF with homogenisation on extraction solution
5.2.6 Effect of drug feed contact time on recovery of TMP
Since TMP was recovered soon after dosing, it was decided to investigate the recovery of TMP with time.

5.2.6.1 Short-Term TMP Recovery Study
Since TMP was recovered soon after dosing, it was decided to investigate the recovery of TMP as a function of contact time. Three 2 g portions of feed were dosed with a TMP in methanol solution and were then extracted after contact times of 1, 3.5 and 48 hours. The chromatograms obtained from the injection of the extraction solutions are shown in Figures 5.19-5.21. Results for the recoveries of TMP from each of the samples are shown in Table 5.5.
Figure 5.18a
Chromatogram of feed extract using DMF/NH₃ with homogenisation on extraction solution

Figure 18b
Chromatogram of feed extract using DMF with homogenisation on extraction solution
Figure 5.19
Short-term TMP recovery study – chromatogram of feed extract after 1 hour drug-feed contact time

Figure 5.20
Short-term TMP recovery study – chromatogram of feed extract after 3.5 hours drug-feed contact time

Figure 5.21
Short-term TMP recovery study – chromatogram of feed extract after 48 hours drug-feed contact time
Table 5.5
Short-Term TMP Dosing and Recovery Study

<table>
<thead>
<tr>
<th>Sample</th>
<th>Contact Time (h)</th>
<th>TMP Concentration (μg g⁻¹)</th>
<th>Percentage Recovery (±4%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Added</td>
<td>Found</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>361</td>
<td>328</td>
</tr>
<tr>
<td>2</td>
<td>3.5</td>
<td>350</td>
<td>307</td>
</tr>
<tr>
<td>3</td>
<td>48</td>
<td>382</td>
<td>321</td>
</tr>
</tbody>
</table>

5.2.6.2 Long-Term TMP Recovery Study
The TMP recovery with time study was repeated with the intention of increasing the analysis intervals after dosing. The results from this study are shown in Table 5.6. The chromatograms obtained from the injection of the extraction solutions are shown in Figures 5.22-5.23

Table 5.6
Long-Term TMP Dosing and Recovery Study

<table>
<thead>
<tr>
<th>Sample</th>
<th>Contact Time (days)</th>
<th>TMP Concentration (μg g⁻¹)</th>
<th>Percentage Recovery (±4%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Added</td>
<td>Found</td>
</tr>
<tr>
<td>T1</td>
<td>4</td>
<td>319</td>
<td>233</td>
</tr>
<tr>
<td>T2</td>
<td>4</td>
<td>327</td>
<td>230</td>
</tr>
<tr>
<td>T3</td>
<td>27</td>
<td>319</td>
<td>–</td>
</tr>
<tr>
<td>T4</td>
<td>27</td>
<td>325</td>
<td>69</td>
</tr>
</tbody>
</table>
Figure 5.22
Long-term TMP recovery
study – chromatogram of feed extract after 4 days
drug-feed contact time

Figure 5.23
Long-term TMP recovery
study – chromatogram of feed extract after 27 days
drug-feed contact time
5.3 ANALYSIS OF FEED SAMPLES DOSED WITH SDZ AND TMP

A commercially obtained, unmedicated pig feed was obtained and dosed by Cheminex Laboratories with Uniprim to give four batches of feed dosed to levels of between 103-397 μg g\(^{-1}\) for SDZ and between 26-79 μg g\(^{-1}\) for TMP.

5.3.1 Experimental

A 250 ml beaker was scooped through the sample bucket to collect ~160 g of sample. This sample was then ground to pass through a 1 mm sieve and then placed in the hopper of a small-scale rotary sample riffler which divided the samples into 16 approximate 10 g samples. Three of these sub-samples were then collected at random and transferred and weighed into separate 250 ml wide-mouth disposable plastic screw-top bottles. The normal extraction and clean-up procedure was then followed. This sampling procedure was followed for all batches with all apparatus being thoroughly cleaned and dried between batches and the order of batch sampling being with increasing concentration to remove the possibility of any contamination.

As a control, two samples of the non-medicated feed were taken and analysed. The results showed that there was contamination of the non-medicated feed with SDZ to levels of 38 μg g\(^{-1}\) for the two samples. Contamination of the non-medicated feed could either have occurred during manufacture or during analysis, but the fact that the two samples were contaminated to such similar levels seemed to imply that the contamination was caused during manufacture.

To rule out the possibility of contamination having occurred during analysis, two fresh 10 g samples were taken and weighed into disposable plastic sample bottles. It was decided that as few steps as possible would be performed between sampling and injection onto the HPLC column. No grinding of the sample was performed to remove the chance of cross-contamination, even though the grinder had been thoroughly cleaned before the analysis of the previous two samples of the non-medicated feed. Fresh extraction solution was prepared, and the normal extraction procedure followed. Filtering the samples through the glass-fibre filter paper was performed, using a thoroughly rinsed and dried filter funnel and Buchner flask. The filtrates were then filtered through 0.45 μm filters and injected onto the HPLC column. Use of the diode array software allowed the spectrum of the region of the chromatogram where SDZ eluted to be taken. This was then compared to the spectrum of the region in the chromatogram from the blank feed where SDZ was thought to be present. Good correlation between the two spectra
confirmed that the peak observed in the blank feed was in fact SDZ.

5.3.2 Results

The results of the analyses are shown in Table 5.7. It can be seen that the results for the SDZ levels show considerable variation from the theoretical dosing level, which can be explained by insufficient mixing. Since levels of SDZ were found in the non-medicated feed used to dose with SDZ at a level of 38 mg g⁻¹, this figure was subtracted from the experimentally determined SDZ levels found in the various batches to give a corrected value. No TMP peaks were observed.

Table 5.7

<table>
<thead>
<tr>
<th>Batch No.</th>
<th>SDZ Concentration / µg g⁻¹</th>
<th>TMP Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Added</td>
<td>Found</td>
</tr>
<tr>
<td>1A</td>
<td>397</td>
<td>301</td>
</tr>
<tr>
<td>1B</td>
<td>397</td>
<td>263</td>
</tr>
<tr>
<td>1C</td>
<td>397</td>
<td>370</td>
</tr>
<tr>
<td>2A</td>
<td>310</td>
<td>335</td>
</tr>
<tr>
<td>2B</td>
<td>310</td>
<td>467</td>
</tr>
<tr>
<td>2C</td>
<td>310</td>
<td>442</td>
</tr>
<tr>
<td>3A</td>
<td>203</td>
<td>265</td>
</tr>
<tr>
<td>3B</td>
<td>203</td>
<td>268</td>
</tr>
<tr>
<td>3C</td>
<td>203</td>
<td>254</td>
</tr>
<tr>
<td>4A</td>
<td>103</td>
<td>159</td>
</tr>
<tr>
<td>4B</td>
<td>103</td>
<td>157</td>
</tr>
<tr>
<td>4C</td>
<td>103</td>
<td>130</td>
</tr>
</tbody>
</table>

5.3.3 Discussion

The fact that no TMP was detected on the HPLC column could have been due to two reasons. Either TMP was not extracted from the feed, or it was extracted from the feed but was lost in the clean-up stage. Losses in the clean-up stage could be due to several reasons namely, that TMP was not being quantitatively retained on the CEC; TMP was quantitatively retained on the column, but was eluted by the MeOH or the water in the washing stages; or, TMP was retained by the cartridge even after elution with the mobile phase. Previous studies, investigating the efficiency of various extraction solutions, showed that no TMP was extracted. It, therefore, appeared that no TMP was detected because it was not extracted from the feed.
5.4 INDUSTRIAL DOSING AND RECOVERY STUDY

5.4.1 Experimental
To investigate the recovery problems of SDZ, a study at a feed mill was carried out. The study took the form of incorporating a drug premix, containing both SDZ and TMP, into a 3 tonne feed batch contained in a mixing vessel. The feed batch was then transferred to a holding bin via a pipeline, from which three samples of feed were taken at five minute intervals to investigate the distribution of the drug within the feed. After the transfer to the holding bin was complete, the batch was then pelleted and stored in three 1 tonne product holding bins. A sample from each of the bins was taken. A non-medicated feed batch was then produced immediately afterwards to investigate the degree of drug carry-over.

5.4.2 Results
The results from this study are shown in Table 5.8.

Table 5.8
Analysis of commercially produced feed samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>SDZ Concentration (µg/g)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Theoretical</td>
<td>Found</td>
<td>Average</td>
</tr>
<tr>
<td>Unmedicated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>36</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>Meal 1</td>
<td>250</td>
<td>168</td>
<td>168</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>167</td>
<td></td>
</tr>
<tr>
<td>Meal 2</td>
<td>250</td>
<td>183</td>
<td>182</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>181</td>
<td></td>
</tr>
<tr>
<td>Meal 3</td>
<td>250</td>
<td>182</td>
<td>182</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>181</td>
<td></td>
</tr>
<tr>
<td>Pellet 1</td>
<td>250</td>
<td>185</td>
<td>164</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>182</td>
<td></td>
</tr>
<tr>
<td>Pellet 2</td>
<td>250</td>
<td>181</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>159</td>
<td></td>
</tr>
<tr>
<td>Pellet 3</td>
<td>250</td>
<td>134</td>
<td>153</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>172</td>
<td></td>
</tr>
</tbody>
</table>
It can be seen that the non-medicated feed produced after the medicated batch contained SDZ at a concentration of 36 µg g\(^{-1}\), indicating that some drug carry-over had occurred. The samples of the meal varied in SDZ concentrations between 167-182 µg g\(^{-1}\), while the concentrations found in the pelleted samples varied from 153-184 µg g\(^{-1}\). If the carry-over of the premix to the unmedicated feed batch is ignored, then recoveries for SDZ range from 54-74 % of the total amount of SDZ added. But since carry-over did occur, then allowance for this fact increases SDZ recoveries to between 63-87 %. There did not appear to be any real differences between SDZ recoveries from pelleted and non-pelleted feeds. This might have been expected, if Figures 1.1 and 1.2 are considered, since both pelleted and non-pelleted feeds give similar recoveries soon after manufacture.

5.5 DISCUSSION AND CONCLUSIONS

The experimental results have shown that SDZ can be extracted from the feed with a variety of different extraction solutions. TMP was more problematical, since it could not be extracted from commercial feed samples and was only extracted from feed samples that had been dosed with the drug in methanol, and then recoveries varied inversely with the time of drug-feed contact.

The fact that SDZ was extracted from commercially produced feeds, while TMP was not, needs to be addressed. The two explanations for the non-extraction of TMP are that either it undergoes degradation, or it is irreversibly bound to feed constituents. Although there was insufficient time in the study to investigate the stability of TMP, the hypothesis presented earlier could explain the non-extraction of TMP, if the solubility of TMP is considered. With TMP reported as being soluble in water (1 part in 2500) compared to SDZ, more TMP could dissolve in the water present in the feed and then diffuse into the interior of the particles, thus prevented from being extracted.

1. The extraction of SDZ has been achieved using a variety of different organic solvents with recoveries of up to 90 %.

2. The extraction of TMP has not been possible despite the use of a variety of solvents with different polarities, extraction with homogenisation and Soxhlet extraction.
3. The fact that TMP has not been extracted from commercially produced feeds of between 2-3 months old, but has been successfully extracted from blank feeds dosed with a solution of TMP in methanol might indicate that contact time is important in terms of recovery. That this is the case has been seen in the long-term recovery study where the recovery drops from some 70% to 21% in 23 days.

4. The work performed to date has not been able to develop a method for the simultaneous analysis of TMP and SDZ. This has been due to the inability to find a suitable extraction solvent to extract TMP from commercially produced feeds of between 2-3 months old. Using feeds recently dosed with TMP in a solution of methanol produces more encouraging results, since TMP can be extracted at recoveries of ~90% within 1 hour of dosing.
Chapter 6

DINITOLMIDE
CHAPTER 6 - DINITOLMIDE

6.1 DINITOLMIDE

Dinitolmide, or 3,5-dinitro-o-toluamide, (DOT), as shown in Figure 6.1, is a drug used in poultry feed to prevent Coccidiosis, which is an intestinal disease caused by a protozoan of the genus Eimeria, and results in death. Dinitolmide should not be used with chickens that have reached laying age, and the withdrawal period is at least three days before slaughter.

Figure 6.1
The structure of Dinitolmide

There are usually two levels of inclusion, as reported by Crosby [107], 125 µg g⁻¹ for chickens and 125-187 µg g⁻¹ turkeys. Conway also reported that Dinitolmide is bound to tissue constituents unless ficin is used to digest the sample, which indicates its high binding capacity.

6.1.1 Chemical Properties

It is a cream to light tan-coloured powder which is practically insoluble in water, soluble in alcohol (1 in 100), soluble in acetone (1 in 15), and slightly soluble in chloroform and ether. The melting point is reported as being 177-181 °C.

6.1.2 Pharmacokinetics

The metabolism of dinitolmide was studied by several researchers [108-112] some of whom used ¹⁴C-labelled dinitolmide. It was observed that the compound underwent biological reduction of the 3-nitro group to give 3-amino-5-nitro-o-toluamide. Also found in faecal samples were: 3-amino-5-nitro-o-toluic acid, 5-amino-3-nitro-o-toluamide, 5-amino-3-nitro-
o-toluic acid, 3,5-diamino-o-toluic acid, 3,5-diamino-o-toluic acid and 3,5-dinitro-o-toluic acid. Only trace amounts of both dinitolmide and the 3-amino-5-nitro-o-toluamide were found in the tissues of the liver and muscles. This indicates that the scope for degradation products is large.

6.2 METHOD OF ANALYSIS

With regard to the methods of analysis, the following papers all rely upon the extraction of the drug with an organic solvent with water, followed by injection of the solution onto a HPLC column directly or after some form of clean-up step.

In the method described by Burns and Jones [113], the dinitolmide was extracted with acetonitrile-water (17 + 3 v/v), with the extraction flask placed in a water bath at 50 °C and then mechanically shaken. A filtered extract was then injected onto a 5 μm Partisil column with uv detection at 270 nm. Results reported indicated that quantitative recoveries were obtained from both commercially produced samples and samples spiked with a solution of dinitolmide in acetone. No consideration of the drug-feed contact time on drug recovery was made.

Morawski and Kyle [114] reported a reverse-phase HPLC method where the dinitolmide is extracted with acetonitrile-water (85 + 15 v/v) on a water bath maintained at 50 °C. Alumina is then added to the flask and the mixture transferred to a sintered funnel. Successive washings with extraction solution are then combined, filtered through a 0.45 μm filter and injected onto a μBondpack C18 column (10 μm) with uv detection at 254 nm. Validation of the method only occurred with spiked samples, where recoveries were reported as ranging from 93-99 %.

Cody et al [58] described a multi-drug detection method that could detect 25 different drugs found in animal feeding stuffs including dinitolmide. The drugs were extracted with aqueous acetonitrile, subjected to clean-up on a silica cartridge, then analysed by HPLC. Dinitolmide was analysed using a Rosil RP-18 column with a mobile phase of acetate buffer, acetonitrile and water. A recovery of 91% was reported for feeds spiked with dinitolmide.

Dinitolmide, like SDM, has given problems in recoveries with incomplete extraction of the
drug. In view of the success in autoradiographic experiments with $^{14}$C-labelled SDM, it was decided to use $^{14}$C-labelled dinitolmide in analytical studies to investigate the causes of poor recoveries.

6.3 STRESSING OF DOT

In view of the fact that poor recoveries of DOT had been noted from feed samples, and that it underwent extensive degradation in vivo, the stability of the drug when subjected to elevated temperatures and steam pressures was investigated. DOT (5 g) was placed in a 50 ml beaker, placed inside a domestic pressure cooker and stressed for 5 minutes. Following removal of the beaker from the pressure cooker, DOT (9.6 mg) was removed from the beaker, weighed into a 100 ml volumetric flask and diluted to the mark with methanol. A 1 ml aliquot of this solution was then diluted to give solution of concentration 0.96 $\mu$g ml$^{-1}$. An unstressed DOT solution was also prepared with a concentration of 1.14 $\mu$g ml$^{-1}$. No differences in the chromatograms of the stressed and non-stressed injected solutions were observed. The chromatogram obtained from the injection of the stressed DOT solution is shown in Figure 6.2. Also injected were standard solutions of some of the possible DOT degradation products: 2-methyl-3-nitrobenzoic acid and 2-methyl-5-nitrobenzoic acid. Chromatograms of these solutions are shown in Figures 6.3 and 6.4 respectively. The results show that DOT does not undergo degradation when stressed in the pressure cooker and, therefore, could be presumed to be stable when subjected to the industrial process of pelleting.

6.4 PROPOSED SYNTHESIS OF $^{14}$C-DINITOLMIDE

In view of the success in using $^{14}$C-SDM in analytical studies, it was decided to label DOT with $^{14}$C. Unfortunately, a commercial supplier of $^{14}$C DOT could not be found, and it was, therefore, necessary to devise a suitable reaction scheme for the synthesis of $^{14}$C-DOT. The same criteria as used in the selection of a suitably labelled precursor for the synthesis of $^{14}$C-SDM were used to select a labelled precursor for the synthesis $^{14}$C-DOT. Ortho-toluic acid, which was labelled uniformly with $^{14}$C in the benzene ring, was chosen.

The proposed reaction sequence is shown in Figure 6.5 and involves the dinitration of the o-toluic acid, followed by conversion of the carboxylic acid group to an acyl chloride, which is then reacted with ammonia to form the toluamide. Initial synthesis used unlabelled o-toluic acid to allow optimisation of reaction conditions before $^{14}$C-o-toluic acid was used.
Figure 6.2
Chromatogram of stressed DOT

Figure 6.3
Chromatogram of 2-methyl-3-nitrobenzoic acid reference standard

Figure 6.4
Chromatogram of 2-methyl-5-nitrobenzoic acid reference standard
6.4.1 Step I - The nitration of o-toluic acid [115]
O-toluic acid (3g) was slowly added to a nitrating mixture of fuming nitric acid (25 ml) and concentrated sulphuric acid (5 ml) over a period of 40 minutes, with the reaction mixture kept at a constant 20 °C during the addition stage and also during the standing period of 180 minutes. The reaction mixture was then poured into an ice/salt water solution (250 ml), filtered, washed free from mineral acid, dissolved in hot water, and allowed to recrystallise overnight. The product that had crystallised out was filtered, dried and the melting point determined. This was found to be 209 °C compared to a published value of 210 °C. 1H NMR spectra were taken of o-toluic acid, the isolated product after nitration and 3,5-dintro-o-toluic acid obtained from Sigma Chemical Co. as a reference material. The spectra are shown in Figures 6.6-6.8 respectively. It can be seen that the nitration step was successful in introducing two nitro groups into the 3 and 5 positions. The carboxylic acid group is meta activating and the effect of the methyl group is to direct to the position para to the methyl group. Thus both nitration positions are favoured, and there appeared to be little or no mono (3 or 5 nitrated products) produced, judging by the very pure NMR spectrum that was produced. The yield for this reaction was 80%.

6.4.2 Step II - Conversion of carboxylic acid group to acyl chloride group [116]
Synthesis of the acyl chloride has been achieved using phosphorous pentachloride or thionyl chloride in dry pyridine which serves as a catalyst. The latter has the advantage over the phosphorous pentachloride method in that there is no need to isolate and purify the acyl chloride before proceeding to the next step. The acyl chloride produced in this step is very sensitive to moisture and reacts violently with water and also fumes in moist air. Figure 6.9 shows the relative merits of the two methods. 3,5-dinitro-o-toluic acid (4.5g) together with thionyl chloride (8 ml) and dry pyridine, (4 drops) were added to a 50 ml round-bottomed flask
Figure 6.5

Synthesis of "C-Dinitrotolmide

Step I - Nitration of o-toluic acid

\[
\text{COOH} \quad \begin{array}{c} \text{H}_2\text{SO}_4 / \text{FUMINGNO}_3 \end{array} \quad \text{COOH} \quad \begin{array}{c} \text{O}_2\text{N} \text{H} \text{NO}_2 \end{array}
\]

Step II - Conversion of Carboxylic acid group to acyl chloride

\[
\text{COOH} \quad \begin{array}{c} \text{SOCl}_2 / \text{Pyridine} \end{array} \quad \text{COCl}_2 \quad \begin{array}{c} \text{O}_2\text{N} \text{H} \text{NO}_2 \end{array}
\]

Step III - Conversion of acyl chloride to amide

\[
\text{COCl}_2 \quad \begin{array}{c} \text{NH}_3 \end{array} \quad \text{CONH}_2 \quad \begin{array}{c} \text{O}_2\text{N} \text{H} \text{NO}_2 \end{array}
\]
Figure 6.6

$^1$H-nmr spectrum of o-toluic acid (Aldrich Chemical Co.)
Figure 6.7

$^1$H-nmr spectrum of product after nitration
Figure 6.8

$^1$H-nmr spectrum of 3,5-dinitro-o-toluic acid (Aldrich Chemical Co.)
connected to a reflux condenser to which a drying tube was attached. Gentle refluxing was then carried out for 2 hours. The initial whitish-coloured solution that formed turned yellow and became visibly clearer after 10 minutes of refluxing. Following refluxing, a clear, pale yellow solution was formed. Removal of excess thionyl chloride was achieved by addition of 3 x 10 ml portions of petroleum ether (40-60) and distillation under vacuum with the ether boiling off at 29 °C to leave a golden coloured solution.

Figure 6.9

Conversion of carboxylic acid group to acyl chloride

Advantages of SOCl₂ over PCl₅

\[
\begin{align*}
&\text{SOCl}_2 \quad R \equiv \text{O} \quad + \quad \text{SO}_2 \quad + \quad \text{HCl} \\
&\text{(easier since gaseous products)} \\
&\text{PCl}_5 \quad R \equiv \text{O} \quad + \quad \text{POCl}_3 \quad + \quad \text{HCl}
\end{align*}
\]

6.4.3 Step III - Conversion of the acyl chloride to the amide [117]

In a 250 ml conical flask, the acyl chloride from step II was added dropwise to ammonia (0.88, 15.2 ml). Large quantities of fumes were evolved and the solution turned a deep red colour with a red solid being formed. Upon addition of all the acyl chloride, the flask was gently swirled and the remaining fumes allowed to dissipate. Filtration through a Buchner funnel allowed the separation of a light pink coloured solid from the crimson coloured filtrate. Thorough washing of the solid with distilled water reduced the intensity of the colour of the solid. Transfer of the solid to a conical flask and addition of aqueous ethanol (EtOH: H₂O, 3:1 v/v), followed by gentle heating on a steam bath dissolved the vast majority of the solid. The solution was then poured into a recrystallising dish and allowed to stand in the fume hood overnight. Pale yellow, very fine monoclinic crystals (1.64 g) were obtained whose melting point was recorded as 180-182 °C which compared favourably to the published range of 177-181 °C. The product spectrum was then recorded using an FT-IR spectometer and compared to a reference standard of dinitolmide supplied by the Laboratory of The Government Chemist. The two spectra can be seen in Figure 6.10 and show that the product was 3,5-dinitro-o-toluamide. The overall yield for the conversion of o-toluic acid to 3,5-dinitro-o-toluamide was 30%.
Figure 6.10
FT-IR spectra of product and DOT reference standard
The reaction sequence devised was shown to be successful in synthesising $^{14}\text{C DOT}$. The only factor that prevented work at Loughborough continuing with the synthesis was the fact that the product licence was withdrawn in this country shortly after the reaction sequence was developed. Since no commercial feed samples were produced after this date, consultation with the product sponsors resulted in work on DOT being suspended.

6.5 CONCLUSIONS

Although a full experimental study using DOT was not possible, due to its withdrawal from the market, experimental work performed has shown that:

1. DOT was stable when stressed in a process simulating the conditioning process.

2. The synthesis of DOT was successful and could be used to synthesise $^{14}\text{C DOT}$.

3. Since degradation of DOT was not observed during stressing, then drug-feed binding could be the cause the poor drug recoveries.
Chapter 7

CONCLUSIONS
CHAPTER 7 - CONCLUSIONS

7.1 The causes of poor analytical drug recoveries

The work presented in this thesis has investigated the causes of poor analytical drug recoveries from medicated animal feeds. Although the majority of this work has been concerned with the recovery of SDM from animal feeds, the hypothesis presented for poor drug recoveries can be used to account for poor recoveries of other drugs which do not undergo degradation.

The hypothesis is that dissolution of the drug by moisture in the feed is followed by diffusion of the solution deep into the internal regions of the feed particles via cracks and pores. The deep penetration of the drug solution prevents the drug from being recovered by the extraction solution. Results from experimental work performed on the recovery of SDM from feeds, and also the adsorption of moisture by feeds, appear to substantiate the hypothesis. The most important results are discussed below.

Drug recoveries were found to decrease as the contact time between the drug and the feed increased. It was noted that there appeared to be large initial decreases in drug recoveries with time, but then the recoveries tended to level off. An inference was made that some sort of equilibrium was being attained.

The inverse relationship between feed moisture content and drug recovery demonstrated that moisture was involved in the mechanism accounting for the non-recovery of SDM. Results showed that the higher the feed moisture content, the lower was the SDM recovery. Additionally, studies investigating the effect of varying the amount of SDM added to a feed sample on drug recoveries showed that the more SDM added, the higher the recovery. Relating this to the hypothesis, this would be as expected because the more SDM in the feed sample, the larger would be the proportion that would not be dissolved in the feed moisture.

Autoradiography studies performed with $^{14}$C-SDM showed that the non-extracted $^{14}$C-SDM was distributed generally throughout the feed. When individual feed constituents were used, the non-extracted $^{14}$C-SDM was found to be associated with all the feed constituents, with the exception of calcium carbonate. Examination of the feed constituents using a scanning electron microscope showed that they were all heterogeneous and were porous with the
exception of calcium carbonate. This again fits in with the hypothesis that the drug solution diffuses into pores/cracks in the constituent particles.

It was thought that increased feed moisture levels, resulting in decreased SDM recoveries, allowed the hydrolysis of SDM, but studies on the stability of aqueous SDM showed that this was not the case. Another important result from this study was that the solubility of SDM in water was determined to be ~400 μg g⁻¹. Using this figure in calculations demonstrated that appreciable amounts of the SDM in a feed could be dissolved by the moisture present.

Studies investigating the adsorption of moisture by feeds showed that diffusion was the rate controlling mechanism. Theoretical consideration of moisture in biological materials predicted three locations for the water. Desorption experiments involving the removal of water from feed samples showed that there appeared to be three locations for the water, with the majority of water being present as normally condensed or bulk water. This water could then dissolve the drug and diffuse into the interior of the feed particles.

An important consequence of the moisture adsorption and desorption work was that it was shown that solvent pretreatment of feeds altered the adsorption/desorption characteristics when compared to an untreated feed. This could explain the fact that extraction solutions composed entirely of organic solvents were not as good at extracting drugs from feeds as an extraction solution composed of organic solvents and water. In Chapter 3, the role of water in the extraction of drugs from feeds was discussed in detail and it was suggested that the water could penetrate deeper into the feed matrix than an organic solvent, because of its smaller molecular size, and therefore extract more drug.

Although the hypothesis suggests that the poor drug recoveries are due to the penetration of drug solutions deep into the constituent particles which prevents the extraction solution from extracting the drug, the possibility of binding occurring to specific sites cannot be ruled out due to the heterogeneity of the feed and the existence of vastly different chemical sites on constituent surfaces. It is thought, however, that specific binding only accounts for a very small proportion of non-recoverable drug.
7.2 Further work

Suggested further work should involve investigating the effect of moisture content on drug recoveries for SDZ and TMP. If an inverse relationship between moisture and drug recovery was found, then the hypothesis of drug dissolution and diffusion is further supported.

The adsorption of moisture by individual feed constituents should be measured to determine the relative rates of moisture adsorption. The experimental work should also be extended to include other medicinal additives and the possibility of radiolabelling them investigated. Should labelling be possible, then they could be used in studies similar to the ones carried out using $^{14}$C-SDM.

Experimental studies should investigate the effect of drying a number of feeds, containing different medicinal additives on drug recoveries. The method of drug dosing should be addressed since when initial work was undertaken the best method of dosing samples was found to be by using a solution of the drug in methanol. Subsequent work on the adsorption of moisture by feeds pretreated with solvents showed that the pretreatment did in fact alter the adsorption characteristics of the feed.

Once a thorough understanding of the mechanisms accounting for poor drug recoveries has been gained, attention should be turned to improving the extraction procedures to ensure that greater drug extraction efficiencies are obtained. A suggestion for improving the recovery of drugs from feeds might be to reduce the moisture content of the feed using silica gel before the extraction stage.
REFERENCES


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Recovery of sulphadimidine from pig feeds

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Abstract
The poor analytical recovery of sulphadimidine from medicated pig feeds was investigated by high performance liquid chromatography using unlabelled and 14C-labelled sulphadimidine. In addition, autoradiographic studies of 14C-labelled sulphadimidine binding in feeds was investigated. HPLC studies using unlabelled sulphadimidine showed that an inverse relationship between feed moisture content and sulphadimidine recovery existed. High moisture content feeds (18% m/m) gave lower recoveries than both control feeds (12% m/m) and low moisture content feeds (5% m/m). Both HPLC studies using uv and radioactivity detection showed no evidence for degradation products of sulphadimidine. Autoradiographic studies showed that the non-recovered drug was dispersed throughout the feed. A hypothesis is proposed to account for the poor recoveries.

Keywords: sulphadimidine determination, animal feed, 14C-sulphadimidine, autoradiography.

Introduction
Medicinal additives are routinely incorporated into medicated animal feeds as prophylactics or as growth promoters. The nature and levels of inclusion of such compounds must be legally declared by the feed manufacturer and therefore checks are necessary to ensure these declarations are correct. A problem frequently encountered with this type of analysis is that medicinal additives are only partially recovered from the feed, particularly after storage. It has been suggested that the poor recoveries are due to degradation of the drug or binding of the drug to feed constituents. Additionally, many feeds are supplied in pelleted form and have been subjected to elevated moisture levels, temperatures and pressures during the production process which have been thought to accentuate any degradation or binding tendency.

Sulphadimidine (SDM) [4-amino-N-(4,6-dimethyl-2-pyrimidinyl) benzenesulphonamide] is a widely used antibacterial agent included in pig feeds, and several analytical methods are reported in the literature for SDM. Several workers have shown that the recovery of SDM from feeds is inversely proportional to the time SDM is in contact with the feed, but many have not taken this into account when validating their methods and have used only freshly spiked samples.

Problems for the regulatory analyst occur when SDM levels are found to be below the permitted lower tolerance since this could simply be poor analytical recovery of the drug and not the result of accidental low level of drug addition by the manufacturers. Conversely, an over addition of drug by the manufacturer may pass undetected due to incomplete drug extraction.

The work presented in this paper investigates the causes of poor SDM recoveries by the novel use of 14C-labelled SDM.
Experimental

Reagents: As specified by Conway

Liquid Scintillant: Ecoscint A (National Diagnostics, Aylesbury, Buckinghamshire, U.K.)

Chemicals: Sulphadimidine-Phenyl-Ring-UL-^{14}C, 10 MBq (Sigma Chemical Company Ltd., Poole, Dorset, U.K.)

Autoradiography Film: Hyperfilm β-Max (Amersham International plc, Amersham, Buckinghamshire, U.K.)

Pig feed: Ultra Finisher Meal (Dalgety Agriculture Ltd., South Wigston, Leicestershire, UK).

Apparatus:
HPLC System: Philips PU 4021 Multi Channel Detector.
Philips PU 4100 Liquid Chromatograph fitted with 20 ml Rheodyne injection loop.
Philips P 3202 Computer with PU 6003 Diode Array Software and PU 6000 Integration Software.

HPLC Columns: Apex Octadecyl, 5 μm, 250 mm x 4.6 mm i.d. (Fisons).
Apex Octadecyl, 5 μm 50 mm x 4.6 mm i.d. guard column (Fisons).

Fraction Collector: RediFrac (Pharmacia Ltd., Milton Keynes, Buckinghamshire, U.K.)

Liquid Scintillation Counter: LKB Wallac 1215 Rackbeta Liquid Scintillation Counter (Pharmacia Ltd., Milton Keynes, Buckinghamshire, U.K.)

Sample Tubes: Falcon Polypropylene Disposable Sample Tubes (50 ml) (Northern Media, Nottingham, UK.)


Method
The analysis method used throughout this paper is that described by Conway and is based upon the extraction of SDM with aqueous acetonitrile from the feed, followed by cation-exchange clean-up and analysis of the resulting solution with a reversed-phase HPLC system. During our work, various dosing methods to introduce SDM into the feed were investigated as to their suitability. These methods included dosing a bulk quantity of feed with a premix of SDM in calcium carbonate, and dosing both a bulk quantity of feed and individual 2 g portions with a solution of SDM in methanol. It was found that dosing bulk quantities of feed with either a premix or a solution introduced errors due to uneven distribution of the SDM throughout the bulk, even after significant mixing times (6 hours). The most satisfactory dosing method was found to be the addition of a solution of SDM in methanol to small portions of feed. However, this method of dosing has disadvantages as indicated in the discussion.
To facilitate the preparation of large numbers of dosed feed samples for use in analytical studies, 2 g feed samples were weighed into 50 ml polypropylene sample tubes. Conway specified that 10 g of feed containing 100 μg g⁻¹ of SDM was extracted with 100 ml of extraction solution. To ensure that the ratio of the mass of feed used in the analysis and the volume of extraction solution remained constant when using 2 g feed samples, 20 ml of extraction solution was used. To ensure that the amounts of SDM injected onto the column were the same as used by Conway, dosing levels were between 300-500 μg g⁻¹.

Preparation of ¹⁴C-SDM dosing solution
¹⁴C-SDM (10 MBq) and carrier SDM (21.5 mg) were transferred into 25 ml volumetric flask and dissolved in methanol and made up to the mark. This was the labelled SDM dosing solution and was of specific activity of 401 kBq ml⁻¹ and SDM concentration of 0.96 mg ml⁻¹.

Analytical recovery of ¹⁴C labelled SDM from pig feed
Two grams of commercially obtained pig feed were accurately weighed into 6 sample tubes (1-6). To each was added 0.5 ml of the ¹⁴C labelled SDM dosing solution to give SDM dosing levels of 232-237 μg g⁻¹. The tubes were then capped and mixed using a vortex mixer for 10 seconds to allow interaction between the dosing solution and the feed. The drug was then extracted with solvent from tubes 1 and 2 after a drug-feed contact time of 1 hour. After 7 days drug-feed contact time, the drug was extracted from tubes 3 and 4 and after 16 days contact time, the drug was extracted from tubes 5 and 6. SDM recoveries were measured by HPLC using uv detection as well as radiochemical detection by fraction collection and liquid scintillation counting. The results shown in Table 1 show that SDM recoveries decreased with time.

To ensure that loss of activity did not occur during the extraction, clean-up and chromatographic stages of the analysis, the experimental specific activity of the solutions were compared to theoretical specific activities which enabled a mass balance study to be carried out. Thus, all the activity extracted could be monitored to ensure that no losses occurred during the subsequent processing stages. The results are shown in Table 2.

Autoradiography studies
In order to investigate the distribution of the non-extracted ¹⁴C- SDM, autoradiography studies were initiated. After the feed solid was filtered off from the extraction solution, the filter paper and the solid were carefully dried, and sellotape strips were then used to remove small portions of the feed from the filter paper which were then fixed onto microscope slides. This method was found to be much easier than trying to embed the feed solid in wax and taking sections using a microtome. Quantitation of the amount of activity associated with a particular sample due to the blackening of the film was not possible due to the uneven thicknesses of the particles on the slides.

The feed solids from Sample 5 were transferred onto slides and all were then exposed to the autoradiographic film for 4 days after which time they were developed. The results shown in Figure 1 indicate that SDM, after the extraction process, was generally distributed on all the feed particles.

Dosing individual feed components with ¹⁴C-labelled SDM
To investigate further whether the non-extracted ¹⁴C-SDM was generally distributed on the feed constituents, individual constituents were dosed with the labelled ¹⁴C-SDM solution and then extracted at time intervals. Feed constituents (wheat, barley, meat and bone meal,
HiPro Soya, ricebran, wheatfeed, malt culms, fishmeal and full fat soya) were obtained from a local mill. Calcium carbonate was also chosen since it is used in some laboratories to prepare a SDM premix. Eight samples of each constituent (0.1g) were accurately weighed into scintillation vial inserts and 20 ml of the $^{14}$C labelled SDM was added to each vial. Duplicate samples from each constituent batch were then extracted using 70% aqueous acetonitrile by shaking mechanically for 1 hour, after which time the samples were centrifuged at 6000g for 1 minute to separate the extraction solution from the solid. 20 ml samples of the extraction solutions were then taken and added to scintillation vials containing 4.2 ml of Ecoscint. Following mixing, all samples were counted in the $^{14}$C channel of the liquid scintillation counter. The activity values were quench corrected and background subtracted. Calculations of the extraction efficiencies were made by dividing the experimentally found specific activity by the theoretical specific activity. The extraction process was repeated after 7, 14 and 25 days contact time between SDM and the feed and the results are shown in Table 3. Samples extracted after 7 days contact time were then prepared for autoradiography as described previously. Each sample was contacted with the film for 10 days. The results of the autoradiography can be seen in Figure 2. Reference to Table 3 and Figure 2 show that calcium carbonate does not sorb $^{14}$C-SDM and that the soya based materials have little affinity for $^{14}$C-SDM. The former explanation may be explained by the lack of porosity of the calcium carbonate and the latter by the high oil content of the soya based materials which prevented penetration of the water into the pores.

**Effect of storage conditions on SDM recovery**

The feed moisture content of a feed already dosed with SDM was modified by placing samples in either a high humidity water saturated environment or a low humidity dry environment, in the following way. Two grams of commercial non-medicated pig feed were accurately weighed into 40 sample tubes (labelled 1-40). To each tube was added 0.5 ml of a solution of SDM in methanol to give an average dosing level of 300 Ilg g$^{-1}$. Mixing of the methanol dosing solution with the feed samples was as described previously. Tubes 1-20 were then placed uncapped in an air-tight box containing 500 g of self-indicating silica gel to absorb moisture. Samples 21-40 were placed in another air-tight box containing 500 ml of water to provide a water saturated environment. Both air-tight boxes were placed inside polystyrene insulation containers which were both stored in an air-conditioned room maintained at 20 ± 3°C. After 11, 25 and 37 days in their respective environments, four samples were removed from each box. Two samples were analysed to determine SDM recovery with the results shown in Figures 3 and 4. The remaining two samples were dried to constant mass in an oven maintained at 105°C to determine the feed moisture content. After oven drying, the samples were then analysed for SDM recovery to determine the effect of oven drying on drug recovery with the results shown in Table 4. The results showed that with the high moisture content samples, the longer the drying time, the greater was the recovery of SDM. Conversely, the low moisture content samples were adversely affected by prolonged drying with SDM recoveries showing a decrease with increasing drying time.

The above study was repeated with the following modifications to the method. To ensure that the relative humidities in the environments remained constant, saturated salt solutions, as reviewed by Young[12], were used. Additionally, the interval between analyses was reduced to three days to see whether the initial change in SDM recovery after dosing was gradual over the entire period of the study or whether the recovery changed sharply to become more constant subsequently. Two gram portions of feed were weighed into 112 sample tubes. Mixing of the methanol dosing solution with the feed samples was as described previously. Samples 1-28 were placed in an air-tight box on their own to serve as controls. Samples 29-56 were placed in contact with a saturated solution of sodium
chloride (75% relative humidity). Samples 57-84 were placed in contact with silica gel and samples 85-112 were placed in contact with a saturated solution of magnesium chloride (33% relative humidity). Samples were removed at 3 day intervals, with two samples being used in the respective moisture determinations and two samples being analysed for SDM recoveries. SDM recoveries are shown in Figure 5. Results for the samples stored in 33% relative humidity are incomplete due to some samples having been contaminated with the magnesium chloride saturated solution.

Effect of modifying feed moisture content before dosing with SDM
As the results from the study above had shown an inverse relationship between feed moisture content and SDM recovery, it was decided to investigate whether modifying the feed moisture content before dosing would also show such a relationship.

Two grams of a non-medicated feed were accurately weighed into 60 sample tubes (labelled 1-60). Samples 1-20 and 21-40 were placed in air-tight boxes as in the previous study containing silica gel and water respectively. Samples 41-60 were placed in an empty air-tight box to serve as controls. All three boxes were placed inside polystyrene insulation containers and placed in an air-conditioned room as before, for 6 days. Representative samples were taken from the respective environments after 6 days and each sample dried for six hours at 105°C. From previously measuring the feed moisture content as 12% m/m, it was found that samples 1-20 had decreased to 5% m/m, samples 21-40 had increased to 18% m/m and samples 41-60 remained unchanged. All samples were then dosed with 0.5 ml of SDM in methanol solution and the procedure from the previous study was followed. Following removal from the fume hood, the sample tubes were capped then stored in an air-conditioned room and protected from light. Four samples were removed after 0, 7, 28 and 42 days drug-feed contact time and 2 samples were analysed for SDM. The results are shown in Figure 6. The remaining two samples from each environment were dried for 6 hours at 105°C to determine the feed moisture content, then stored for a further week and analysed for SDM. The results are shown in Figure 7.

The fact that in high moisture content feeds the SDM recovery decreased compared to low moisture content feeds led us to the consideration that the increased moisture content might accelerate hydrolysis of SDM. To investigate this, the stability of a saturated aqueous solution of SDM was determined.

Stability of aqueous SDM solutions
SDM (65.7 mg) was weighed into a 100 ml volumetric flask and 80 ml of HPLC grade water was added. The flask was stoppered, shaken and placed in an ultrasonic bath for 2 minutes to aid dissolution. On removal from the ultrasonic bath, the solution was made up to the mark and filtered through a 0.45 μm filter and aliquots were transferred to two 30 ml glass storage bottles, labelled 1 and 2. Bottle 1 was wrapped in aluminium foil to protect it from light, bottle 2 was left exposed to light. Samples of each solution were taken after 0, 1, 8 and 62 days and injected onto the HPLC to determine the concentration of SDM in the solution. The results are shown in Table 5 and indicate that SDM was stable in solution over the period of the study.

Sorption of SDM on feed
The experimental results showed that binding of SDM to feed constituents was occurring and that drug degradation was not in evidence. In order to investigate whether there was a limited number of binding sites in the feed, an experiment was set up to investigate the effect of varying the amount of SDM added to the feed.
Sample tubes labelled A 1-16, B 1-16 and C 1-16 containing feed (2 g) were prepared and the previously described dosing protocol was followed using known dilutions of a solution of SDM in methanol. The dosing levels ranged from 11-500 µg g⁻¹. Control samples were dosed with 0.5 ml of methanol. Analyses were then performed at 7, 28 and 42 days on the sets of samples A, B and C respectively. Figure 8 shows that the higher the SDM dose, the higher the percentage SDM recovery and that the recovery decreases with drug-feed contact time.

The effect of reducing particle size of a feed on SDM recovery
The results from the sorption study suggested that SDM was sorbing to the feed particles. To investigate this further, the specific surface area of a feed was increased by reducing the particle size of the feed in a grinder. Reduction in particle size was measured using sieve analysis and by laser diffraction in a Malvern 2600c laser particle sizer. Although the particle size range (10 µm to 1000 µm) did not change during the grinding, particle size was reduced as was evidenced by the observation that in the original feed 50 % of the particles were < 350 µm whereas after grinding, 50 % of particle were < 150 µm. Recovery studies were then performed on both the feed and reduced particle size feed.

A quantity of feed (~10 g) was ground for 1 minute using a domestic coffee grinder. Three 2g portions were accurately weighed into sample tubes (1-3). The same feed but unground was used to weigh out a further three 2 g samples (4-6). Dosing the samples with a solution of SDM in methanol was performed to give SDM levels of 662-695 µg g⁻¹. The samples were then stored in a polystyrene insulation box and left for 18 days to allow interaction between the SDM and the feed. Recoveries from the two feed samples were found to be the same. If it is assumed that no alteration of the surface groups took place during the grinding process, then it could be deduced that SDM is not predominantly confined to the feed particle surfaces. Alternatively, if there is an excess of sorption sites on the surface of the particles, size reduction would not necessarily effect an increased surface sorption.

Discussion
Considering the moisture content of the feed, calculations concerning the maximum amount of SDM that can dissolve in the moisture in the feed were made using the following assumptions:

(i) the nominal feed moisture content is 12% m/m
(ii) all the water in the feed is capable of dissolving SDM
(iii) the maximum solubility of SDM is ~400 µg g⁻¹ (determined in the stability study).

If a feed contains SDM at a level of 100 µg g⁻¹, then in an aged feed approximately 50 mg (50%) can dissolve in the water present in the feed. During some extractions, low recoveries of 50% were observed.

Extending this further, a hypothesis is proposed whereby dissolution of the SDM by moisture in the feed results in solutions being formed which could penetrate into the feed matrix, either by absorption or diffusion. Scanning electron microscopic examination of the feed constituents showed that all exhibit a degree of porosity. If drug dissolution / diffusion into the feed particles occurs then the non-extractable SDM could be explained by penetration of the solution deep into the pores of the feed particles. This hypothesis may be tested by using alternative extraction solvents. This work is in progress.
The moisture content of the feed was altered from its original value of 12% to artificial values of 5% and 18% in order to provide samples of feed with different moisture contents. The high moisture content samples could dissolve more SDM than the corresponding low moisture content samples, which therefore allowed more of the SDM present to be transferred into the pores of the feed.

The dosing method used in this study used a solution of SDM in methanol. Although this method is not the same as that used in industry, the method did allow accurate addition of SDM to the samples. One feature noted about the methanol dosed samples was that the decrease in drug recoveries was larger in magnitude and occurred at a faster rate than in commercially manufactured samples. This may be explained by not removing completely the methanol after dosing and by the increased solubility of SDM in methanol compared to water. Better recoveries from commercial samples could also result from (a) overage ie adding more than the declared content; (b) addition of SDM in the solid state resulting in increased time for dissolution from premix into the feed moisture; or (c) use of granular form of SDM.

The feed moisture content - SDM recovery correlation could explain why pelleted feeds give lower recoveries than non-pelleted feeds due to the higher moisture, temperature and pressures experienced by the feed in the conditioning or ripening stage of the production process immediately before extrusion.

Conclusions
These studies have shown an inverse relationship between the feed moisture content and recoveries of SDM. SDM was found to be stable in aqueous solution and degradation products were not observed after SDM had been in contact with feed. A hypothesis has been proposed to account for the extraction behaviour of SDM from feed and also to account for the fact that pelleted feeds give lower recoveries than the same feed before pelleting.

Acknowledgements
The authors would like to thank the Laboratory of the Government Chemist for funding this research [Grant No. EMRC/35]. Also Mr A. Arafa at Dalgety Agriculture Ltd, South Wigston, Leicestershire for kindly supplying the feeds.

References
Table 1  HPLC and radiochemical results showing decrease in SDM recovery from feed with time

<table>
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<tr>
<th>Sample</th>
<th>Contact Time (days)</th>
<th>SDM Recovery (%)</th>
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<tr>
<td></td>
<td></td>
<td>uv detection (±4%)</td>
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<tr>
<td>1</td>
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<td>94</td>
</tr>
<tr>
<td>2</td>
<td>0.04</td>
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<td>6</td>
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Table 2  Analytical method mass balance

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<tr>
<th>Sample</th>
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<th>3</th>
<th>4</th>
<th>5</th>
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<tr>
<td>Contact Time (days)</td>
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<td>0</td>
<td>7</td>
<td>7</td>
<td>16</td>
<td>16</td>
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<td>Analytical Stage (±5%)</td>
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<tr>
<td>Percentage\textsuperscript{a} extraction efficiency</td>
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<td>85</td>
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<tr>
<td>Percentage\textsuperscript{b} retention of SDM on cation-exchange column</td>
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<td>96</td>
<td>96</td>
<td>96</td>
<td>90</td>
<td>88</td>
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<tr>
<td>Percentage\textsuperscript{c} activity removed by washing column with water</td>
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<td>0</td>
<td>0</td>
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<td>0</td>
<td>0</td>
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<tr>
<td>Percentage\textsuperscript{c} activity removed by washing column with methanol</td>
<td>0</td>
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<tr>
<td>Percentage\textsuperscript{d} recovery of SDM from column</td>
<td>98</td>
<td>96</td>
<td>81</td>
<td>86</td>
<td>88</td>
<td>83</td>
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Extraction Efficiency \textsuperscript{a} is the amount of activity found in the extraction solution as a percentage of the total amount of activity added to feed.

Retention \textsuperscript{b} is the percentage of the extracted activity retained on the cation-exchange column calculated by measuring the activity of the load solution after passing through the column.

Wash \textsuperscript{c} is the percentage of activity retained on the cation-exchange column removed by washing with either water or methanol.

Elution \textsuperscript{d} is the percentage of the retained activity on the cation-exchange column that was removed by elution with 25ml of HPLC mobile phase.
Figure 1 $^{14}$C-SDM autoradiograph of feed sample following drug extraction after 16 days drug-feed contact time.

Figure 2 $^{14}$C-SDM autoradiograph of feed constituents following drug extraction after 7 days drug-feed contact time.
Figure 3
SDM recovery from low moisture content feed samples

Figure 4
SDM recovery from high moisture content feed samples

xi
### Table 3  14C-SDM recovery from constituents

<table>
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<tr>
<th>Constituent</th>
<th>Contact Time/days</th>
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<td>Wheat</td>
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<td>Ricebran</td>
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<td>Barley</td>
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<td>72</td>
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<td>HiPro Soya</td>
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<td>97</td>
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<td>100</td>
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<tr>
<td>Calcium Carbonate</td>
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<td>Full Fat Soya</td>
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<td>73</td>
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<td>Malt Culms</td>
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<td>94</td>
<td>79</td>
<td>69</td>
</tr>
<tr>
<td>Meat and Bone Meal</td>
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<td>Wheatfeed</td>
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### Table 4  Effect of storage conditions and oven drying on SDM recovery

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<th>Contact Time/days</th>
<th>Drying Time/days</th>
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<td>7</td>
<td>66</td>
<td>54</td>
</tr>
<tr>
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<td>5</td>
<td>54</td>
<td>39</td>
</tr>
<tr>
<td>37</td>
<td>11</td>
<td>48</td>
<td>91</td>
</tr>
</tbody>
</table>

### Table 5  Stability of saturated aqueous solution of SDM

<table>
<thead>
<tr>
<th>Sample</th>
<th>Average SDM Peak Area at Time/days</th>
<th>Average RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Light excluded</td>
<td>1042.8</td>
<td>1077.2</td>
</tr>
<tr>
<td>Light exposed</td>
<td>1071.3</td>
<td>1095.4</td>
</tr>
</tbody>
</table>
Figure 5
SDM recovery as a function of storage humidity

Figure 6
SDM recovery as a function of feed moisture content
Figure 7
SDM recovery from feed samples after oven drying

Figure 8
Sorption Study