Supercritical Fluid Extraction of Veterinary Drug Residues from Meat
SUPERCRITICAL FLUID EXTRACTION
OF VETERINARY DRUG RESIDUES
FROM MEAT

Authors

Michael O’Keeffe B.Sc., Ph.D.

The National Food Centre, Dunsina, Castleknock,
Dublin 15

This research was partly funded by grant aid from the
Department of Agriculture and Food, under the US-
Ireland Co-Operation Programme in Agricultural
Science and Technology

ISBN 1 84170 076 2
October 1999

Teagasc, 19 Sandymount Avenue, Ballsbridge, Dublin 4
## CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Summary</td>
<td>1</td>
</tr>
<tr>
<td>Introduction</td>
<td>2</td>
</tr>
<tr>
<td>Supercritical fluid applications in residue analysis</td>
<td>3</td>
</tr>
<tr>
<td>Characteristics of β-agonists</td>
<td>5</td>
</tr>
<tr>
<td>Clenbuterol method</td>
<td>11</td>
</tr>
<tr>
<td>Multi-residue β-agonist method</td>
<td>15</td>
</tr>
<tr>
<td>Conclusions</td>
<td>16</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>16</td>
</tr>
<tr>
<td>References</td>
<td>16</td>
</tr>
</tbody>
</table>
Extraction is a key step in the analysis of food samples for residues of veterinary drugs. Over the last ten years there has been increasing interest in alternatives to the use of organic solvents for sample extraction. The development of supercritical fluid extraction (SFE) for the determination of residues in meat provides alternative methods, which may be automated and which have attractive properties such as avoidance of organic solvents and increased speed of extraction. This project is a jointly-funded United States Department of Agriculture and Department of Agriculture and Food activity with the objective of developing robust SFE procedures suitable for the quantitative recovery of β-agonists (illegal growth promoters) from animal tissue. Fundamental aspects of SFE in its application to residue analysis are also addressed.

An SFE method for the extraction of the principal β-agonist, clenbuterol, from beef liver was developed and validated. The optimised procedure involves a combination of SFE with enzyme immunoassay. Good recovery (greater than 80%) and low variation (coefficient of variation of less than 15%) were achieved for a range of clenbuterol contents in liver down to the maximum residue limit (MRL) for clenbuterol of 0.5 ppb (parts per billion) or ng/g. The developed procedure was shown to be applicable to the determination of clenbuterol in liver samples from treated cattle. Use of organic modifiers, effect of sample moisture, extraction temperature, and other SFE conditions were found to have an important role in determining extractability of clenbuterol by SFE.

A multi-residue procedure has also been developed and validated for the extraction of different classes of β-agonists from beef liver samples. The method is suitable for both the substituted aniline-type compounds (e.g. clenbuterol) and the more polar phenolic-type compounds (e.g. salbutamol), including conjugated forms of the latter. Again, SFE coupled to enzyme immunoassay resulted in good recovery and low variation (coefficient of variation of less than 25%) for both clenbuterol and salbutamol in liver tissue, at low ppb levels. These studies show that manipulation of the sample matrix prior to SFE is important in order to extract residues from complex samples such as liver.
Supercritical Fluid Extraction (SFE) is becoming an important alternative to
the use of organic solvents for the extraction of samples prior to analysis. A
supercritical fluid is neither a liquid nor a gas, but occurs at a density
somewhere between the two. A supercritical fluid, therefore, offers the
unique advantage of being able to diffuse into a sample like a gas, while still
having the solvating power of a liquid. Supercritical carbon dioxide (SF,
CO₂) is the most widely used supercritical fluid and, in the area of residue
analysis, has been used successfully to extract many agrochemical residues
such as pesticides, herbicides, and fungicides from samples. However, the
most important limitation of SF-CO₂ as an extraction solvent is that its
polarity is often too low to provide efficient extraction of more polar
residues.

The extraction of residues from animal tissues is difficult because target
compounds are present at trace levels and must be recovered from a
complex background of potentially interfering tissue components. The
presence of fat in tissue is particularly problematic as all tissues contain
some level of fat which has a relatively high solubility in SF-CO₂. The co-
extraction of fat with the target analyte may require lengthy post SFE clean-
up steps to allow accurate quantitation of the analytes. In addition, many
veterinary drug residues are relatively polar and, therefore, are not very
soluble in SF-CO₂. This problem may be overcome by use of a polar
modifier to increase the solubility of the analytes in the supercritical fluid
and assist in breaking strong analyte-matrix interactions. However, the
inclusion of a modifier in the supercritical fluid may increase the problem of
interference from co-extractives. Earlier SFE work on compounds such as
the growth promoter, trenbolone (Din et al., 1996), reported good recovery
(98%) using SF-CO₂ with methanol as a modifier. Freeze-drying of samples
prior to extraction was also suggested, to increase accessibility of the
analytes to the supercritical fluid.
The importance of instrument design on the recovery of veterinary drug residues from tissue was illustrated by Maxwell (1995), who designed an SFE instrument that enables deposition of the analytes on a standard solid phase extraction (SPE) column, thus simplifying post SFE operations. This SFE/SPE equipment was used for a number of applications, such as the extraction of nitrosamines from frankfurters (Maxwell et al., 1995). This type of equipment was used at The National Food Centre in the development of SFE methods for clenbuterol and for β-agonists, generally. The successful application of analytical SFE to residue analysis requires an understanding of the interactions occurring during the SFE process between the supercritical fluid, the analytes being extracted and the sample matrix. In order to successfully apply SFE to residue analysis, manipulation of the supercritical fluid alone is not sufficient. Sample pretreatment prior to SFE and the mode of trapping of the analyte post SFE are important considerations for successful method development.

Characteristics of β-agonists

β-Agonists are synthetic derivatives of naturally occurring compounds, such as adrenaline and noradrenaline. Clenbuterol is the only β-agonist licensed for the therapeutic treatment of respiratory conditions in animals. At multiples of the recommended therapeutic dose β-agonists act as so-called repartitioning agents, the net result of which is the production of a leaner animal carcass. They have been widely used throughout Europe as illegal growth promoters in beef and veal production following the ban on anabolic agents in the mid-1980s. There are two main classes of β-agonists; the substituted anilines, which include clenbuterol and cimaterol, and the more polar substituted phenols, which include salbutamol and terbutaline. The structure of the substituted phenol type increases the polarity of the molecule and renders it less extractable by SF-CO\textsubscript{2}. These compounds are also a target for formation of even more polar glucuronide or sulphate conjugates, which require hydrolysis prior to extraction.
Liver sample
(1.5g fresh, 0.5g oven dried)
- add hydromatrix (2 g)
- mix for 30 sec
- pack SFE vessel
- add 1.5 ml methanol (if required)

SFE
- temperature: 40 or 100°C
- pressure: 690 Bar
- flow rate: 2 litres per min
- extraction time: 10 or 40 min
- off-line SPE trap: alumina (1.0 or 2.0 g)

SPE
- collect residue on alumina
- elute with methanol/water (70+30, 4 or 8 ml)
- evaporate a fraction (1/5 or 1/10) of the eluate
- reconstitute in assay buffer

Residue determination
EIA

Figure 1: SFE method for the determination of clenbuterol in beef liver.
(Abbreviations: SFE – supercritical fluid extraction; SPE – solid phase extraction; EIA – enzyme immunoassay)
Effective conventional methods for the analysis of these compounds in animal tissues have incorporated procedures such as immunoaffinity chromatography (Haasnoot et al., 1990), matrix solid phase dispersion (Blyod et al., 1993) and solid phase extraction (Collins et al., 1994), and determination systems such as immunoassay (Degand et al., 1992) and gas chromatography-mass spectrometry (Montrade et al., 1993).

In this report, the work on development of SFE methods for the extraction of β-agonist residues from liver samples is described; the residue content was then determined by enzyme immunoassay (EIA).

CLENBUTEROL METHOD

Liver was chosen as the most suitable matrix for clenbuterol analysis, because it is the edible tissue in which β-agonist residues persist for longest (Meyer and Rinke, 1991). SFE was applied previously to the extraction of clenbuterol from liver but was unsuccessful; Guyer (1991) combined SFE with gas chromatography and showed that liver fortified at 50 ppb (parts per billion, or ng/g) clenbuterol could be extracted with recovery of 76%, but the high level of co-extracted matrix components prevented quantitative determination at the maximum residue limit (MRL) of 0.5 ppb. Other researchers also reported difficulties in achieving the sensitivity required for clenbuterol analysis using SFE. (Van Vyncht et al., 1995, Jimenez-Carmona et al., 1995). In this work, a robust SFE method was developed for the quantitative recovery of clenbuterol at the MRL of 0.5 ppb (O’Keeffe et al., 1998, Figure 1).
A series of studies were undertaken to evaluate the effect of organic modifiers, inherent sample moisture, extraction temperature and length of extraction on the recovery of clenbuterol from bovine liver. A summary of the findings are as follows:

Figure 2: Effect of methanol and ethanol addition on clenbuterol recovery from oven-dried liver, fortified by addition of 5 parts per billion clenbuterol, using supercritical carbon dioxide at 40°C and 690 Bar.

- Methanol is a superior extraction aid to ethanol (Figure 2).
- Increasing the extraction temperature increases the recovery from both fresh and oven-dried tissue (Figure 3).
- Using methanol-modified SF-CO₂ at low extraction temperatures (40°C) and shorter extraction times (10 min), sample moisture has a negative effect on recovery of clenbuterol (a recovery greater than 80% could only be achieved from oven-dried tissue; Figure 3).
- Increasing the extraction temperature to 100°C and elongating the extraction time to 40 min causes sample moisture to facilitate the recovery of clenbuterol (to greater than 90%) by acting as an inherent modifier of the SF-CO₂ (Figure 4).
Therefore, two methods were developed for the extraction of clenbuterol by SFE from oven-dried and fresh tissue, respectively. Both of these SFE procedures offer the advantages of lower solvent usage and less sample.

Figure 3: Effect of temperature on the recovery of clenbuterol from beef liver fortified at 5 ppb, using supercritical carbon dioxide with 1.5 ml of methanol over a 10 min dynamic extraction period.
clean-up than other methods. The method based on fresh tissue was used for method validation as less sample preparation time was involved. The performance of this method was assessed through recovery studies, i.e. addition of a known quantity of clenbuterol to residue free liver samples and determination of the amount of the added residue recovered by the method. The results obtained are shown in Figure 5. Mean recoveries are shown together with the coefficient of variation (CV) of the mean (which measures reproducibility of the assay). Good mean recoveries (greater than 80%) were obtained for all levels of clenbuterol added. The within assay results show acceptable variation for samples fortified at 0.5 and 2.0 ppb.

Figure 4: Effect of extraction time, at an extraction temperature of 100°C, on the recovery of clenbuterol from beef liver fortified at 5 ppb, using unmodified supercritical carbon dioxide.
Figure 5: Recovery of clenbuterol from liver by supercritical fluid extraction followed by enzyme immunoassay.
Figure 6: Clenbuterol level in liver samples from treated animals, determined by supercritical fluid extraction (SFE) followed by enzyme immunoassay (EIA). Between assay variation is slightly higher but acceptable, being at less than 15%.

The performance of the method was further assessed on samples of liver obtained from animals which had been treated experimentally with clenbuterol. Figure 6 shows a comparison between the values obtained by this method and values obtained for the same samples by an alternative (immunoaffinity chromatography/gas chromatography-mass spectrometry) method. Three samples were analysed which contained low, medium and high levels of clenbuterol. The SFE method is capable of determining clenbuterol at the three levels and the values obtained are in good agreement with the values determined by the alternative method.
MULTI-RESIDUE β-AGONIST METHOD

Because a range of different β-agonists might be used illegally for growth promotion in beef animals, a multi-residue procedure capable of determining β-agonists from the two main classes is required. The SFE conditions developed for the extraction of clenbuterol from fresh bovine liver were evaluated for the analysis of the other principle type of β-agonist, represented by salbutamol, but were unsuccessful. This may be attributed to the more hydrophilic nature of salbutamol such that it has a higher affinity than clenbuterol for the water fraction of the liver sample. Subsequently, the SFE conditions applied to the extraction of clenbuterol from oven-dried liver were evaluated and gave good recovery of salbutamol from fortified tissue (greater than 85%). More polar β-agonists like salbutamol require the removal of sample moisture and addition of methanol modifier for efficient extraction by SF-CO₂.
Figure 8: Supercritical fluid extraction method for all classes of β-agonists in beef liver.

Liver sample (10g)
- add enzyme in sodium acetate buffer (15 ml)
- agitate (5 min)
- enzymatic deconjugation

Freeze-drying
- grind sample

SFE
- weigh dried sample (0.5 g)
- add hydromatrix (2 g) and mix
- pack into SFE vessel
- add methanol modifier (1.5 ml)
- extract (100°C, 690 Bar, 2 litres per min, 10 min)

SPE
- collect residue on off-line alumina trap (2 g)
- elute with methanol/water (70+30, 8 ml)
- evaporate a fraction (1/10) of the eluate
- reconstitute in buffer

Residue determination
EIA

(Abbreviations: SFE - supercritical fluid extraction; SPE - solid phase extraction; EIA - enzyme immunoassay)
The method of drying was shown to have a large impact on the extractability of β-agonists by SFE. Recoveries were higher when sample moisture was removed by freeze-drying rather than by oven-drying. Studies using tissue samples from animals treated with clenbuterol were carried out to examine this effect. It was concluded that the difference in recovery can be attributed to the difference in mean particle size of the samples resulting from the two drying procedures. Freeze-drying resulted in a reduced mean particle size (~250 µm) relative to oven-dried samples (~450 µm), which leads to increased exposure of the target analytes to the SF-CO₂ thereby increasing extraction efficiency. Increased recovery of clenbuterol from oven-dried samples could be obtained by grinding the dried samples to a smaller mean particle size (Figure 7). Reducing the mean particle size of the oven-dried material below 250 µm does not increase the amount of clenbuterol determined significantly. This suggests that there may be a threshold value for particle size below which extractability of clenbuterol with SF-CO₂ is significantly enhanced.
Unlike clenbuterol, salbutamol and similar β-agonists have been reported to occur in liver primarily as polar conjugates and, therefore, for a multi-residue SFE method a deconjugation step is required. This step was undertaken on fresh tissue prior to drying such that all water, both from the sample itself and from the deconjugation buffer, was removed prior to SFE. The multi-residue SFE/EIA procedure for β-agonists is outlined in Figure 8 (O’Keeffe et al., 1999). Recoveries of greater than 85% and greater than 60% were achieved for clenbuterol and salbutamol, respectively (Figure 9). Acceptable levels of variation were obtained for within assay (CV of less than 7%) and between assay variation (CV of less than 24%). When the procedure was applied to samples from treated animals, the results compared well with those obtained using alternative methods (Figure 10).
CONCLUSIONS

- Supercritical fluid extraction has been successfully applied to the isolation of clenbuterol from beef liver at levels as low as the MRL (maximum residue limit), which was previously unattainable by SFE (supercritical fluid extraction). Two possible methods were developed, one of which is fully validated. A multi-residue SFE method, applicable to both types of β-agonists was developed and validated. These new methods are efficient and robust procedures and have a reduced usage of hazardous solvents.

- Fundamental aspects of this new technology in the area of drug residues have been highlighted, which will assist in the development of SFE models for the isolation of other veterinary drug residues from food samples.
ACKNOWLEDGEMENTS

Mr. A. Lightfield and Dr. R. Maxwell (ARS-USDA, Philadelphia, PA, USA), are thanked for their help in establishing the SFE instrumentation at the NFC and for their assistance throughout the project. Prof. G. Maghuin-Rogister (Faculty of Veterinary Medicine, University of Liege, Belgium), Dr. L. Stolker (RIVM, Bilthoven, The Netherlands) and Dr. B. Jülicher (BgVV, Berlin, Germany) are thanked for supply of liver samples from treated animals. Dr. S. Quilty (Particular Sciences, Dublin, Ireland) is thanked for assistance with determination of the particle size distribution of samples.

REFERENCES


