DEVELOPMENT OF AN ANALYTICAL METHOD
FOR THE ANALYSIS OF FLUMETSULAM IN
SOYBEAN BY HPLC-UV & LC-MS/MS

By

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Presented to the University of Western Sydney
in partial fulfilment of these requirements
for the degree of
Doctor of Philosophy

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<td>°C</td>
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<tr>
<td>cm</td>
<td>Centimetre</td>
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<td>cm⁻¹</td>
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<td>H₂O</td>
<td>Ultra high purity Milli Q water</td>
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ABSTRACT

Flumetsulam, or \( N-\text{(2,6-difluorophenyl)}-\text{5-methyl-}[1,2,4] \text{triazolo}[1,5-a]\text{pyrimidine-2-sulfonamide}, \) is a member of the triazolopyrimidine sulfonanilide family of herbicides. It is used for pre or post-emergence broadleaf weed control in wheat, corn, barley and soybean. It has low toxicity and is readily degradable.

A HPLC-UV method for the analysis of flumetsulam with the use of a surrogate and internal standard was developed and validated for soybean. The surrogate and internal standards are diclosulam and benzoic acid respectively. A Phenomenex Synergi Fusion RP C-18 column was used with UV detection at 225 nm. The soybean was extracted and cleaned up either by solvent-solvent or solid phase extraction. The mobile phase was 50 mM pH 2.2 phosphate buffer:ACN (68:32, % v/v). Unless stated otherwise, all mobile phase mixtures refer to their volume ratios (%v/v), not their weight ratios.

A LC-ESI-MS/MS confirmation method in the positive ion mode was also developed for flumetsulam. This method also uses a Phenomenex Synergi Fusion RP C-18 column. The mobile phase was 5% aqueous glacial acetic acid:ACN (85:15, % v/v). The \( m/z \) ions monitored were 326 [M+H]^+, 262,197 and 128.

In the solvent-solvent extraction method, flumetsulam was extracted from the soybean with MeOH and the extract partitioned with a hexane:MTBE mixture (which was discarded). The MeOH layer, containing the flumetsulam was
evaporated to dryness and made up in ACN. This was then and partitioned with a hexane:EtOAc mixture and discarded. The ACN layer was then evaporated to dryness and the residue dissolved in a neutral pH buffer and partitioned with DCM. The DCM was discarded. The aqueous phase was acidified and partitioned into DCM and then repartitioned into a neutral buffer. The aqueous phase was then acidified to pH 2 and partitioned back into DCM before evaporating to dryness and making up with LC mobile phase for HPLC analysis.

In the solid phase extraction and clean up method, C-18, SAX and SCX cartridges were used. Flumetsulam was extracted from the soybean as for the solvent-solvent extraction method. Flumetsulam in the organic phase was evaporated to dryness and reconstituted in MeOH:H2O before passing into a C-18 SPE cartridge which was washed with a series of organic solvents before elution into a SAX cartridge.

The SAX cartridge was washed with neutral buffer and alkaline MeOH before eluting with an acidic buffer into a SCX cartridge. The analyte was not retained by the SCX cartridge and the eluent that passes through was analysed.

The solvent-solvent extraction method was used for flumetsulam determination at fortification levels of 10, 20, 50 and 100 ng (g soybean)\(^{-1}\). A coefficient of determination (R\(^2\)) of >0.998 was achieved. The method has average absolute recoveries of 80% (range 72 to 92%). The absolute precision of recovery is about 16% and the detection limit is 4.8 ng g\(^{-1}\).
The solid phase extraction method was used for flumetsulam determination at fortification levels of 20, 50 and 100 ng (g soybean)$^{-1}$. A $R^2$ of >0.998 was achieved. The method has average recoveries of 85% (range 79 to 94%). The precision of recovery is about 11%. The detection limit is 7.2 ng g$^{-1}$. The MRL for flumetsulam recommended by the Australian Pesticides and Veterinary Medicines Authority is 50 ng g$^{-1}$ for pulses.
Acknowledgments

My gratitude goes to my supervisor Dr Cheang Khoo for his guidance, support, advice, friendship and encouragement throughout the years of this project.

I would also like to thank members of the Department of Chemistry, School of Food, Science and Horticulture, Campbelltown campus for their continuing support through the years.

I am grateful to Dr R. Crumbie, Dr L. Tarasoff, Dr R. Kaziro, and Dr T. Bailey for their advice and support. I would like also to thank Mr T. Nguyen for his help in the laboratory.

I would like to thank my fellow postgraduates for their help and friendship throughout the university’s years.

I would also like to thank the chemistry team at Novartis for their support throughout my Ph.D candidature.
Statement of Authentication

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in whole or in part, for a degree at this or any other institution.

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Michael Moawad

B.Sc. Advanced (Chemistry), B.Sc. (Honours)

August, 2005
CHAPTER 1

GENERAL INTRODUCTION
This chapter discusses the history of pesticide development, introduction to chromatographic techniques, methods of extraction, aim of the project and analytical method validation.

1.1 BRIEF HISTORY OF PESTICIDE DEVELOPMENT

The agricultural use of chemicals for crop protection has advanced rapidly in the twentieth century in modern agriculture. These chemicals, which include insecticides, fungicides and herbicides, are collectively termed pesticides (1). The use of chemicals to control pests dates back to ancient Greek and Roman times when the use of fumigant by burning sulphur and the use of soda and olive oil for the seed treatment of legumes was practiced (2).

The middle of the nineteenth century marked the beginning of scientific studies into the use of crop protection chemicals. In 1867 ‘Paris Green’, an impure copper arsenite was introduced and used to check the spread of the Colorado beetle in the USA. By 1900 the use was so widespread that the first pesticide legislation was introduced. In 1885 a chemical combination known as ‘Bordeaux mixture’, a lime sulphur mix, became the first fungicide to receive large scale use in Europe and California for the control of downy mildew in vine and fruit trees (3).

In 1896 a French grape grower observed that the leaves of charlock weed growing nearby turned black, when Bordeaux mixture was applied to his vines. This chance observation demonstrated the possibility of chemical weed control. Afterwards, it was found that iron sulphate, when sprayed onto a mixture of cereal and dicotyledonous weeds, killed the weeds without damaging the crop.
In the years between the two world wars the number and complexity of pesticides increased. Dinitro-orthocresol was patented in France in 1932 for the control of weeds in cereals and in 1934 Thiram, a dithiocarbamate fungicide, was patented in the USA. The birth of modern pesticides occurred during the second world war, beginning with the discovery of DDT (dichloro-diphenyl-trichloroethane) in Switzerland and the development of organophosphorus compounds in Germany (4).

In 1945 the first soil-acting carbamate herbicide was discovered in Britain and the organochlorine insecticide Chlordane was introduced in the USA and Germany. The period between 1950 and 1955, saw the introduction of the urea-derived herbicides Captan, Glyodin and Malathion. The period between 1955 and 1960, newcomers included the triazines and quaternary ammonium herbicides. In 1968 a systemic fungicide Benomyl and the herbicide Glyphosphate were discovered.

After World War II, rapid urbanisation and increased food prices saw an increase in consumption of pesticides with herbicide representing a major portion that is still increasing at a more rapid rate than other herbicides. In the developing world, herbicides are the dominant class of pesticide used and its use is rising due to labour shortage, substitution for tillage due to higher fuel price and equipment cost and shortage. Herbicides are used to minimise nutrient loss from competing weeds.
1.2 **CLASSIFICATION OF HERBICIDES**

The classification of herbicides allow farmers, advisors and researchers to know which herbicides are best suited to combat specific weeds in resistance management. The herbicides are classified alphabetically according to their primary sites of action.

Group A herbicides include the aryloxyphenoxypropionates and cyclohexanedione families. The mode of action of group A herbicides is by lipid synthesis inhibition. The herbicide inhibits a single enzyme, acetyl CoA carboxylase (ACC) involved in fatty acid synthesis. This herbicide prevents the formation of fatty acids, components essential for the production of plant lipids that are vital to the integrity of cell membranes and to new plant growth.

Group B herbicide labels are applied to herbicides that work by inhibition of the enzyme acetolactate synthase. Trade names applying to this group include Ally, Brush-off, Cut-Out, Glean, Harmony, Logran, Londax, Monza, Oust, Renovate, Titus, Arsenal, Flame, OnDuty, Spinnaker, Broadstrike and Eclipse. There are sets of rules about the application of Group B herbicides to crops. For example, you can only use Group B herbicides once in a season and twice in four years.
1.3 HERBICIDES APPLIED TO SOIL AND LEAF

Soil applied herbicides are applied to the ground after ploughing and before the weeds appear. The herbicide must be positioned in the top 2 to 5 cm of the soil surface because the seeds of many weed species are quite small and germinate within 1 to 3 cm of the soil surface. Once the herbicide comes in contact with the weed, it will be absorbed through the roots or shoots and move within the plant to the site of herbicidal activity.

Physical and environmental factors that promote rapid crop emergence reduce the length of time that a plant is in contact with the herbicide and therefore reduce the possibility of crop injury.

Post-emergence herbicides are foliage-applied type of herbicides, which are sprayed onto the weeds after they emerge from the ground. For post-emergence herbicides, the chemical and physical relationships between the leaf surface and herbicide often determine the rate and amount of uptake. There are two groups of post-emergence herbicides:

- non-mobile herbicides, which must thoroughly cover the plant and
- mobile herbicides, which can move from the site of application to the root system and then to the growing point of the plant.
1.4 HERBICIDE SELECTIVITY

Some plants can rapidly degrade or deactivate a herbicide and escape its toxic effects. Corn is tolerant to triazine herbicides because it quickly deactivates these by binding them to naturally occurring plants chemicals. Soybean tolerance to some herbicides is at least partially due to the deactivation of the herbicide by conjugating them to plant sugar molecules.

However, situations may occur in which a crop may be injured by a herbicide to which it is normally tolerant. This can occur when environmental stress or excessive application causes the crop’s herbicide degradation and deactivation systems to be overwhelmed.

1.5 HERBICIDE FAMILIES BASED ON MODE OF ACTION

An understanding of how herbicides kill weeds may be useful in selecting and applying the proper herbicide for a given weed control problem. There are a large number of herbicides available on the market and several have similar chemical properties. Herbicides with a common chemistry are grouped into “families” allowing a convenient way of organising information about them. Two or more herbicide families may have the same mode of action within the plant and thus express the same herbicide activity and injury symptoms. The term ‘mode of action’ refers to the sequence of events from absorption by the plant to plant death. There are seven major mode of action groups. These are growth regulation, amino acid synthesis inhibition, lipid synthesis inhibition, seedling growth inhibition, photosynthesis inhibition, cell membrane disruption, and pigment inhibition.
1.6 FLUMETSULAM MODE OF ACTION

Flumetsulam, or N-(2,6-difluorophenyl)-5-methyl-[1,2,4]triazolo[1,5-a]pyrimidine-2-sulfonamide, is a member of the potent triazolopyrimidine sulfonanilide family of herbicides and the structure is presented in Figure 1.1. It was developed for the pre- or post-emergence broadleaf weed control in wheat, corn, barley and soybean by Dow AgroSciences (formally known as DowElanco) (5). It acts by disrupting branched chain amino acid biosynthesis through the inhibition of the enzyme acetolactate synthase, which is essential for the production of the amino acids, valine, leucine and isoleucine. It has high herbicidal activity at low application rates between 10 and 50 g ha\(^{-1}\) (6). Herbicides containing flumetsulam as an active ingredient are marketed by Dow AgroSciences under the names Broadstrike® (used in Australia), Hornet®, Python®, Preside® and Scorpion® (used overseas).

![Figure 1.1 Chemical structure of flumetsulam](image)

**Figure 1.1 Chemical structure of flumetsulam**

For a herbicide to be effective, it must adequately contact the plant, be absorbed by the plant, move within the plant to the site of action without being deactivated, and reach toxic levels at the site of action. The mode of action of the herbicide influences how the herbicide is applied. Contact herbicides that disrupt the cell membrane are
applied to the leaves after they emerge (post-emergence) while seedling inhibitors are soil applied to control seedling germination. Thus the two types of herbicides, classified according to their mode of application, are soil-applied herbicides and leaf-applied herbicides.

Flumetsulam is a broadleaf herbicide with no ryegrass activity (7). It is absorbed in a few hours by both roots and shoots of germinating broadleaf weeds. Leaves of annual weeds turn yellow, 2 to 3 days after administration. Stem and leaf are necrotised in 4 to 7 days and the whole body withers and dies in 10 days.

Flumetsulam is applied as a water dispersible granule formulation. Sicklepod and morningglory are among the most common and most troublesome weeds in soybean. Although control by soil-applied herbicides is inconsistent and often inadequate, morningglory can be controlled with several post-emergence herbicides. Options to control sicklepod are more limited although it is not as competitive as some other broadleaf weeds. However, infested fields normally have high populations of sicklepod and soybean yield reductions greater than 50% have been observed when sicklepod is not controlled (8). A more effective soil-applied herbicide for sicklepod and morningglory would be an advantage. Flumetsulam has activity on sicklepod, morningglory and other broadleaf weeds.

Weeds compete with soybeans for soil moisture, nutrients, sunlight and space in the field. One type of weed may occupy four to eight square feet of area, thereby reducing the space available for soybean growth. When weeds shade the soybean plant, less sunlight is available for soybean production. Most of the soybean’s yield-reduction
from velvetleaf and pigweeds is due to shading by the weed leaves above the soybean canopy.

Askew et al (9) has shown that a period of 4 to 6 weeks without weed competition at the beginning of the growing season will allow production of maximum yields under most environmental conditions. Any weed emerging in the crop after this initial weed free period will not compete effectively with soybeans and will not affect yield potential. Similarly, a period of 4 to 6 weeks of weed interference at the beginning of the season usually can be tolerated by soybeans with no significant yield loss provided that the crop is maintained weed free for the remainder of the season.

1.7 Amino Acid Synthesis Inhibitors

The amino acid synthesis inhibitors include the herbicide family’s sulfonylurea, imidazolinone, sulfonanilides and amino acid derivatives. Flumetsulam belongs to the sulfonanilide group. Amino acid synthesis inhibitors act on a particular enzyme to prevent the production of specific amino acids, essential for normal plant growth and development.

Sulfonylurea, imidazolinone and sulfonanilide herbicides prevent the production of three essential branch-chain amino acids by inhibiting one key plant enzyme. The amino acid derivative herbicides inhibit the production of three essential aromatic amino acids by inhibiting a different plant enzyme. In general, injury symptoms are slow to develop (one to two weeks) and include stunting or slowing of plant growth and a slow plant death.
Herbicides in the sulfonylurea, imidazolinone and sulfonanilide families can move in both the xylem and phloem to areas of new growth and can be taken up through plant foliage and roots. Herbicides in these families vary greatly in selectivity and may control annual and perennial broadleaf or grass weeds and may be soil or foliar applied.

1.8 INTRODUCTION TO HPLC

Liquid chromatography (LC) is a separation technique where analytes are separated by virtue of differing solubility between a liquid mobile phase and a liquid or solid stationary phase. High performance liquid chromatography (HPLC) is a type of LC where the mobile phase is forced through a reusable column by means of a pumping system and the stationary phase is usually a ligand bonded onto a porous particle that is densely packed inside the column. In reverse-phase HPLC (RP-HPLC) the stationary phase is a hydrophobic ligand chemically bonded onto a particulate support. RP-HPLC is generally used to separate small polar to semi-polar molecules (MW < 2000). All references to HPLC here refer to RP-HPLC unless otherwise noted.

A simplified schematic diagram of a HPLC system is presented in Figure 1.2. Analytical HPLC generally uses column inner diameters of 2.1 to 4.6 mm and flow rates from 0.5 to 2 mL min\(^{-1}\).
1.8.1 LIQUID-LIQUID CHROMATOGRAPHY

Classical liquid-liquid chromatography uses stationary phases with a predominantly polar functional group. Therefore, polar compounds are more strongly retained and less polar compounds more easily eluted. In HPLC, this classical situation is reflected in the term ‘normal phase’ for the use of a polar stationary phase and ‘reversed phase’ for the use of a non-polar phase. However, reversed phase is used by 90% of HPLC users. Some examples of HPLC solvents are presented in Table 1.1. The $P'$ factor is a classical view of solvent strength, which gives more weight to polar solvents with water being the strongest.
Table 1.1 Characteristics of HPLC separation modes and solvent systems

<table>
<thead>
<tr>
<th>Reversed Phase</th>
<th>Solvent</th>
<th>Strength ($P'$)</th>
<th>Normal Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weak</td>
<td>Water</td>
<td>10.2</td>
<td></td>
</tr>
<tr>
<td>Strong</td>
<td>Acetonitrile</td>
<td>5.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>5.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tetrahydrofuran</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Propanol</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Methylene Chloride</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hexane</td>
<td>0.1</td>
<td></td>
</tr>
</tbody>
</table>

This corresponds to the normal-phase view of solvent strength. There are two equally useful definitions for a strong solvent in HPLC:

1. A strong solvent is one whose polarity is most like the stationary phase.
2. A strong solvent is one that decreases analyte retention on the column.

1.9 **SOLID PHASE EXTRACTION**

Solid-phase extraction is the most important technique used in sample pretreatment for HPLC. SPE can be used in a fashion similar to liquid-liquid extraction (LLE). Whereas LLE is a one-stage separation process, SPE is a chromatographic procedure that resembles HPLC and has a number of potential advantages over LLE:

1. More complete extraction of the analyte.
2. More efficient separation of interferences from analytes.
3. Reduced organic solvent consumption.
4. Easier collection of the total analyte fraction.
7. More easily automated.
It is easier to obtain a higher recovery of the analyte by SPE than LLE, because it is a more efficient separation process. With SPE, it is possible to obtain a more complete removal of interferences from the analyte fraction. Reversed phase SPE techniques are most popular, as only small amounts of organic solvent are required for elution, maintaining a high concentration of analyte. Because there is no need for phase separation in SPE, the total analyte fraction is easily collected, eliminating errors associated with variable or inaccurately measured extract volumes. Finally, larger particulates are trapped by the SPE cartridge and do not pass through into the analyte fraction.

Some disadvantages of SPE include:

1. Variability of SPE cartridges.
2. Irreversible adsorption of some analytes on SPE cartridges.

The solvents used in LLE are usually pure and well defined, so that LLE separations are quite reproducible. Conversely, the solid phase used in SPE cartridges tend to vary from lot to lot, so that method reproducibility is sometimes a concern with SPE procedures.

1.9.1 SPE METHOD DEVELOPMENT

The application of SPE generally involves four steps:

1. Conditioning the packing.
2. Sample application.
3. Washing the packing (removal of interferences without loss of analyte).

4. Recovery of the analyte.

In step 1, carried out prior to addition of sample, the packing is conditioned by the passage of a few bed volumes of solvent, typically methanol or acetonitrile, through the cartridge. The role of the conditioning step is to remove any impurities that may have collected while the cartridge was exposed to the laboratory environment and allows the sorbent to be solvated. Solvation is important because reversed-phase packing that has been allowed to dry out, often exhibits decreased sample retention. In addition, varying states of SPE phase dryness lead to non-reproducible analyte recoveries. A preconditioning water wash also serves to ready the SPE cartridge for introduction of an aqueous sample.

Step 2 is the SPE sample application (loading) where the sample, dissolved in a weak solvent, is added to the cartridge. This weak solvent allows strong retention of the analyte. For RP-SPE operations, a weak solvent is water or buffer, with a small amount of organic modifier. The sample solution should be passed through the cartridge without allowing it to dry out. A critical control point in this step is to precisely control the flow rate.

Step 3 is the removal of interferences by washing the cartridge with a solvent of intermediate strength. Optimally, the wash step is discontinued just before the analyte begins to leave the cartridge. In this way, interferences that are more weakly retained than the analyte are washed from the cartridge, without loss of the analyte. Water or a
buffer is often used for the wash solvent in RP-SPE, but this may not provide maximum removal of interferences from the analyte fraction that is collected. A small controlled amount of organic solvent may be added to wash the solution to aid the removal of more hydrophobic substances.

Step 4 involves the elution and collection of the analyte fraction. If detection sensitivity is a major goal, the analyte is collected in as small a volume as possible. This can be achieved with a strong elution solvent. Alternatively, the use of a weaker solvent that still provides elution of the analyte will minimise the elution of more strongly retained interferences. This is an important consideration when late eluters are present in significant amounts, since these compounds may increase the required run time for the HPLC separation.

In the case that the analyte is an acid or base, the pH of the sample can be adjusted to suppress analyte ionisation and maximise RP-SPE retention in steps 2 and 3. Elution of the analyte in step 4 can then be effected by a change in pH so as to ionise the sample, allowing it to be eluted. After the analyte fraction is collected, the pH of the fraction can be readjusted to suppress ionisation of the analyte for it to show retention on a hydrophobic (reversed phase) column.

SPE also can be used to retain impurities but allow the analyte of interest to pass through the cartridge. Although this option is far less common, however if combined with the traditional RP-SPE, it can prove to be beneficial in the removal of interferences.
1.10 **INTRODUCTION TO LC-ESI-MS/MS**

Mass spectrometry/mass spectrometry (MS/MS) is the term used to describe the process of mass analysing fragments of a mass-selected precursor ion. This process could be applied to a fragment ion that was produced by an initial fragmentation of the ion that represents the intact analyte molecule or the fragmentation of the ion representing the intact molecule itself.

The MS/MS technique is often referred to as a “tandem-instrument” process. This is because ion separation is done for selection of a precursor ion followed by a dissociation stage, and then the resulting product ions undergo \( m/z \) analysis. The concept of MS/MS is illustrated schematically in Figure 1.3. A mass spectrum can be obtained from an ion in a primary mass spectrum. The primary mass spectrum is formed in the conventional manner by an ion source such as ESI together with some means of mass analysis. The first mass analyser serves to select ions at a specific \( m/z \) value from the primary mass spectrum for dissociation. The commonly used dissociation process is collision-induced dissociation (CID) or collision activated dissociation. In the Varian 1200L triple quadrupole MS, the CID process is accomplished in the 180 degree collision cell in which the partial pressure of an inert gas, such as argon is allowed to reach a pressure of approximately \( 10^{-5} \) torr. Collisions between the collision gas molecules and the ions of the selected \( m/z \) value increase the internal energy of the precursor ion causing some of these ions of the selected \( m/z \) value increase the internal energy of the precursor ion causing some of these ions to fragment into ions of lower mass. A second mass analyser then is used to analyse the fragment ions or product ions from the CID process according to mass.
Figure 1.3 The concept of MS/MS fragmentation
The Varian 1200L electrospray interface employs an off-axis spray needle, which is at 74° to the inlet capillary. This has the same effect as the orthogonal sprayers allowing precipitates and large droplets to bypass the inlet to the mass spectrometer. In addition, the Varian interface also has the inlet capillary positioned 6° off-axis from the hexapole ion lens, which leads to the mass analyser. Residual liquid droplets that may have entered the capillary are projected straight along the axis of the capillary, making it impossible for them to enter the mass analyser. This ESI interface is presented in Figure 1.4.

Figure 1.4 Varian 1200L off-axis spray needle and an off-axis inlet capillary
(From Varian Inc. 1200L Quadrupole MS product literature, 2003)
1.10.1 IONISATION MODES

‘Electrospray ionisation’ (ESI) is unique among LC-MS interfaces in being able to address the entire wide range of flow rates found in modern analytical HPLC. The ions commonly formed in electrospray are the typical ions expected to form in solution. Protonated ([M+H]+) and de-prontated ([M-H]-) molecules may be seen in the mass spectrum depending on whether positive or negative ion analysis is selected. In addition to protonated molecules, sodiated and potassiated molecules are also commonly seen due to the prevalence of Na and K in the laboratory environment. In addition to Na and K, other atoms or groups (e.g. NH₂) can also form adducts with the analyte molecule. Electrospray ion formation is a low energy process. Only a small amount of energy is imparted to the analyte by the ion evaporation process. This is, therefore, a suitable technique for formation of ions of intact molecules that would easily fragment in other energy ionisation modes such as EI. In this work, positive and negative modes were tried but it was found that the positive mode gave greater sensitivity and an abundant mass spectrum. The sodiated and potassiated parent ions were observed and proved to be useful for identification and confirmation purposes. Some of the advantages and disadvantages are presented in Table 1.2.
Table 1.2 Characteristics of electrospray ionisation

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight information</td>
<td>No information from controlled fragmentation¹</td>
</tr>
<tr>
<td>Good for both volatile and non-volatile</td>
<td>Problems with non-volatile buffers</td>
</tr>
<tr>
<td>analytes</td>
<td></td>
</tr>
<tr>
<td>Good for ionic and polar analytes</td>
<td>Analyte must be capable of forming an ion in solution</td>
</tr>
<tr>
<td>High sensitivity (low detection limit)</td>
<td>Ion suppression for a variety of mobile-phase constituents is possible</td>
</tr>
<tr>
<td>Allows for determination of high molecular</td>
<td></td>
</tr>
<tr>
<td>weight</td>
<td></td>
</tr>
<tr>
<td>Ideally suited for packed capillary LC columns</td>
<td></td>
</tr>
<tr>
<td>Various formats (ionspray, microelectrospray,</td>
<td></td>
</tr>
<tr>
<td>nanoelectrospray) allows use with a wide range</td>
<td></td>
</tr>
<tr>
<td>of LC flow rates (10 nL min⁻¹ to 1 mL min⁻¹)</td>
<td></td>
</tr>
</tbody>
</table>

¹ This problem was overcome because the Varian instrument was a triple quadrupole MS which allows the use of CID to yield structural information.

The other form of atmospheric pressure ionisation is ‘atmospheric pressure chemical ionisation’ (APCI). This is gas-phase rather than liquid-phase ionisation. APCI is a reaction between a reagent ion and an analyte molecule in the gas phase where the LC mobile phase acts as the CI reagent. A corona-discharge needle in the atmospheric pressure portion of the interface has a high potential applied to it, and this supplies electrons that ionise the surrounding gas molecules. The ionised gases, in turn, ionise the LC solvent vapor, which acts as the ionisation reagent for the analyte.

APCI is used when the analyte contains no acidic or basic sites. The analyte has to be in the vapor phase. A high temperature (200 – 400 °C) is required to desolvate and vaporise the analyte and the mobile phase, but too high a temperature can lead to analyte decomposition. APCI is a rugged technique that is less dependent on solvent
choice, flow rates, or additives, compared to ESI. The advantages and disadvantages of APCI are presented in Table 1.3.

### Table 1.3 Characteristics of atmospheric pressure chemical ionisation

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight information</td>
<td>No information from controlled fragmentation</td>
</tr>
<tr>
<td>Good for moderately volatile analytes</td>
<td>Thermal degradation of analytes can result</td>
</tr>
<tr>
<td>Easy to use</td>
<td>Potential problems with non-volatile buffers</td>
</tr>
<tr>
<td>Accommodates high flow rates (&gt;1 mL min(^{-1}))</td>
<td>Low molecular weight (&lt;100 Da) analytes are a problem due to chemical noise</td>
</tr>
<tr>
<td>Sensitive (compound dependant)</td>
<td>Single charge ions limit range to 1000 Da</td>
</tr>
<tr>
<td>Single charge ions</td>
<td>Limited to flow rates of 250 µL min(^{-1}) and greater</td>
</tr>
<tr>
<td>Adduct ion formation</td>
<td></td>
</tr>
<tr>
<td>Good results in negative and positive ion modes</td>
<td></td>
</tr>
<tr>
<td>Works well with water</td>
<td></td>
</tr>
<tr>
<td>Good reproducibility for quantitation</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

1.11 SOYBEAN PHYSIOLOGY

Soybean is a valuable agricultural commodity due to its unique chemical composition. The soybean seed consists of lipids, proteins, carbohydrates and minerals. Other valuable components found in soybeans include phospholipids, vitamins. On average, oil and protein together constitute about 60% of dry soybeans. The remaining dry matter is composed mainly of 35% carbohydrates and 5% ash.

Nine fatty acids make up the lipid content of soybeans. During seed development, soybeans store their lipids, mainly in the form of triglycerides, in organelles known as oil bodies. The soybean contains 20% oil, the second highest oil content among food legumes. The highest oil content is found in peanut, with about 48% oil; the third highest oil content is found in chickpeas, with about 5%. Crude soy oil contains impurities such as phosphatides, free fatty acids, gummy substances, color bodies, hydrocarbons and a waxy fraction. Refining operations remove these impurities.
1.12 AIMS AND OBJECTIVES

There are few published methods available for the determination of flumetsulam in biological matrices. The aim of this project is to develop an analytical method for the sensitive and reliable determination of flumetsulam in soybean using HPLC with UV detection. The HPLC method is convenient for the parent compound because it is not easily volatilised for GC analysis. Soybean was chosen, as it is a major agricultural crop. It has 15 to 20% oil content and is a crop that has been successfully used with this herbicide.

The objectives of the study are:

1. To develop a method for the extraction and clean up of flumetsulam from soybean by solvent-solvent extraction and by solid-phase extraction.
2. To determine the optimum conditions for the chromatographic analysis of flumetsulam.
3. To find a surrogate and internal standard suitable for use in the developed method.
4. To validate the proposed methods.
5. To develop a LC-MS method for the confirmation and quantitation of flumetsulam in soybean.
1.13 **ANALYTICAL METHOD VALIDATION**

In validating an analytical procedure several validation parameters have to be quantified so that the limits of the method’s performance are well known. The main parameters for method validation are selectivity, precision, accuracy, linearity, range, limit of detection and quantitation and ruggedness.

1.13.1 **ANALYTE**

This is a component of a sample that has to be detected, identified and/or quantified. The term ‘analyte’ includes, where appropriate, derivatives formed from the analyte during the analysis. The quantitative measure of an analyte has to be reported as an amount, expressed as a mass quantity or as a concentration expressed as a mass fraction or a mass concentration.

1.13.2 **PURE STANDARD**

The pure standards of the analyte, surrogate and internal standard were purchased from the appropriate suppliers for this work. These standards were used to fortify a blank sample matrix with a known concentration. After extraction of the analyte from the matrix and injection into the analytical instrument, its recovery can be determined by comparing the response of the extract with the response of the reference material dissolved in pure solvent.
1.13.3 Selectivity

The terms selectivity and specificity are often used interchangeably but there is a small difference. The “specificity” of a method refers to the ability of the method to distinguish between the analyte being measured (produce a response to only a single analyte) and other substances. The term “selectivity” of a method refers to its response to a number of chemical entities, which may or may not be distinguished. If the response for the method of interest is distinguished from all other responses, the method is said to be selective. The selectivity of an analytical method is its ability to measure accurately an analyte in the presence of interference that may be expected to be present in the sample. In method validation, selectivity is tested by comparing results obtained for the test sample with and without impurities present.

1.13.4 Precision

The precision of a method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samples. Precision is measured by analysing a series of samples and standards and calculating the standard deviation of a set of results. Peak areas are used for quantitation, while retention time identifies the peak. If a mass spectrometer (MS) is used, the MS spectrum provides additional identity confirmation. Precision can be subdivided into two categories, repeatability and reproducibility. Repeatability is obtained if one operator, using one piece of equipment over a relatively short time span, carries out the analysis in one laboratory. Reproducibility is the ability to achieve the same results with different operators and equipment in different laboratories or run within a single laboratory but on a different
day. Repeatability is often considered to be the “intra-day” precision and reproducibility the “inter-day” precision.

Where the analytical procedure involves an extraction and/or purification step(s), its contribution to overall precision can be distinguished from that of the instrumental contribution by making multiple injections of a single extract and comparing it to the precision obtained for several replicate spiked samples subjected to extraction and analysis.

1.13.5 **Accuracy**

The accuracy of an analytical method is the degree of agreement of test results generated by the method to the true value. Accuracy assessment can be obtained by comparing results of the method with results of an established reference method, or by comparison to expected results of a standard reference material. In this study, neither an established reference method nor certified reference material is available. In this study it was possible to fortify flumetsulam into soybean that is free of this substance, which gives measure of the accuracy of the method.

1.13.6 **Linearity**

The linearity of an analytical method is its ability to produce test results that are directly, or by means of well-defined mathematical transformations, proportional to the concentration of analyte(s) in the sample within a stated range. Instrumental linearity is determined by a series of injections of standards at about five different concentrations that span 50 to 150% of the expected working range assay. The
response should be linearly related to the concentration of standards. A linear regression equation applied to the calibration curve should have an intercept not significantly different from zero. If a significant non-zero intercept is obtained, it should be demonstrated that there is no effect on the accuracy of the method. Under most circumstances, the correlation coefficient ($R^2$) should be $> 0.990$.

1.13.7 RANGE

The range of an analytical method is the interval between the upper and lower levels that have been demonstrated to be determined with precision, accuracy, and linearity using the method as written.

1.13.8 LIMIT OF DETECTION AND LIMIT OF QUANTITATION

The limit of detection is the point at which a measured value is larger than the uncertainty associated with it. It is the lowest concentration of analyte in a sample that can be detected, but not necessarily quantified. In chromatography, the detection limit is the amount injected which results in a peak with a height at least three times that of the standard deviation of the noise level. This definition however does not factor into account the standard deviation contribution of the analyte extraction and purification procedure. The limit of quantitation has a similar definition to the limit of detection except that the limit of quantitation is calculated based on ten times the standard deviation (10).
1.13.9 Stability

Solution stability of the test substances after preparation according to the test method should be evaluated according to the test method. The stability of the standard solutions as well as sample extracts needs to be determined and this is achieved by analysing these solutions over a period of time under predetermined storage conditions. Analyte decomposition is usually indicated by decreasing analyte peak height accompanied by the appearance of extraneous peaks.

1.13.10 Ruggedness

Ruggedness is the effect that operational and environmental conditions have on the analytical result. It is the degree of variance in the test results obtained by the analysis of the same sample under a variety of different test conditions, such as different laboratories, different analysts and different instruments. In the laboratory used for this study there is limited opportunity for ruggedness testing other than switching HPLC column brand and using the same solvent from different suppliers.

1.13.11 Robustness

Robustness tests examine the effect that operational parameters have on the analysis results. For the determination of a method’s robustness, a number of method parameters, for example pH, flow rate, column temperature, injection volume, detection wavelength or mobile phase composition, are varied within a realistic range, and the quantitative influence of the variables determined. If the influence of the parameters is within a previously specified tolerance, the parameter is said to be...
within the method’s robustness range. Obtaining data on these effects helps to assess whether a method needs to be revalidated when one or more parameters are changed.

### 1.14 INTERNAL STANDARD

The highest precision for quantitative chromatography is obtained by using an internal standard because the uncertainties introduced by sample injection are avoided. An internal standard is also useful when unavoidable sample losses are expected. If a standard is added to a sample prior to any losses, then the fraction of standard lost is the same as the fraction of the sample lost, and the ratio (concentration of analyte):(concentration of internal standard) remains constant. An internal standard is a pure analyte added to a solution in known amount and used to measure the relative responses of other analytes and surrogate that are components of the same solution. In practice the internal standard is added when the solution is finally made up to volume for injection compared to a surrogate standard, which is added at the start of the extraction process. The internal standard used in this work is benzoic acid and the structure is presented in Figure 1.5.

An internal standard should ideally meet the following requirements:

(i) it should chromatograph well and be separated from the sample components without being markedly different from them in retention time,

(ii) it should be stable and not react with the sample or any column component under the experimental conditions and

(iii) it should not be present in the test sample.
**1.15 Surrogate Standard**

In an analytical method, the addition of an ideal surrogate standard will render the detection, quantitation and recovery of analyte more reliable. The surrogate standard is a pure analyte which is extremely unlikely to be found in the sample, and which is added to the sample in a known amount before extraction and is measured in the same chromatographic run. The surrogate standard should be an analogue of the analyte or the deuterated form of the analyte if a MS detector is used.

The quantitation and recovery determined using the ratio of analyte to surrogate standard would then compensate for losses during the extraction and analytical process.

The surrogate standard, unlike the internal standard, is not clearly defined in the literature and the terms are sometimes used interchangeably. In this report the surrogate standard is taken to be a substance that is a homologue of the analyte, but not found in the sample, and is introduced into the sample at the beginning of the extraction procedure to compensate for its loss in the extraction process. This study incorporates both an internal standard and surrogate standard to make the method more robust. The surrogate standard used in this work is diclosulam and the structure is presented in Figure 1.6.

![Figure 1.5 Chemical structure of internal standard, benzoic acid](image-url)
Figure 1.6 Chemical structure of surrogate standard, diclosulam

Though diclosulam is also a registered herbicide, it has not been reported used together with flumetsulam.
1.16 REFERENCES

Chapter 2: Literature Review

2.1 INTRODUCTION

Flumetsulam is a member of a relatively new class of selective herbicides used for pre- or post-emergence broadleaf weed control in wheat, corn barley and soybeans. There are few methods available for the determination of flumetsulam in biological matrices like food crops and environmental matrices like water and soil. The isolation procedures described in the available literature are usually based on an alkaline treatment of the sample followed by acidification and isolation of the analyte in an organic phase. The extracts are then cleaned up by solvent-solvent partitioning alone or in combination with solid-phase extraction (SPE) followed by analysis of the extracts by HPLC-UV, LC-MS or MS/MS, GC-MS after suitable derivatisation, in the case of the GC methods.

Although one of the first published methods to analyse flumetsulam in a biological matrix used GC-MS, it is no longer a method of choice due to the need for derivatisation. LC-MS/MS is now preferred for sulphonanilide compounds. The number of publications describing reversed-phase LC-MS/MS has increased steadily but due to the high cost of instrumentation, HPLC-UV is still a desirable technique to be developed.

In pesticide/herbicide analysis, the traditional liquid-liquid extraction and clean up is gradually being replaced by SPE where not only is the SPE technique used to extract the analyte, but also to clean the sample.
2.2 METHODS FOR THE DETERMINATION OF FLUMETSULAM IN AGRICULTURAL GRAINS

DowElanco developed flumetsulam, which is a selective acetolactate synthase inhibiting herbicide. After development, DowElanco provided a method (1) for the analysis of flumetsulam in corn grain, forage and fodder. For the fortification study, a ground portion of the sample was fortified with flumetsulam. Forage and fodder samples were acidified (0.1 M HCl) before extracting with acidified acetone (10% 0.1 M HCl) and grain samples were extracted with acidified acetone (10% 0.1 M HCl) directly. After blending and centrifuging the extraction solvent was transferred to a vial and concentrated before acidification and extraction with hexane. The hexane was discarded and the aqueous phase subjected to C-18 and alumina SPE extraction. The C-18 SPE was washed with aqueous acid. The analyte from the C-18 SPE was rinsed with acidified dichloromethane (1% acetic acid) and eluted with methanol into an alumina SPE cartridge before washing it with acidified dichloromethane (1% acetic acid). The analyte was then eluted with acidified dichloromethane (3% acetic acid). The eluate was evaporated to dryness and reconstituted with acetonitrile before adding triethylamine and methyl iodide to form the N-methyl derivative. After the formation of the derivative, the solution was evaporated and reconstituted with methyl-t-butyl ether and 5% NaCl solution. The aqueous layer was discarded and the organic layer evaporated to dryness before the addition of the deuterated internal standard and analysis by capillary GC-MS.
A Durabond-17 capillary column was used (10 m x 0.18 mm i.d., 0.3 µm film thickness). The carrier gas was helium and the column oven temperature program was 120 °C and ramped up to 325 °C at a rate of 20 °C min⁻¹. The linear velocity was 25 cm s⁻¹ and the injection volume was 2 µL splitless. The retention time of the internal standard and flumetsulam derivative was 12.5 min. The flumetsulam derivative had a m/z 134 and the internal standard has a m/z 145. Therefore, both ions could be quantified separately using the MS. The method reported an average recovery of 98% (range 91% to 97%), and average SD of 5% (range 3% to 7%). The LODs, calculated as three times the SD of the lowest fortification level, were 1.29, 0.31 and 0.17 ng g⁻¹ for grain, forage and fodder respectively. It is more usual practice to determine the LOD at a fortification level less than 10 times higher than the LOD as linearity assumptions are less likely to be valid. The details of method performance are summarised in Table 2.1, reference 1.

Unlike GC, HPLC methods are especially attractive due to their high specificity in addition to avoiding the need for derivatisation, even for low-level determinations. The only method reported in a journal which included flumetsulam as one of five analytes in a multi-residue method and maize as the matrix was by Lagana et al (2). The authors reported the development of a solids extraction unit for analyte extraction from maize followed by LC-MS/MS analysis. The extraction parameters that were studied included temperature, duration of the static extraction, sample amount, solvent volume and flow rate. After extraction, herbicides present in the methanolic phase were isolated and cleaned up by a C-18 SPE followed by a Carbograph-1 cartridge.
For the fortification samples, an appropriate volume of standard working solution was added to sieved maize. The fortified and unfortified maize samples were then quantitatively transferred to the empty extraction column-cells. Methanol was then introduced into the column-cell and collected from the other end of the column. The methanolic extract was then subjected to SPE clean up. The pre-washed C-18 SPE was used to filter the methanolic extract which had been diluted with H₂O and the SPE washed with a methanol:H₂O mixture which was collected and combined with the filtrate. This filtrate was further diluted with H₂O and passed through a preconditioned Carbograph-1 cartridge. A back-flushing elution was performed by turning the cartridge upside-down and washed with methanol and the analytes back-eluted with a acidified dichloromethane:methanol mixture. The eluate was evaporated to a small volume for LC-MS analysis.

For the recovery studies, measuring peak areas and comparing them with those obtained from standard solutions, determined the concentration of the analytes. The analytes were injected onto a C-18 column with an acetonitrile:H₂O mobile phase basified with ammonium acetate. Gradient elution was performed by linearly increasing the percentage of acetonitrile from 10% to 80% in 10 min and up to 95% in a further 10 min. The retention time of flumetsulam was 8.3 min. The flow rate of the HPLC was 1 mL min⁻¹. The addition of ammonium acetate to the mobile phase suppresses the production of Na⁺ and K⁺ adduct ions during MS analysis and possibly enhanced detector response. The MS was operated in the positive ion mode to obtain the full scan spectra of all the analytes. This gave rise to the [M + H]⁺ ion as the base peak. For quantitation, collision induced dissociation (CID) spectra were obtained with their respective peak base ions as a precursor at individual collision energies.
The LODs of the herbicides in maize, calculated as three times the SD of the lowest fortification level, were between 8.9 and 17.2 ng g\(^{-1}\). The average recovery was 85% (range 81 to 89%). The average RSD was 10% (range 7 to 13%). The details of method performance are summarised in Table 2.1, reference 2. This method of analysis mainly focused on the use of hot (subcritical) methanol and the use of a solids extraction unit, to extract from the matrix. However, this method would be difficult to adopt because the equipment used was specially constructed for the project by the investigators.
2.3 METHODS FOR THE DETERMINATION OF FLUMETSULAM IN SOIL

Baskaran et al (3) described a HPLC-UV method for measuring flumetsulam residue in soil. The soil was extracted with aqueous NaHCO$_3$ solution and the extract acidified before passing through a C-18 SPE disc. The concentrated extract obtained was then cleaned up by gel permeation chromatography (GPC) on Bio-Beads SX-3 prior to analysis by reversed phase HPLC-UV.

The fortified and unfortified soil samples were extracted with aqueous NaHCO$_3$. After centrifugation, the aqueous solution was decanted and the extraction procedure repeated two more times. The combined extract was acidified and passed through a preconditioned C-18 SPE disc. The disc was then dried and the analyte eluted from the disc with ethyl acetate. The ethyl acetate was evaporated to dryness and reconstituted with ethyl acetate:cyclohexane (1:1) before GPC clean up. The GPC column was eluted with ethyl acetate:cyclohexane (1:1). The first fraction was discarded and the second fraction containing the analyte, was collected, evaporated to dryness and redissolved in methanol:0.5% aqueous acetic acid (10:90) for HPLC-UV ($\lambda=250$ nm) analysis. The retention time of flumetsulam was 13 min.

The analyte was analysed on a Zorbax SB-C-18 reversed phase column at 35 °C and using a mobile phase of methanol:0.5% aqueous acetic acid (25:75) run isocratically at a flow rate of 1 mL min$^{-1}$. The injection volume was 100 µL.

The average recovery was 67.5% (range 66.4 to 69.1%) with an average RSD of 4.8% (range 3.7 to 6.6%) and the reported LOD was 4 µg kg$^{-1}$. Details of the method performance are summarised in of Table 2.2, reference 3.
Qu et al (4) reported a method for the analysis of flumetsulam in soil by GC-FID and MS. The analyte was extracted from soil by acetone:0.1 M HCl (90:10, % vv) and purified by liquid-liquid partitioning in acidic and basic (2-8) pH. The analyte was derivatised with methyl iodide to form the N-methyl derivative. The average recovery was between 90.5 to 97.6% and the LOD was 2.5 $\mu$g kg$^{-1}$. The details of method performance are summarised in Table 2.2, reference 4.

Lagana et al (5) reported a multi-residue method for the confirmation and quantitation of 16 herbicides, which included two triazolopyrimidine sulfonanilides in soil. The analytes were extracted using soil column extraction and the extracts cleaned up using a Carbograph-1 cartridge. The extract was evaporated to dryness, redissolved in H$_2$O:acetonitrile (80:20) acidified with formic acid and analysed using LC-MS/MS.

The fortified and unfortified soil samples were transferred to a filtration tube stacked on top of a Carbograph-1 cartridge and extracted by passing through potassium dihydrogen phosphate with sodium hydroxide at pH 8. The soil column was disconnected and the Carbograph-1 cartridge washed with H$_2$O, methanol and dichloromethane:methanol (80:20). As with Lagana’s other maize method, a back flushing elution was performed with dichloromethane:methanol (80:20) acidified with trifluoroacetic acid. The eluate was evaporated to dryness, reconstituted with H$_2$O:acetonitrile (80:20) acidified with formic acid before HPLC-MS/MS.

The herbicides were chromatographed on a reversed phase Alltima C-18 column with a gradient solvent program. The initial composition of the mobile phase was acetonitrile:H$_2$O (22:78) acidified with formic acid, then linearly increased to 80%
acetonitrile in 20 min. The flow rate was 1 mL min\(^{-1}\). The retention time of flumetsulam was 12 min.

The MS was operated under the negative mode where the deprotonated molecular ion \([M – H]\)\(^-\) was predominant with low abundance fragmentation. The analysis of herbicides was performed in the MRM mode. With the sulfonanilides, deprotonation of the anilinic nitrogen produced the precursor ion from which, due to the cleavage of the link between the sulfonylic group and the triazolopyrimidinic ring, gives the \(m/z\) 133 fragment.

The average recovery for flumetsulam was 73% (range 67 to 77%) and the average RSD was 5% (range 4 to 7%). The reported LOD was calculated as three times the SD of the lowest fortification level was 1.41 ng g\(^{-1}\). Details of the method performance are summarised in Table 2.2, reference 5. This method allowed the simultaneous determination of 16 herbicides in soil. The recoveries were low and the method used specially constructed equipment.

Rouchaud et al (6) developed a method for the analysis of flumetsulam in soil using GC-electron capture detection (GC-ECD) and GC-MS after methylation by diazomethane to form the N-methyl derivative. The method cleaned up the soil extracts by repeated TLC. The method was applied to the measurement of flumetsulam in the soil from several corn crops treated with flumetsulam.

For the analysis of flumetsulam, the soil was extracted with aqueous NaHCO\(_3\) and the mixture was centrifuged. The supernatant was removed and the soil re-extracted once
more. The combined supernatant was washed with dichloromethane and discarded before the aqueous phase was acidified and extracted twice with ethyl acetate. The ethyl acetate was concentrated to a small volume and applied as a band to a TLC plate along with the flumetsulam standard applied as a spot in a separate lane. Elution with diethyl ether:ethyl acetate (50:50) gave a band at $R_f = 0.54$ where it was scraped off and the silica gel extracted with acetone and the extract concentrated to a small volume before adding ethyl acetate and a solution of diazomethane in ether. The solution was then concentrated to a small volume and applied to a second TLC plate together with the standard of the N-methyl derivative in a separate lane. Elution with hexane:acetone (67:33) gave the N-methyl derivative band at $R_f = 0.39$. The band was removed and extracted with acetone and the extract concentrated and analysed by GC-ECD. The column used was an Alltech SE30 glass column set at 225 °C and thereafter increased to 240 °C. The retention time of the N-methyl derivative was 3.1 min.

GC-MS analysis was made with a 15 m Chromsorb capillary column, with an initial temperature of 50 °C, which was increased to 250 °C after 20 min. Electron impact was set at 30 eV and source temperature 200 °C. The retention time of flumetsulam was not reported.

Although analysis was performed using the method, no method performance results were reported. The main focus of the method was to study soil persistence and mobility of flumetsulam rather than method performance. It is uncertain whether the results obtained were accurate and reproducible. The details of method performance are summarised in Table 2.2, reference 6.
2.4 METHODS FOR THE DETERMINATION OF FLUMETSULAM IN WATER

Krynitsky (7) developed a capillary zone electrophoresis (CZE)–UV method and a LC-ESI-MS confirmatory method to analyse twelve-sulfonylurea herbicides and one sulfonanilide (flumetsulam) herbicide in runoff water. The water used for fortification was collected from a local marsh that contained high levels of potentially interfering compounds.

For the fortified and unfortified water samples, 500 mL was first acidified with glacial acetic acid. A reversed phase C-18 SPE cartridge was conditioned with methanol and acetic acid before loading the sample. The cartridge was then rinsed with H$_2$O and allowed to dry under vacuum. The analytes were eluted with methanol and the eluate evaporated to dryness before redissolving in acetonitrile. The solution was once again dried and redissolved in methanol to ensure complete removal of moisture from the extract prior to SAX/alumina SPE clean up because moisture affected the retention properties of alumina. The SAX SPE was stacked on top of the alumina SPE, both preconditioned with methanol. The sample was loaded onto the SAX SPE and allowed to drip through both cartridges. The cartridges were then rinsed twice with methanol and allowed to dry under vacuum before discarding the SAX SPE and eluates. The alumina SPE was rinsed with a small volume 0.5\% acetic acid in dichloromethane and the analytes eluted with a larger volume of 0.5\% acetic acid in dichloromethane before drying and reconstituting the eluate with 0.05 M aqueous acetic acid for CZE and LC-ESI-MS analysis.
The samples were quantified using peak height response. Individual compounds were identified by migration time. Detection was set at 240 nm. The CZE conditions were 161 nL injection, 50 mM ammonium acetate buffer at pH 4.75 with 12% aqueous acetonitrile, 30 KV, 30 µA and 30 °C oven temperature. The capillary length was 122 cm x 75 µm i.d. bare fused silica with a UV optical cell. The retention time of flumetsulam was 17.5 min.

Because CZE-UV is non-specific, there is potential for interferences. Therefore LC-MS was used as a confirmatory method. The LC column was a Zorbax RX C-8 with a 0.25 mL min⁻¹ flow rate and 75 µL was injected. The gradient mobile phase was acetonitrile:0.15% aqueous acetic acid (20:80) increasing to 50:50 after 31 min. The retention time of flumetsulam was 13.5 min.

The MS was operated in the positive mode where the protonated molecular ion [M + H]⁺ was predominant. The analysis of the herbicides was performed in the selective ion-monitoring (SIM) mode. Flumetsulam was identified by its [M +H]⁺ ion as well as one product ion. The author reported the use of source CID were the precursor ion was collisionally activated to form the product ion.

For the CZE-UV method, the average recovery for flumetsulam was 111% (range 106 to 115%) and the average RSD 11.9% (range 4.7 to 19%). The LOD calculated as three times the SD of the lowest fortification level, was 0.13 ng mL⁻¹. For the LC-MS method, limited data was generated because the LC-MS was used only as a confirmatory method. The author reported a recovery of 95% for flumetsulam with a RSD of 16%. The details of method performance are summarised in Table 2.3,
reference 7. This method allowed the simultaneous determination of 13 herbicides in water by LC-ESI-MS. The method had high sensitivity because; with a relatively clean aqueous matrix, it was possible to concentrate the analyte from a large volume of water sample onto the SPE cartridge. Analytes from the 500 mL sample were concentrated to 0.161 µL for CZE and 75 µL for LC-MS giving an enormous concentration increase. However, this concentration power is not available for complex matrices like plant or biological samples due the presence of large quantities of co-extractives, which will quickly saturate the SPE.

Rodriguez and Orescan (8) developed a multi-analyte method for the confirmation and quantitation of 16 herbicides in surface water by LS-ESI-MS. Samples are acidified and the analytes extracted using RP-102 cartridges and the extracts cleaned up using an anion exchange SPE stacked on top of an alumina SPE. The final extracts are evaporated to dryness, redissolved in acetonitrile:H₂O and analysed by LC-ESI-MS.

The RP-102 SPE is a polystyrene divinylbenzene resin. The SPE was preconditioned with methanol and acidified H₂O before loading the acidified water samples. The SPE was then washed with acidified H₂O before eluting the analytes with methanol. The eluate was evaporated to dryness and any traces of H₂O were removed for the next step.
An alumina and SAX SPE cartridge were separately conditioned with methanol before loading the extract dissolved in methanol from the RP-102 extraction. After washing the SPE stacks with methanol, the SAX SPE was discarded and the alumina SPE dried under vacuum. The alumina SPE was then washed with a small volume of acidified dichloromethane and the analytes eluted with a larger volume of acidified dichloromethane. The eluate was evaporated to dryness and the residue redissolved in acetonitrile:H$_2$O for LC-ESI-MS analysis.

For the quantitation of the individual analytes, calibration curves were prepared by plotting the average total ion peak area versus compound concentration. The compounds were separated on a Zorbax C-8 column and the initial mobile phase was acetonitrile:0.15% aqueous acetic acid (20:80) followed by a gradient increase from 20% to 50% acetonitrile which gave flumetsulam a retention time of 18.5 min. The flow rate was 0.2 mL min$^{-1}$ and the injection volume 100 µL.

A single quadrupole MS was used with positive ion ESI employing time-programmed capillary exit voltages to increase sensitivity. Selected ion monitoring (SIM) was used for quantitation. Since a single quadrupole MS was used, the authors had to generate fragment ions in addition to the [M + H]$^+$ ion by CID. The CID generates enough energy to break bonds by accelerating ions into collisions with molecules of the drying gas.
The average recovery for flumetsulam was 114% with a RSD of 9%. The recoveries reported for their other analytes ranged from 71% to 114% with RSDs less than 13%. The LOD calculated as three times the SD of the lowest fortification level was 0.031 ng mL\(^{-1}\). The low LOD is due to the large amount of water sample (250 g) being concentrated down to a small volume (100 µL). The details of method performance are summarised in Table 2.3, reference 8.

Furlong et al (9) developed a two-cartridge, SPE method for isolating 16 herbicides, including flumetsulam, for LC-ESI-MS analysis to identify and quantify these herbicides. The method was used to analyse 196 surface and ground water samples collected throughout the Midwestern United States, and more than 100 quality assurance and quality control samples.

For each sample extraction, two SPE cartridges were stacked in series. The first cartridge was a SAX SPE and the second was a RP-102 styrene-divinyl benzene polymeric SPE. The stacked SPEs were conditioned with acidified acetone and then acidified H\(_2\)O. 1 L of sample was used for all extractions and acidified prior to loading onto the SPE cartridges. After the sample passed through, the SPEs were dried under vacuum and a small volume of methanol added to the cartridges to remove any traces of water. After drying the stacked SPEs again, the analytes were eluted with acidified acetone and evaporated to dryness. The sample extract was reconstituted with acetonitrile:10 mM pH 3.7 buffer (10:90) for LC-ESI-MS analysis.

Analytes were quantified by comparing the integrated peak area from an environmental sample to the integrated peak area from a calibration line. The analytes
were separated on a Metasil Basic C-8 column with a acetonitrile:ammonium formate:aqueous formic acid pH 3.7 buffer (20:80) mobile phase with gradient elution from 20% to 90% acetonitrile. The flow rate was 0.2 mL min$^{-1}$ and the injection volume 50 µL. The column temperature was 30 °C and the retention time of flumetsulam was 7.5 min.

Full scan ESI mass spectra were collected for each analyte. The single quadrupole MS was operated in positive ion mode. Fragmentation voltages between the source capillary and the initial skimmer were controlled so that at least three ions were formed for each analyte. From the full scan spectra, the three ions were chosen for SIM analysis and the three ions, including the [M + H]$^+$ ion were monitored for quantitation. The ions monitored were m/z 129, 262 and 326.

The average recovery for flumetsulam was reported to be 73% with a RSD of 20%. However, in total for all the analytes, the recoveries ranged from 39% to 92% and the RSDs ranged from 14% to 26%. The LOD calculated as three times the SD of the lowest fortification level was 22 ng mL$^{-1}$. The details of method performance are summarised in Table 2.3, reference 9.

Voyksner et al (10) reported a method that employed turbulent flow chromatography integrated with ion trap mass spectrometry for the automated analysis of 13 herbicides in marsh water. A modified high throughput LC system with switching valves, C-18 SPE column, and a Metasil Basic C-8 column were assembled with computer automation. The method employed a H$_2$O:acetonitrile solvent system for extraction, clean up and elution from the Cohesive C-18 SPE cartridge. The author claimed that
this online automated system achieved resolution of the 13 herbicides in marsh water, but substantial detail was left out from the paper. The sample volume was 1.8 mL but it was not reported how the samples were treated or how they were introduced into the system and what the system conditions were.

Lake, pond and runoff water were fortified at two levels with the 13 herbicides. The [M + H]$^+$ ion for each compound was first isolated at maximum intensity with full scan ion trap MS. The reported average recovery for flumetsulam was 58% with a RSD of 6.7% with a range of 3.3% to 23%. The LOD calculated as three times the SD of the lowest fortification level was 0.023 ng mL$^{-1}$. For routine qualitative analysis, this method was able to cut preparation time, but in terms of quantitation, the results would not meet E.U. or other generally accepted guidelines. This may explain the lack of results, precision and accuracy information. The other disadvantage of this method is the use of specialised and costly equipment that is not readily available in most laboratories. The details of method performance are summarised in Table 2.3, reference 10.
Table 2.1 METHODS FOR THE DETERMINATION OF FLUMETSULAM IN AGRICULTURAL GRAINS

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Extraction solvent</th>
<th>Preconcentration and SPE clean up</th>
<th>Instrument &amp; Detector</th>
<th>Chromatographic column</th>
<th>Mobile phase</th>
<th>Detection limit(s)</th>
<th>Recoveries ± RSD</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean</td>
<td>Acidified acetone</td>
<td>C-18 and alumina SPE clean up</td>
<td>GC-MS</td>
<td>DB-17 capillary column</td>
<td>Helium</td>
<td>0.17 to 1.29 ng g(^{-1})</td>
<td>98% ± 5%</td>
<td>1</td>
</tr>
<tr>
<td>Corn</td>
<td>MeOH</td>
<td>C-18 &amp; Carbograph-1</td>
<td>LC-ESI-MS/MS</td>
<td>C-18</td>
<td>ACN: H(_2)O Gradient</td>
<td>8.9 to 17.2 ng g(^{-1})</td>
<td>85% ± 10%</td>
<td>2</td>
</tr>
</tbody>
</table>

\(^{1}\)Deuterated internal standard was used.
<table>
<thead>
<tr>
<th>Matrix</th>
<th>Extraction solvent</th>
<th>Preconcentration and SPE cleanup</th>
<th>Instrument &amp; Detector</th>
<th>Column</th>
<th>Mobile Phase</th>
<th>Detection limit(s)</th>
<th>Recovery ± RSD</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil</td>
<td>NaHCO$_3$ aqueous solution</td>
<td>C-18 cartridge and GPC</td>
<td>HPLC-UV</td>
<td>C-18</td>
<td>MeOH/aqueous 0.5% CH$_3$CO$_2$H</td>
<td>4 ng g$^{-1}$</td>
<td>67.5% ± 4.8%</td>
<td>3</td>
</tr>
<tr>
<td>Soil</td>
<td>Acidified (HCl) Acetone</td>
<td>Liquid-liquid$^1$ partition</td>
<td>GC-FID</td>
<td>C-18</td>
<td>ACN/ aqueous 0.1 M HCl</td>
<td>2.5 ng g$^{-1}$</td>
<td>90.5% ± 15.5%</td>
<td>4</td>
</tr>
<tr>
<td>Soil (pH 8)</td>
<td>KH$_2$PO$_4$ aqueous solution</td>
<td>Carbograph-1 cartridge</td>
<td>LC-ESI-MS/MS</td>
<td>C-18</td>
<td>ACN:H$_2$O</td>
<td>1.41 ng g$^{-1}$</td>
<td>73% ± 5%</td>
<td>5</td>
</tr>
<tr>
<td>Soil (pH 8)</td>
<td>NaHCO$_3$ aqueous solution</td>
<td>Liquid-liquid$^1$ partition &amp; TLC</td>
<td>GC-ECD &amp; GC-MS</td>
<td>Capillary column</td>
<td>Nitrogen</td>
<td>Not reported</td>
<td>Not reported</td>
<td>6</td>
</tr>
</tbody>
</table>

$^1$See text for details
### Table 2.3 METHODS FOR THE DETERMINATION OF FLUMETSULAM IN WATER

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Extraction solvent</th>
<th>Preconcentration and SPE clean up</th>
<th>Instrument &amp; Detector</th>
<th>Column</th>
<th>Mobile Phase</th>
<th>Detection limit(s)</th>
<th>Recovery ± RSD</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>N/A</td>
<td>C-18, SAX, alumina SPE cartridges</td>
<td>CZE-UV &amp; LC-MS</td>
<td>C-8</td>
<td>ACN/H₂O/0.15% aqueous HCO₂H</td>
<td>0.13 ng mL⁻¹ for CZE &amp; 0.1 ng mL⁻¹ for LC-MS</td>
<td>111% ± 11.9% for CZE &amp; 95% ± 16% for LC-MS</td>
<td>7</td>
</tr>
<tr>
<td>Water</td>
<td>N/A</td>
<td>Polystyrene divinylbenzene, SAX &amp; alumina SPE cartridges</td>
<td>LC-MS</td>
<td>C-8</td>
<td>ACN/H₂O/aqueous 0.15% HCO₂H</td>
<td>0.031 ng mL⁻¹</td>
<td>114% ± 9%</td>
<td>8</td>
</tr>
<tr>
<td>Water</td>
<td>N/A</td>
<td>SAX, styrene-divinyl benzene SPE cartridges</td>
<td>LC-MS</td>
<td>C-8</td>
<td>ACN:ammonium formate/ HCO₂H buffer</td>
<td>21.9 ng mL⁻¹</td>
<td>73% ± 20%</td>
<td>9</td>
</tr>
<tr>
<td>Water</td>
<td>N/A</td>
<td>C-18 SPE cartridge</td>
<td>LC-MS</td>
<td>C-8</td>
<td>ACN:HO₂</td>
<td>0.023 ng mL⁻¹</td>
<td>58% ± 6.7%</td>
<td>10</td>
</tr>
</tbody>
</table>
2.5 REFERENCES


CHAPTER 3

INITIAL METHOD DEVELOPMENT
3.1 **INTRODUCTION**

Before beginning method development, we need to review what is known about the sample and analyte. Ideally, a complete description of the sample and analyte is available, for example, soybean is a lipid containing matrix and flumetsulam is an acidic herbicide, soluble in H$_2$O at neutral pH. The goal of high performance liquid chromatography (HPLC) separation in this case is to determine flumetsulam content in soybean, so the primary interest is in the properties of flumetsulam that will affect its HPLC separation. The chemical composition of the sample can provide valuable clues for the best choice of initial conditions for a HPLC separation. Two somewhat different approaches to HPLC method development are possible. The first approach is to try to exploit the different characteristics of the analyte and the matrix. The analyte is a polar and acidic compound soluble in H$_2$O, and the matrix is somewhat a neutral lipid-like organic substance, chemically different to the analyte. To do this, the chromatographer relies heavily on past experience (separation of compounds of similar structure) and/or they supplement this information with data from the literature. The second approach is to proceed directly to an initial chromatographic separation. These two kinds of HPLC method development might be characterised as theoretical vs. empirical. Either a theoretical or an empirical approach to HPLC method development can be successful, and best strategy is often some blend of these two procedures. In this work, both approaches were tried. The first goal was to determine the chromatographic conditions required for the flumetsulam standard to show a retention time of 15-25 min and for the retention time to be reproducible and peak shape to be sharp and symmetrical using HPLC-UV. The wavelength of detection was obtained from literature and confirmed by running a UV spectrum of the standard. The traditional C-18 column was initially tried but very little retention of
the analyte was observed with a methanol:H$_2$O mobile phase. A C-8 column was then tried but it also gave a short retention time. The peak was, however, sharp and symmetrical. Different mobile phases were tried, including methanol or acetonitrile with H$_2$O or aqueous buffers at low pH (<3) with the aim of ionisation suppression. Figures 3.1 and 3.2 show some chromatograms showing the short retention times of the flumetsulam standard. The structure of the stationary phase is also presented on the chromatograms.

![Chromatogram of 0.5 µg mL$^{-1}$ flumetsulam standard on Phenomenex Luna C-18 column with a 50:50 (methanol:H$_2$O) mobile phase. λ detection = 213 nm](image)

Figure 3.1 Chromatogram of 0.5 µg mL$^{-1}$ flumetsulam standard on Phenomenex Luna C-18 column with a 50:50 (methanol:H$_2$O) mobile phase. λ detection = 213 nm

The C-18 and C-8 columns tried were the Luna by Phenomenex and the XTerra by Waters respectively. Various combinations and compositions of mobile phase were tried. An Agilent C-18 Zorbax column was also tried and initially gave good results.
with a retention time of 20 min. However, after 20-30 injections, the retention time started to shift to a shorter time and the column was unable to be restored.

A 100% H₂O mobile phase was also tried. Although the retention of the analyte did increase to 13 min, the peak was broad and asymmetrical. A fronting peak was observed – this peak profile occurs when there is column overload. One possible explanation for the fronting peak profile is that the C-18 ligands on the HPLC column may have collapsed, and therefore surface area decreased leading to column overload. A schematic diagram in Figure 3.3 is presented highlighting the column overload along with the chromatogram obtained.

Figure 3.2 Chromatogram of 0.5 µg mL⁻¹ flumetsulam standard on Waters C-8 column with a 50:50 (methanol:H₂O) mobile phase. λ detection = 213 nm
After 10 column volumes of 100% aqueous mobile phase

**Figure 3.3 Chromatogram of 0.5 µg mL⁻¹ flumetsulam standard on Phenomenex Luna C-18 column with 100% H₂O mobile phase. λ detection = 213 nm**

It was then decided to try a different type of column from the C-18 stationary phase. Flumetsulam is acidic (pKa = 4.6) and polar in nature. Therefore, if the mobile phase is neutral or basic, flumetsulam becomes negatively charged. In this case, if an ion exchange column was used, reasonable retention should be observed between the analyte and the positively charged stationary phase. A strong anion exchange (SAX) column by Supelco was then tried. The mobile phase used was 20:80 (methanol:50 mM aqueous pH 8 phosphate buffer) premixed before used. An example of a chromatogram obtained is presented in Figure 3.4 (A).
Figure 3.4 Chromatogram of 0.5 µg mL⁻¹ flumetsulam on SAX column with mobile phase 20:80 (methanol:aqueous pH 8 phosphate buffer) (A) 1-10 injection (B) 10-20 injections (C) after > 20 injections. λ detection = 213 nm

As presented in Figure 3.4 (A), a sharp and symmetrical peak was obtained with good retention time. This 23 min retention time is desirable because most of the co-extractives elute in the 5 to 15 min region. This column worked well initially but after 20-30 injections, the peak shape and retention time started to shift and split peaks appeared. The column was then put through the manufacturers clean up procedure to restore the column but without success. Another column was then purchased from Supelco but after multiple injections the same problems started to develop as presented in Figures 3.4 (B) and (C).
There is very little literature available on the performance of SAX columns. The split peaks may arise if some of the quaternary ammonium groups were to cleave to expose silica or silica hydroxide groups would act as a secondary stationary phase as presented in Figure 3.5. The (acidic) flumetsulam may cause this cleavage.

![Figure 3.5 Proposed theory for SAX column degradation](image)

The decision was made to pursue the use of a C-18 column and to use an acidic eluent, which would suppress ionisation of flumetsulam. Research was conducted into finding a suitable column that can tolerate a pH of ≤ 2. The Synergi Fusion RP C-18 column by Phenomenex was tried because it has a wide pH range (1-11) and also has non-nitrogen polar embedded groups on the silica backbone. A phosphate buffer (50 mM) was tried at pH 2.15 (first pKa of phosphate) with acetonitrile (68:32). This combination of mobile phase and column gave good results. Two pH units below the pKa of flumetsulam (pKa = 4.6) provided 99% ionisation suppression. A retention time of 25 min was obtained and the peak was sharp and symmetrical as presented in Figure 3.6. The retention time and peak shape profile was reproducible after many injections. This mobile phase used on the Agilent Zorbax and Phenomenex C-18
columns resulted in column damage after multiple runs but did not damage the Synergi Fusion C-18 column.

- Sharp and symmetrical
- Reproducible retention and peak shape
- Good baseline noise

Figure 3.6 Chromatogram of 0.5 µg mL\(^{-1}\) standard on the Phenomenex Synergi Fusion C-18 column with a pH 2.15 68:32 (buffer:ACN) mobile phase, \(\lambda\) detection = 225 nm
3.2 Sample Clean Up Procedure

In developing the extraction and clean up procedure, the first test was to quantitatively extract the analyte from the soybean matrix after fortification. A high concentration of analyte was fortified into the blank soybean and several extraction methods and solvents were tried. Methanol gave the best extraction efficiency. The critical control point in this step was to add fresh methanol to the fortified soybean and to place the mixture in an incubator shaker set at 60 °C and 250 rpm. Different temperatures were tried but 60 °C was found to give the highest recoveries.

It was also found that after extraction with methanol, the extract required filtering through a 0.45 µm membrane filter. If this step were to be left out, precipitation of a white (possibly a protein) substance causes an increase in the extraneous peaks observed in the blank and sample chromatograms.

During the liquid-liquid partitioning, emulsions formed between the two layers in the separating funnel. Due to the high lipid (16%) and protein (32%) content in soybean, emulsions were common. This was overcome by two strategies. The first was performing a methanol and hexane partition to remove some of the lipids. The second was the use of saturated NaCl to increase the ionic strength and density of the aqueous phase. The use of a neutral phosphate buffer rather than a basic solution (NaOH) to extract flumetsulam reduced emulsion formation. Using a base (to ionise the analyte) resulted in saponification of the lipids. Once an emulsion forms, it is very difficult to breakup, even by heating. Low recoveries were also observed during the initial stages.
of the method development, due to emulsions causing inconsistency in the solvent partitioning steps.

After the liquid-liquid extraction method was developed and validated, some time was given to developing a solid phase extraction (SPE) method. The SPE method uses less solvent and is easier to automate. This technique is fairly inexpensive and does not require the use of specialised equipment. A vacuum manifold SPE tank is most convenient.

Three different cartridges were used in the SPE method and all provided different advantages. The C-18 SPE was used to clean up the lipids as well as most of the hydrophobic co-extractives found in the soybean matrix. After trying several C-18 cartridges by different suppliers, it was found that the Supelco cartridges gave the best recoveries and precision. It also provided a cleaner extract. Alltech SPE cartridges gave non-reproducible results. Phenomenex cartridges showed poor consistency because some cartridges would drip fast and some would not drip unless pressure was applied. The Supelco cartridges did not show any of these faults.

Due to global regulatory agencies such as European Commission stating in their Council Directive 2002/657/EC (1) that more than one chromatographic system is needed for an LC method, a LC-electrospray ionisation-mass spectrometry/mass spectrometry (ESI-MS/MS) procedure was also developed for the confirmation of flumetsulam in soybean.
The LC-ESI-MS/MS method offers the advantage of rapid analysis and identity confirmation. This confirmation method provides information on the chemical structure of the analyte. The use of selected ion monitoring gave sharp peaks with minimal matrix interferences. The tandem quadrupole gave specific identification of the analyte by performing product ion scan. The initial challenge was to establish a mobile phase suitable for MS (no non-volatile salts typically used in buffers). After direct infusion into the MS, the optimum voltages were determined for highest sensitivity.
3.3 Reference

CHAPTER 4

FLUMETSULAM ANALYSIS USING

SOLVENT PARTITIONING CLEAN UP
4.1 **INTRODUCTION AND PRINCIPLE OF EXTRACTION METHOD**

Solvent-solvent extraction is a classic method of extraction, which can be carried out without specialised equipment. The main disadvantage is the labour intensive nature of the process and the generation of more solvent waste than with solid phase extraction.

In this work the optimum extraction solvent for flumetsulam and diclosulam was found to be methanol with 5% acetic acid. The solvent is sufficiently polar to extract the analyte and surrogate standard (s-STD) while minimising non-polar co-extractives, especially lipids. The 5% acetic acid suppresses the ionisation of flumetsulam and diclosulam so that they can be extracted into an organic phase.

Having the analyte, s-STD and internal standard (i-STD) as acids means that they should all behave similarly during the extraction and partitioning process. After extraction with acidic methanol, the volume is reduced and partitioned with hexane saturated with methanol to remove the non-polar co-extractives from the methanol extract. Flumetsulam and diclosulam show little solubility in hexane. The hexane layer is discarded and the methanol layer reduced to dryness under reduced pressure using a rotary evaporator. The residue is then dissolved in pH 7.0 aqueous phosphate buffer (50 mM) making the analyte and s-STD negatively charged. The aqueous phase is then partitioned with dichloromethane and the dichloromethane discarded. The dichloromethane extracts moderately polar co-extractives found in the extract. The aqueous phase is then made acidic, converting the analyte and s-STD to their uncharged form to be extracted with dichloromethane. The dichloromethane is then partitioned with pH 7.0 buffer (50 mM), which extracts the analyte and s-STD in their
negatively charged form. The aqueous phase is made acidic again and the analyte and s-STD are partitioned into fresh dichloromethane and evaporated to dryness. The residue is then dissolved in acetonitrile and partitioned with hexane to remove more of the non-polar co-extractives. The acetonitrile is evaporated to dryness to be made up with mobile phase for HPLC analysis.

4.2 APPARATUS

(a) Eyela rotary vacuum evaporator. – HD Scientific (NSW, Australia).

(b) Digital pH meter with a Ionode pH combination electrode (model PBFC). – TPS Pty Ltd (Queensland, Australia).

(c) Graphite furnace. – Carbolite (Sheffield, England).

(d) Innova 4000 incubator shaker. – John Morris Scientific (NSW, Australia).

(e) Filter paper. – 15 cm diameter Whatman from Interpath Services (NSW, Australia).

(f) Nylon filters. – 0.45 µm porosity, 47 mm diameter-Millipore (NSW, Australia).

(g) Vortex mixer. – Lab Line Instruments (ILL, USA).

(h) Volumetric pipettes. – 1, 5 and 10 mL Duran from Selby Biolab (NSW, Australia).

(i) Volumetric syringes. – 50, 100 and 500 µL Hamilton Co. from Alltech Associates (NSW, Australia).

(j) Volumetric flasks. – 100 mL Schott Garsco (NSW, Australia).

(k) Separatory funnels. – 100 and 250 mL Schott Garsco (NSW, Australia)

(l) Round bottomed flasks for rotary vacuum evaporator. – 100, 250 and 500 mL Supelco (NSW, Australia).
(m) Glass vials. – 8 mL Schott Garsco (NSW, Australia).

(n) Schott bottles. – 500 mL Schott Garsco (NSW, Australia).

(o) Blender/food processor. – Breville Kitchen Wizz. Model number FP20 was purchased from Big W (NSW, Australia).

(p) Buchner funnel and flask. – Pyrex Sigma-Aldrich (NSW, Australia).

4.3 MATERIALS AND REAGENTS

Reagents for HPLC analysis were HPLC grade; other chemicals were of analytical reagent (AR) grade or the highest purity available. Purified water (H$_2$O) was obtained from a Millipore ultra pure Milli-Q water purification system (NSW, Australia).

(a) Reference standards. – Flumetsulam, standards grade (99% ± 0.5% purity) and diclosulam, standards grade (98% ± 0.5% purity) were purchased from Alltech Associates Pty. Limited (NSW, Australia). Identity and purity of the standards were checked by NMR (Appendix I for flumetsulam and Appendix II for diclosulam), IR (Appendix III for flumetsulam) and UV (Appendix IV for flumetsulam) spectroscopy. The certificates of analysis (Appendix V for flumetsulam and Appendix VI for diclosulam) and MSDS data (Appendix VII for flumetsulam and Appendix VIII for diclosulam) are also attached.

(b) Benzoic acid. – (98% purity) by Fluka Chemika was purchased from Sigma-Aldrich (NSW, Australia).

(c) Solvents HPLC grade. – Methanol, acetonitrile, hexane, methyl tert-butyl ether, ethyl acetate dichloromethane were purchased from Lomb Scientific (NSW, Australia).

(d) Orthophosphoric acid. – Selby Biolab (NSW, Australia).
(e) Dibasic sodium hydrogen orthophosphate anhydrous. – Ajax Chemicals (NSW, Australia).

(f) Monosodium dihydrogen orthophosphate monohydrate. – Merck Pty. Ltd. (VIC, Australia).

(g) AR grade solid inorganic materials. – NaCl, NaOH pellets and HCl were purchased from APS Finechem (NSW, Australia).

(h) Solvents AR grade. – Acetone and acetic acid were purchased from APS Finechem (NSW, Australia).

(i) Whole soybean. – McKenzie’s soybean was purchased from Woolworths Supermarket (NSW, Australia). The specifications for the soybean are given in Appendix IX.

(j) Gas. – Nitrogen, high purity, from BOC Gases (NSW, Australia).

(k) Dimethyl-dichlorosilane (DMDCS). – Sigma-Aldrich (NSW, Australia).

### 4.4 Preparation of Extraction and Partitioning Solvents and Chromatographic Reagents

All pH adjustments were performed with a pH meter.

(a) 0.05 M pH 7.0 phosphate buffer. – A 0.05 M phosphate buffer solution, pH 7.0 was prepared. 7.1 g anhydrous dibasic sodium hydrogen orthophosphate was dissolved in 500 mL H₂O, its pH was adjusted to 7.0 with concentrated orthophosphoric acid, and the solution made up to 1000 mL with H₂O.

(b) 0.05 M pH 2.2 phosphate buffer. – A 0.05 M phosphate buffer solution, pH 2.2 was prepared. 6.9 g monosodium dihydrogen orthophosphate monohydrate was dissolved in 500 mL H₂O, its pH was adjusted to 2.2 with concentrated orthophosphoric acid, and the solution made up to 1000 mL with H₂O.
(c) Extraction solvent. – Methanol:acetic acid (95:5). To 950 mL methanol was added 50 mL glacial acetic acid to give a resultant volume ratio of 95:5, % v/v.

(d) Partitioning solvents

1. hexane:methyl tert-butyl ether (90:10). 900 mL hexane was mixed with 100 mL methyl tert-butyl ether.

2. hexane:ethyl acetate (95:5). 950 mL hexane was mixed with 50 mL ethyl acetate.

3. Dichloromethane:methyl tert-butyl ether (70:30). 700 mL dichloromethane was mixed with 300 mL methyl tert-butyl ether.

(e) Acetonitrile saturated with hexane. – 500 mL acetonitrile and 100 mL hexane were added to a separatory funnel and shaken before collecting the bottom acetonitrile layer.

(f) Saturated NaCl. – 500 g NaCl was made up to 1 L with H₂O. The solution was decanted when required.

(g) HPLC mobile phase. – 2720 mL 0.05 M pH 2.2 phosphate buffer solution and 1280 mL acetonitrile (68 + 32, v/v) was combined in a 4 L glass vessel. The mobile phase was then filtered and degassed with vacuum through a 0.45 μm Nylon filter using an all glass HPLC solvent filtration unit.

4.5 Preparation of Glassware

After every use the glassware was washed with detergent and H₂O followed by rinsing with H₂O and acetone. After washing, the glassware was decontaminated by heating in an oven at 450 °C for at least 4 h. The heating is to remove contaminants from the glassware that are adsorbed during the extraction procedure, which is not possible to clean by washing alone.
Round bottomed flasks, volumetric flasks, volumetric pipettes and glass vials used were silanised to deactivate –OH or silanol groups, which can adsorb analyte and impurities, giving poor precision. The glassware was deactivated by rinsing with acetone and heating in an oven at 180 °C for at least 1 h. The cooled glassware was placed in a 5% solution of DMDCS in toluene and allowed to soak for 10 min before rinsing with toluene. After rinsing, it was soaked in methanol for 10 min and allowed to dry before use.

### 4.6 HPLC INSTRUMENTATION

(a) HPLC system. – Varian 9012 solvent delivery system (NSW, Australia) equipped with a Rheodyne injector port (USA), a Rheodyne 100 μL injector loop and a Varian 9050 variable wavelength UV-Vis detector were from Varian Australia Pty. Ltd. (NSW, Australia).

(b) Phenomenex Synergi Fusion. – RP C18 column 250 x 4.6 mm id, 5 μm particle size was from Phenomenex (NSW, Australia).

(c) HPLC column temperature controller. – from ICI Instruments (VIC, Australia) set at 30 °C.

(d) HPLC syringes. – 50, 100 and 200 μL by Hamilton Co. from Alltech Associates (NSW, Australia).

(e) Hewlett Packard laser printer controlled by a Windows based software installed on an IBM personal computer. – from Computer world (NSW, Australia).

(f) Data acquisition system. – Data Acquisition was via a Data Acquisition and Plotting Analysis (DAPA) system from DAPA scientific Pty. Ltd. (WA, Australia).
4.7 **HPLC CONDITIONS**

The Varian HPLC and C-18 column used are as described in section 4.6 (a)-(c). The column temperature was set at 30 °C. A 100 μL sample loop was used with UV detection at 225 nm. The HPLC mobile phase was 0.05 M pH 2.2 phosphate buffer solution and acetonitrile (68 + 32, v/v). The flow rate was 0.5 mL min\(^{-1}\) with a linear composition of 68:32 for 20 min, followed by a gradient increase of acetonitrile from 32% to 50% in 5 min and from 50% to 70% in 10 min and maintained at this composition for 3 min.

4.8 **PREPARATION OF ANALYTICAL STANDARD SOLUTIONS**

4.8.1 **PREPARATION OF STANDARD STOCK SOLUTIONS**

Individual stock solutions of 1000 μg mL\(^{-1}\) flumetsulam, diclosulam (surrogate standard, s-STD) and benzoic acid (internal standard, i-STD) were prepared by accurately weighing 10 mg of the respective standards into separate 10 mL volumetric flasks and making up to the mark with acetonitrile. Where applicable, the amount of analyte was adjusted according to the stated purity of the standard material. The stock solutions were used for preparing the fortification and calibration solutions. These stock solutions were stored in a refrigerator at -4 °C and prepared freshly every month.
4.8.2 PREPARATION OF FORTIFICATION STANDARD SOLUTIONS

A 10 µg mL⁻¹ flumetsulam standard solution was prepared by diluting 1 mL of the 1000 µg mL⁻¹ stock solution to 100 mL with acetonitrile. A 1 µg mL⁻¹ flumetsulam standard solution was prepared by diluting 10 mL of the 10 µg mL⁻¹ solution to 100 mL with acetonitrile.

Individual standards of 100 µg mL⁻¹ of diclosulam and benzoic acid standard solutions was prepared by diluting 10 mL of each of the 1000 µg mL⁻¹ stock solutions to 100 mL with acetonitrile. 5 mL of the 100 µg mL⁻¹ diclosulam and benzoic acid solutions were diluted to 100 mL with acetonitrile to give 5 µg mL⁻¹ of each standard solution. 100 and 200 µL of the 1 µg mL⁻¹ flumetsulam standard were used for fortifying 10 g of ground soybean to give 0.01 and 0.02 µg g⁻¹ fortification levels of flumetsulam respectively. 50 and 100 µL of the 10 µg mL⁻¹ flumetsulam standard were used for fortifying 10 g of ground soybean to give 0.05 and 0.1 µg g⁻¹ fortification levels respectively. 100 µL of 5 µg mL⁻¹ diclosulam standard was also added to the soybean to give 0.05 µg mL⁻¹ of the s-STD diclosulam in the final solution for the flumetsulam analysis.

These standards were stored in a refrigerator and prepared freshly every week.

4.8.3 PREPARATION OF CALIBRATION STANDARD SOLUTIONS

Individual standard solutions containing 0.005, 0.01, 0.02, 0.05, 0.1, 0.5 and 1 µg mL⁻¹ of flumetsulam and 1 µg mL⁻¹ diclosulam were prepared by transferring 0.05, 0.1, 0.2, 0.5, 1, 5 and 10 mL of the 10 µg mL⁻¹ flumetsulam respectively and 1 mL of the
100 µg mL\(^{-1}\) diclosulam into seven 100 mL volumetric flasks and making up to volume with the LC mobile phase.

For each flumetsulam standard, the s-STD diclosulam and i-STD benzoic acid were added to give a final concentration of 1 µg mL\(^{-1}\) each.

These calibration standards were stored in a refrigerator (-4 °C) and prepared freshly every week.

### 4.9 Sample Preparation

500 g of dry soybean seeds was homogenised to a fine powder using an electric blender. The sample was allowed to cool slightly after 3 to 5 min of blending to prevent the sample from heating more than 5 °C above ambient temperature. The soybean was passed through a 500 µm sieve. The larger particles were reground till it also passed through the sieve. The sample was shaken and mixed thoroughly before being stored in a screw top polypropylene container.

### 4.10 Choice of Internal Standard (i-STD) and Surrogate Standard (s-STD)

For flumetsulam extraction, the s-STD, diclosulam, was included in the fortification solution. The fortification level was 1 µg ml\(^{-1}\), which gave a peak height comparable to the 100% fortification level of flumetsulam.

The i-STD benzoic acid was also fortified at 1 µg mL\(^{-1}\) to give a peak height comparable to the 100% fortification level of flumetsulam.
Chapter 4: Solvent partitioning cleanup

4.11 FORTIFICATION, EXTRACTION AND FRACTIONATION OF FLUMETSULAM

4.11.1 FORTIFICATION OF SOYBEAN

Five portions of 10 g of ground soybean were accurately weighed into separate 500 mL Shott bottles and fortified with flumetsulam and diclosulam to give the desired fortification concentrations. Flumetsulam fortification levels of 0, 0.01, 0.02, 0.05 and 0.1 µg g⁻¹ were obtained by adding 0, 100, and 200 µL of the 1 µg mL⁻¹ flumetsulam standard and 50 and 100 µL of the 10 µg mL⁻¹ flumetsulam standard. Finally 100 µL of the 5 µg mL⁻¹ diclosulam standard was added to each sample. Seven replicates for each fortification level were performed to give a total of 35 samples. After fortification, at least 10 min was allowed for the solvent to evaporate.

4.11.2 METHANOL EXTRACTION

To each sample was added 100 mL acidified methanol and the bottle capped and placed on a shaker set at 250 rpm and 60 °C for 15 min. The mixture was allowed to rest for at least 15 min before decanting the methanol supernatant into a clean 250 mL conical flask. The soybean was re-extracted with another 100 mL methanol, and the combined methanol extracts vacuum filtered through a 0.45 µm Nylon filter. The combined filtrate was placed in a 250 mL round bottomed flask and reduced to approximately 20 mL using a rotary vacuum evaporator set at 60 °C and transferred to a 50 mL separatory funnel. The methanol concentrate was extracted with 3 x 20 mL hexane:methyl tert-butyl ether (90:10) and the hexane:methyl tert-butyl ether discarded. The methanol was evaporated to dryness and made up with 20 mL hexane saturated acetonitrile before extraction with 1 x 20 mL hexane:ethyl acetate (95:5). The hexane:ethyl acetate was discarded. Any emulsion formed was broken by stirring.
with a glass rod. The acetonitrile was transferred to a 100 mL round bottomed flask and evaporated to dryness on a rotary vacuum evaporator set at 60 °C.

The extraction procedure is summarised as a flow chart in Figure 4.1.

![Flowchart of extraction of flumetsulam from soybean](image)

**Figure 4.1 Flowchart of extraction of flumetsulam from soybean**

### 4.11.3 Fractionation of extract

The residue from section 4.11.2, (extraction procedure) was dissolved in 15 mL pH 7.0 phosphate buffer, and transferred to a 100 mL separatory funnel. The procedure was repeated with a fresh 15 mL of the buffer to ensure all the residue was
transferred. 10 mL of saturated NaCl was added to the separatory funnel and partitioned with 3 x 40 mL dichloromethane and the dichloromethane discarded. The aqueous layer was then acidified with orthophosphoric acid to pH 2.2 and extracted with 3 x 50 mL dichloromethane. The aqueous layer was discarded and the combined dichloromethane reduced to 20 mL on a rotary vacuum evaporator set at 60 °C. The dichloromethane concentrate was partitioned with 3 x 20 mL pH 7.0 phosphate buffer and the layers were allowed to separate clearly before discarding the dichloromethane layer. The aqueous layer was acidified to pH 2.2 with orthophosphoric acid and extracted with 3 x 60 mL dichloromethane. The aqueous layer was discarded and the combined dichloromethane extracts washed with 100 mL H2O. The dichloromethane layer was then dried with anhydrous Na2SO4 and gravity filtered to remove the drying agent. The dichloromethane extract was then transferred to a 250 mL round bottomed flask and evaporated to dryness on a rotary vacuum evaporator set at 60 °C. The residue was dissolved in 2 mL fresh dichloromethane and transferred to a 8 mL silanised glass vial. The procedure was repeated with two more volumes of 2 mL dichloromethane and added to the glass vial before evaporating the solvent to dryness under a stream of nitrogen. 50 µL of 10 µg mL⁻¹ i-STD benzoic acid in acetonitrile was added to give a final concentration of 1 µg mL⁻¹. The acetonitrile was evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved in 500 µL of mobile phase using a vortex mixer and filtered through a 0.45 µm Nylon syringe filter for LC analysis. The mobile phase was run through isocratically at 0.5 mL min⁻¹ with the detector set at 225 nm. 100 µL was injected for analysis.
4.11.4 C-18 CLEAN UP PROCEDURE

If additional clean up is required as evidence from the blank chromatogram, an additional SPE clean up step may be incorporated. A Supelco Discovery C-18 cartridge was conditioned with 2 mL methanol, 2 mL H₂O, 2 mL methanol, and finally 2 mL dichloromethane:hexane (50:50). The dichloromethane residue from section 4.11.3, before the addition of the 50 µL of 10 µg mL⁻¹ i-STD benzoic acid, was dissolved in 5 mL dichloromethane:hexane (50:50) and transferred to the preconditioned cartridge. The cartridge was washed with 2 x 2 mL hexane and 2 x 2 mL ethyl acetate:hexane (10:90). The analyte was eluted with 2 x 2 mL dichloromethane:methyl tert-butyl ether (70:30) and collected in a 8 mL sample vial. 50 µL of 10 µg mL⁻¹ i-STD benzoic acid in acetonitrile was added to give a final concentration of 1 µg mL⁻¹. This eluate was evaporated to dryness under a gentle stream of nitrogen and the residue dissolved in 500 µL of mobile phase. 100 µL was injected for analysis.

A flow chart summarising the fractionation and analysis of flumetsulam and the s-STD diclosulam is summarised in Figure 4.2 and described in section 4.11.3.
Chapter 4: Solvent partitioning cleanup

Add pH 7 buffer to the residue containing flumetsulam & s-STD

Add sat. NaCl and partition with DCM

Discard DCM phase

Acidify aqueous phase and add sat. NaCl & partition with DCM

Discard aqueous phase

Reduce DCM volume and partition with pH 7.0 buffer

Discard aqueous phase

Discard DCM phase

Acidify aqueous phase & partition with DCM

Discard aqueous phase

Wash DCM layer with H₂O and discard H₂O

Dry DCM layer with Na₂SO₄

Discard Na₂SO₄

Evaporate DCM layer to dryness

Dissolve residue in DCM and transfer to 8 mL sample vial

If necessary load onto C-18 cartridge and elute with DCM

Add benzoic acid (i-STD) to dissolved residue or eluate, evaporate to dryness and dissolve in mobile phase for LC analysis

Figure 4.2 Flow chart of fractionation of flumetsulam from soybean
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4.12 RESULTS AND DISCUSSION

The analytical method developed for the determination of flumetsulam using solvent-solvent extraction with LC-UV analysis was validated using the parameters described in Chapter 1.

Seven replicate samples of 10 g soybean were fortified with flumetsulam to give concentrations of 0, 0.01, 0.02, 0.05 and 0.1 µg g\(^{-1}\).

4.12.1 RECOVERY STUDIES FOR FLUMETSULAM

A calibration curve was constructed from 0.005, 0.01, 0.02, 0.05, 0.1, 0.5 and 1 µg g\(^{-1}\) flumetsulam in LC mobile phase. Five replicate injections of each concentration were performed. The final concentration of the s-STD and i-STD were 1 µg mL\(^{-1}\) each prior to LC analysis.

Four types of recoveries were calculated, namely recovery calculated based on:
- the analyte only
- the analyte with s-STD
- the analyte with i-STD
- the analyte with s-STD and i-STD
4.12.1.1 Recoveries of flumetsulam without s-STD and i-STD

4.12.1.1.1 Calibration curve

Table 4.1 presents the mean peak areas for flumetsulam for each of the seven standards. The flumetsulam calibration curve is a plot of flumetsulam concentration versus the mean peak area of flumetsulam.

<table>
<thead>
<tr>
<th>Flumetsulam (µg mL⁻¹)</th>
<th>Mean peak area x 10⁴</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.005</td>
<td>0.93</td>
<td>7.9</td>
</tr>
<tr>
<td>0.01</td>
<td>2.09</td>
<td>6.8</td>
</tr>
<tr>
<td>0.02</td>
<td>5.50</td>
<td>5.2</td>
</tr>
<tr>
<td>0.05</td>
<td>17.15</td>
<td>3.6</td>
</tr>
<tr>
<td>0.1</td>
<td>50.88</td>
<td>2.1</td>
</tr>
<tr>
<td>0.5</td>
<td>204.74</td>
<td>1.3</td>
</tr>
<tr>
<td>1</td>
<td>431.65</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Calculated from 5 replicate injections.

The flumetsulam standard calibration curves (Table 4.2) are linear from 0.005 to 1 µg mL⁻¹ with a mean coefficient of determination (R²) of 0.9992. The linear regression equation is y = 429.77x - 1.6032 for the fortification level 0.01 µg g⁻¹. This calibration curve is presented in Figure 4.3.
4.12.1.1.2 Recoveries for flumetsulam

The concentrations of flumetsulam in fortified soybean were calculated from the mean peak area of seven replicate extractions and analyses at each fortification level of 0.01, 0.02, 0.05 and 0.1 µg g\(^{-1}\). The equation of the calibration curve for the 0.01 µg g\(^{-1}\) fortification level is given in Table 4.2 along with the equations obtained for the 0.02, 0.05 and 0.1 µg g\(^{-1}\) fortification levels. The R\(^2\) were all > 0.998.

The equations obtained are summarised in Table 4.2.
Chapter 4: Solvent partitioning cleanup

### Table 4.2 Equations used to determine the recoveries for the four fortification levels

<table>
<thead>
<tr>
<th>Fortification level (µg g⁻¹)</th>
<th>Equation¹ (y = mx + b)</th>
<th>Coefficient of determination (R²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>y = 429.77x - 1.6032</td>
<td>0.9988</td>
</tr>
<tr>
<td>0.02</td>
<td>y = 385.15x - 0.8830</td>
<td>1.0</td>
</tr>
<tr>
<td>0.05</td>
<td>y = 378.57x - 3.5944</td>
<td>0.9981</td>
</tr>
<tr>
<td>0.1</td>
<td>y = 400.91x - 2.0903</td>
<td>0.9999</td>
</tr>
</tbody>
</table>

¹Without using i-STD and s-STD.

The ‘m’ is the slope (429.77) of the line and b (-1.6032) refers to the y-axis intercept.

The recoveries calculated from the equations are presented in Table 4.3.

### Table 4.3 Recoveries for flumetsulam determined without the i-STD¹ and s-STD²

<table>
<thead>
<tr>
<th>Fortification level (µg g⁻¹)</th>
<th>% Recovery³</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>71.8</td>
<td>22.1</td>
</tr>
<tr>
<td>0.02</td>
<td>78.3</td>
<td>19.7</td>
</tr>
<tr>
<td>0.05</td>
<td>78.2</td>
<td>14.7</td>
</tr>
<tr>
<td>0.1</td>
<td>91.7</td>
<td>8.0</td>
</tr>
</tbody>
</table>

¹i-STD: internal standard, benzoic acid.
²s-STD: surrogate standard, diclosulam.
³Calculated from 7 replicate fortified samples.

The results show that the method has good and reproducible recoveries for flumetsulam. The recoveries are between 71.8% and 91.7% (mean = 80%) with a RSD of between 8% and 22.1% (mean = 16.1%).
4.12.1.2 Recoveries for flumetsulam determined using the i-STD

4.12.1.2.1 Calibration curve

In this section, the flumetsulam concentration is plotted against the ratio of [flumetsulam:i-STD].

Table 4.4 presents the results of the mean peak area ratio of flumetsulam to the i-STD benzoic acid for each flumetsulam concentration. The flumetsulam calibration curve is a plot of flumetsulam concentration versus the ratio of the mean peak area of flumetsulam to the i-STD benzoic acid.

<table>
<thead>
<tr>
<th>Flumetsulam (µg mL(^{-1}))</th>
<th>Mean peak area ratio(^2) [Flumetsulam:i-STD]</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.005</td>
<td>0.04</td>
<td>6.4</td>
</tr>
<tr>
<td>0.01</td>
<td>0.07</td>
<td>4.9</td>
</tr>
<tr>
<td>0.02</td>
<td>0.15</td>
<td>3.3</td>
</tr>
<tr>
<td>0.05</td>
<td>0.37</td>
<td>1.8</td>
</tr>
<tr>
<td>0.1</td>
<td>0.74</td>
<td>1.2</td>
</tr>
<tr>
<td>0.5</td>
<td>3.21</td>
<td>0.9</td>
</tr>
<tr>
<td>1</td>
<td>6.57</td>
<td>0.7</td>
</tr>
</tbody>
</table>

\(^1\)i-STD: internal standard, benzoic acid.
\(^2\)Calculated from 5 replicate injections.

The flumetsulam standard calibration curves are linear from 0.005 to 1 µg mL\(^{-1}\) with a mean \(R^2\) of 0.9995. The linear regression equation is \(y = 7.826x – 0.0342\) for the lowest fortification level. The calibration curve is presented in Figure 4.4.
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Figure 4.4 Flumetsulam standard calibration curve determined with the i-STD

Note: Peak area ratio of [flumetsulam:i-STD]

4.12.1.2.2 Recoveries for flumetsulam

The concentrations of flumetsulam in soybean were calculated from the mean peak area ratios of seven replicate extractions and analyses each at fortification levels of 0.01, 0.02, 0.05 and 0.1 µg g⁻¹. The equation of the calibration curve for the 0.01 µg g⁻¹ fortification level is given in Table 4.5 along with equations obtained for the 0.02, 0.05 and 0.1 µg g⁻¹ fortification levels. The $R^2$ are all > 0.998.

The equations used are presented in Table 4.5.
Chapter 4: Solvent partitioning cleanup

Table 4.5  Equations used to determine the recoveries for the four fortification levels

<table>
<thead>
<tr>
<th>Fortification level (µg g⁻¹)</th>
<th>Equation¹ (y = mx + b)</th>
<th>Coefficient of determination (R²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>y = 7.826x + 0.0342</td>
<td>0.9995</td>
</tr>
<tr>
<td>0.02</td>
<td>y = 5.122x + 0.0106</td>
<td>0.9999</td>
</tr>
<tr>
<td>0.05</td>
<td>y = 10.044x + 0.0807</td>
<td>0.9987</td>
</tr>
<tr>
<td>0.1</td>
<td>y = 6.516x + 0.026</td>
<td>0.9997</td>
</tr>
</tbody>
</table>

¹Without using s-STD.

The recoveries calculated from the equations are presented in Table 4.6.

Table 4.6 Recoveries for flumetsulam determined with the i-STD¹

<table>
<thead>
<tr>
<th>Fortification level (µg g⁻¹)</th>
<th>% Recovery²</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>70.1</td>
<td>20.6</td>
</tr>
<tr>
<td>0.02</td>
<td>73.1</td>
<td>16.2</td>
</tr>
<tr>
<td>0.05</td>
<td>83.6</td>
<td>12.3</td>
</tr>
<tr>
<td>0.1</td>
<td>85.8</td>
<td>9.7</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td><strong>78.2</strong></td>
<td><strong>14.7</strong></td>
</tr>
</tbody>
</table>

¹i-STD: internal standard, benzoic acid.
²Calculated from 7 replicate fortified samples.

The results show that the method has good and reproducible recoveries of flumetsulam. The recoveries were between 70.1% and 85.8% (mean = 78.2%) with % RSDs of between 9.7% and 20.6% (mean = 14.7%). The results are largely comparable to those determined without the i-STD but the % RSDs are slightly improved. This observation is in harmony with other studies, which observe an improvement in precision when an i-STD is incorporated in the method.
4.12.1.3 Recoveries for flumetsulam determined using the s-STD

4.12.1.3.1 Calibration curve

In this section, the flumetsulam concentration is determined using the area ratio of [flumetsulam:s-STD] versus flumetsulam concentration.

Table 4.7 presents the results of the mean peak area ratio of flumetsulam to the s-STD diclosulam for each flumetsulam concentration. The flumetsulam calibration curve is a plot of flumetsulam concentration versus the mean peak area ratio of flumetsulam to the s-STD diclosulam.

<table>
<thead>
<tr>
<th>Flumetsulam (µg mL⁻¹)</th>
<th>Mean peak area ratio(^2) [Flumetsulam:s-STD]</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.005</td>
<td>0.015</td>
<td>6.0</td>
</tr>
<tr>
<td>0.01</td>
<td>0.029</td>
<td>5.3</td>
</tr>
<tr>
<td>0.02</td>
<td>0.056</td>
<td>4.3</td>
</tr>
<tr>
<td>0.05</td>
<td>0.154</td>
<td>2.7</td>
</tr>
<tr>
<td>0.1</td>
<td>0.287</td>
<td>1.1</td>
</tr>
<tr>
<td>0.5</td>
<td>1.206</td>
<td>0.5</td>
</tr>
<tr>
<td>1</td>
<td>2.299</td>
<td>0.8</td>
</tr>
</tbody>
</table>

\(^1\) s-STD: surrogate standard, diclosulam.  
\(^2\) Calculated from 5 replicate injections.

The calibration curve is linear from 0.005 to 1 µg mL\(^{-1}\) with a mean \(R^2\) of 0.9994. The linear regression equation is \(y = 3.0217x - 0.0119\) for the lowest fortification level.

The calibration curve is presented in Figure 4.5.
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Figure 4.5 Flumetsulam standard calibration curve determined with the s-STD
Note: Peak area ratio of [flumetsulam:s-STD]

4.12.1.3.2 Recoveries for flumetsulam

The concentrations of flumetsulam in soybean were calculated from the mean peak area ratio of seven replicate extractions and analyses each at a fortification level of 0.01, 0.02, 0.05 and 0.1 µg g⁻¹. The equations of the calibration curve for the four fortification levels are given in Table 4.8 along with their equations. The R² are all > 0.999.

Table 4.8 Equations used to determine the recoveries for the four fortification levels

<table>
<thead>
<tr>
<th>Fortification level (µg g⁻¹)</th>
<th>Equation¹ (y = mx + b)</th>
<th>Coefficient of determination (R²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>y = 3.0217x + 0.0119</td>
<td>0.9992</td>
</tr>
<tr>
<td>0.02</td>
<td>y = 2.2504x + 0.0085</td>
<td>0.9995</td>
</tr>
<tr>
<td>0.05</td>
<td>y = 3.7992x + 0.0251</td>
<td>0.9998</td>
</tr>
<tr>
<td>0.1</td>
<td>y = 2.2929x + 0.0261</td>
<td>0.9991</td>
</tr>
</tbody>
</table>

¹Without using i-STD.
The recoveries calculated from the equations are presented in Table 4.9.

Table 4.9 Recoveries for flumetsulam determined with the s-STD$^1$

<table>
<thead>
<tr>
<th>Fortification level (µg g$^{-1}$)</th>
<th>% Recovery$^{2}$</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>80.7</td>
<td>18.5</td>
</tr>
<tr>
<td>0.02</td>
<td>84.0</td>
<td>9.4</td>
</tr>
<tr>
<td>0.05</td>
<td>94.8</td>
<td>7.6</td>
</tr>
<tr>
<td>0.1</td>
<td>93.9</td>
<td>5.4</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td><strong>88.4</strong></td>
<td><strong>10.2</strong></td>
</tr>
</tbody>
</table>

$^1$s-STD: surrogate standard, diclosulam.
$^2$Calculated from 7 replicate fortified samples.

The recoveries obtained were between 80.7% and 94.8% (mean = 88.4%) with a % RSD of between 5.4% and 18.5% (mean = 10.2%). The recoveries are improved by at least 2% (absolute value) compared to using the i-STD alone but the precisions of recoveries are similar.
4.12.1.4 Recoveries for flumetsulam determined using both the i-STD and s-STD

4.12.1.4.1 Calibration curve

In this section, the flumetsulam concentration is plotted against the ratio of [flumetsulam:s-STD]:[i-STD].

Table 4.10 presents the results of the mean peak area ratio of flumetsulam to the s-STD diclosulam then to the i-STD benzoic acid for each flumetsulam concentration. The flumetsulam calibration curve is a plot of flumetsulam concentration versus the ratio of the mean peak area of [flumetsulam:s-STD diclosulam]:[i-STD benzoic acid].

<table>
<thead>
<tr>
<th>Flumetsulam (µg mL(^{-1}))</th>
<th>Mean peak area ratio(^3) \text{[Flumetsulam:s-STD]:[i-STD]} (x (10^3))</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.005</td>
<td>0.26</td>
<td>6.0</td>
</tr>
<tr>
<td>0.01</td>
<td>0.48</td>
<td>5.3</td>
</tr>
<tr>
<td>0.02</td>
<td>0.93</td>
<td>4.3</td>
</tr>
<tr>
<td>0.05</td>
<td>2.4</td>
<td>2.7</td>
</tr>
<tr>
<td>0.1</td>
<td>4.7</td>
<td>1.1</td>
</tr>
<tr>
<td>0.5</td>
<td>19.1</td>
<td>0.5</td>
</tr>
<tr>
<td>1</td>
<td>37.5</td>
<td>0.8</td>
</tr>
</tbody>
</table>

\(^1\)i-STD: internal standard, benzoic acid.
\(^2\)s-STD: surrogate standard, diclosulam.
\(^3\)Calculated from 5 replicate injections.

The calibration curve is linear from 0.005 to 1 µg mL\(^{-1}\) with a mean \(R^2\) of 0.9996. The linear regression equation is \(y = 0.055x - 0.0002\) for the lowest fortification level. The calibration curve is presented in Figure 4.6.
Chapter 4: Solvent partitioning cleanup

Figure 4.6 Flumetsulam standard calibration curve determined with the i-STD and s-STD

Note: Peak area ratio of [flumetsulam:s-STD]:[i-STD benzoic acid].

4.12.1.4.2 Recoveries for flumetsulam

The concentrations of flumetsulam in soybean were calculated from the mean peak area ratio of seven replicate extractions and analyses each at fortification levels of 0.01, 0.02, 0.05 and 0.1 µg g⁻¹. The equation of the calibration curve for the 0.01 µg g⁻¹ fortification level is given in Table 4.11 along with their equations. The R² are all > 0.999.

Table 4.11 Equations used to determine the recoveries for the four fortification levels

<table>
<thead>
<tr>
<th>Fortification level (µg g⁻¹)</th>
<th>Equation (y = mx + b)</th>
<th>Coefficient of determination (R²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>y = 0.0550x + 0.0002</td>
<td>0.9997</td>
</tr>
<tr>
<td>0.02</td>
<td>y = 0.0356x + 0.0002</td>
<td>0.9998</td>
</tr>
<tr>
<td>0.05</td>
<td>y = 0.0569x + 0.0005</td>
<td>0.9993</td>
</tr>
<tr>
<td>0.1</td>
<td>y = 0.0373x + 0.0004</td>
<td>0.9995</td>
</tr>
</tbody>
</table>
The recoveries calculated from the equations are presented in Table 4.12.

### Table 4.12 Recoveries for flumetsulam determined with the i-STD\(^1\) & s-STD\(^2\)

<table>
<thead>
<tr>
<th>Fortification level (µg g(^{-1}))</th>
<th>% Recovery(^3)</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>70.4</td>
<td>22.0</td>
</tr>
<tr>
<td>0.02</td>
<td>76.0</td>
<td>16.4</td>
</tr>
<tr>
<td>0.05</td>
<td>80.3</td>
<td>14.0</td>
</tr>
<tr>
<td>0.1</td>
<td>92.9</td>
<td>9.6</td>
</tr>
<tr>
<td>Mean</td>
<td>79.9</td>
<td>15.5</td>
</tr>
</tbody>
</table>

\(^1\)i-STD: internal standard, benzoic acid.
\(^2\)s-STD: surrogate standard, diclosulam.
\(^3\)Calculated from 7 replicate fortified samples.

The recoveries are between 70.4% and 92.9% (mean = 79.9%) with a % RSD of between 9.6% and 22% (mean = 15.5%). The recoveries are improved by at least 2% (absolute value) compared to using the i-STD alone but the precisions of recoveries are similar when compared to not using the i-STD.
Comparison of the flumetsulam recovery results determined with and without the i-STD and/or s-STD

Table 4.13 presents the results for flumetsulam recoveries determined using the analyte alone, analyte with i-STD, analyte with s-STD and analyte with both i-STD and s-STD.

Table 4.13 Summary of flumetsulam recoveries determined with and without the i-STD\(^1\) and/or s-STD\(^2\)

<table>
<thead>
<tr>
<th>Fortification level (µg g(^{-1}))</th>
<th>Flumetsulam only(^3) % ± RSD</th>
<th>Flumetsulam, i-STD % ± RSD</th>
<th>Flumetsulam, s-STD % ± RSD</th>
<th>Flumetsulam, i-STD, s-STD % ± RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>71.8 ± 22.1</td>
<td>70.1 ± 20.6</td>
<td>80.7 ± 18.5</td>
<td>70.4 ± 22.0</td>
</tr>
<tr>
<td>0.02</td>
<td>78.3 ± 19.7</td>
<td>73.1 ± 16.2</td>
<td>84.0 ± 9.4</td>
<td>76.0 ± 16.4</td>
</tr>
<tr>
<td>0.05</td>
<td>78.2 ± 14.7</td>
<td>83.6 ± 12.3</td>
<td>94.8 ± 7.6</td>
<td>80.3 ± 14.0</td>
</tr>
<tr>
<td>0.1</td>
<td>91.7 ± 8.0</td>
<td>85.8 ± 9.7</td>
<td>93.9 ± 5.4</td>
<td>92.9 ± 9.6</td>
</tr>
</tbody>
</table>

(%) Mean recovery 80.0 78.2 88.4 79.9

\(^1\) i-STD: internal standard, benzoic acid.
\(^2\) s-STD: surrogate standard, diclosulam.
\(^3\) Recovery ± RSD of 7 replicate extractions.

The results in Table 4.13 show that all four methods of quantitation have good recoveries and precision. The methods with and without the i-STD have similar recoveries. However, the use of the s-STD improves the recovery by between 5% and 8% on an absolute basis. Clearly, the use of a s-STD to compensate for the amount of analyte losses during the extraction process is worthwhile, but if either the i-STD benzoic acid or s-STD diclosulam is not available, or not able to be used, good recoveries and precision are still achievable. Like flumetsulam, diclosulam is a registered commercial herbicide but after an extensive search, no formulations were found that used a combination of flumetsulam and diclosulam. If there are any
reservations about using diclosulam, an unfortified soybean sample could be analysed to confirm that there is no peak at the retention time of diclosulam.

4.12.2 Other validation parameters

4.12.2.1 Selectivity

The selectivity of an analytical method is its ability to accurately measure an analyte in the presence of possible interferences that may be present in the matrix. The chromatograms of a blank soybean extract and fortified soybean sample are presented in Figures 4.7 and 4.8 respectively. The analyte, i-STD and s-STD peaks are well resolved from each other and from co-extractive peaks. Other sources of soybean, such as one from China show an essentially similar blank. A chromatogram of an extract of a Chinese soybean blank is presented in Appendix X.
Chapter 4: Solvent partitioning cleanup

Figure 4.7 Chromatogram of extract of soybean blank. (1), (2) and (3) indicates the retention times of i-STD, flumetsulam and s-STD respectively.

Figure 4.8 Chromatogram of extract of soybean fortified at 0.05 µg g⁻¹. (1), (2) and (3) are peaks for i-STD, flumetsulam and s-STD respectively.
Chapter 4: Solvent partitioning cleanup

4.12.3 Precision of Analysis

4.12.3.1 Intra-day precision of replicate injections of one extract

Seven injections of soybean extract fortified with 0.05 µg g⁻¹ (the MRL concentration) of flumetsulam, i-STD and s-STD were performed on the same day. This provides the instrumental precision or the instrumental contribution to the overall (instrumental plus extraction) precision.

Table 4.14 presents the results for precision of the peak area of flumetsulam alone, peak area ratio of [flumetsulam:s-STD], [flumetsulam:i-STD], and [(flumetsulam:s-STD):(i-STD)]. The instrumental precision calculated using flumetsulam peak area alone is 1.58%. The instrumental precision calculated using the ratio of [flumetsulam:s-STD] is 2.91%. The instrumental precision calculated using the ratio of [flumetsulam:i-STD] is 2.18%. The instrumental precision calculated using the ratio of [(flumetsulam:s-STD):(i-STD)] is 2.02%. Therefore the four methods of calculation yield essentially similar precision.

<table>
<thead>
<tr>
<th>Injection number</th>
<th>Peak area from Flumetsulam</th>
<th>Peak area ratio calculated from [Flumetsulam:s-STD]¹</th>
<th>Peak area ratio calculated from [Flumetsulam:i-STD]²</th>
<th>Peak area ratio calculated from [(Flumetsulam:s-STD):(i-STD)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>66.8146</td>
<td>3.5314</td>
<td>9.3212</td>
<td>0.0477</td>
</tr>
<tr>
<td>2</td>
<td>67.5147</td>
<td>3.6351</td>
<td>9.5319</td>
<td>0.0452</td>
</tr>
<tr>
<td>3</td>
<td>67.8963</td>
<td>3.6447</td>
<td>9.6318</td>
<td>0.0455</td>
</tr>
<tr>
<td>4</td>
<td>66.2356</td>
<td>3.5359</td>
<td>9.6234</td>
<td>0.0461</td>
</tr>
<tr>
<td>5</td>
<td>68.9514</td>
<td>3.7545</td>
<td>9.7324</td>
<td>0.0473</td>
</tr>
<tr>
<td>6</td>
<td>66.7568</td>
<td>3.7650</td>
<td>9.8521</td>
<td>0.0468</td>
</tr>
<tr>
<td>7</td>
<td>68.9173</td>
<td>3.7859</td>
<td>9.9602</td>
<td>0.0459</td>
</tr>
<tr>
<td>Mean</td>
<td>67.5838 ± 1.0680 (1.58% RSD)</td>
<td>3.6646 ± 0.1068 (2.91% RSD)</td>
<td>9.6647 ± 0.2103 (2.18% RSD)</td>
<td>0.0464 ± 0.0009 (2.02% RSD)</td>
</tr>
</tbody>
</table>

¹s-STD: surrogate standard, diclosulam.
²i-STD: internal standard, benzoic acid.
4.12.3.2 Inter-day precision of replicate injections of one extract

Seven injections of a soybean extract fortified with 0.05 µg g^{-1} of flumetsulam, i-STD and s-STD were performed over five days. Table 4.15 presents the inter-day precision of seven injections for the first day and five for the remaining days. The method precision calculated from flumetsulam alone was between 1.02% and 2.64% (mean = 1.75%). The method precision calculated from the ratio of [flumetsulam:s-STD] was between 2.21% and 2.97% (mean = 2.58%). The method precision calculated from the ratio of [flumetsulam:i-STD] was between 2.18% and 3.37% (mean = 3.01%). The method precision calculated from the ratio of [flumetsulam:s-STD]:[i-STD] was between 2.02% and 3.61% (mean = 2.84%). The precision calculated using both the i-STD and s-STD is slightly worse than that using i-STD or s-STD alone. This is probably due to the propagation of error associated with using an additional measurement. In contrast, for the intra-day results, the RSDs were improved with use of the s-STD and i-STD.
Table 4.15 Inter-day precision of flumetsulam peak area

<table>
<thead>
<tr>
<th>Inter-day injections</th>
<th>Mean peak area of Flumetsulam ± %RSD</th>
<th>Mean peak area ratio [Flumetsulam: s-STD]$^2$ ± %RSD</th>
<th>Mean peak area ratio [Flumetsulam: i-STD]$^3$ ± %RSD</th>
<th>Mean peak area ratio [Flumetsulam: s-STD]: [i-STD] ± %RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>66.5547 ± 1.59%</td>
<td>3.6646 ± 2.91%</td>
<td>9.6647 ± 2.18%</td>
<td>0.0464 ± 2.02%</td>
</tr>
<tr>
<td>Day 2</td>
<td>65.2388 ± 1.47%</td>
<td>3.5379 ± 2.45%</td>
<td>9.5875 ± 2.93%</td>
<td>0.0434 ± 2.64%</td>
</tr>
<tr>
<td>Day 3</td>
<td>67.9178 ± 2.06%</td>
<td>3.7965 ± 2.36%</td>
<td>9.3284 ± 3.25%</td>
<td>0.0428 ± 2.57%</td>
</tr>
<tr>
<td>Day 4</td>
<td>69.2587 ± 1.02%</td>
<td>3.7257 ± 2.21%</td>
<td>8.9546 ± 3.37%</td>
<td>0.0452 ± 3.38%</td>
</tr>
<tr>
<td>Day 5</td>
<td>68.6572 ± 2.64%</td>
<td>3.5966 ± 2.97%</td>
<td>10.2148 ± 3.34%</td>
<td>0.0415 ± 3.61%</td>
</tr>
<tr>
<td>Mean</td>
<td>67.5254 ± 2.41%</td>
<td>3.6643 ± 2.79%</td>
<td>9.5505 ± 4.86%</td>
<td>0.0439 ± 4.44%</td>
</tr>
</tbody>
</table>

$^1$Calculated from 5 replicates.
$^2$s-STD: surrogate standard, diclosulam.
$^3$i-STD: internal standard, benzoic acid.

4.12.3.3 Precision of retention time

In this work the identification of the analyte is achieved by comparison with the retention time of a known standard. A standard mixture of flumetsulam, benzoic acid and diclosulam was injected into the LC for five days with five injections per day. Table 4.16 lists the retention times of flumetsulam, i-STD and s-STD for twenty-five successive injections over a five-day period.

The intra-day precision of the retention time was between 0.41% and 1.04% (mean = 0.77%) for the i-STD, between 0.51% to 1.4% (mean = 0.87%) for the flumetsulam and between 0.33% and 0.95% (mean = 0.6%) for the s-STD.
The inter-day precision of the retention time for the i-STD, flumetsulam and s-STD are 1.0%, 1.05% and 0.71% respectively. The retention times are therefore reproducible. The results for the precision of the retention times are given in Table 4.16.

**Table 4.16 Precision of retention times for i-STD\(^1\), flumetsulam and s-STD\(^2\)**

<table>
<thead>
<tr>
<th>Intra-day injections(^3)</th>
<th>i-STD (min)</th>
<th>Flumetsulam (min)</th>
<th>s-STD (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>23.75</td>
<td>25.97</td>
<td>35.96</td>
</tr>
<tr>
<td></td>
<td>23.49</td>
<td>25.49</td>
<td>35.97</td>
</tr>
<tr>
<td></td>
<td>23.65</td>
<td>25.67</td>
<td>35.74</td>
</tr>
<tr>
<td></td>
<td>23.57</td>
<td>26.03</td>
<td>36.09</td>
</tr>
<tr>
<td>Mean</td>
<td>23.61 ± 0.41%</td>
<td>25.79 ± 0.86%</td>
<td>36.00 ± 0.52%</td>
</tr>
<tr>
<td>Day 2</td>
<td>23.21</td>
<td>26.10</td>
<td>35.98</td>
</tr>
<tr>
<td></td>
<td>23.35</td>
<td>26.37</td>
<td>35.92</td>
</tr>
<tr>
<td></td>
<td>23.28</td>
<td>25.96</td>
<td>35.85</td>
</tr>
<tr>
<td></td>
<td>23.24</td>
<td>25.88</td>
<td>35.70</td>
</tr>
<tr>
<td>Mean</td>
<td>23.36 ± 0.93%</td>
<td>26.03 ± 0.82%</td>
<td>35.84 ± 0.33%</td>
</tr>
<tr>
<td>Day 3</td>
<td>23.64</td>
<td>25.99</td>
<td>35.81</td>
</tr>
<tr>
<td></td>
<td>23.56</td>
<td>25.85</td>
<td>35.65</td>
</tr>
<tr>
<td></td>
<td>23.97</td>
<td>25.55</td>
<td>35.72</td>
</tr>
<tr>
<td></td>
<td>23.92</td>
<td>25.26</td>
<td>35.48</td>
</tr>
<tr>
<td>Mean</td>
<td>23.75 ± 0.76%</td>
<td>25.56 ± 1.4%</td>
<td>35.73 ± 0.51%</td>
</tr>
<tr>
<td>Day 4</td>
<td>23.45</td>
<td>25.56</td>
<td>35.84</td>
</tr>
<tr>
<td></td>
<td>23.85</td>
<td>25.49</td>
<td>35.93</td>
</tr>
<tr>
<td></td>
<td>23.64</td>
<td>25.46</td>
<td>35.26</td>
</tr>
<tr>
<td></td>
<td>23.47</td>
<td>25.94</td>
<td>35.24</td>
</tr>
<tr>
<td>Mean</td>
<td>23.61 ± 0.69%</td>
<td>25.63 ± 0.76%</td>
<td>35.62 ± 0.95%</td>
</tr>
<tr>
<td>Day 5</td>
<td>24.07</td>
<td>25.74</td>
<td>35.26</td>
</tr>
<tr>
<td></td>
<td>23.65</td>
<td>25.72</td>
<td>35.46</td>
</tr>
<tr>
<td></td>
<td>24.11</td>
<td>25.94</td>
<td>35.91</td>
</tr>
<tr>
<td></td>
<td>23.73</td>
<td>25.69</td>
<td>35.73</td>
</tr>
<tr>
<td>Mean</td>
<td>23.83 ± 1.04%</td>
<td>25.73 ± 0.51%</td>
<td>35.61 ± 0.71%</td>
</tr>
</tbody>
</table>

| Inter-day                  | Mean ± %RSD | 23.63 ± 0.76% | 25.75 ± 0.70% | 35.76 ± 0.46% |

\(^1\)i-STD: internal standard, benzoic acid.
\(^2\)s-STD: surrogate standard, diclosulam.
\(^3\)Calculated from 5 replicates.
4.12.4 **LINEAR RANGE OF CALIBRATION**

The method was found to be linear between 0.005 to 1 µg mL⁻¹ for flumetsulam in soybean. Table 4.17 presents the regression equations of flumetsulam standard solution and in fortified soybean samples determined using the i-STD benzoic acid and s-STD diclosulam. The $R^2$ were 0.998 or better with or without the use of the i-STD and s-STD.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Regression equation (^3) ((y = mx + b))</th>
<th>Coefficient of determination ((R^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard solution</td>
<td>(y = 0.055x + 0.0002)</td>
<td>0.9997</td>
</tr>
<tr>
<td>Soybean sample</td>
<td>(y = 0.046x + 0.0005)</td>
<td>0.9992</td>
</tr>
</tbody>
</table>

\(^1\)i-STD: internal standard, benzoic acid.  
\(^2\)s-STD: surrogate standard, diclosulam.  
\(^3\)\(y = \text{peak area ratio } \left[\frac{\text{Flumetsulam:s-STD}}{\text{i-STD}}\right] \), \(x = \text{concentration in } \mu\text{g mL}^{-1}\), \(m = \text{slope}, b = \text{intercept}\).

The intercepts of the regression equations in standard and soybean sample solutions are similar and not significantly different from zero, showing that the soybean is free of flumetsulam before the standard addition and that there are no co-eluting substances.

The slopes of the regression equations in standard solution and soybean sample solution are similar, indicating that quantitation using a standard curve will yield comparable results as that obtained by the method of standard addition, and that the matrix is not significantly affecting the quantitation of the analyte using the standard calibration curve.
4.12.5 **Range of Method**

Figures 4.3, 4.4, 4.5, 4.6 and Table 4.17 show that the regression equations and calibration curves are linear from 0.005 to 1 µg mL$^{-1}$ for the standard solution and from 0.01 to 0.1 µg g$^{-1}$ of flumetsulam in soybean.

4.12.6 **Limit of Detection and Limit of Quantitation (LOD & LOQ)**

The limit of detection (LOD) of flumetsulam, calculated as three times the standard deviation and the limit of quantitation (LOQ) as ten times the standard deviation of the lowest fortification level (0.01 µg g$^{-1}$) were found to be between 0.0042 to 0.0048 and 0.014 to 0.016 µg g$^{-1}$ respectively using the four different methods of calculation. The LODs and LOQs are presented below in Table 4.18. As a reference, the MRL for flumetsulam is 0.05 µg g$^{-1}$ for pulses (1).

| Table 4.18 LODs and LOQs determined with and without the i-STD and/or s-STD |
|------------------------------|-----------------|----------|
| Method                        | LOD (µg g$^{-1}$) | LOQ (µg g$^{-1}$) |
| Analyte alone                 | 0.0048           | 0.016    |
| Analyte with i-STD            | 0.0042           | 0.014    |
| Analyte with s-STD            | 0.0045           | 0.015    |
| Analyte with both i-STD and s-STD | 0.0047           | 0.016    |
| Mean                          | **0.005**        | **0.015** |
4.12.7 **STABILITY OF STANDARD AND SAMPLE SOLUTIONS**

At room temperature, the sample and standard solutions in acetonitrile are stable for only three days. After one week, the chromatographic peak area diminishes by about 10% and multiple peaks appear around the region of the analyte peak. The appearance of multiple peaks is more obvious in the sample than the standard solution. If the solutions are stored in the refrigerator at –4 °C, the dilute standard and sample solutions are stable for at least one week. After one week, the standard solution showed no additional peaks and only a few small additional peaks appear in the sample solution. Therefore, all sample and standard solutions should be stored in a refrigerator at –4 °C and analysed within one week.
4.13 **REFERENCE**

CHAPTER 5

FLUMETSULAM ANALYSIS USING

SOLID PHASE EXTRACTION CLEAN UP
5.1 INTRODUCTION

Solid phase extraction (SPE) is a method for rapid sample preparation in which a solid stationary phase is typically packed in a syringe barrel and used to selectively extract, concentrate and purify target analytes prior to analysis by HPLC or GC.

During the last two decades, SPE has steadily gained acceptance within the analytical community and is now rapidly replacing traditional liquid-liquid extraction (LLE) as the sample preparation technique of choice for discriminating chromatographers around the world. It has the advantage of requiring less labour and generates less solvent waste than traditional LLE.

The efficacy and economy of SPE is now well documented in a staggering number of peer-reviewed journal articles and reviews. Literally thousands of SPE applications are now available for the extraction of key analytes in the pharmaceutical, clinical/toxicological, environmental and biomedical fields and new methods continue to be developed on a daily basis.

A principal aim of any sample preparation technique is to facilitate analysis and maximise sample throughput by concentrating the target analyte and removing impurities. SPE has the potential to accomplish these goals because it:

1. Eliminates otherwise co-eluted impurities.
2. Concentrates analytes, improves sensitivity and lowers the LOD and LOQ.
3. Facilitates the rapid and efficient, simultaneous processing of multiple samples.
4. Enables solvent switching or buffer exchange prior to analysis.
5. Provides high extraction efficiencies, with quantitative recoveries of analytes and low levels of contaminants.

6. Provides consistent, reproducible results.

The key element to any SPE product is the sorbent. The physicochemical properties of the sorbent determine extraction efficiency and the overall quality of the separation.

Silica-based SPE sorbents are the most popular because they are rigid, inexpensive, easy to derivatise and manufacture reproducibility, stable and immune to shrinking and swelling in common aqueous and organic solvents. The most popular SPE sorbents are chemically modified silica particles with functional groups covalently attached to the surface. Bare silica is polar, retaining analytes via normal phase and cation exchange mechanisms. By attaching a saturated hydrocarbon such as C-18, the surface becomes hydrophobic (non polar). Covalently attaching a variety of other functional groups has been used to produce SPE sorbents with a wide range of chromatographic selectiveness.

Silica based sorbents may still contain unreacted or free silanols. These exposed silanols provide polar, acidic patches on the chromatographic surface that are capable of binding amines via hydrogen bonding and cation exchange mechanisms. Since a number of analytes are ionised under typical extraction conditions, interactions with these silanols may cause undesirable retention or low recoveries. However, it should be noted that in SPE, free silanols could often be used to facilitate the retention of polar analytes on reversed phase sorbents like C-18, C-8 and phenyl.
The other type of sorbent is polymer-based sorbents or resins that are typically composed of highly cross-linked polystyrene-divinyl-benzene (SDB).

In this work, a combination of three SPEs was utilised. The acidic properties of flumetsulam (pKa = 4.6) were an advantage in cleaning up the soybean matrix. The first SPE used was a Supelco Discovery C-18 SPE that effectively removed most of the non-polar co-extractives. Then a strong anion exchange (SAX) cartridge was used to retain the analyte and discard co-extractives such as non-polar and cationic compounds, which are effectively removed by washes with neutral buffers and alkaline methanol. A mixture of water miscible organic solvent with acid modifier can then be used to elute the analyte. Strong acids of pKa less than 2.0 may be difficult to quantitatively elute from a SAX cartridge. The final step involves the use of a strong cation exchange (SCX) cartridge that uses non-retentive mechanism. In this step, the sorbent has no affinity for the analyte but has affinity for the sample contaminants. As a result, the analyte passes directly through the column without being retained, while the contaminants are bound. The chemical structures of silica-based sorbents are presented in Figure 5.1.

![Chemical structures of sorbents](image)

**Figure 5.1 Structures of silica-based sorbents used**
5.2 APPARATUS

The apparatus used is the same as described in Chapter 4, section 4.2 with the addition of:

(a) 12 position vacuum manifold. – SPE vacuum manifold, model number AH0-6023 from Phenomenex (NSW, Australia).

(b) Vacuum pump. – Millipore model number DOA-V130-BN from Waters (NSW, Australia).

(c) Solid phase extraction tubes. – Discovery™ DSC-18, 1000 mg per 6 mL from Supelco (NSW, Australia).

(d) Solid phase extraction tubes. – SAX BOND ELUT®, 500 mg per 3 mL from Varian (NSW, Australia).

(e) Solid phase extraction tubes. – SCX BOND ELUT®, 500 mg per 3 mL from Varian (NSW, Australia).

(f) Reservoir. – 10 mL polypropylene reservoir from Varian (NSW, Australia).

(a) Glass test tubes. – Schott Garsco (NSW, Australia).

(g) Polypropylene test tubes. – 15 mL made by Iwaki, from Crown Scientific (NSW, Australia).

5.3 MATERIALS AND REAGENTS

Reagents for HPLC analysis were HPLC grade; other chemicals were of analytical reagent (AR) grade or the highest purity available. Purified water (H₂O) was obtained from a Millipore ultra pure Milli-Q water purification system (NSW, Australia).
(a) Reference standards. – Flumetsulam, standards grade (99% ± 0.5% purity) and diclosulam, standards grade (98% ± 0.5% purity) were purchased from Alltech Associates Pty. Limited (NSW, Australia).

(b) Benzoic acid. – (98% purity) by Fluka Chemika was purchased from Sigma-Aldrich (NSW, Australia).

(c) Solvents HPLC grade. – Methanol, acetonitrile, hexane, methyl tert-butyl ether, ethyl acetate and dichloromethane were purchased from Lomb Scientific (NSW, Australia).

(d) Solvents AR grade. – Acetone and acetic acid were purchased from APS Finechem (NSW, Australia).

(e) Whole Soybean. – McKenzie’s soybean was purchased from Ward McKenzie Pty. Ltd. (VIC, Australia).

(f) Gas. – Nitrogen, high purity, from BOC Gases (NSW, Australia).

(g) Dimethyl-dichlorosilane (DMDCS). – From Sigma-Aldrich (NSW, Australia).

(h) HCl. – 32% AR grade from APS FineChem (NSW, Australia).

(i) Glacial acetic acid. – AR grade from APS FineChem (NSW, Australia).

(j) Ammonium hydroxide. – AR grade from BDH Merck (VIC, Australia).

5.4 Preparation of SPE washing solutions and eluting solvents and chromatographic reagents

5.4.1 C-18 clean up reagents

(a) Methanol:H₂O:acetic acid (40:60:0.1). – 1600 mL methanol was combined with 2400 mL H₂O and 4 mL glacial acetic acid in a 4 L glass vessel.
(b) Dichloromethane:hexane (50:50). – 2000 mL dichloromethane was combined with 2000 mL hexane in a 4 L glass vessel.

(c) Ethyl acetate:hexane (10:90). – 400 mL Ethyl acetate was combined with 3600 mL hexane in a 4 L vessel.

(d) Dichloromethane:methyl tert-butyl ether (70:30). – 2800 mL dichloromethane was combined with 1200 mL methyl tert-butyl ether in a 4 L vessel.

5.4.2 SAX CLEAN UP REAGENTS

(a) 0.05 M pH 7.0 phosphate buffer. – A 0.05 M phosphate buffer solution, pH 7.0 was prepared. 7.1 g anhydrous dibasic sodium hydrogen orthophosphate was dissolved in 500 mL H₂O and the pH adjusted to 7.0 with concentrated orthophosphoric acid, before diluting to 1000 mL with H₂O.

(b) 0.15 M pH 2.2 phosphate buffer. – A 0.15 M phosphate buffer solution, pH 2.2 was prepared. 20.7 g monosodium dihydrogen orthophosphate monohydrate was dissolved in 500 mL H₂O and the pH adjusted to 2.2 with concentrated orthophosphoric acid, before diluting to 1000 mL with H₂O.

(c) Methanol:0.15 M pH 2.2 buffer (20:80). – 800 mL methanol was combined with 3200 mL pH 2.2 buffer in a 4 L glass vessel.

(d) 0.2% ammonium hydroxide in methanol. – 2 mL NH₄OH was combined with 998 mL methanol in a 1 L glass vessel.

5.4.3 SCX CLEAN UP REAGENTS

(a) Methanol:0.1% acetic acid (90:10). – 900 mL methanol was combined with 100 mL of 0.1% aqueous glacial acetic acid in a 1 L glass vessel.
5.5 Preparation of Glassware

The round-bottomed flasks, volumetric flasks, volumetric pipettes and glass vials were silanised as described in Chapter 4, section 4.5.

5.6 HPLC Instrumentation

The HPLC-UV instrumentation is as described in Chapter 4, section 4.6.

5.7 HPLC Conditions

The HPLC conditions are described in Chapter 4, section 4.7.

5.8 Preparation of Analytical Standard Solutions

5.8.1 Preparation of Standard Stock Solutions

Individual stock solutions of 1000 µg mL\(^{-1}\) flumetsulam and benzoic acid were prepared by accurately weighing 10 mg of the respective standards into separate 10 mL volumetric flasks and making up to the mark with acetonitrile. Where applicable, the amount of analyte and internal standard were adjusted according to the stated purity of the material. These stock solutions were used for preparing the fortification and calibration solutions. The solutions were stored at –4 °C and prepared freshly every month.
5.8.2 PREPARATION OF FORTIFICATION STANDARD SOLUTIONS

A 10 µg mL\(^{-1}\) flumetsulam standard solution was prepared by diluting 1 mL of the 1000 µg mL\(^{-1}\) stock solution to 100 mL with acetonitrile. 10 mL of the 10 µg mL\(^{-1}\) solution was diluted to 100 mL with acetonitrile to give a 1 µg mL\(^{-1}\) standard solution.

A 100 µg mL\(^{-1}\) of benzoic acid standard solution was prepared by diluting 10 mL of the 1000 µg mL\(^{-1}\) stock solution to 100 mL with acetonitrile. 10 mL of the 100 µg mL\(^{-1}\) benzoic acid solution was diluted to 100 mL with acetonitrile to give a 10 µg mL\(^{-1}\) standard solution.

200 µL of the 1 µg mL\(^{-1}\) flumetsulam standard was used for fortifying 10 g of ground soybean to give a 0.02 µg g\(^{-1}\) fortification level of flumetsulam. 50 and 100 µL of the 10 µg mL\(^{-1}\) standard were used for fortifying 10 g of ground soybean to give 0.05 and 0.1 µg g\(^{-1}\) fortification levels each of flumetsulam.

These fortification standards were stored at –4 °C and prepared freshly every week.

5.8.3 PREPARATION OF CALIBRATION STANDARD SOLUTIONS

Individual standard solutions containing 0.01, 0.02, 0.05, 0.1 and 0.5 µg mL\(^{-1}\) of flumetsulam were prepared by transferring 0.1, 0.2, 0.5, 1 and 5 mL of the 10 µg mL\(^{-1}\) flumetsulam standard into five 100 mL volumetric flasks and making up to volume with the LC mobile phase. For each flumetsulam standard, 1000 µL of the 100 µg mL\(^{-1}\) i-STD benzoic acid was added to give a final concentration of 1 µg mL\(^{-1}\).
These calibration standards were stored at –4 °C in a refrigerator and prepared freshly on the day of use.

5.9 **SAMPLE PREPARATION**

Sample preparation is described in Chapter 4, section 4.9

5.10 **FORTIFICATION, EXTRACTION AND FRACTIONATION OF FLUMETSULAM**

5.10.1 **FORTIFICATION OF SOYBEAN**

Four portions of 10 g of the ground soybean were weighed into separate 500 mL Shott bottles and fortified with flumetsulam to give the desired fortification concentrations. Fortification levels of 0, 0.02, 0.05 and 0.1 µg g⁻¹ were prepared by adding 0, 200 µL of the 1 µg mL⁻¹ and 50 and 100 µL of the 10 µg mL⁻¹ flumetsulam standard to each soybean sample. There were three replicates for each fortification level giving a total of 12 samples. After fortification, 10 min were allowed for the solvent to evaporate.

5.10.2 **METHANOL EXTRACTION**

To each sample was added 100 mL acidified methanol, the bottle capped before placing on a shaker set at 250 rpm and 60 °C for 15 min. The mixture was allowed to rest for at least 15 min before decanting the methanol supernatant into a clean 250 mL conical flask. The extraction was repeated with another 100 mL of acidified methanol, and the methanol extracts combined before filtering through a 0.45 µm Nylon filter. The filtrate was placed in a 250 mL round bottomed flask and evaporated to dryness.
on a rotary vacuum evaporator set at 60 °C. The residue was dissolved in 2 x 5 mL methanol:H₂O (40:60) for LC analysis.

The procedure described is summarised in Figure 5.2.

**Figure 5.2 Extraction of flumetsulam from organic phase**

**5.10.2.1 C-18 clean up procedure**

A C-18 cartridge was attached to a 12-position SPE vacuum manifold with a 10 mL reservoir on top of the SPE cartridge. The SPE was preconditioned with 6 mL acetonitrile followed by 6 mL H₂O, 6 mL methanol and finally 6 mL methanol:H₂O:acetic acid (40:60:0.1). Care was taken to ensure the SPE cartridge did not run dry. The tap on the tank was closed to allow the methanol:H₂O solution to remain in the cartridge 1 mm above the frit. 5 mL of the extract from section 5.10.2 was loaded on to the cartridge. A fresh 5 mL methanol:H₂O:acetic acid (40:60:0.1)
was added to the round bottomed flask to dissolve the remainder of the extract in the flask and this was added to the previous 5 mL in the cartridge. The tap of the manifold was opened to allow the solution to drip through the cartridge. The eluant was discarded and the SPE washed with 6 mL ethyl acetate:hexane (10:90) followed by 6 mL of dichloromethane:hexane (50:50). The washings were discarded. The vacuum was turned on for 30 sec to dry the sorbent. A glass test tube was placed inside the manifold underneath the SPE cartridge to collect the eluate eluted with 2 x 3 mL dichloromethane:methyl tert-butyl ether (70:30). This eluate was transferred to a preconditioned SAX cartridge.

The above procedure is summarised in Figure 5.3.

---

**Figure 5.3 C-18 SPE extraction flowchart**
5.10.2.2 SAX clean up procedure

The SAX cartridge conditioned with 6 mL methanol, followed by 6 mL dichloromethane. The 6 mL of dichloromethane:methyl tert-butyl ether (70:30) eluate from section 5.10.2.1 was loaded onto the SAX cartridge and allowed to drip through at a rate of 1 drop sec$^{-1}$. The cartridge was washed with 5 mL 0.05 M pH 7.0 phosphate buffer followed by 5 mL 0.2% NH$_4$OH in methanol and the washings discarded. The vacuum was turned on for 30 sec to dry the sorbent. The analyte was eluted with 2 x 5 mL of methanol:0.15 M pH 2.2 buffer (20:80) into a clean glass test tube. The collected was loaded onto a pre-conditioned SCX cartridge.

The above procedure is summarised in Figure 5.4.

---

Figure 5.4 SAX SPE extraction flowchart
5.10.2.3 SCX clean up procedure

A SCX cartridge was conditioned with 6 mL methanol, followed by 6 mL methanol:0.15 M pH 2.2 buffer (20:80) before loading the eluent from section 5.10.2.2 onto the SCX cartridge. The sample was allowed to drip through at a rate of 1-drop sec\(^{-1}\) and the eluent collected in a 15 mL polypropylene tube. 2 mL of acidified methanol was then added to the cartridge to quantitatively elute the analyte. 5 mL of fresh acidified methanol was added to the polypropylene tube and the mixture vortexed for 1 min before transferring the methanol layer into a glass vial. The methanol was evaporated to dryness under a gentle stream of nitrogen following addition of 50 µL of 10 µg mL\(^{-1}\) i-STD benzoic acid in acetonitrile. The residue was dissolved in 500 µL of mobile phase by vortexing for 1 min for LC analysis. The final concentration of i-STD is 1 µg mL\(^{-1}\).

The above procedure is summarised in Figure 5.5.
Chapter 5: solid phase extraction cleanup

Precondition SCX cartridge

Load eluant MeOH:pH 2.2 buffer from Figure 5.4 into SCX cartridge

Elute the remainder of the analyte with a small volume of acidified MeOH

Collect the eluant in polypropylene tube

Add fresh acidified MeOH and vortex to extract the analyte from the aqueous phase

Discard aqueous layer

Add internal standard and evaporate MeOH layer

Dissolve residue in mobile phase for HPLC analysis

Figure 5.5 SCX SPE extraction flowchart


5.11 RESULTS AND DISCUSSION

5.11.1 OPTIMISATION OF SOLID PHASE EXTRACTION

Different solvent combinations were used to optimise the elution of flumetsulam from the C-18 SPE cartridge. Acidified methanol and acetonitrile at different concentrations were trialed, but low recoveries of analyte were obtained. If the column was acidified before the eluting solvent was added, good recoveries were obtained.

The purpose of this method was to take advantage of the neutral characteristics of the matrix and the acid-base characteristics of the analyte by using the ion exchange SPE cartridges as well as using the reversed phase SPE cartridge to clean up the hydrophobic lipids present in soybean.

5.11.2 RECOVERY STUDIES OF FLUMETSULAM FROM SOYBEAN

Four replicate 10 g samples of soybean were fortified with flumetsulam to give concentrations of 0, 0.02, 0.05 and 0.1 µg g\(^{-1}\) respectively. Standard solutions of flumetsulam containing 0.01, 0.02, 0.05, 0.1 and 0.5 µg mL\(^{-1}\) were prepared as described in section 5.8.3. The i-STD benzoic acid was added to the sample at the last step before LC analysis to give a resultant concentration of 1 µg mL\(^{-1}\).

Two types of recoveries were calculated using:

- the analyte only
- the analyte with i-STD
5.11.2.1 Recoveries of flumetsulam without the i-STD

5.11.2.1.1 Calibration curve

Table 5.1 presents the mean peak areas for flumetsulam for each of the four standards. The flumetsulam calibration curve is a plot of the concentration versus the mean peak area of flumetsulam.

<table>
<thead>
<tr>
<th>Flumetsulam (µg mL⁻¹)</th>
<th>Mean peak area x10⁴</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>3.1</td>
<td>1.5</td>
</tr>
<tr>
<td>0.02</td>
<td>7.3</td>
<td>1.1</td>
</tr>
<tr>
<td>0.05</td>
<td>17.8</td>
<td>0.7</td>
</tr>
<tr>
<td>0.1</td>
<td>34.1</td>
<td>0.9</td>
</tr>
<tr>
<td>0.5</td>
<td>160.9</td>
<td>0.4</td>
</tr>
</tbody>
</table>

¹Calculated from 3 replicate injections.

The flumetsulam standard calibration curve is linear from 0.01 to 0.5 µg mL⁻¹ with a coefficient of determination (R²) of 0.9998. The linear regression equation is y = 320.89x + 0.7206 for the fortification levels. The calibration curve is presented in Figure 5.6.
Chapter 5: solid phase extraction cleanup

5.1.1.2 Recoveries for flumetsulam

The concentration of flumetsulam in fortified soybean was calculated from the mean peak area of four replicate extractions and analyses at each fortification levels of 0.02, 0.05 and 0.1 µg g\(^{-1}\). The recoveries determined are presented in Table 5.2.

![Calibration curve used for the fortification levels 0.02, 0.05 and 0.1 µg g\(^{-1}\)](image)

\[y = 320.89x + 0.7206\]
\[R^2 = 0.9998\]

**Figure 5.6 Flumetsulam calibration curve determined without i-STD**

<table>
<thead>
<tr>
<th>Fortification level (µg g(^{-1}))</th>
<th>% Recovery(^1)</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02</td>
<td>78.5</td>
<td>15.2</td>
</tr>
<tr>
<td>0.05</td>
<td>82.9</td>
<td>9.6</td>
</tr>
<tr>
<td>0.1</td>
<td>94.2</td>
<td>7.8</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td><strong>85.2</strong></td>
<td><strong>10.9</strong></td>
</tr>
</tbody>
</table>

\(^1\)Calculated from 4 replicate fortified samples.
The results show that the method has good and reproducible recoveries for flumetsulam. The recoveries are between 78.5% and 94.2% (mean = 85.2%) with a RSD of between 7.8% and 15.2% (mean = 10.9%).

### 5.11.2.2 Recoveries for flumetsulam determined using the i-STD

#### 5.11.2.2.1 Calibration curve

In this section, the flumetsulam concentration is plotted against the ratio of [flumetsulam:i-STD]. Table 5.3 presents the results of the mean peak area ratio of flumetsulam to the i-STD benzoic acid for each flumetsulam concentration. The flumetsulam calibration curve is a plot of flumetsulam concentration versus the ratio of the mean peak area of flumetsulam to the i-STD benzoic acid.

#### Table 5.3 Flumetsulam standard calibration values and RSDs determined with the i-STD

<table>
<thead>
<tr>
<th>Flumetsulam (µg mL⁻¹)</th>
<th>Mean peak area ratio⁻¹ [Flumetsulam:i-STD]</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>0.15</td>
<td>3.7</td>
</tr>
<tr>
<td>0.02</td>
<td>0.32</td>
<td>3.1</td>
</tr>
<tr>
<td>0.05</td>
<td>0.83</td>
<td>2.3</td>
</tr>
<tr>
<td>0.1</td>
<td>1.7</td>
<td>2.6</td>
</tr>
<tr>
<td>0.5</td>
<td>7.7</td>
<td>1.1</td>
</tr>
</tbody>
</table>

¹i-STD: internal standard, benzoic acid.
²Calculated from 3 replicate injections.
The flumetsulam standard calibration curve is linear from 0.01 to 0.5 $\mu$g mL$^{-1}$ with a $R^2$ of 0.9997. The linear regression equation is $y = 15.35x + 0.0492$. The calibration curve and equation of the line is presented in Figure 5.7.

![Calibration curve used for the fortification levels 0.02, 0.05 and 0.1 $\mu$g g$^{-1}$](image)

**Figure 5.7 Flumetsulam standard calibration curve determined with the i-STD**

Note: Peak area ratio of $[\text{flumetsulam}:i-\text{STD}]$

### 5.11.2.2.2 Recoveries for flumetsulam

The concentrations of flumetsulam in soybean were calculated from the mean peak area ratios of four replicate extractions and analyses each at fortification levels of 0.02, 0.05 and 0.1 $\mu$g g$^{-1}$. The recoveries obtained using the i-STD are presented in Table 5.4.
Chapter 5: solid phase extraction cleanup

Table 5.4 Recoveries for flumetsulam determined with the i-STD

<table>
<thead>
<tr>
<th>Fortification level (µg g⁻¹)</th>
<th>Mean % recovery²</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02</td>
<td>81.3</td>
<td>12.9</td>
</tr>
<tr>
<td>0.05</td>
<td>86.7</td>
<td>8.2</td>
</tr>
<tr>
<td>0.1</td>
<td>89.5</td>
<td>6.6</td>
</tr>
<tr>
<td>Mean</td>
<td>85.8</td>
<td>9.2</td>
</tr>
</tbody>
</table>

¹i-STD: internal standard, benzoic acid.
²Calculated from 4 replicate fortified samples.

The results show that the method has good and reproducible recoveries of flumetsulam. The recoveries were between 81.3% and 89.5% (mean = 85.8%) with % RSDs of between 6.6% and 12.9% (mean = 9.2%). The results are good and largely comparable to those determined without the i-STD but the % RSDs are slightly improved.

5.11.2.3 Comparison of the flumetsulam recovery results determined with and without the i-STD

Table 5.5 presents the comparative results for flumetsulam recoveries determined using the analyte alone and analyte with i-STD.
Table 5.5 Summary of flumetsulam recoveries determined with and without the i-STD\(^1\)

<table>
<thead>
<tr>
<th>Fortification level (µg g(^{-1}))</th>
<th>Recovery(^2) without i-STD</th>
<th>Recovery(^2) with i-STD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% ± RSD</td>
<td>% ± RSD</td>
</tr>
<tr>
<td>0.02</td>
<td>78.5 ± 15.2%</td>
<td>81.3 ± 12.9%</td>
</tr>
<tr>
<td>0.05</td>
<td>82.9 ± 9.6%</td>
<td>86.7 ± 8.2%</td>
</tr>
<tr>
<td>0.1</td>
<td>94.2 ± 7.8%</td>
<td>89.5 ± 6.6%</td>
</tr>
</tbody>
</table>

\(^1\) i-STD: internal standard, benzoic acid.  
\(^2\) Calculated from 4 replicate fortified samples.

The results in Table 5.5 show that both methods of quantitation have good recoveries and precision. They both have similar recoveries. Clearly, the use of an i-STD to compensate for the amount of analyte losses during is worthwhile, but if the i-STD benzoic acid cannot be used, satisfactory recoveries and precision are still achievable.

5.11.3 **OTHER VALIDATION PARAMETERS**

5.11.3.1 **Selectivity**

The selectivity of an analytical method is its ability to accurately measure an analyte in the presence of possible interferences that may be present in the matrix. The chromatograms of a blank soybean extract and a fortified soybean sample are presented in Figures 5.8 and 5.9. The analyte and i-STD peaks are well resolved from each other and from co-extractive peaks.
Figure 5.8 Chromatogram of extract of soybean blank. (1), (2) indicates the retention times of i-STD and flumetsulam respectively.

Figure 5.9 Chromatogram of extract of soybean fortified at 0.05 µg g⁻¹. (1) and (2) are peaks for i-STD and flumetsulam respectively.
Chapter 5: solid phase extraction cleanup

5.11.3.2 Precision of analysis

Three injections of soybean extract fortified with 0.02 µg g⁻¹ of flumetsulam and the i-STD were performed over three days. Table 5.6 presents the intra-day precision with and without the use of the i-STD. The intra-day precision incorporating the i-STD was ± 4.5% RSD and without the i-STD was ± 4.8% RSD. There is therefore no significant difference in precision with or without the use of the i-STD.

Table 5.6 Intra-day precision of flumetsulam peak area

<table>
<thead>
<tr>
<th>Injection number</th>
<th>Peak area [Flumetsulam only]</th>
<th>Peak area ratio [Flumetsulam:i-STD]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.5</td>
<td>0.32</td>
</tr>
<tr>
<td>2</td>
<td>6.1</td>
<td>0.35</td>
</tr>
<tr>
<td>3</td>
<td>6.2</td>
<td>0.33</td>
</tr>
<tr>
<td>4</td>
<td>6.1</td>
<td>0.32</td>
</tr>
<tr>
<td>5</td>
<td>6.2</td>
<td>0.36</td>
</tr>
<tr>
<td>6</td>
<td>6.9</td>
<td>0.34</td>
</tr>
<tr>
<td>7</td>
<td>6.6</td>
<td>0.33</td>
</tr>
<tr>
<td>Mean</td>
<td>6.4 ± 0.3 (4.8% RSD)</td>
<td>0.34 ± 0.02 (4.5% RSD)</td>
</tr>
</tbody>
</table>

i-STD: internal standard, benzoic acid.

Table 5.7 presents the inter-day precision with and without the use of the i-STD. The inter-day precision was determined in a similar way to that of the intra-day precision but with three injection per day. The precision was 2.9% and 0.9% RSD with and without the use of i-STD respectively. While the use of the i-STD has increased the RSD, its incorporation could increase the robustness of the method.
Table 5.7 Inter-day precision of flumetsulam peak area

<table>
<thead>
<tr>
<th>Injection number(^1)</th>
<th>Peak area [Flumetsulam only] ± RSD</th>
<th>Peak area ratio [Flumetsulam:i-STD(^2)] ± RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.5 ± 0.6%</td>
<td>0.35 ± 0.8%</td>
</tr>
<tr>
<td>2</td>
<td>6.4 ± 0.5%</td>
<td>0.33 ± 0.5%</td>
</tr>
<tr>
<td>3</td>
<td>6.4 ± 0.6%</td>
<td>0.34 ± 0.7%</td>
</tr>
<tr>
<td>Mean ± % RSD</td>
<td>6.4 ± 0.9%</td>
<td>0.34 ± 2.9%</td>
</tr>
</tbody>
</table>

\(^1\)Calculated from 3 replicates.
\(^2\)i-STD: internal standard, benzoic acid.

5.11.3 Precision of retention time

Table 5.8 lists the mean retention times of flumetsulam and the i-STD benzoic acid for fifteen successive injections over a three-day period. The retention time was found to be 23.27 min ± 0.39% for benzoic acid and 26.72 min ± 0.68% for flumetsulam.
Chapter 5: solid phase extraction cleanup

Table 5.8 Precision of retention time of i-STD\(^1\) and flumetsulam retention time (min) ± % RSD

<table>
<thead>
<tr>
<th>Inter-day injections(^2)</th>
<th>i-STD</th>
<th>Flumetsulam</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>23.17 ± 0.45%</td>
<td>26.55 ± 0.36%</td>
</tr>
<tr>
<td>Day 2</td>
<td>23.35 ± 0.62%</td>
<td>26.69 ± 0.57%</td>
</tr>
<tr>
<td>Day 3</td>
<td>23.29 ± 0.51%</td>
<td>26.91 ± 0.41%</td>
</tr>
<tr>
<td>Mean</td>
<td>23.27 ± 0.39%</td>
<td>26.72 ± 0.68%</td>
</tr>
</tbody>
</table>

\(^1\)i-STD: internal standard, benzoic acid.
\(^2\)Calculated from 3 replicates.

The intra-day precision of the retention time is between 0.45% and 0.62% RSD (mean = 0.53%) for the i-STD benzoic acid and 0.36% and 0.57% RSD (mean = 0.45%) for flumetsulam. The inter-day precision of the retention times for the i-STD and the analyte are 0.39% and 0.68% respectively.

5.11.3.4 Linear range of calibration

The method was found to be linear within the range of 0.01 to 0.5 µg mL\(^{-1}\) of flumetsulam in standard solution. Table 5.9 presents the regression equations for flumetsulam in standard solution and in fortified soybean samples determined using the i-STD, benzoic acid. The R\(^2\) were 0.9997 or better with or without the use of the i-STD. The intercepts of the regression equations in the standard solution and soybean sample solutions are similar and not significantly different from zero and the intercepts are comparable.
Table 5.9 Regression equations for flumetsulam in standard solution and in soybean determined with the i-STD\(^1\)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Regression equation(^2)</th>
<th>Coefficient of determination (R(^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard solution</td>
<td>(y = 15.35x + 0.0492)</td>
<td>0.9997</td>
</tr>
<tr>
<td>Soybean sample</td>
<td>(y = 12.87x - 0.0259)</td>
<td>1.00</td>
</tr>
</tbody>
</table>

\(^1\)i-STD: internal standard, benzoic acid.

\(^2\)\(y = \text{peak area ratio [Flumetsulam:i-STD]}, x = \text{Concentration (µg mL}^{-1}\), m = \text{slope}, b = \text{intercept.}\)

5.11.3.5 Range of method

Figures 5.6 and 5.7 show that the regression equation of the calibration curves are linear from 0.01 to 0.5 µg mL\(^{-1}\) for the standard solution and between 0.02 to 0.1 µg g\(^{-1}\) of flumetsulam in soybean.

5.11.3.6 Limit of detection and limit of quantitation (LOD & LOQ)

The limit of detection (LOD) of flumetsulam, calculated as three times the standard deviation and the limit of quantitation (LOQ) as ten times the standard deviation of the lowest fortification level (0.02 µg g\(^{-1}\)) was found to be 0.0072 µg g\(^{-1}\) and 0.024 µg g\(^{-1}\) respectively. The LOD and LOQ are slightly lower if the i-STD is used. By comparison the MRL (1) for flumetsulam and is 0.05 µg g\(^{-1}\) for pulses.
5.11.3.7 Stability of standard and sample solutions

At room temperature, the sample and standard solutions in acetonitrile are stable for only three days. After one week, the chromatographic peak area diminishes by about 10%. If the solutions were stored in the refrigerator at −4 °C, the solutions are stable for at least one week.
5.12 Reference

CHAPTER 6

CONFIRMATION AND ANALYSIS OF

FLUMETSULAM USING LC-ESI-MS/MS
6.1  **INTRODUCTION AND PRINCIPLE OF LC-ESI-MS/MS METHOD**

The liquid chromatography-mass spectrometry (LC-MS) system can be used both as a qualitative and quantitative tool. The detection limits for quantitation are unsurpassed in mass spectrometry of amenable analytes when compared with other techniques. Significant information can also be gained about the structure of an organic molecule. The most popular LC-MS techniques are electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI), which is a softer ionisation than ESI and produces less fragmentation. Through the use of MS/MS, (Figure 6.1) fragmentation information can be obtained which will allow for a reasonable degree of structural determination.

**Figure 6.1 Top view of the Varian 1200L tandem mass spectrometer**
(From Varian Inc. 1200L Quadrupole MS product literature, 2003)
Because ESI and APCI are both softer ionisation techniques than the traditional electron impact (EI) MS, a significant amount of the ion current is concentrated in the ion that represents the intact molecule (often the protonated molecule). LC-MS analytes, as a group, are usually of a higher molecular weight (and hence less volatile) than GC-MS analytes. This lesser degree of fragmentation produced by APCI and ESI often allow for a lower limit of quantitation in LC-MS than can be obtained by EI GC-MS. This is especially true of APCI, which has a much lower degree of ionisation efficiency than does EI.

ESI generates ions directly from solution (usually an aqueous or aqueous/organic solvent system) by creating a fine spray of highly charged droplets in the presence of a strong electric field (typically 3.5 kV). As the droplet decreases in size, the electric charge density on its surface increases. The mutual repulsion between like charges on this surface becomes so great that it exceeds the forces of surface tension, and ions begin to leave the droplet through what is known as a "Taylor cone". The ions are then electrostatically directed into the mass analyser. Vaporisation of these charged droplets results in the production of singly or multiply-charged gaseous ions. The number of charges retained by an analyte can depend on such factors as the composition and pH of the electro-sprayed solvent as well as the chemical nature of the sample. Because mass spectrometers measure the mass-to-charge ($m/z$) ratio, the resultant ESI mass spectrum contains multiple peaks corresponding to the different charged states.
ESI allows for very sensitive analysis of small, large and labile molecules such as peptides, proteins, organometallics, oligosaccharides, and polymers. While past attempts to couple LC with mass spectrometry resulted in limited success, ESI has made LC-MS routine.

In contrast to EI, which produces many fragment ions, the new ionisation techniques are relatively gentle and do not produce a significant amount of fragment ions. To obtain more information on the molecular ions generated in the electrospray ionisation source, it has been necessary to apply techniques such as tandem mass spectrometry (MS/MS) to induce fragmentation. Tandem mass spectrometry allows one to induce fragmentation and mass analyse the fragment ions. This is accomplished by collisionally generating fragments from a selected ion and then mass analysing the fragment ions. Fragmentation can be achieved by inducing ion/molecule collisions by a process known as collision-induced dissociation (CID) (also known as collision-activated dissociation). CID is accomplished by selecting an ion of interest with a mass analyser and introducing that ion into a collision cell. The selected ion then collides with a collision gas (typically argon or helium) resulting in fragmentation. The fragments are then analysed to obtain a fragment ion spectrum. Tandem mass analysis is primarily used to obtain structural information and to provide an adequate amount of confirmatory ions.

In this study, a triple quadrupole LC-MS/MS by Varian was used. A triple quadrupole is also useful for differentiation of two molecules that have different structures and the same molecular weight, but give unique fragment ions upon collision. The triple quadrupole,
through the use of MS/MS, can give much better selectivity and sensitivity with samples with dirty or complex matrices. For improved sensitivity, selected ion monitoring (SIM) is a technique that uses only a single quadrupole for the mass filtering. MS/MS performed on a transmission quadrupole filters the desired precursor ion on the first quadrupole (Q1), allowing only that ion to travel to the second quadrupole (Q2) for the CID, and finally the mass filtering on the third quadrupole (Q3) of the product ions. While SIM can give better sensitivities in a clean sample or standards, MS/MS can give additional confirmation of the analyte identity and better sensitivities.

Background noise is reduced because the cell curvature positions the electron multiplier is off-axis from the source. This improves the sensitivity of the MS by improving the signal to noise. This is true for any operation mode, single or triple quadrupole.
If full scan spectra are recorded in single MS, a minimum of four ions must be present with a relative intensity of \( \geq 10\% \) of the base peak for confident identity confirmation (1). The molecular ion should be included if it is present in the reference spectrum, with relative intensity of \( \geq 10\% \). If LC-MS/MS selected reaction monitoring (SRM) technique is used and one precursor and two daughter ions are observed, this is equivalent to four identification points (1). However, in order to qualify for the identification points required for confirmation, a minimum of at least one ion ratio must be measured and a maximum of three separate techniques can be combined to achieve the same number of identification points. In this study, a LS-MS/MS identity confirmation method was developed as an independent means to analyse flumetsulam extracts at the lowest \((0.02 \, \mu\text{g g}^{-1})\) fortification level. Since most global regulatory agencies now require some form of identity confirmation, this confirmatory method was used to analyse flumetsulam in soybean.

### 6.1.1 Quadrupole

Quadrupoles are four precisely parallel rods with a direct current (DC) voltage and a superimposed radio-frequency (RF) potential. By scanning a pre-selected RF field one effectively scans a mass range.

Quadrupole mass analysers have been used in conjunction with electron ionisation sources since the 1950s and are the most common mass spectrometers in existence today. Quadrupoles have three primary advantages. First, they are tolerant of relatively poor vacuums \((\sim 5 \times 10^{-5} \text{ torr})\), which make them well suited to electrospray ionisation since the ions are produced under atmospheric pressure conditions. Secondly, quadrupoles are now capable of routinely analysing up to a \( m/z \) of 3000,
which is useful because electrospray ionisation of proteins and other biomolecules commonly produces a charge distribution below $m/z$ 3000. Finally, the relatively low cost of single quadrupole mass spectrometers makes them attractive as electrospray analysers. Considering these mutually beneficial features of electrospray and quadrupoles, it is not surprising that most of the successful commercial electrospray instruments thus far have been coupled with quadrupole mass analysers.

6.1.2 ION DETECTION

Once the ion passes through the mass analyser, the ion detector detects it, which is the final element of the mass spectrometer. The detector allows a mass spectrometer to generate a signal current from incident ions by generating secondary electrons, which are further amplified. Alternatively, some detectors operate by inducing a current generated by a moving charge. Among the detectors described, the electron multiplier (Varian) and scintillation counter are the most commonly used and convert the kinetic energy of incident ions into a cascade of secondary electrons.

6.1.3 ELECTRON MULTIPLIER

An electron multiplier is one of the most common means of detecting ions, achieving high sensitivity by extending the principle used with a Faraday cup. Whereas a Faraday cup uses one dynode, an electron multiplier is made up of a series of dynodes maintained at increasing potentials. Ions strike the dynode surface, resulting in the emission of electrons. These secondary electrons are then attracted to the next dynode where more secondary electrons are generated, ultimately resulting in a cascade of
electrons. Typical amplification or current gain of an electron multiplier is one million. A picture of the Varian electron multiplier is presented in Figure 6.2.

![Varian electron multiplier detector](From Varian Inc. 1200L Quadrupole MS product literature, 2003)

**Figure 6.2 Varian electron multiplier detector**

**6.2 APPARATUS**

The apparatus is as described in Chapter 4, section 4.2 (a) to (o).

**6.3 MATERIALS AND REAGENTS**

Reagents for HPLC analysis were HPLC grade; other chemicals were of analytical reagent (AR) grade or the highest purity available. Purified water (H₂O) was obtained from a Millipore ultra pure Milli-Q water purification system (NSW, Australia).

Refer to Chapter 4, section 4.3 (c) to (k).

(a) Reference standards. – Flumetsulam, standards grade (99% ± 0.5% purity) was purchased from Alltech Associates Pty. Ltd. (NSW, Australia).

**6.4 PREPARATION OF EXTRACTION AND PARTITIONING SOLVENTS AND CHROMATOGRAPHIC REAGENTS**

All pH adjustments were performed with a pH meter.

Details of pH meter described in Chapter 4, section 4.4 (a) to (f).

(a) 5% glacial acetic acid. – A 5% glacial acetic acid solution was prepared by diluting 200-mL of glacial acetic acid to 4-L with H₂O.
(b) **LC-ESI-MS mobile phase.** – 3400-mL 5% aqueous glacial acetic acid solution was combined with 600-mL acetonitrile (85 + 15, v/v) in a 4 L glass vessel and filtered through a 0.45-µm Nylon filter using an all glass LC solvent filtration unit.

### 6.5 Preparation of Glassware

The round-bottomed flasks, volumetric flasks, volumetric pipettes and glass vials were silanised as described in Chapter 4, section 4.5.

### 6.6 LC-MS Instrumentation

(a) **LC-MS system.** – Varian ProStar Model 210 solvent delivery module equipped with a Varian ProStar Model 410 auto injector with a 500-µL injector loop and Varian ProStar Model 500 column oven module from Varian Australia (NSW, Australia).

(b) **Electrospray ionisation (ESI) MS.** – Varian Model 1200L triple quadrupole MS with ESI ion source module and Varian rotary vane pumps Model DS-602 (NSW Australia).

(c) **Drying gas.** – Provided by a Domnic Hunter nitrogen generator (NSW, Australia).  
(d) **LC column.** – Phenomenex Synergi Fusion-RP C-18 column 150 x 4.6-mm id, 4-µm particle size from Phenomenex (NSW, Australia).

(e) **Data acquisition.** – Varian Prostar Microsoft Windows based software for data acquisition set up on a Dell PC and connected to a HP laser printer (NSW, Australia).

(f) **Infusion Pump.** – Harvard Apparatus Model 11 (NSW, Australia).
6.7 **LC CONDITIONS**

The injection volume was set at 50 µL. The HPLC mobile phase was 5% aqueous glacial acetic acid and acetonitrile (85 + 15, v/v). The flow rate was 0.25 mL min\(^{-1}\) with a linear concentration of 85:15 for 12 min. The mobile phase was pumped through isocratically at 0.25 mL min\(^{-1}\). The HPLC and MS conditions used are summarised in Table 6.1. A picture of the LC-MS system is presented in Figure 6.3.

![ProStar Binary 210 Solvent, ProStar 430 AutoSampler Module, 1200L LC-MS]

**Figure 6.3 Varian LC system coupled to the tandem MS**
(From Varian Inc. 1200L Quadrupole MS product literature, 2003)
Table 6.1 LC and MS operating conditions for the determination of flumetsulam

<table>
<thead>
<tr>
<th>LC operating conditions</th>
<th>MS operating conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile phase 5% glacial acetic acid solution and acetonitrile (85 + 15, v/v)</td>
<td>Ionisation mode Electrospray</td>
</tr>
<tr>
<td>Flow rate 0.25 mL min(^{-1})</td>
<td>Polarity Positive</td>
</tr>
<tr>
<td>Auto injector temperature 8 °C</td>
<td>Capillary voltage 70 V</td>
</tr>
<tr>
<td>Pre column filter Supelco 0.45 µm</td>
<td>Needle voltage 5000 V</td>
</tr>
<tr>
<td>Column temperature 30 °C</td>
<td>Drying gas N(_2)</td>
</tr>
<tr>
<td>Injection volume 50 µL</td>
<td>Drying gas temperature 300 °C</td>
</tr>
<tr>
<td>Analysis time 15 min</td>
<td>Drying gas pressure 1.05 x 10(^6) mTorr</td>
</tr>
<tr>
<td></td>
<td>Nebulizer gas N(_2)</td>
</tr>
<tr>
<td></td>
<td>Nebulizer gas pressure 2.76 x 10(^6) mTorr</td>
</tr>
<tr>
<td></td>
<td>Electrospray voltage 5 kV</td>
</tr>
<tr>
<td></td>
<td>Collision energy voltage -25 V</td>
</tr>
<tr>
<td></td>
<td>Electron multiplier voltage 1300 V</td>
</tr>
<tr>
<td></td>
<td>Shield voltage 600 V</td>
</tr>
<tr>
<td></td>
<td>Ion source pressure 3 mTorr</td>
</tr>
</tbody>
</table>

### 6.8 Preparation of Analytical Standard Solutions

#### 6.8.1 Preparation of Standard Stock Solutions

An individual stock solution of 1000 µg mL\(^{-1}\) flumetsulam was prepared by weighing 10 mg of the standard into a 10 mL volumetric flask and making up to the mark with acetonitrile. Where applicable, the amount of analyte was adjusted according to the stated purity of the standard material. This stock solution was used for preparing the fortification and calibration standard solutions. This stock solution was stored in a refrigerator at −4 °C and prepared freshly every month.
6.8.2 Preparation of Fortification Standard Solution

A 10 µg mL\(^{-1}\) flumetsulam standard solution was prepared by diluting 1 mL of the 1000 µg mL\(^{-1}\) stock solution to 100 mL with acetonitrile.

These standards were stored in a refrigerator at –4 °C and prepared freshly every week.

6.8.3 Preparation of Calibration Standard Solutions

Individual standard solutions containing 0.02, 0.05, 0.1, 0.5 and 1 µg mL\(^{-1}\) of flumetsulam were prepared by transferring 0.2, 0.5, 1, 5 and 10 mL respectively of the 10 µg mL\(^{-1}\) standard into five 100 mL volumetric flasks and making up to volume with the LC mobile phase.

These calibration standards were stored in a refrigerator at –4 °C and prepared freshly every week.

6.9 Sample Preparation

The soybean sample was prepared as described in Chapter 4, section 4.9.

6.10 Fortification, Fractionation and Extraction of Flumetsulam

6.10.1 Fortification of Soybean

The 10 µg mL\(^{-1}\) standard was used for fortifying 10 g of ground soybean to give 0.05 and 0.1 µg g\(^{-1}\) fortification levels respectively. Three portions of 10 g of the ground
soybean were weighed into separate 500 mL Shott bottles and fortified with flumetsulam to give the desired fortification concentrations. The flumetsulam fortification levels of 0, 0.05 and 0.1 µg g⁻¹ were prepared by adding 0, 50 and 100 µL of the 10 µg mL⁻¹ flumetsulam standard to the soybean samples. Each fortification level was carried out in triplicate. After fortification, the solvent was allowed to evaporate for at least 10 min.

6.10.2 Methanol extraction

To each fortified sample and blank was added 100 mL acidified methanol. The bottle was capped and placed on a shaker set at 250 rpm and 60 °C for 15 min. The mixture was allowed to rest for at least 15 min before decanting the methanol supernatant into a clean 250 mL conical flask. The sample was re-extracted with another 100 mL acidified methanol, and the extracts combined and vacuum filtered through a 0.45 µm Nylon filter. The filtrate was transferred to a 250 mL round bottomed flask and the volume reduced to approximately 20 mL using a rotary vacuum evaporator set at 60 °C. The methanol concentrate was then transferred to a 50 mL separatory funnel and extracted with 3 x 20 mL hexane. The hexane was discarded. Any emulsion formed was broken by stirring with a glass rod. The methanol was transferred to a 100 mL round bottomed flask and evaporated to dryness on a rotary vacuum evaporator set at 60 °C.

The above procedure is summarised as a flow chart in Figure 6.4.
Figure 6.4 Flowchart of extraction of flumetsulam from soybean

6.10.3 Fractionation of extract

15 mL pH 7.0 phosphate buffer was added to dissolve the residue from section 6.10.1 and transferred to a 100 mL separatory funnel. The procedure was repeated with a fresh 15 mL buffer to ensure that all the residue is transferred. 10 mL of saturated NaCl was added to the separatory funnel, and the mixture extracted with 3 x 40 mL of dichloromethane. The dichloromethane was discarded. The aqueous layer was acidified with orthophosphoric acid to pH 2.2 and extracted with 3 x 50 mL dichloromethane. The aqueous layer was discarded, and the combined dichloromethane reduced to 20 mL on a rotary evaporator set at 60 °C. The dichloromethane concentrate was partitioned with 3 x 20 mL pH 7.0 phosphate buffer and the dichloromethane layer discarded. The aqueous layer was adjusted to pH 2.2 with orthophosphoric acid and extracted with 3 x 60 mL dichloromethane. The
aqueous layer was discarded and the combined dichloromethane extract washed with 100 mL H₂O. The dichloromethane layer was then dried over Na₂SO₄ and gravity filtered to remove the Na₂SO₄. The dichloromethane was then placed in a 250 mL round bottomed flask and evaporated to dryness on a rotary evaporator set at 60 °C.

The residue was dissolved in 2 mL fresh dichloromethane and transferred to a 8 mL silanised glass vial. The procedure was repeated with two more volumes of 2 mL dichloromethane and added to the glass vial before evaporating to dryness under a stream of nitrogen gas. The residue was dissolved in 1 mL of mobile phase with the aid of a vortex mixer and filtered through a 0.45 µm Nylon syringe filter for LC analysis.

6.10.4 C-18 CLEAN UP PROCEDURE

If additional clean up is required as evidenced by the blank chromatogram, an additional SPE clean up step may be incorporated. A C-18 cartridge was conditioned with 2 mL each of methanol, H₂O and methanol again, before adding 2 mL 50:50 dichloromethane:hexane. The dichloromethane residue from section 6.10.3 was dissolved in 5 mL dichloromethane:hexane (50:50) and transferred to the preconditioned cartridge. The cartridge was washed with 2 x 2 mL hexane and 2 x 2 mL EtOAc:hexane (10:90). The analyte was eluted with 2 x 2 mL dichloromethane and collected in a 8 mL sample vial. This eluate was evaporated to dryness under a gentle stream of nitrogen gas and the residue dissolved in 1 mL of mobile phase.

A flow chart summarising the extraction and clean up of flumetsulam is presented in Figure 6.5 and described in sections 6.10.3 and 6.10.4.
Chapter 6: LC-ESI-MS/MS

Add pH 7 buffer to the residue containing flumetsulam

Add sat. NaCl and partition with DCM

Discard DCM

Acidify aqueous phase and add sat. NaCl & partition with DCM

Discard aqueous phase

Reduce DCM volume and partition with pH 7.0 buffer

Discard DCM phase

Acidify aqueous phase & partition with DCM

Discard aqueous phase

Wash DCM layer with water and discard water

Dry DCM layer with Na₂SO₄

Discard Na₂SO₄

Evaporate DCM layer to dryness

If necessary load onto C-18 cartridge and elute with DCM

Dissolve residue in DCM and transfer to 8 mL sample vial

Evaporate to dryness and dissolve in mobile phase

Filter for LC/MS analysis

Figure 6.5 Flow chart of fractionation of flumetsulam from soybean
6.10.5 Determination of MS voltages by direct infusion

In direct infusion operation, a syringe pump is used to continuously deliver a solution of the pure standard for ESI analysis. The solvent used should match what will be entering the interface in the actual analysis. A 0.1 µg mL⁻¹ flumetsulam standard prepared in mobile phase, was used to fill a 100 µL syringe. The syringe was inserted into the syringe pump, which was set to deliver 10 µL min⁻¹. During the direct infusion, the position of the corona needle was adjusted vertically and horizontally for highest sensitivity. After 10 min, the optimum quadrupole voltages were determined by automatic voltage scanning and the detector ready for the analysis. A schematic diagram of the mass analyser with the voltages is presented in Figure 6.6.

The syringe pump used for direct infusion can be used with syringe sizes of 0.5 µL-140 mL. The flow rate can be between 0.0014 µL hr⁻¹ and 26.56 mL min⁻¹. It has an accuracy of 1% and reproducibility of 0.1%.
Figure 6.6 Schematic diagram of mass analyser with voltages shown for flumetsulam

6.11 RESULTS AND DISCUSSION

The analytical method developed for the determination of flumetsulam using solvent-solvent extraction with LC-MS/MS analysis was validated using the validation parameters described in Chapter 1.

Triplicate samples of 10 g soybean were fortified with flumetsulam to give concentrations of 0.05 and 0.1 µg g⁻¹.

6.11.1 RECOVERY STUDIES FOR FLUMETSULAM

Standard solutions containing 0.02, 0.05, 0.1, 0.5 and 1 µg g⁻¹ flumetsulam in mobile phase were prepared. Three replicate injections for each concentration were performed. The absolute recoveries were calculated.

6.11.1.1 Absolute recoveries of flumetsulam

6.11.1.1.1 Calibration curve

Table 6.2 presents the mean peak areas for flumetsulam for each of the four standards. The flumetsulam calibration curve is a plot of flumetsulam concentration versus the mean peak area of flumetsulam.
Table 6.2 Flumetsulam standard calibration values and RSDs

<table>
<thead>
<tr>
<th>Flumetsulam (µg mL(^{-1}))</th>
<th>Mean peak area(^1) x 10(^6)</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02</td>
<td>4.368</td>
<td>4.1</td>
</tr>
<tr>
<td>0.05</td>
<td>10.392</td>
<td>3.2</td>
</tr>
<tr>
<td>0.1</td>
<td>21.284</td>
<td>2.5</td>
</tr>
<tr>
<td>0.5</td>
<td>109.874</td>
<td>1.3</td>
</tr>
<tr>
<td>1</td>
<td>214.042</td>
<td>0.8</td>
</tr>
</tbody>
</table>

\(^1\)Calculated from 3 replicate injections.

The flumetsulam standard calibration curve determined by LC-MS was linear from 0.02 to 1 µg mL\(^{-1}\) with a coefficient of determination (R\(^2\)) of 0.9998.

6.11.1.1.2 Recoveries for flumetsulam

The concentrations of flumetsulam in fortified soybean were calculated from the mean peak area of three replicate extractions and analyses at fortification levels of 0.05 and 0.1 µg g\(^{-1}\). The equation of the calibration curve for 0.05 and 0.1 µg g\(^{-1}\) fortification levels is given in Figure 6.7. Flumetsulam fortification levels of 0.05 and 0.1 µg g\(^{-1}\) were prepared and three replicate injections for each concentration were performed.

The recoveries obtained are presented in Table 6.3.
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Figure 6.7 Flumetsulam calibration curve determined by LC-MS

Table 6.3 Recoveries for flumetsulam determined by LC-MS

<table>
<thead>
<tr>
<th>Fortification level (µg g⁻¹)</th>
<th>% Recovery¹</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>80.6</td>
<td>13.6</td>
</tr>
<tr>
<td>0.1</td>
<td>89.0</td>
<td>9.1</td>
</tr>
</tbody>
</table>

Mean 84.8 11.35

¹Calculated from 3 replicate fortified samples.

The results show that the method has good and reproducible recoveries. The recoveries are 80.6 ± 13.6% RSD and 89.0 ± 9.1% RSD for the 0.05 µg g⁻¹ and 0.1 µg g⁻¹ fortification levels respectively.
6.11.2 SELECTIVITY

Other sources of soybean show an essentially similar blank. The chromatograms of a blank soybean extract and flumetsulam fortified soybean are presented in Figures 6.12 and 6.13 respectively (pages 166-167). The analyte peak is well resolved from the co-extractive peaks. These co-extractives can be present at higher concentrations and still be resolved from the analyte peak.

6.11.3 PRECISION OF ANALYSIS

6.11.3.1 Intra-day precision of replicate injections of one extract

Four injections of the soybean extract fortified with 0.05 µg g⁻¹ flumetsulam were performed on the same day to obtain the instrumental precision or the instrumental contribution to the overall (instrumental plus extraction) precision.

Table 6.4 presents the intra-day results obtained. The instrumental precision is quite good at 1.7%.

<table>
<thead>
<tr>
<th>Injection number</th>
<th>Peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>93.658</td>
</tr>
<tr>
<td>2</td>
<td>95.369</td>
</tr>
<tr>
<td>3</td>
<td>94.986</td>
</tr>
<tr>
<td>4</td>
<td>97.573</td>
</tr>
</tbody>
</table>

Mean \( 95.397 \pm 1.63 \) (1.7% RSD)
6.11.3.2 Inter-day precision of replicate injections of one extract

Four injections of soybean extract fortified with 0.05 µg g\(^{-1}\) of flumetsulam were performed over a three-day period. Table 6.5 presents the inter-day precision of three injections for the three days.

The instrument precision calculated from the flumetsulam peak area is between 1.1% and 1.7% (mean = 1.4%).

Table 6.5 Inter-day precision of flumetsulam peak area

<table>
<thead>
<tr>
<th>Inter-day injections</th>
<th>Peak area(^1) ± RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>95.397 ± 1.7%</td>
</tr>
<tr>
<td>Day 2</td>
<td>92.671 ± 1.1%</td>
</tr>
<tr>
<td>Day 3</td>
<td>89.919 ± 1.5%</td>
</tr>
<tr>
<td>Mean ± RSD</td>
<td>92.66 ± 2.96%</td>
</tr>
</tbody>
</table>

\(^1\)Calculated from 4 replicates.

6.11.3.3 Precision of retention time

In this part of the work, identification of the analyte is achieved by LC-MS/MS as well as comparison with the retention time of a known standard. A standard of flumetsulam was injected into the LC-MS for four days with five injections per day. Table 6.6 lists the retention times of flumetsulam for twenty successive injections over a four-day period.

The intra-day precision of the retention time is between 0.3 to 0.5% (mean = 0.45%) for the flumetsulam. The mean inter-day precision of the retention time for flumetsulam is 1.5%.
Table 6.6 Precision of retention times of flumetsulam determined by LC-MS

<table>
<thead>
<tr>
<th>Intra-day injections(^1)</th>
<th>Retention time (min)</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Mean</th>
<th>Inter-day mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>11.42</td>
<td>11.45</td>
<td>11.51</td>
<td>11.43</td>
<td>11.42</td>
<td>11.53 ± 1.5%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.51</td>
<td>11.44</td>
<td>11.32</td>
<td>11.38</td>
<td>11.35</td>
<td>11.36 ± 0.5%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>Mean</td>
<td>Mean</td>
<td>Mean</td>
<td>Mean</td>
<td>11.55 ± 0.5%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.43</td>
<td>11.38</td>
<td>11.48</td>
<td>11.62</td>
<td>11.59</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.42</td>
<td>11.35</td>
<td>11.48</td>
<td>11.74</td>
<td>11.69</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.45 ± 0.3%</td>
<td>Mean</td>
<td>11.36 ± 0.5%</td>
<td>Mean</td>
<td>11.55 ± 0.5%</td>
<td>Mean</td>
</tr>
</tbody>
</table>

\(^1\)Calculated from 5 replicates.

6.11.4 Linearity range of calibration

Table 6.2 and Figure 6.7 show the LC-MS method to be linear between 0.02 and 1 µg mL\(^{-1}\) of flumetsulam in soybean. Table 6.7 presents the regression equations for flumetsulam standard solution and in soybean samples. The R\(^2\) for the standard and sample curves were 0.9981 and 0.9998 respectively.
Table 6.7 Regression equations for flumetsulam in standard solution and in soybean

<table>
<thead>
<tr>
<th>Sample</th>
<th>Regression equation(^1)</th>
<th>Coefficient of determination (R^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard solution</td>
<td>y = 214.84x + 0.2356</td>
<td>0.9998</td>
</tr>
<tr>
<td>Soybean sample</td>
<td>y = 211.37x + 0.0174</td>
<td>0.9981</td>
</tr>
</tbody>
</table>

\(^1\) y = Peak area, x = concentration (µg mL\(^{-1}\)), m = slope, b = y-axis intercept.

The intercepts of the regression equations in standard and soybean sample solutions are similar and not significantly different from zero, showing that the soybean is free of flumetsulam before the standard addition and that there is no contribution from co-eluting substances.

The slopes of the regression equations are comparable, indicating that quantitation using a standard curve will yield a similar result to that obtained by the method of standard addition, and that the matrix is not significantly affecting the quantitation of the analyte.

6.11.5 **Range of method**

Table 6.2 and Figure 6.7 show that the method is linear from 0.02 to 1 µg mL\(^{-1}\) for the standard solutions and from 0.05 to 0.1 µg g\(^{-1}\) for flumetsulam in soybean.
6.11.6 LIMIT OF DETECTION AND LIMIT OF QUANTITATION (LOD & LOQ)

The limit of detection (LOD) of flumetsulam, calculated as three times the standard deviation, and the limit of quantitation (LOQ) as ten times the standard deviation of the lowest fortification level (0.05 µg g⁻¹), were found to be between 0.017 and 0.055 µg g⁻¹ respectively. By comparison the MRL (2) for flumetsulam is 0.05 µg g⁻¹ for pulses. The MRL was set at the lowest LOD previously available.

6.11.7 STABILITY OF STANDARD AND SAMPLE SOLUTIONS

At room temperature, the sample and working standard solutions in acetonitrile or mobile phase are stable for one week. After one week, the chromatographic peak area diminishes by about 10% and multiple peaks appear around the region of the analyte peak. The appearance of multiple peaks is more noticeable in the sample solution than the standard solution. Therefore, all sample and standard solutions should be stored in a refrigerator at –4 °C and analysed within one week.
6.12 MASS SPECTROMETRY RESULTS AND DISCUSSION

A tandem mass spectrometer can be thought of as two mass spectrometers in series connected by a chamber that can break a molecule into pieces. This chamber is known as a collision cell. A sample is “sorted” and “weighed” in the first mass spectrometer, then broken into pieces in the collision cell, and a piece or pieces sorted and weighed in the second mass spectrometer. For highest sensitivity, optimum voltages of the quadrupoles need to be determined. Therefore, by use of direct infusion of the standard into the MS, the instrument can determine the optimum voltages for highest sensitivity in detecting the analyte.

SIM is a technique that uses only a single quadrupole for the mass filtering. MS/MS performed on a transmission quadrupole, filters the desired precursor ion on Q1, allowing only that ion to travel to Q2 for the CID, and finally the mass filtering on Q3 of the product ions. While SIM can give better sensitivities in a clean sample or standards, MS/MS can give additional confirmation of the analyte identity and better sensitivities in samples with dirty or complex matrices. Therefore, MS/MS was used for this work, since the soybean matrix is complex. The long collision cell is curved and without lenses for wide CID energy range and for low noise and high sensitivity. The collision cell also removes neutrals from the CID process and promotes efficient dissociation of stable ions while decreasing ion losses.
6.12.1 **Scan Modes**

Several scan modes were tried. The first scan mode was full scan, which provided the fragmentation pattern of flumetsulam and gave structural confirmation. Full scan mode allows the RF/DC quadrupole filter to be scanned. This may cover part or all of the \( m/z \) range of the MS. Triple quadrupole MS has the advantage (compared to a single quadrupole) of either using Q1 or Q3 to scan as presented in Figure 6.8. The second experiment was SIM, which scanned for only the parent ion \([M+H]^+\). This gave higher sensitivity than full scan but with less spectral information. The SIM mode allows the RF/DC quadrupole filter to be set at one (or more) RF/DC settings to filter for a specific \( m/z \) value(s).

6.12.2 **Qualitative Results**

Experiments such as product, precursor and neutral loss scans can be performed on the instrument used. In product ion scan, the precursor ion is focussed in the first mass analyser and transferred into the collision cell where it interacts with a collision gas and fragments. The fragments are then measured by scanning the second mass analyser. This results in a typical MS/MS spectrum and is the method most commonly employed with ESI ionisation LC-MS.

In precursor ion scan, the first \( m/z \) analyser is set to allow each ion of successive \( m/z \) values pass into the collision cell where they are dissociated. The second \( m/z \) analyser is set to a single \( m/z \) value so that only a dissociation fragment of a given mass-to-charge ratio can pass to the detector.
In neutral loss scan, the two mass analysers are scanned at the same rate, however, the second analyser is offset by a fixed $m/z$ value from the first. In this way, only ions that have a specific $m/z$ loss in the collision cell will be passed by the second analyser and recorded. This mode is more useful for EI and CI ionisation. Neutral loss scan is useful to identify common function groups for several analytes. Figure 6.8 summarises the three different experiments (3).

![Figure 6.8 Schematic representation of the types of qualitative MS/MS analyses](image)

The results from the above experiments gave sufficient results for qualitative analysis and initial structural confirmation. The above experiments were performed and the results are summarised in Table 6.8.
### Table 6.8 Qualitative MS results from the product ion scan

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Ion range or selected ion (m/z)</th>
<th>Ion selected for CID (m/z)</th>
<th>Daughter ion(s) (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Product ion scan</td>
<td>326 [M+H]^+ 348 [M+Na]^+</td>
<td>326</td>
<td>129, 262</td>
</tr>
<tr>
<td>Precursor ion scan</td>
<td>80-450</td>
<td>All</td>
<td>129</td>
</tr>
<tr>
<td>Neutral loss scan</td>
<td>80-450</td>
<td>All offset</td>
<td>197</td>
</tr>
</tbody>
</table>

In the product ion scan, the parent ion was selected for CID then the second mass analyser was set to scan for the daughter ions which are given in Table 6.8. In the precursor scan, a range was selected for scanning by the first mass analyser, in which the generated ions were put through the collision cell, but only one daughter ion was selected to pass to the detector by the second mass analyser. In the neutral loss scan, only those ions that exhibit this \( m/z \) loss are selected and detected by scanning both the first and second mass analysers simultaneously with a 129-\( m/z \) unit offset for the second mass analyser.

In this study quantitation was carried out using multiple reaction monitoring (MRM). MRM mode is a MS/MS technique that is identical to SRM but is performed on multiple precursor ions. The first MS cycle filtered for the first precursor ion and measured the related product ion(s), then the second MS cycle filtered for the second precursor ion and measure the related product ion(s). Four precursor ions is the limit because of cycle time and sensitivity. MRM is not as sensitive as SIM but it is more specific to the analyte especially in a complex matrix like soybean. Prior to MRM, a full scan experiment was carried out. The full scan and the MRM mass spectra are given in Figures 6.9 and 6.10 respectively.
Figure 6.9 Full scan mass spectrum of a 0.05 µg mL$^{-1}$ flumetsulam standard

Figure 6.10 Multiple reaction monitoring of a 0.05 µg mL$^{-1}$ flumetsulam standard
Chapter 6: LC-ESI-MS/MS

The full scan mass spectrum is relatively rich in ions with abundances of \( \geq 15\% \). Generating structural information is the main aim to applying ESI for residue work. Identification cannot be based solely on the presence of the \([M+H]^+\) ion. In ESI, CID to yield product ions can occur either in the transport region (source CID) or in the Q2 in a triple quadrupole MS/MS.

6.12.2.1 LC-ESI-MS/MS confirmation

The positive ion ESI-MS/MS conditions for the identity confirmation of flumetsulam in fortified soybean are summarised in Table 6.1. The retention time for flumetsulam in the standard and sample were 11.48 min and 11.41 min respectively. Under the MS conditions used, the \( m/z \)'s of 129 (60\%), 197 (26\%), 262 (36\%), 326 (100\%) and 348 (90\%) were observed for the standard and \( m/z \)'s of 129 (58\%), 197 (20\%), 262 (34\%), 326 (100\%) and 348 (86\%) were obtained for the sample. The relative abundances of each ion are listed parenthetically next to the ion. The \( m/z \) of 326 is the \([M+H]^+\) ion, the \( m/z \) of 348 is the \([M+Na]^+\) ion and the \( m/z \) of 129 is the protonated \( N-(2,6\)-difluorophenyl) ion. Representative full scan total ion chromatograms of the pure standard and unfortified soybean (blank) sample are presented in Figures 6.11 and 6.12 respectively. A representative full scan total ion chromatogram and its corresponding mass spectrum for a 0.05 \( \mu g \) g\(^{-1}\) fortified soybean sample are presented in Figures 6.13 and 6.14 respectively. The ‘Nitrogen Rule’ states that “single protonated molecules (the type of positive ions that are often encountered in LC-MS) that have an odd number of nitrogen atoms have an even nominal mass”, hence flumetsulam has five nitrogen atoms and the observed \( m/z \) ion was 326, therefore the ‘Nitrogen Rule’ provides additional evidence to confirm that the \([M+H]^+\) parent ion is present.
Figure 6.11 Total ion chromatogram of 0.05 µg mL⁻¹ flumetsulam standard

Figure 6.12 Total ion chromatogram of extract of soybean blank
Figure 6.13 Total ion chromatogram of extract of soybean fortified at 0.05 µg g\(^{-1}\)

Figure 6.14 Mass spectrum of extract of soybean fortified at 0.05 µg g\(^{-1}\)
During positive ion electrospray MS, all soybean extracts and flumetsulam standards formed an abundant \([\text{M+Na}]^+\) ion and potassium adducts. The abundant \([\text{M+Na}]^+\) ions were investigated as possible precursors for MRM during LC-MS/MS but were too stable to produce useful product ions during CID. Furthermore, the relative amounts of \([\text{M+Na}]^+\) and \([\text{M+H}]^+\) species were variable depending on the trace amounts of sodium in the sample or mobile phase.

### 6.12.2.2 LC-ESI-MS/MS quantitation

LC-ESI-MS/MS enables the monitoring of prominent product ions formed during multiple reactions. The first mass analyser was set to pass only the \([\text{M+H}]^+\) ion. This is subject to CID in the 180-degree collision cell and the fragments produced are then passed along the second mass analyser. The product ions are sequentially passed along to the detector set in the MRM mode. A response is registered by the detector when the analyte with the correct ion mass elutes from the LC system. For flumetsulam quantitation by LC-ESI-MS/MS, the \(m/z\) ions used were 129, 197 and 262 and were observed in the mass spectrum in Figure 6.15. Table 6.9 presents the MRM experiments of the \([\text{M+H}]^+\) ion to the product ions.

<table>
<thead>
<tr>
<th>Analyte (MW)</th>
<th>Precursor ion ((m/z))</th>
<th>Product ion ((m/z))</th>
<th>Capillary voltage (V)</th>
<th>CID collision voltage (eV)</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flumetsulam (325)</td>
<td>326</td>
<td>129</td>
<td>62</td>
<td>-25</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>326</td>
<td>197</td>
<td>70</td>
<td>-30</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>326</td>
<td>262</td>
<td>70</td>
<td>-25</td>
<td>4</td>
</tr>
</tbody>
</table>
Figure 6.15 Mass spectrum of MS/MS experiment showing the formation of the three daughter ions from the \([M + H]^+\) parent ion.
6.12.2.3 Proposed fragmentation mechanism

The proposed structures of the observed m/z ions in the mass spectrum are given in Figure 6.16.

Figure 6.16 Proposed fragmentation mechanism for the m/z structures observed
The ion fragments produced from ESI-MS/MS occurs at those points were bond breakage is more favourable. The $m/z$ of 262 may arise because organic molecules favour the loss of inorganic functional groups such as $\text{H}_2\text{O}$, $\text{CO}_2$, CO, and, in this case, $\text{SO}_2$ (4). After the loss of the $\text{SO}_2$, the remaining mass ion is $m/z$ 262. A proposed mechanism (5) for its formation is given below in Figure 6.17.

![Proposed Mechanism](image)

**Figure 6.17 Proposed mechanism of the formation of $m/z$ 262, daughter ion**

Attack of the sulfonamide nitrogen on the carbon at position 2 of the triazolopyrimidine leads to the formation of the intermediate complex (I). Collapse of this tricyclic intermediate and expulsion of the $\text{SO}_2$ follows, leaving the resultant $m/z$ structure (II), which is observed in the mass spectrum as the charged species as presented in Figure 6.18.
Figure 6.18 Structure of m/z 262 ion
6.13 REFERENCES


CHAPTER 7

CONCLUSIONS
A method of the analysis for the herbicide flumetsulam using two different methods of extraction and clean up (solvent-solvent extraction and solid phase extraction) and two different chromatographic systems (LC-UV and LC-ESI-MS/MS) have been developed and validated.

The solvent-solvent extraction method was used for the determination flumetsulam at fortification levels of 10, 20, 50 and 100 ng (g soybean)$^{-1}$. The solvent-solvent partitioning method for flumetsulam uses the internal standard (i-STD) benzoic acid and the surrogate standard (s-STD) diclosulam for quantitation. The flumetsulam method has good recoveries with a mean recovery of 80.0 ± 16.1% (range 71.8 to 91.7%) without the use of i-STD or s-STD, 78.2.3 ± 14.7% (range 70.1 to 85.1%) with the use of i-STD only, 88.4 ± 10.2 (range 80.7 to 93.9%) with the use of s-STD only and 79.9 ± 15.5% (range 70.4 to 92.9%) with the use of both the i-STD and s-STD. The use of the s-STD significantly improves the recovery by an absolute value of 8%. Clearly, the use of an s-STD to compensate for analyte losses during the extraction process is worthwhile, but if either the i-STD or s-STD cannot be used, for whatever reason, satisfactory recoveries and precision are still possible.

The limit of detection (LOD) and limit of quantitation (LOQ) of flumetsulam, taken as three times and ten times the standard deviation are 5 and 15 ng g$^{-1}$ respectively (calculated from the lowest fortification level of 10 ng (g of soybean)$^{-1}$.

The solid phase extraction (SPE) method uses a C-18, SAX and SCX cartridge in series for extract clean up. The solid phase extraction method used for flumetsulam at fortification levels of 20, 50 and 100 ng (g soybean)$^{-1}$ gave good recoveries with a
mean recovery of 85.2 ± 10.9% (range 78.5 to 94.2%) without the use of the i-STD and 83.8 ± 9.2% (range 81.3 to 89.5%) with the use of the i-STD. The LOD and LOQ are 7.2 ng g\(^{-1}\) and 24 ng g\(^{-1}\) respectively (calculated from the lowest fortification level of 20 ng g\(^{-1}\) of soybean).

The recommended maximum residue limit (MRL) for flumetsulam is 50 ng g\(^{-1}\) for pulses. By comparison the solvent-solvent extraction method developed has a LOD and LOQ of 5 and 15 ng g\(^{-1}\) respectively and the SPE method has a LOD and LOQ of 7 and 24 ng g\(^{-1}\) respectively. The methods are thus sufficiently sensitive for the detection of this substance in soybean. The method is also rugged as changes in environment and batches of reagents have minimal effect on the results.

The estimated time per analysis is approximately 2 h for the solvent-solvent extraction method. This includes the sample grinding, extraction and purification steps, which takes about 1.30 h followed by 0.35 h for the HPLC analysis. The solid phase extraction method takes the same amount of time but it gives a more efficient clean up process resulting in cleaner chromatograms. The s-STD was not used for the SPE method because irreversible retention on the SAX cartridge was observed.

A positive ion LC-ESI-MS/MS confirmation method was developed for flumetsulam in soybean. Under the MS conditions used, the \(m/z\)'s of 326 (100%), 348 (90%) and 129 (60%) were observed for the standard and \(m/z\)'s of 326 (100%), 348 (86%) and 129 (58%) for the sample. The \(m/z\) of 326 is the [M+H]\(^+\) ion, the \(m/z\) of 348 is the [M+Na]\(^+\) ion and the \(m/z\) of 129 is the protonated \(N- (2,6\text{ difluorophenyl})\) ion. The method demonstrated good recoveries (81-89%), good precision (9-13%), good
sensitivity (LOD: 17 ng g\(^{-1}\)) and ability to confirm the presence of flumetsulam. The work presented has been accepted for publication (1).

Future investigations could include:

- Application of the proposed method for other seeds, oils and plants.
- Application of the method for other sulfonanilide herbicides.
- Development of a simultaneous method of analysis for flumetsulam and other sulfonanilide herbicides.
- Search for a suitable s-STD for the SPE method or find an eluent that is able to elute diclosulam from the SAX cartridge.
REFERENCE

**Appendices**

**1H NMR spectrum (CD$_3$)$_2$SO**: $\delta$ 10.83 (s, 1H), 9.37 (d, 1H, $J = 6.8$ Hz), 7.46 (d, 1H, $J = 7.2$ Hz), 7.38 (s, 1H), 7.11 (t, 2H, $J = 8.0$ Hz), 2.68 (s, 3H).

Appendix I. NMR spectrum of flumetsulam (Varian NMR 400 MHz)

**1H NMR spectrum (CD$_3$)$_2$SO**: $\delta$ 11.06 (s, 1H), 7.51 (d, 2H, $J = 7.2$ Hz), 7.36 (s, 2H), 4.69 (q, 2H, $J = 7.2$ Hz, $J = 14.4$ Hz), 1.46 (t, 3H, $J = 7.2$ Hz).

Appendix II. NMR spectrum of diclosulam (Varian NMR 400 MHz)
Appendix III. Infrared spectrum of flumetsulam run as a KBr pellet
Appendix IV. UV spectrum of 0.003 M flumetsulam in MeOH
Appendix V. Certificate of analysis for flumetsulam
Appendix VI. Certificate of analysis for diclosulam

CERTIFICATE OF ANALYSIS

INVOICE #: CS245718
PO #: 74615

CATALOG #: PS-2208
DESCRIPTION: Diclosulam

LOT #: 298-118B
PURITY: 98%
EXPIRATION DATE: 02/07

Chem Service, Inc. guarantees the purity of this chemical ± 0.5% deviation prior to the expiration date shown on the label and exclusive of any customer contamination.

Two or more of the following methods of analysis are used to determine purity: Melting point, refractive index, titration, FTIR, IR, TLC, GC/FID, GC/TCD, GC/ECD, GC/MS, HPLC or DSC.

Our standards are suitable for use with all EPA methods.

Certified By:

John Conrad
CSM/TC

---

Appendix VI. Certificate of analysis for diclosulam
Appendices

Chem Service, Inc.

MATERIAL SAFETY DATA SHEET

PS-2111

Alltech Associates (Aust) P/L
P.O. Box 6005, Baulkham Hills, NSW 2153
P.O. Box 599, West Chester, PA 19381  (610)-692-3026

SECTION 1 - CHEMICAL PRODUCT and COMPANY IDENTIFICATION

Catalog Number: PS-2111
Description: Flumetsulam
Other Name(s): 2’,6’-Difluoro-5-methyl[1,2,4]triazolo[1,5-a]pyrimidine-2-sulfonanilide

Supplied by CHEM SERVICE, Inc. PO BOX 599, WEST CHESTER, PA 19381  (610)-692-3026
EMERGENCY PHONE: 1-610-692-3026

SECTION 2 - COMPOSITION, INFORMATION ON INGREDIENTS

CAS No.: 99447-46-9
Description: Flumetsulam
HNESCS No.: Not Available
Hazard Symbols: Not Available

SECTION 3 - HAZARDS IDENTIFICATION

Contact lenses should not be worn in the laboratory.
All chemicals should be considered hazardous - Avoid direct physical contact!
May be harmful by inhalation, ingestion, or skin absorption.
Overall chemical, physical and toxic data has not been thoroughly investigated.

SECTION 4 - FIRST AID MEASURES

An antidote is a substance intended to counteract the effect of a poison. It should be administered only by a physician or trained emergency personnel. Medical advice can be obtained from a POISON CONTROL CENTER.

In case of contact: Flush eyes continuously with water for 10-20 minutes. Flush skin with water for 15-20 minutes. If no burns have occurred, use soap and water to cleanse skin.
If inhaled remove patient to fresh air. Administer oxygen if patient is having difficulty breathing. If patient has stopped breathing administer artificial respiration.
If patient is in cardiac arrest administer CPR.
Continue life supporting measures until medical assistance has arrived.
If patient is exhibiting signs of shock - Keep warm and quiet.
Contact Poison Control Center immediately if necessary.
Do not administer liquids or induce vomiting to an unconscious or convulsing person.
If patient is vomiting-watch closely to make sure airway does not become obstructed by vomit.
If swallowed, rinse out mouth with water, providing the person is conscious.
Get medical attention if necessary. Remove and wash contaminated clothing.

SECTION 5 - FIRE and EXPLOSION DATA

Flash Point: 95+ C  This is a combustible compound.
Extinguishing Media:
Water spray, carbon dioxide, dry chemical powder, alcohol or polymer foam.

Appendix VII. MSDS for flumetsulam
Appendix VIII. MSDS for diclosulam

<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SECTION 1 - CHEMICAL PRODUCT and COMPANY IDENTIFICATION</strong></td>
<td></td>
</tr>
<tr>
<td>Catalog Number:</td>
<td>PS-2208</td>
</tr>
<tr>
<td>Description:</td>
<td>Diclosulam</td>
</tr>
<tr>
<td>Other Name(s):</td>
<td>Strongarm(TM)/N-(2,6-Dichlorophenyl)-5-ethoxy-7-fluoro-[1,2,4]-triazolo-[1,5-c]pyr</td>
</tr>
<tr>
<td>Supplied by:</td>
<td>CHEM SERVICE, Inc. PO BOX 599, WEST CHESTER, PA 19381 (610)-692-3026</td>
</tr>
<tr>
<td>Emergency Phone:</td>
<td>1-610-692-3026</td>
</tr>
</tbody>
</table>

| **SECTION 2 - COMPOSITION, INFORMATION ON INGREDIENTS** | |
| CAS No.: | 145701-21-9 |
| Description: | Diclosulam |
| EINECS No.: | Not Available |
| Hazard Symbols: | Not Available |

| **SECTION 3 - HAZARDS IDENTIFICATION** | |
| Contact lenses should not be worn in the laboratory. | All chemicals should be considered hazardous - Avoid direct physical contact! |
| May be harmful by inhalation, ingestion, or skin absorption. | |

| **SECTION 4 - FIRST AID MEASURES** | |
| An antidote is a substance intended to counteract the effect of a poison. | It should be administered only by a physician or trained emergency personnel. Medical advice can be obtained from a POISON CONTROL CENTER. |
| In case of contact: Flush eyes continuously with water for 15-20 minutes. | Flush skin with water for 15-20 minutes. |
| If no burns have occurred-use soap and water to cleanse skin. | |
| If inhaled remove patient to fresh air. Administer oxygen if patient is having difficulty breathing. | If patient has stopped breathing administer artificial respirations. |
| If patient is in cardiac arrest administer CPR. | |
| Continue life supporting measures until medical assistance has arrived. | |
| If patient is exhibiting signs of shock - Keep warm and quiet. | Contact Poison Control Center immediately if necessary. |
| Do not administer liquids or induce vomiting to an unconscious or convulsing person. | |
| If patient is vomiting-watch closely to make sure airway does not become obstructed by vomit. | |
| If swallowed, rinse out mouth with water, providing the person is conscious. | Get medical attention if necessary. Remove and wash contaminated clothing. |

| **SECTION 5 - FIRE AND EXPLOSION DATA** | |
| Flash Point: | Not Available |
| Extinguishing Media: | Water spray, carbon dioxide, dry chemical powder, alcohol or polymer foam. |
| Upper Explosion Limit: | Not Available |
| Lower Explosion Limit: | Not Available |
Soybean description

Variety: Glycine max
Product code: 021428
Product description: Small yellow brown oblong/round beans.
Pack size: 25 kg net polypropylene sacks
Shelf life: In excess of 2 years
Ingredients list: soybeans
GMO status: non GMO
Allergens: Soybeans
Origin: Australia
Recommended storage conditions: Store in a cool dry area free from toxic chemicals, odours, insect and rodent infestation. Stored under these conditions, this product will have a shelf life of at least 2 years.

Analytical Specification:

Purity: 99.95% min.
Tolerance for non-vegetable matter: virtually nil
Moisture: 14% max.
Total defects - damaged, stained beans: 1% max.

Nutritional Information:

(Per 100g)
Energy: 1460 kJ
Protein: 31.6 g
Carbohydrate: Total 28.8 g
Fat: Total 15.8 g (saturated)
Dietary fibre: 10.8 g
Sodium: 44 mg
Potassium: 1616 mg

This product must conform to the requirements of the ANZFA Food Standards Code.
Appendix X. Chromatogram of extract of Chinese soybean blank
PUBLICATIONS


