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Service contract for markers for nitrofurazone and on semicarbazide analysis

Lot 1: Structural elucidation of an alternative indicator (marker residue) for nitrofurazone use in animal derived food products and development of a routine method of analysis to identify this marker that fullfills the requirements of Decision 2002/657/EC

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SUMMARY

In order to control the illegal use of nitrofurans in food producing animals, an analytical method based on the detection of the side-chain molecules as marker residues has been in use. In analogy with furazolidone and its marker residue 3-amino-2-oxazolidinone (AOZ), semicarbazide (SEM) has been adopted as the marker residue for nitrofurazone. There is abundant evidence on the formation of semicarbazide from nitrofurazone (NFZ). Recently the confidence in SEM as an unambiguous marker residue has been questioned, due to the fact that alternative sources of SEM have been identified. Therefore, to ensure efficient and reliable control of nitrofurazone abuse, an alternative marker analyte, which can be unequivocally linked to nitrofurazone abuse, needs to be identified. For this purpose in this research project the following main objectives were defined:

- Identification and structure elucidation of an alternative indicator (marker residue) for nitrofurazone use in animal derived food products
- Development of a routine method of analysis to identify this marker that fulfils the requirements of Decision 2002/657/EC.

Key to finding a new marker residue has been the study of the metabolism of nitrofurazone and the structure identification of the free metabolites most abundantly formed. *In-vitro* incubations were conducted using chicken liver microsomes and S9 fraction as well as *Escherichia Coli* ATCC 8739 cultures. Two ^{14}C radio-labelled analogs and a $^{13}C,^{15}N_2$-labelled stable analog of NFZ were synthesized to assist in the identification of metabolites. Owing to the fact that the synthesis of these standards was successful, the metabolic pathway of nitrofurazone could be elucidated in considerable detail.

The combination of state-of-the-art high-resolution ToF mass spectrometry with MetAlign™ software for analysis of the full scan mass data proved very fruitfull in the detection and identification of a substantial number of metabolites. Based on the detected accurate mass elemental compositions could be derived for a number of potential metabolites. LC-MS/MS analysis of the incubates by means of neutral loss scanning yielded complementary information to LC-ToF-MS. Many of the metabolites detected by LC-ToF-MS were also identified by LC-MS/MS. MS/MS fragmentation yielded important information on the chemical structure of some of the newly identified metabolites.

The major metabolite identifed was the cyano-metabolite. It was detected in all test systems and under all circumstances. In addition, two other interesting metabolites were identified: a low molecular weight metabolite (aminofurfural hydrazone) in microsome and S9 incubates and an amide metabolite in S9 and *E. Coli* incubates. Several dimeric compounds were also detected in the microsome and *E. Coli* incubates, but these are of limited interest for *in vivo* systems. Furthermore, several cysteine and mercaptoethanol adducts were (tentatively) identified. However, these metabolites were only formed in detectable amounts when large amounts of the thiol component were added to the incubates. Based on the experiments conducted and the metabolites identified it can be concluded that several modes of metabolism occur simultaneously in the *in vitro* systems. The formation of the CN-metabolite is in good agreement with what is known from the metabolism of furazolidone and nitrofurazone. The CN-metabolite could therefore be considered as a potential alternative marker for NFZ.

Analysis of incurred material showed unequivocally that the CN-metabolite is present in substantial amounts (approx. 500 µg/kg) in porcine muscle tissue during medication with nitrofurazone. In liver tissue the concentration was significantly lower. Probably the metabolite is quickly metabolised to other yet unidentified metabolites. Of the other metabolites selected, the aminofurfural hydrazone metabolite was qualitatively detected in incurred muscle tissue at zero withdrawal time. All other potential metabolites, if detected at all, were present in muscle as well as liver tissue only at low concentrations, even at zero withdrawal time. It could not be excluded that adducts are formed by reaction of thiol-containing substances (amino acids, gluthatione, proteins), but these adducts may be of limited stability under the extraction conditions used, or extraction recoveries for these compounds may be low.

Accumulation of the CN-metabolite largely occurs in muscle tissue during medication. In muscle tissue it is detectable for at least one week after the end of medication. Although depletion in muscle tissue is relatively quick compared to the persistent tissue-bound adducts, its presence can be detected up to one week after the end of medication. It should be realized that many authorized veterinary drugs have depletion half-lives ranging from only a few hours up to a few days at the most. This is also the case for other banned substances like nitroimidazoles and various growth promoting agents.

Overall it can be concluded that the main objective of the project, being “The identification and structure elucidation of an alternative indicator (marker residue) for nitrofurazone use in animal derived food products” has been completed successfully. The second objective, “Development of a routine method of analysis to identify this marker that fulfils the requirements of Decision 2002/657/EC”, could not be completed fully, due to the time-consuming procedure to elucidate the metabolic pathway of nitrofurazone. Nonetheless, the preliminary results obtained indicate that such a method can be developed for the CN-metabolite of NFZ. It is recommended that a future prolongation of this research should include improvement of the LC-MS/MS method for the CN-metabolite and that the substance should be made available as a reference material (including an isotopically-labelled analogue as IS).

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1. INTRODUCTION

Nitrofurazone is demonstrated to be in use in certain countries in shrimp farming and poultry production. However, this substance has been banned for use in animal production within the EU. Like the other nitrofuran antibiotics, it is readily available from numerous suppliers e.g. via the Internet in bulk quantities and at low price. Hence there is a clear risk of illegal use of this substance in the production of animal products intended for human consumption. Consequently the need for adequate control methods to detect the illegal use of nitrofurazone is evident.

In order to control the illegal use of nitrofurans in food producing animals, an analytical method based on the detection of the side-chain molecules as marker residues has been in use. In analogy with furazolidone and its marker residue 3-amino-2-oxazolidinone (AOZ), semicarbazide (SEM) has been adopted as the marker residue for nitrofurazone.

There is abundant evidence on the formation of semicarbazide (SEM) from nitrofurazone (NFZ) (McCracken *et al,* 2005). Recently the confidence in SEM as an unambiguous marker residue has been questioned, due to the fact that alternative sources of SEM have been identified (Becalski *et al*, 2004, Stadler *et al*, 2004, Nooman *et al*, 2005). Therefore, to ensure efficient and reliable control of nitrofurazone abuse, an alternative marker analyte, which can be unequivocally linked to nitrofurazone abuse, needs to be identified.

For this purpose the following main objectives were defined:

- Identification and structure elucidation of an alternative indicator (marker residue) for nitrofurazone use in animal derived food products
- Development of a routine method of analysis to identify this marker that fulfils the requirements of Decision 2002/657/EC.

To achieve these objectives, the work was divided into 6 tasks:

1. *In-vitro* techniques for metabolism studies
2. Structure elucidation by advanced state-of-the-art mass spectrometric techniques of an alternative marker for nitrofurazone and further verification of the structure by using additional analytical instrumentation
3. Conducting animal experiments for metabolism studies
4. Synthesis of the pursued alternative marker
5. Isolation and separation of residues at the µg/kg and sub-µg/kg level and development of an analytical method for the determination of an alternative marker compliant with CD 2002/657/EC
6. Reporting and dissemination of results

Key to finding a new marker residue will be in the study of the metabolism of nitrofurazone and the structure identification of the most prominent free metabolites. For furazolidone such studies have been performed, resulting in the identification of a cyano-containing compound as one of the major

metabolites (van Koten-Vermeulen *et al*, Vroomen *et al*, 1987a, 1987c, Abraham *et al*, 1984). In addition to that, protein-bound metabolites of furazolidone were identified which has eventually resulted in the banning of nitrofurans, but also in the current methodology to detect residues of nitrofurans.

For the other nitrofurans including nitrofurazone, much less is known with regard to their metabolism. As mentioned, SEM has been chosen because of the resemblance of nitrofurazone to furazolidone and the experimental confirmation of the existence of SEM as a residue resulting from the treatment of animals with nitrofurazone. It is anticipated that a more detailed insight in the formation and identity of the major metabolites of nitrofurazone may offer opportunities to select another marker residue. Based on the information available on the metabolism of nitrofurazone, furazolidone and other nitrofurans an overview of potential metabolites of NFZ was made. This overview is presented in Annex I. Reduction of the nitro group by the action of nitroreductases is thought to occur *in vitro* as well as *in vivo* (De Angelis *et al, 1999*, Vroomen *et al*, 1987a, 1987b, 1987c, Abraham *et al*, 1984, Yeung *et al*, 1983). Reduction leads to the formation of aminofurazone, a reactive intermediate, that will quickly rearrange to a cyano-metabolite (CN-metabolite) (Yeung *et al*, 1983, Ebertino *et al*, 1962). The CN-metabolite is relatively stable and has been detected in urine of treated rats and swine (Vroomen *et al*, 1987c, Yeung *et al*, 1983). An alternative reduction route has been postulated resulting in the formation of an acrylonitrile metabolite (AN-metabolite) (Vroomen et al, 1988, Vroomen et al, 1987b). This reactive intermediate can bind with thiol groups of peptides, amino acids and glutathione (Vroomen *et al*, 1988). Furthermore, for furazolidone in some cases a dimeric compound has been observed *in vitro* systems (Vroomen *et al*, 1987a, Tatsumi *et al*, 1981). The relevance of dimeric compounds is considered to be very limited, as concentrations of NFZ used during medication will result in far lower levels *in vivo* than those applied *in vitro*.

Furazolidone metabolism has been studied using a radio-labelled compound and identifying the major metabolites using radio-chromatography. Radio-labelled NFZ combined with radio-chromatography could be extremely helpful in identifying relevant substances that result from exposure of microsomes for further identification and structure elucidation. However, radio-labelled NFZ is not commercially available. Part of the research in this task will therefore aim at the synthesis of such a radio-labelled substance (Chapter 2).

To perform a detailed study of nitrofurazone metabolism experiments using *in-vitro* techniques (e.g. liver microsomes and *E. Coli* cultures) will be conducted (Chapter 3). Cultures will be exposed to nitrofurazone and radio-labelled nitrofurazone to reveal differences in metabolism and in the formation of specific metabolites that could be useful in distinguishing nitrofurazone abuse from other sources of semicarbazide. Furthermore, cysteine and mercaptoethanol will be added to the incubates to determine whether these (in part endogenous) substances effect the metabolic pathway of nitrofurazone.

Metabolites that are generated by means of the *in-vitro* techniques will be characterised using state-of-the-art mass spectrometric techniques. As a first step chromatographic separation will be employed.

Incubates will be analysed using radio-chromatography as well as chromatographic separation combined with mass spectrometric analysis (LC-MS/MS and high resolution LC-ToF-MS).

A stable isotope-labelled analog of NFZ will be synthesized and used in the incubations to determine the metabolic pathway. Dedicated software will be used for the comparison of full-scan spectra to identify specific differences in those spectra originating from microsome incubates with either native or isotope-labelled compound (Chapter 4).

Once a candidate alternative marker residue is identified, a method of analysis should be elaborated. To determine whether the identified metabolites are present in incurred samples an analytical method will be developed. A number of standards of the identified metabolites has been provided by the partner involved in lot 2 (Eurofins, Germany). Due to their availability a straightforward extraction procedure and LC-MS method can be developed. (Chapter 5).

To confirm whether the *in-vitro* metabolism complies with the *in-vivo* metabolism, incurred samples originating from animal studies performed by the partner involved in lot 3 (Chemical Surveillance Department, Veterinary Science Department, Belfast) will be analysed for the presence of the identified metabolites of nitrofurazone (Chapter 5).

The report will conclude with the major results obtained by the research conducted in lot 1. Some recommendations for further study, directed towards the implementation of a confirmatory method using an alternative marker of nitrofurazone will be presented (Chapter 6).

2. PREPARATION OF RADIO ISOTOPE LABELLED AND STABLE ISOTOPE LABELLED NITROFURAZONE

In the past the metabolism of furazolidone (FZD) has been studied using a radio-labelled analogue in combination with radio-chromatography to identify the major metabolites (Vroomen *et al*, 1987a, 1987b, Abraham *et al*, 1984, Tatsumi *et al*, 1981). It was envisaged that the inclusion of radio-labelled NFZ in combination with radio-chromatography could be very helpful in identifying relevant metabolites, produced during exposure to microsomes or *E. coli*. Radio-labelled NFZ could also assist in the identification and structure elucidation by LC-MS. However, as a radio-labelled compound, NFZ is not commercially available, therefore it had to be prepared. The obtained radio-labelled NFZ was used for the *in-vitro* metabolic study of NFZ using microsomes and *Escherichia coli* (chapter 3). A stable isotope labelled isomer of NFZ was also included, in particular for the LC-MS studies of microsome and *E. coli* incubates.

The isotopically labelled analogues of NFZ (Figure 1) that were synthesized, include a stable isotope labelled NFZ containing a carbon-13 and two nitrogen-15 atoms in the semicarbazide side-chain (NFZ-$^{13}C,^{15}N_2$), and two radio-labelled NFZ analogs, containing a carbon-14 either at the methylidene carbon (NFZ-$^{14}C_{met}$) or at the semicarbazide carbon (NFZ-$^{14}C_{sem}$). Introduction of one carbon-14 atom increases the molecular weight with two mass units, while the molecular mass of the stable isotope analogue is increased with three units.

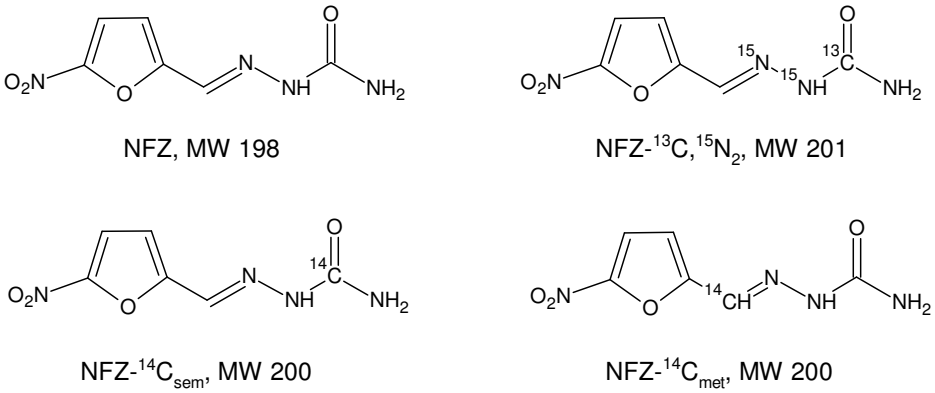

Figure 1: Chemical structure of NFZ and radioactive and stable isotope-labelled analogues.

The synthesis of the stable isotope analog, NFZ-$^{13}C,^{15}N_2$, was carried out by condensation of commercially available isotope-labelled SEM (SEM-$^{13}C,^{15}N_2$, CSS, Belfast, UK) with 5-nitrofurfural (5-NF) in dilute hydrochloric acid (Figure 2). NFZ-$^{13}C,^{15}N_2$ was isolated in good yield (80%) and purity (>90%). Yield and purity were estimated by LC-MS analysis.

SEM, containing a carbon-14 atom, is commercially available as well (ARC, St. Louis, USA). SEM-^{14}C, with a specific activity of 40 mCi/mmol was reacted with 5 equivalents of 5-NF in dilute hydrochloric acid. After extraction with ethyl acetate, purification was performed on a Waters Sep-Pak plus silica cartridge. The product was dissolved in methanol. Yield (130 µg) and purity (65%) were estimated by means of LC-PDA-radio-chromatography and LC-MS analysis.

The synthesis of NFZ containing the carbon-14 label at the methylidene position (NFZ-$^{14}C_{met}$) was conducted by side-chain exchange starting from ^{14}C-labelled FZD (FZD-$^{14}C_{met}$), specific activity 44 mCi/mmol). FZD-$^{14}C_{met}$ was reacted with a large excess of SEM in dilute hydrochloric acid (Fig. 2). After extraction with ethyl acetate, purification was performed on a Waters Sep-Pak plus silica cartridge. The product was dissolved in methanol. Yield (75 µg) and purity (90%) were estimated by means of LC-PDA-radiometric analysis. In Figure 3 radio-chromatograms of NFZ-$^{14}C_{sem}$ and NFZ-$^{14}C_{met}$ are shown.

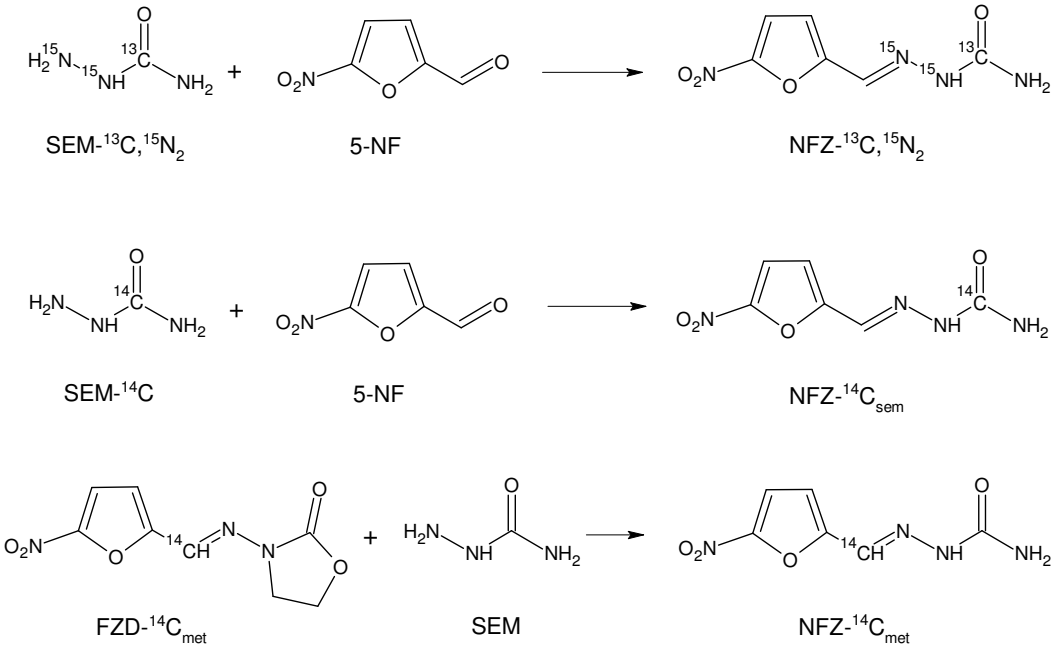

Figure 2: Synthesis of the stable and radio isotope-labelled analogs of NFZ.

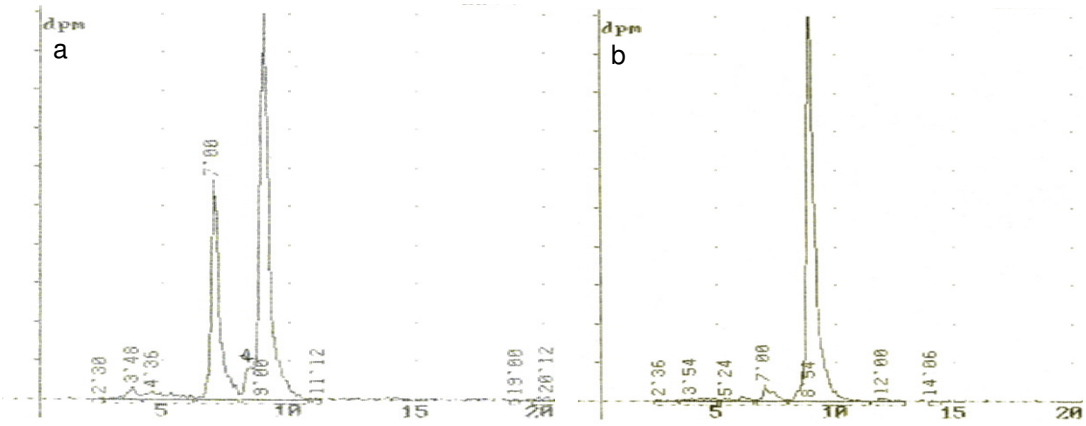

Figure 3: Radio-chromatograms of standard solutions of (a) NFZ-$^{14}C_{sem}$ and (b) NFZ-$^{14}C_{met}$.

3. IN-VITRO TECHNIQUES FOR METABOLISM STUDIES

3.1. *In-vitro* metabolic study of NFZ using chicken liver microsomes

In-vitro techniques (e.g. incubates using liver microsomes) have been used to perform a detailed study of NFZ metabolism. For this purpose liver microsomes obtained from broilers were exposed to FZD and NFZ. Microsomes were isolated according to the procedure described by Vroomen *et al*. (1987a, 1987b). FZD was included in the experiments, for the purpose of comparison.

Microsome incubates use ß-nicotinamide adenine dinucleotide phosphate (NADPH) as cofactor. In the initial experiments a large background signal for SEM was observed in the LC-MS/MS analysis of the positive as well as in the control samples. A detailed investigation was carried out to locate the origin of this background signal. Eventually, the source of the SEM background could be pinpointed to a specific batch of NADPH. When a different batch of NADPH was used, the background of tissue-bound SEM originating from NADPH was only 0.1% of the total tissue-bound SEM.

Tests were conducted to optimise the experimental conditions of the microsome incubates, in particular the incubation time, the concentration of NFZ and the amount of microsomes. Incubates were analysed for NFZ and for tissue-bound SEM by LC-MS/MS. The results are presented in Figure 4.

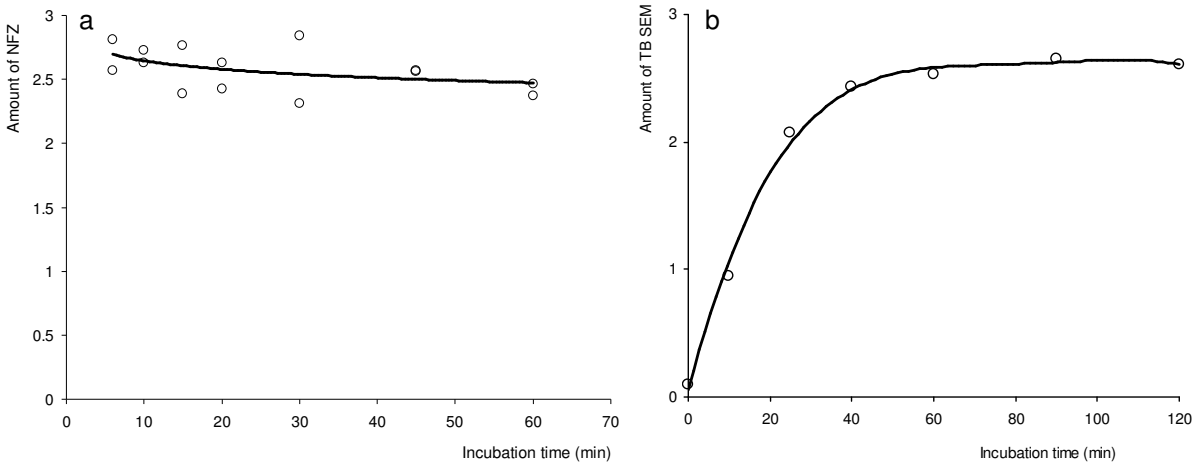

Figure 4. (a) Decrease of NFZ and (b) formation of tissue-bound semicarbazide (TB SEM) during the incubation of microsomes (arbitrary units).

From Figure 4 it is evident that the concentration of tissue-bound SEM reaches a plateau after approximately 60 minutes of incubation at 37°C. There is however only a 10-15% decrease in NFZ concentration in the incubate mixture. Apparently the microsomes become inactivated after 60 min and the conversion to metabolites stops. Increasing one or more of the ingredients of the incubate mixture did not result in a significantly improved conversion. The procedure presented below describes the optimum conditions for the *in-vitro* microsomal metabolism of NFZ.

Microsomal incubation

A standard incubation mixture was prepared by mixing 3 mM $MgCl_2$ (750 µl), 0.1 M potassium phosphate buffer, pH 7.4 (600 µl) and 500 µl of a freshly thawed chicken liver microsome suspension (containing ca. 2.5 mg protein). Freshly prepared 1mM NADPH (100 µl) was added to the mixture. The incubation mixture was pre-incubated at 37°C for two minutes. Incubation was started by the addition of NFZ (20 µg) dissolved in methanol (50 µl). The final volume was 2 ml. After incubation for 60 minutes at 37°C the reaction was terminated by the addition of ice-cold methanol (1 ml) or by cooling the incubation tube using ice.

Additionally, incubations were also carried out using the complete S9 liver fraction incubates. This fraction contains more liver cells and proteins and can therefore result in additional metabolites or adducts compared to the microsome incubates.

3.2. *In-vitro* metabolic study of NFZ using *E. Coli* incubates

In the past FZD exposed to *Escherichia Coli* ATCC 8739 has been used to study its metabolism (Abraham *et al,* 1984*).* In order to determine whether *E. Coli* could be helpful in the search for an alternative marker residue for NFZ, incubations with NFZ were carried out. To 10 ml of freshly prepared *E. Coli* culture 100 µg of NFZ or FZD (for comparison) was added and the mixture was incubated at 37°C. Aliquots were taken at different points in time (0 – 15 – 30 and 60 minutes) and extracted using ethyl acetate. The ethyl acetate fractions were evaporated and redissolved in mobile phase. The samples were analysed by LC-MS/MS for the presence of NFZ and the cyano-metabolite (CN-metabolite). The results are shown in Figure 5. A rapid metabolism of NFZ occurred resulting in the almost complete disappearance of this drug after 1 hour. At the same time the formation of the CN-metabolite as a prominent component is evident.

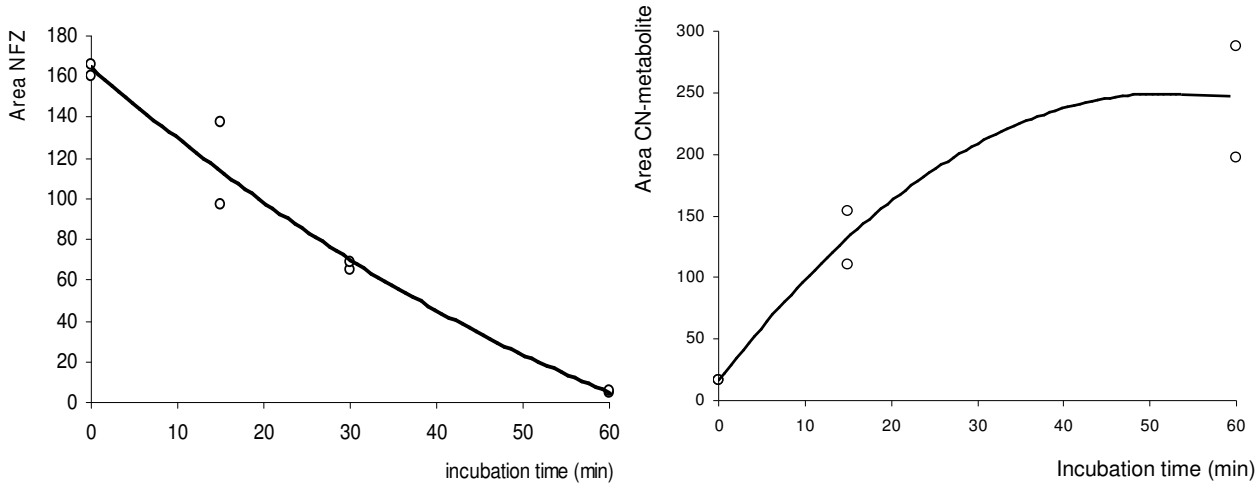

Figure 5: (a) Decrease of NFZ and (b) formation of the cyano-metabolite during the E. Coli ATCC 8739 incubation (arbitrary units). The results are based on the analysis of the ethyl acetate fractions.

At the time these *E. coli* experiments were conducted, a synthesized standard of the CN-metabolite was not available yet. Identification was based on LC-MS/MS data and on analogy with the formation of a CN-metabolite in the metabolism of furazolidone in *E. Coli* incubates. The samples were analysed using tentative precursor-fragment ion transitions for the presumed CN-metabolite. Later on the identity of the CN-metabolite in *E. Coli* as well as microsome incubates was confirmed by means of comparison with a chemically synthesized standard, kindly provided by Dr. S. Weigel and Dr. R. Gatermann (Eurofins, Hamburg, Germany).

4. STRUCTURE ELUCIDATION OF NITROFURAZONE METABOLITES

In the search for a potential marker residue of NFZ-metabolism, the detection and structure-elucidation of potential metabolites forms an essential part of research. To achieve the identification of metabolites several techniques were applied:

1. Analysis of radio-labelled NFZ incubates by means of radio-chromatography.
2. Analysis of incubates of NFZ, radio-labelled NFZ and stable isotope-labelled NFZ for the presence of metabolites using LC - Time of Flight - mass spectrometry (LC-ToF-MS) in combination with MetAlign^{TM} data processing software.
3. Analysis of incubates of NFZ in combination with stable isotope-labelled NFZ for the presence of metabolites using LC – tandem – mass spectrometry (LC-MS/MS).

In Table 1 an overview is given of the standards, test systems and detection techniques which have been used in this study to generate, identify and elucidate metabolites of nitrofurazone, that could serve as alternative markers for semicarbazide. The radio-isotope labelled analogs have been incubated in the three *in-vitro* systems and analysed by radio-chromatography and by LC-ToF-MS. Nitrofurazone and its stable-isotope analogue have been metabolised in the same three test systems, in the absence and in the presence of cysteine or mercaptoethanol. Detection and identification of metabolites in these incubates has been performed by LC-ToF-MS and LC-MS/MS techniques. The synthesized standards have been incorporated to assist in the identification of potential metabolites.

Table 1: Overview of the relevant compounds, test systems and detection techniques used in this study

Compounds	*In-vitro* test systems	Addititives	Detection Techniques	Synthesized standards
NFZ $^{13}C^{15}N_2$-NFZ	Chicken liver microsomes, S9 *E. Coli ATCC 8739*	Cysteine Mercaptoethanol	LC-ToF-MS (MetAlign) LC-MS/MS (neutral loss and MS/MS)	Cyano-metabolite Acrylonitrile-metabolite Cysteine-cyano-metabolite Gluthatione-cyano-metabolite
^{14}C-NFZ_{met} ^{14}C-NFZ_{sem}	Chicken liver microsomes, S9 *E. Coli ATCC 8739*		Radio-chromatography LC-ToF-MS	

4.1 Radio-chromatography

Radio-chromatography is used to identify the fractions of the chromatogram that contain NFZ metabolites. Furthermore, LC-radiometry can provide information about the relative amounts and the polarity of the metabolites formed during *in vitro* experiments.

4.1.1 Applied methodology

Extracts of microsomal, S_9 and *E. Coli* incubates exposed to ^{14}C-NFZ$_{sem}$ or ^{14}C-NFZ$_{met}$ were produced and analysed by LC-radiometry as described below.

LC-Radiometric analysis of microsome and E. Coli incubates

Extracts of microsome and *E. Coli* incubates obtained according to the protocols described in section 3.1 and 3.2 were injected into the LC-PDA-Radiometric system equipped with a Chrompack Hypersil 5 ODS column (25 x 4.6 mm, 5 µm). A gradient changing from 2% to 90% acetonitrile in water-1.0M acetate buffer pH 5.0 was used at a flow of 1 ml. The PDA detector (Waters991) was set at 254 nm. Ultima Flo M (Packard bioscience) was added post column as scintillation reagent at 3.5 ml/min. After mixing with the column effluent, the beta-radiation was measured with a flow counting detector (Berthold LB 506C).

Inspection of the radio-chromatograms enabled identification of fractions containing possible metabolites of NFZ. The next step was to collect fractions of the incubates for detailed analysis by LC-MS. However, the addition of scintillation fluid to the LC-eluent, necessary to perform the radiometric detection, prohibited the in-line collection of fractions. Alternatively, fractions were collected with a LC-fractionation system (Hewlet Packard, HP1100) using a Novapak C_{18} (150 x 3.0 mm, 5 µm) analytical column at a flow of 400 µl/min. Gradient elution was applied from 0 to 90% acetonitrile in 31 minutes. Aliquots of 100 µl were injected on the fractionation system and fractions of 1 min were collected. The fractions collected were analysed by UPLC-ToF-MS to identify potential metabolites of NFZ (section 4.1.2).

4.1.2 Results and Discussion

Radio-chromatograms of microsome incubates with the two ^{14}C-NFZ radio-labelled compounds are presented in Figure 6. The results show that incubates to which ^{14}C-NFZ$_{met}$ was added contained more and higher concentrations of metabolites than the incubates containing ^{14}C-NFZ$_{sem}$. Considering also the fact that the purity of ^{14}C-NFZ$_{met}$ is much better (Chapter 2) it was decided to conduct additional *in-vitro* experiments solely with ^{14}C-NFZ$_{met}$. From Figure 6 it can be seen that several metabolites are produced. The peak eluting at 12.1 min could be attributed to the CN-metabolite.

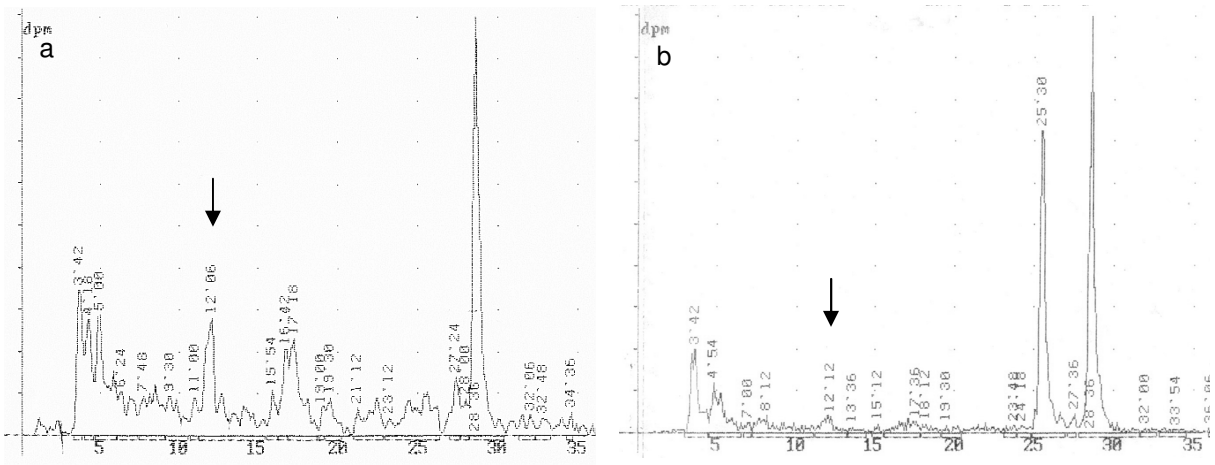

Figure 6: Radio-chromatograms of (a) a chicken liver microsome incubate containing ^{14}C-NFZ$_{met}$ and (b) an incubate containing ^{14}C-NFZ$_{sem}$. ^{14}C-NFZ$_{met}$ and ^{14}C-NFZ$_{sem}$ elute at 28.4 minutes and the CN-metabolite at 12.1 min. The peak eluting at 25.3 min in the ^{14}C-NFZ$_{sem}$ incubate is not a metabolite but an impurity present in the starting material.

In Figure 7 a radio-chromatogram of an *E. Coli* and a S9 incubate with addition of ^{14}C-NFZ$_{met}$ is presented. The extracts were analysed under slightly different chromatographic conditions compared to the microsome incubates given in Figure 6, resulting in somewhat reduced retention times. Under these conditions ^{14}C-NFZ$_{met}$ eluted at a retention time of 22.2 min. Compared to the radio-chromatogram of the microsome incubates, a much larger decrease of the ^{14}C-NFZ$_{met}$ signal was observed in the S9 and even more prominently in the *E. Coli* incubates. The observation for the *E. Coli* incubate is in close agreement with the results obtained during the optimisation of the *E. Coli* incubation procedure (chapter 3). These results indicate a more efficient metabolism using *E. Coli* compared to microsomes. Furthermore, according to the radio-chromatogram, the *E. Coli* incubation with NFZ produces one major peak at a retention time of 8.2 min. In analogy to the predominant formation of a CN-metabolite from furazolidone and furaltadone in *E. Coli* incubates (Vroomen *et al*, 1987c, Hoogenboom *et al*, 1994) and taking into account the results obtained during the optimisation process described in section 3.2, this peak can be attributed to the CN-metabolite of NFZ.

The radio-chromatogram of the S9 incubate also shows a significant decrease of ^{14}C-NFZ$_{met}$. A small peak is observed at RT = 8.2 min., very likely corresponding to the CN-metabolite. Furthermore, the majority of radioactivity elutes at very short retention times (< 6 min.), indicating the formation of highly polar metabolites in S9 incubates.

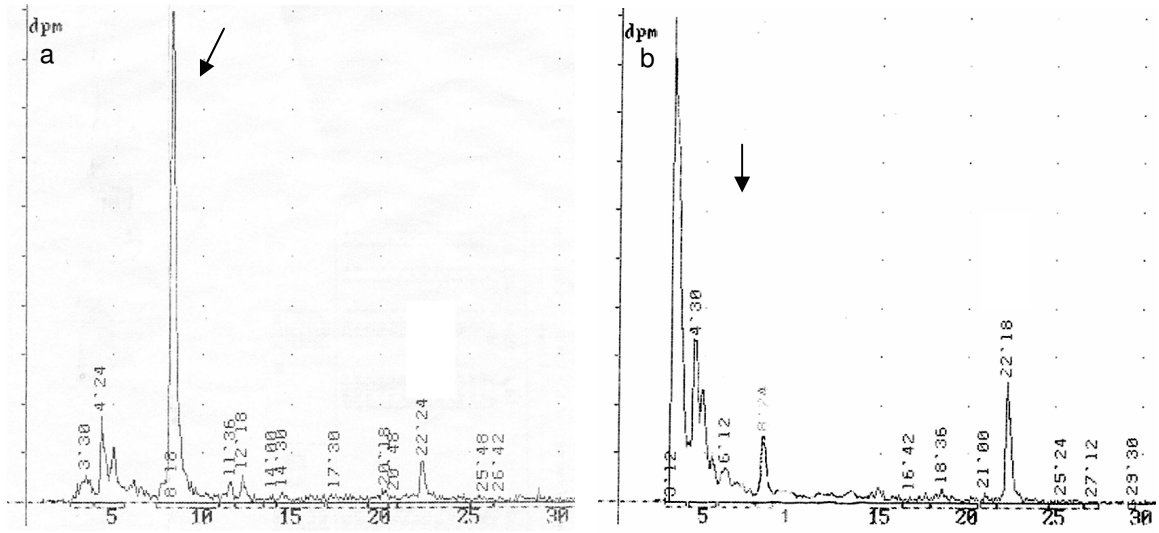

Figure 7: Radio-chromatograms of (a) an E. Coli incubate containing ^{14}C-NFZ$_{met}$ and (b) a S9 incubate containing ^{14}C-NFZ$_{met}$. ^{14}C-NFZ$_{met}$ elutes at 22.2 minutes and the CN-metabolite at 8.2 min.

Following the radio-chromatography results, several incubates were fractionated. These fractions were analysed for metabolites of NFZ using LC-ToF-MS (section 4.2). Unfortunately, fractionation resulted in rather diluted sample extracts, which in practise prevented the detection and identification of all except the most prominent metabolites. Furthermore it proved difficult to 'translate' the chromatographic conditions of the LC-PDA-radiometric system to that of the LC-PDA-fractionation system. This was due to the fact that both systems have rather different dimensions (column size, eluent flow, dead volumes, etc). As a result the fractionation envisaged could not be achieved with the LC-fractionating system. Considering the complications encountered it was decided not to proceed in this direction, but instead to analyse the complete extracts of incubates directly.

4.2 LC-ToF-MS

For the detailed analysis of incubates of NFZ, LC-ToF-MS in combination with MetAlign™ software provides a powerful combination. Comparison of the full scan mass data obtained for blank incubates with incubates to which native NFZ, radio-labelled or stable-isotope labelled NFZ has been added, may lead to the identification of potentially interesting metabolites. The analysis of these full scan data will result in elementary compositions generated for metabolites. The availability of an elementary composition will assist in the assignment of a chemical structure to an unknown metabolite.

Compounds containing thiol groups (glutathione, cysteine, mercaptoethanol, proteins) have been implicated to react with the acrylonitrile metabolite to form conjugates (Vroomen *et al*, 1987c). Adding an excess of a thiol-containing compound may have a significant effect on the metabolism of NFZ, as it may direct the system towards the production of the corresponding sulphur-containing adducts. In this study the formation of thiol-containing metabolites was simulated by the addition of cysteine and mercaptoethanol to the reaction mixture.

4.2.1 Applied methodology

Freshly prepared extracts of microsome, *E. Coli* and S9 incubates of NFZ, ^{14}C-NFZ$_{met}$, $^{13}C^{15}N_2$-NFZ or a mixture of NFZ and ^{14}C-NFZ$_{met}$ were analysed using LC-ToF-MS in positive as well as negative electrospray mode. The experimental conditions are described below.

LC-ToF-MS analysis of microsome, S9 and E. Coli incubates

Extracts of microsome, S9 and *E. Coli* incubates were obtained according to the protocols described in section 3.1 and 3.2. 10-20 µl aliquots were injected into the LC-ToF-MS system. Two different types of column were applied for chromatographic separation.

In the first method a Waters Acquity C_{18} (50 x 2.1 mm, 1.7 µm) column was used at a flow rate of 200 µl/min. The metabolites were separated using a linear gradient changing from 100% water to 90% acetonitrile in 19 minutes. With this method NFZ was observed at a retention time of 12 minutes and the CN-metabolite at 6 minutes.

The second method was developed specifically for the separation of more polar compounds and consisted of a Waters Atlantis C_{18} (150 x 2.1 mm, 3 µm) column, operated at a flow rate of 200 µl/min. The metabolites were separated using a linear gradient changing from 100% water to 90% acetonitrile in 22 minutes. With this method NFZ was observed at a retention time of 23 minutes and the CN-metabolite at 16 minutes.

In both methods water and acetonitrile of LC-MS quality (Biosolve, Valkenswaard, the Netherlands) was used to reduce background interferences.

ToF-MS settings:

Parameter	Positive mode	Negative mode
Ionisation technique	ESI	ESI
Capillary voltage (kV)	2.0	3.0
Sample cone (V)	35	35
Desolvation temperature (°C)	450	450
Source temperature (°C)	120	120
Cone gas flow (l/h)	50	50
Desolvation gas flow (l/h)	450	450
Mode (resolution, FWHM)	W (>10000)	W (>10000)
Scan time (s)	0.5	0.5
Lock mass	Sulfadiazine (*m/z*=251.0603)	Sulfadiazine (*m/z*=249.0480)
Attenuated lock mass	Sulfadimethoxine (*m/z*=311.0814)	Sulfadimethoxine (*m/z*=309.0658)

The LC-ToF-MS is capable of collecting full scan data at high resolution (>10.000 in W-mode). Typically, datasets of several gigabytes are produced during an overnight run. For the interpretation of these data sets specialized software is required which removes interferences and reduces the amount of data produced. MetAlign™ software is a program specifically designed to perform these tasks (see below).

MetAlign™ (**www.metalign.nl**) has been designed to distinguish between and filter out statistically significant differences between pre-defined classes of full scan LC or GC mass spectrometry data sets. As such, MetAlign™ has broad application in the fields of metabolomics, pharmaceuticals (lead and target identification), agrochemicals (lead identification, mode of action analysis), biological science (biochemical profiling, breeding), food processing (quality control, substantial equivalence) and GM product analysis (substantial equivalence).

MetAlign™ can be used to identify differences between the chromatograms of blank incubates and incubates of NFZ. Examples of MetAlign™ output are given in Figures 8 and 9. In Figure 8 total ion current (TIC) chromatograms are given of an *E. Coli* incubate of NFZ, a blank *E. Coli* incubate and the corresponding MetAlign™ output. In this example the difference between the TIC chromatograms is quite clear. However, the chromatograms of incubates of microsomes show a very different situation (Figure 9) and indicate the necessity of using MetAlign™ to pinpoint the relevant differences. The compound(s) linked to a specific difference can be identified by further processing of the full scan data.

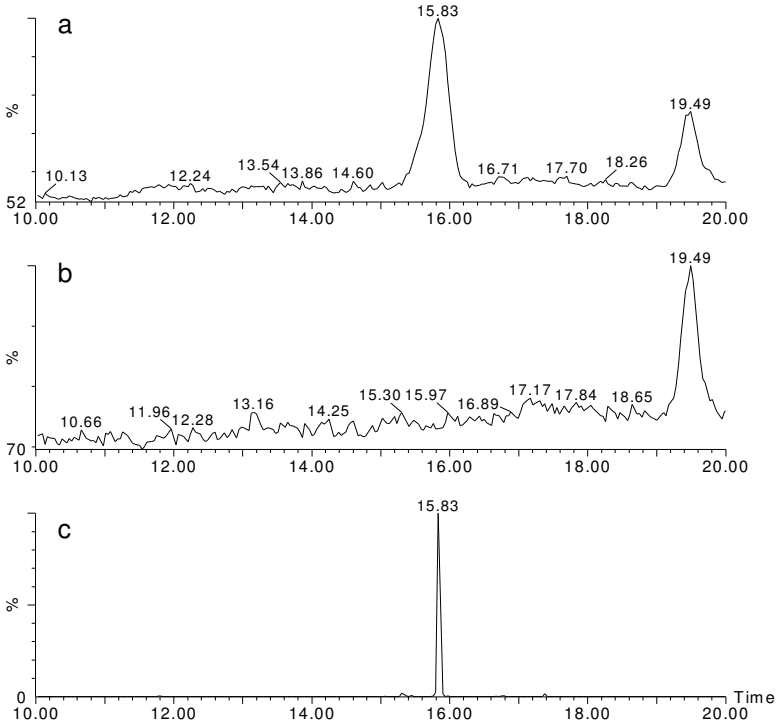

Figure 8. Section of the TIC chromatograms (pos ESI) of (a) an E. Coli incubate of NFZ, (b) a blank E. Coli incubate and (c) the corresponding MetAlign™ output indicating a major difference at RT = 15.8 min. Chromatograms have been obtained with LC-MS gradient method 2.

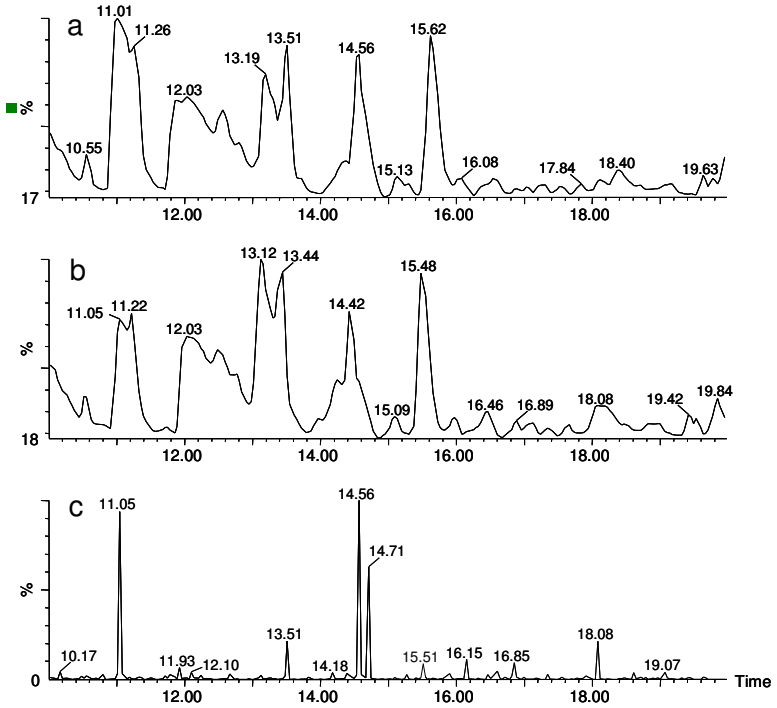

Figure 9. Section of the TIC chromatograms (pos ESI) of (a) a microsome incubate of NFZ, (b) a blank microsome incubate and (c) the corresponding MetAlign™ output indicating several significant differences. Chromatograms have been obtained with LC-MS gradient method 2.

An example of the procedure depicted is presented in Figures 9 and 10. Using MetAlign™, the TIC chromatograms (representing full scan data!) of the blank and NFZ microsome incubate (Figure 9) have been processed and the significant differences are shown in the lower trace. Each difference present in this trace has been investigated. For instance, the mass spectrum corresponding to the peak at RT=15.5 min of Figure 9 is shown in Figure 10. From the full scan data the single ion chromatograms can be extracted. This has been done for the m/z=169 ion trace of the blank and NFZ-microsome incubates as an example. The single ion chromatograms show the presence of a compound with mass of 169 (as protonated molecular ion) in the NFZ-incubate at the selected retention time. Finally the high resolution mass spectrum of the selected peak at RT=15.5 is depicted, revealing the accurate mass of the selected compound.

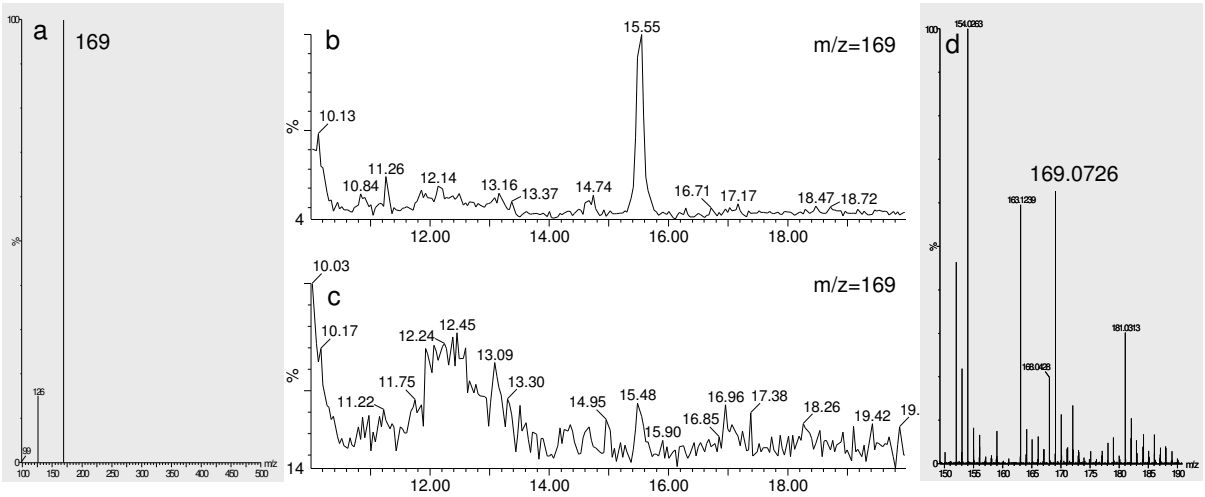

Figure 10. Procedure for the determination of the exact mass of an unknown compound showing (a) the spectrum of the MetAlign™ output obtained from the Total Ion Current chromatograms presented in Figure 9 corresponding with the peak at RT=15.5 min, (b) the single ion chromatogram (m/z=169) of the microsome incubate of NFZ showing the peak of the unknown compound, (c) the single ion chromatogram (m/z=169) of the blank microsome incubate and (d) the mass spectrum of the selected peak at RT=15.5 showing the accurate mass of the unknown compound.

The final step in the identification of unknown compounds is to work out possible elemental compositions (molecular formulas) by using the Masslynx 4.0 software. To this end possible elements (C, H, O, N, S, Na, K) and specific ranges for each element, as well the maximum allowed mass error have to be included. Under normal circumstances the instrument operates with a mass error of 5 ppm or less. However, to make sure no metabolites are overlooked the maximum allowed mass error has been set at 10 ppm. An example of the output of the calculation of possible elemental compositions is presented in Figure 11. The combined information on the retention time, the accurate mass and the calculated elemental compositions can be used to carry out a tentative identification.

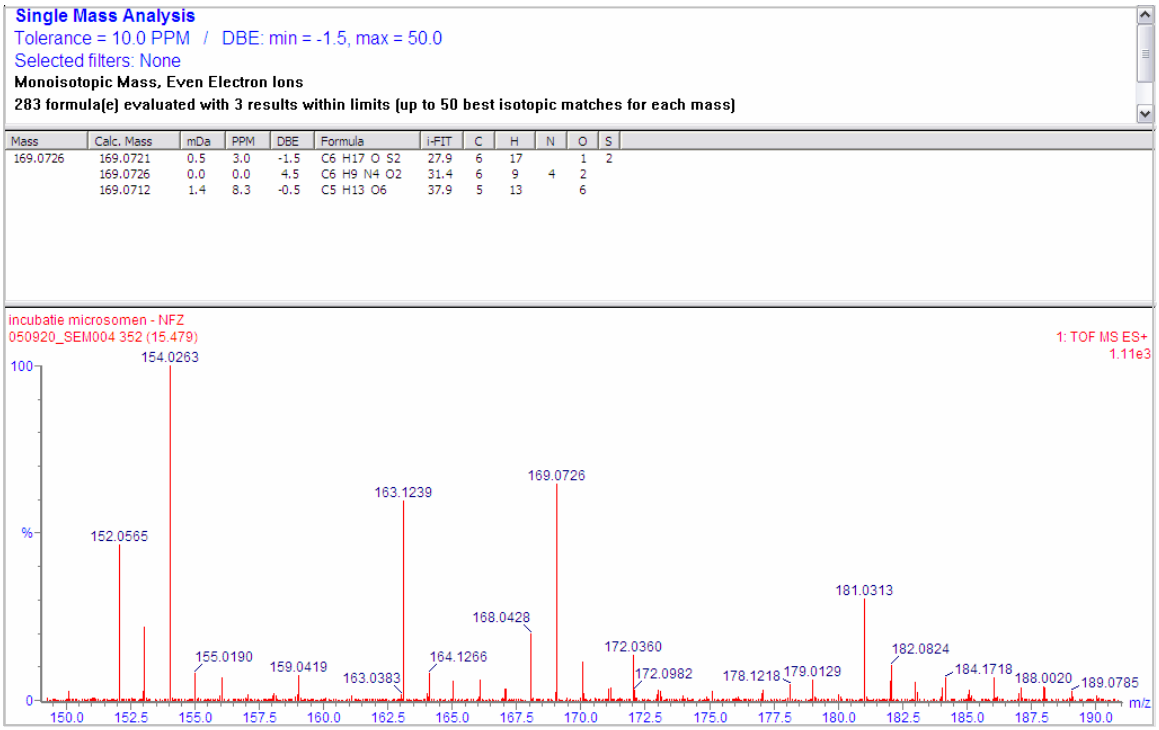

Figure 11: Masslynx elemental composition output indicating the possible elemental compositions with a theoretical mass within a given range of 10 ppm from the experimentally determined mass. As an example the output is shown for the component selected in Figure 10.

The determination of an accurate high resolution mass and (a list of) elemental composition(s) in many cases may not be sufficient to derive a chemical structure for a particular component. An additional problem occurs for components observed at elevated molecular weights. The theoretical number of possible elemental compositions increases exponentially with increasing mass.

However, additional information whether an unknown compound originates from NFZ or not can be obtained by comparing the obtained mass data of the native NFZ incubate with corresponding metabolites and degradation products originating from radio-labelled NFZ incubate and the stable-isotope labelled NFZ incubate. The presence of a ^{14}C-atom in ^{14}C-NFZ$_{met}$ produces a mass difference of 2.0032 units with that of native NFZ, while the isotopes present in $^{13}C^{15}N_2$-NFZ will yield a mass difference of 2.9974 units. If a component is detected in the incubates of NFZ, ^{14}C-NFZ$_{met}$ and $^{13}C^{15}N_2$-NFZ at identical retention time and with correct theoretically predicted mass increments it is a compound highly likely to originate from NFZ. An example is presented in Figure 12.

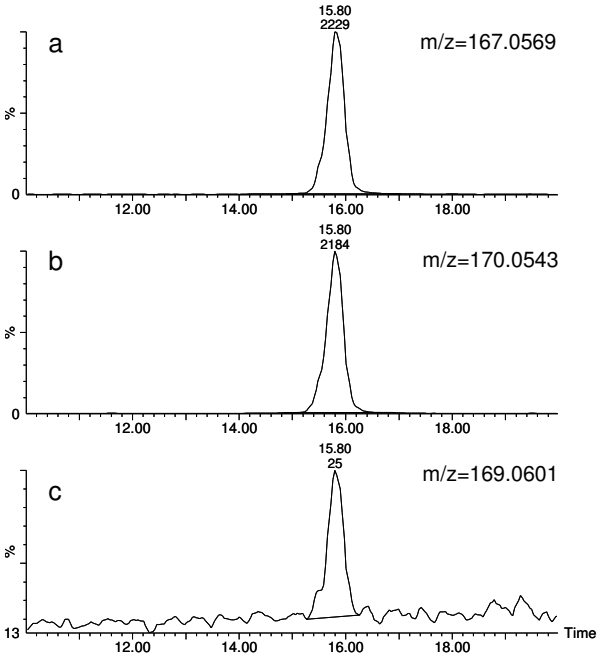

Figure 12. Corresponding single ion chromatograms of the E. Coli incubates of (a) NFZ, (b) $^{13}C^{15}N_2$-NFZ and (c) ^{14}C-NFZ$_{met}$. The presence of a peak in each of the chromatograms of the NFZ incubates, at the same retention time and at a mass matching the predicted elemental composition and mass increments, is highly indicative for a metabolite originating from NFZ.

The mass increase for metabolites originating from labelled NFZ, will only be observed if the labelled atoms are still present in the metabolite. Hence, the results obtained for the NFZ-$^{13}C^{15}N_2$ incubate will provide information about the presence (or absence) of the SEM side-chain in the unknown molecule, whereas the presence or absence of the methylidene carbon in the unknown compound can be concluded from the results of the NFZ-$^{14}C_{met}$ incubate. Likewise, wehn dimeric products are formed, this is reflected in a double increment of the molecular mass in the isotopically labelled analogs.

4.2.2 Identification of metabolites by LC-ToF-MS

Analysis of the chicken liver microsomal, S9 and *E. Coli* incubates by LC-ToF-MS (positive and negative electospray mode) resulted in numerous full scan chromatograms. Hundreds of differences were indicated by comparing blank incubate full scan chromatograms with corresponding NFZ incubate full scan chromatograms using MetAlign^{TM} software. Most differences were only found once and were therefore not consistent over replicate analyses. Those differences were considered as artefacts.

For the remaining, consistent differences, the accurate mass was determined resulting in a list of possible metabolites for every type of incubate for both positive and negative mode analysis. Typically these 'extended' lists contained 10 to 30 candidate compounds. For each candidate compound possible elemental compositions were calculated that fitted with the accurate mass determined. Some of those calculations resulted in unrealistic elemental compositions (e.g. $C_2H_{18}NS$) and based on the

accurate mass, no further link to the theoretical metabolism of NFZ could be made. These candidate compounds were not investigated further.

Under the experimental conditions, in positive mode the metabolites were most commonly detected as protonated molecular ions (theoretical molecular mass + 1.0078 Da), but sometimes as their sodium (+ 22.9898 Da) and potassium (+ 38.9637 Da) adducts as well. On some occasions (partial) in-source fragmentation was observed. The formation of protonated ammonia (+ 18.0338 Da) or acetonitrile (+ 42.0038 Da) adducts was also observed. In negative mode the deprotonated molecular ion (theoretical molecular mass - 1.0078 Da) in general was the most prominent and little or no in-source fragmentation was observed.

Information regarding the presence of the SEM side-chain and the methylidene carbon in the remaining candidate metabolites was derived by comparison of the NFZ incubate with the labelled NFZ incubates. A list of metabolites (tentatively) identified by LC-ToF-MS is presented in Table 2 and in Annex II. Of the metabolites listed, only the presence of the CN-metabolite could be confirmed in all incubates.

Table 2. The theoretical mass, retention time and suggested identity of NFZ metabolites obtained by LC-ToF-MS in the microsome, S9 fraction and E. Coli incubates.

Microsome S9 fraction *E. Coli*	Theoretical mass (m/z)	Retention time (min)		Positive / Negative ESI	SEM side-chain	Suggested elemental composition	Suggested identity
		gradient 1	gradient 2				
M, S, E	168.065	5.9	15.8	P + N	Single	$C_6H_8N_4O_2$	Cyano-metabolite
M, S	316.103	6.5, 10.3	16.3, 18.1	P + N	Double	$C_{12}H_{12}N_8O_3$	Dimeric compound
M, S	366.104	10.2, 11.1		P + (N)	Double	$C_{12}H_{14}N_8O_6$	Dimeric compound, mono-nitro derivative?
E	187.059		6.3	P	Single	$C_8H_9N_3O_4$	Carboxylic acid metabolite (trace)
E	332.098	11.2		N	Double	$C_{12}H_{12}N_8O_4$	Dimeric compound

A number of different dimeric compounds was formed in these incubates; the most important are listed in Table 2. The structures of these dimeric compounds remain highly speculative. Moreover, each structure may be present in the incubates in more than one isomeric form. Additional information (NMR, MS/MS fragmentation) will be required to propose a molecular structure for these compounds. However, some remarks can be made here. For instance, the dimeric compound with mass 366 present in microsomal and S9 incubates may be related to a similar dimer that has been found in furazolidone incubates. The dimer reported in rat liver microsome incubates (Tatsumi *et al*, 1981 and Vroomen *et al*, 1987a, 1987b) was thought to originate from the condensation of furazolidone and its corresponding acrylonitrile metabolite (AN-metabolite). However, the metabolite described also contained an additional hydroxyl group, which would in case of nitrofurazone result in a mass of 382. The structure of the furazolidone dimer proposed (Tatsumi *et al*, 1981), was mainly based on its elementary composition and proton NMR spectrum; no mass spectra of this metabolite could be obtained at the time.

In the microsome and S9 incubates of nitrofurazone two other dimeric compounds - with mass 316 - are present, which do not seem to have an equivalent with the studies on furazolidone. These metabolites are formed abundantly in the incubates and they are observed in positive as well as in negative electrospray mode. The derived elemental composition and the information obtained from the isotopically-labelled analogs (the presence of two SEM side-chains) suggests a dimeric compound formed by condensation of an acrylonitrile metabolite and the CN-metabolite or the amino metabolite, followed by loss of an oxygen atom in the form of water. The proposed structure remains highly speculative, however. In *E. Coli* preparations the above mentioned dimers could not be detected. Instead, another dimeric compound (mass 332) is present in low concentrations. Dimeric compounds may occur in *in vitro* incubates due to the relatively high concentration of nitrofurazone in these systems. There is little chance, howerer, that i*n vivo* these dimers will be detected, because NFZ concentrations are much lower.

Even with gradient 2 and the high polarity Waters Atlantis column, the possibility remains that one or more polar metabolites escape detection by LC-ToF-MS. According to the radio-chromatograms of the various incubates, there may be several polar metabolites eluting very early in the chromatogram. However, many other interfering matrix components elute in this region, making the selection and identification of potential metabolites by means of ToF-MS rather difficult.

When excess cysteine or mercaptoethanol is added to the incubates, this has a pronounced effect on the metabolic pathways and as a result many new metabolites have been identified (Table 3). In general the CN-metabolite is still present in these incubates, although in somewhat reduced amounts (especially when cysteine is added). Perhaps more striking is the observation that dimerisation is strongly suppressed, only trace amounts can be found in some of the microsome incubates.

Addition of cysteine to the incubates produces at least three different cysteine adducts. Some tentative structures are presented in Annex III. The cysteine metabolite with mass 287 detected in the *E. Coli* preparations is very likely the same compound as the cysteine-cyano-metabolite synthesized by Eurofins (section 4.3.3). The theoretical mass, suggested elemental composition and retention time all matches each other. In microsome and S9 preparations this metabolite is probably produced in trace amounts only. Instead, two new cysteine containing metabolites are observed, with MW 288 and 290, respectively. The elemental compositions calculated for these compounds (Table 3) indicate the presence of a cysteine adduct. Furthermore, based on the mass increment in the corresponding $^{13}C^{15}N_2$- analogs it is clear that the SEM side-chain is still intact in these metabolites. The mass difference of 1 and 3 units with the cysteine-cyano-metabolite synthesized provides an indication that one of the nitrogen atoms has been lost. According to the calculated elemental compositions this nitrogen atom has been replaced by an oxygen atom. At the moment the most likely explanation is that the nitro (or amino) group of the furan ring is attacked by the thiol group and, subsequently after ring opening and addition of water, a thioester is formed (see Annex III).

Table 3. The theoretical masses, retention times and suggested elemental composition of the metabolites found in the microsome, S9 and E. Coli incubates in the presence of excess cysteine and mercaptoethanol[a]

Microsome S9 fraction *E. Coli*	Addition of Cysteine / Mercapto-ethanol	Theoretical mass (m/z)	Retention time (min)		Positive/ Negative mode	Suggested elemental composition	Suggested identity
			Gradient 1	Gradient 2			
M, S, E	C, M	168.065	5.9	15.8	P + N	$C_6H_8N_4O_2$	Cyano-metabolite
(M), E	C	287.069	0.9 / 1.1	3.3 / 5.1	P + N	$C_9H_{13}N_5O_4S$	Cysteine-Cyano-metabolite
M, S	C	290.068		11.2	P + N	$C_9H_{14}N_4O_5S$	Cysteine ester metabolite
M	C	288.053		21.1	P	$C_9H_{12}N_4O_5S$	Cysteine ester-dehydro-metabolite
M	M	186.075		11.5	P + N	$C_6H_{10}N_4O_3$	Amide-metabolite
M	M	228.068	12.2		P	$C_8H_{12}N_4O_2S$	Mercaptoethanol adduct pyrrole ring
M, (S)	M	229.075	6.2	14.9	P + (N)	$C_8H_{11}N_3O_3S$	Mercaptoethanol-furazone
M, S, (E)	M	244.063	9.1	19.3 / 19.8	P + N	$C_8H_{12}N_4O_3S$	Mercaptoethanol-Cyano-metabolite
M, S, E	M	324.093 / 246.079	6.4 / 6.7	14.9 / 15.8	P	$C_{10}H_{20}N_4O_4S_2$ / $C_8H_{14}N_4O_3S$	Dimercaptoethanol adduct (amide)[1]

[a] All metabolites presented contain an intact semicarbazide side-chain. [1] The possibility of a non-covalent complex consisting of a mono mercaptoethanol adduct with MW 246 and mercaptoethanol cannot be ruled out.

Addition of mercaptoethanol produces at least 4 different adducts (excluding isomers), comprising mono- and – possibly – a di-adduct. The mercaptoethanol metabolite with mass 244 is detected in most of the incubates and is likely the fusion product of mercaptoethanol (ME) with the AN-metabolite. As for the cysteine incubates, the mercaptoethanol incubates produced a number of 'unexpected' metabolites. One of them, at RT = 11.5 min has a MW of 186 and its elemental composition matches with the CN-metabolite plus water. This corresponds to a compound in which the nitrile (CN) is replaced by an amide group ($O=C-NH_2$). Two more interesting metabolites have been identified: one with a MW of 228 and another one with MW 229. According to the elemental composition both contain a mercaptoethanol substituent, but the former appears to have lost an oxygen atom and the latter a nitrogen atom. Possibly these metabolites contain an intact furan or a pyrole ring, respectively. Tentative structures are given in Annex III.

These results indicate that thiol-containing substances (including amino acids, peptides, proteins, glutathione) may react in a number of ways with the reactive species formed during NFZ metabolism. Some of these products may be quite polar and elute early in the chromatogram. This is in agreement with the results obtained in the radio-chromatograms of the incubates, showing high activity at short

retention times. When metabolic intermediates react with thiol groups of proteins this will lead to tissue-bound adducts, both *in vitro* and *in vivo*.

4.3 LC-MS/MS

In addition to the LC-ToF-MS analysis the extracts obtained from microsomes, S9, as well as *E. Coli* incubates with NFZ and $^{13}C^{15}N_2$-NFZ were analysed by LC-MS/MS. As a first step the extracts were analysed by neutral loss scanning assuming the presence of an intact SEM side-chain in the metabolites. Potential metabolites were identified by comparison of spectra obtained for blank, NFZ and $^{13}C^{15}N_2$-NFZ containing incubates. The second step was to verify the obtained results by regular LC-MS/MS analysis.

4.3.1 Applied methodology neutral loss scanning

The semicarbazide side-chain in nitrofurazone and many of is metabolites may produce product ions upon collision induced dissociation resulting from the loss of *m/z* 17 (NH_3) and 43 (HN=C=O) neutral fragments. Similarly, metabolites originating from $^{13}C^{15}N_2$-NFZ may undergo specific loss of *m/z* 17 (NH_3) and 44 (HN=^{13}C=O) neutral fragments. The loss of these fragments may occur in negative as well as positive ESI mode. Each compound may produce its specific product ions and with its specific abundance. The assumption was made that for most, if not all, semicarbazide-containing substances, when subjected to MS/MS fragmentation, one of more product ions will be produced by loss of *m/z* 17 or 43/44 in either or both positive and negative mode. By operating the LC-MS/MS instrument in neutral loss scanning mode the precursor ions are recorded that produce these typical fragmentations (see below). By comparing the spectra obtained for blank, NFZ and $^{13}C^{15}N_2$-NFZ incubates, those metabolite-related (protonated or deprotonated) precursor ions can be filtered out. The experimental procedure is given below.

LC-MS/MS analysis of microsome, S9 and E. Coli incubates: neutral loss scanning

Extracts of microsome, S9 and *E. Coli* incubates (obtained according to the protocols described in section 3.1 and 3.2), including incubates containing surplus cysteine and mercaptoethanol were injected (20 µl) into the LC-MS/MS system. A Waters Symmetry C_{18} (150 x 3.0 mm, 5 µm) column was used at a flow rate of 400 µl/min. The metabolites were separated using a linear gradient changing from 100% water to 80% acetonitrile in 15 minutes. With this method NFZ was observed at a retention time of 10.9 minutes and the CN-metabolite at 8.4 minutes.

The mass spectrometer was operated in positive as well as in negative ESI mode. For both modes the cone voltage was set at 15 kV and the collision energy at 10 eV. The extracts were scanned (2 sec per scan) for loss of *m/z* 17 and *m/z 43/44* fragments. Precursor ions with a mass ranging from 120 to 375 were recorded. For each run scans were combined to compose spectra covering 1-min windows. This was done to allow easy visual comparison of spectra and providing an indicative retention time of metabolites detected. The composite spectra obtained for the NFZ containing incubates were compared with the spectra obtained for the blank incubates. Each new precursor ion was marked. The same was done for incubates containing $^{13}C^{15}N_2$-NFZ. Next the marked protonated precursor ions for each composite spectrum of NFZ and that of the corresponding $^{13}C^{15}N_2$-NFZ incubate were compared. In case a correct match was obtained between the samples (a mass increase of 3 or 6 units in the isotope-labelled incubate spectra) the corresponding precursor ion was flagged.

For the metabolites identified in this way the precise retention times were obtained by reinjection of the incubates in the LC-MS/MS system. The LC-MS/MS was run in MRM (Multiple reaction monitoring) mode at the transitions identified by the neutral loss scanning experiments. For the more prominent metabolites MS/MS scanning in product mode was performed at 10 – 20 eV in order to obtain additional information on the chemical structure of the metabolites.

In Figure 13 an example is given of the composite mass spectrum collected for the fraction eluting between 8 and 9 min of precursor ions undergoing a neutral loss fragmentation of *m/z* 43 (44 for the isotope-labelled compound) of a blank microsome incubate, an incubate with nitrofurazone and an incubate with $^{13}C^{15}N_2$-NFZ. The protonated precursor ion with mass 169 in the NFZ incubate is absent in the blank incubate, and has shifted to mass 172 in the $^{13}C^{15}N_2$-NFZ incubate, indicating the presence of a single SEM side-chain. In the same fraction a minor component is observed in the NFZ incubate with mass 317 and while a component with mass 323 is present in the $^{13}C^{15}N_2$-NFZ incubate. This mass shift is indicative for a dimeric compound, containing two SEM side-chains.

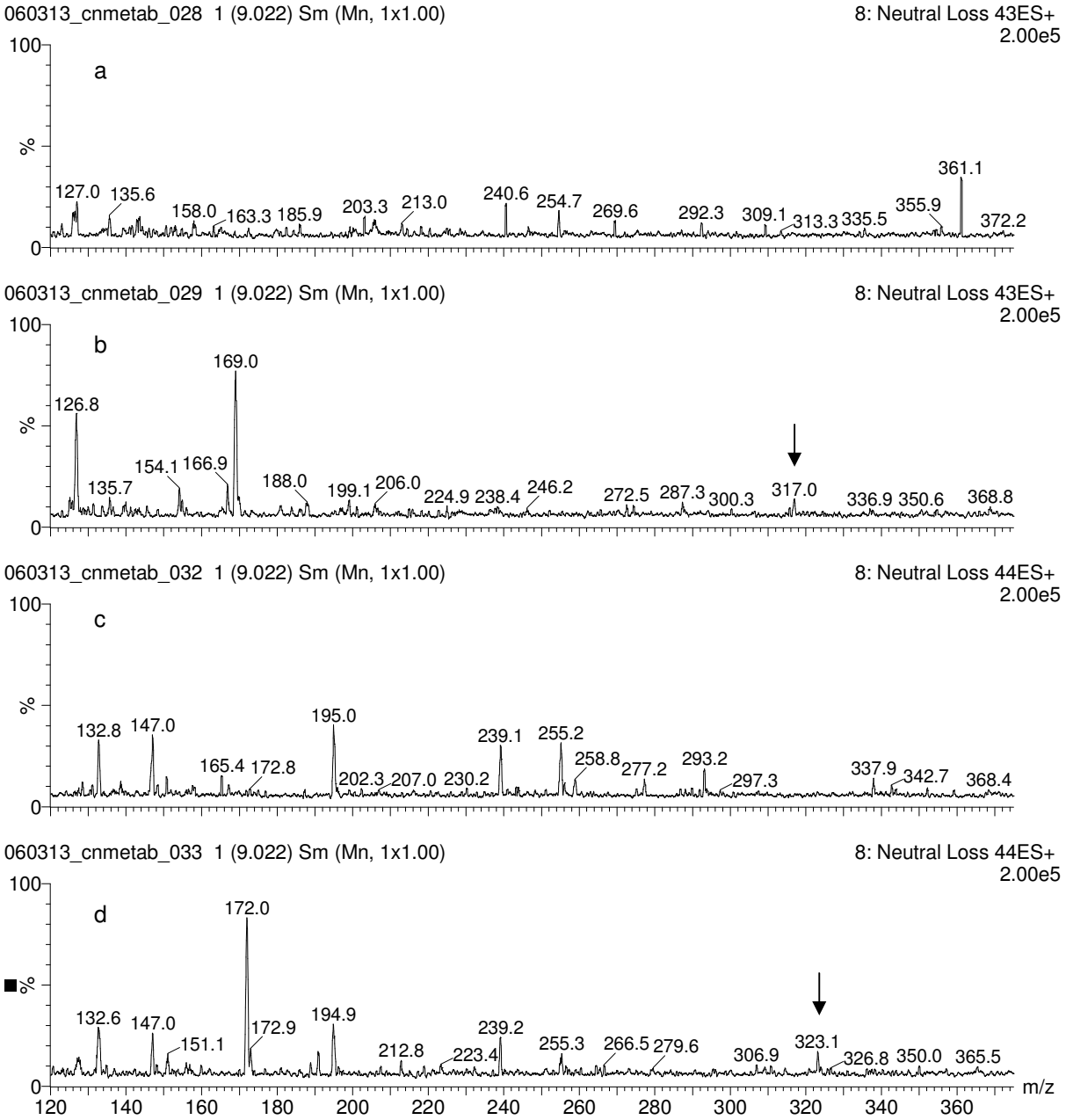

Figure 13: Composite mass spectra of components eluting between 8 and 9 min and undergoing a neutral loss of m/z 43 or 44 under positive ESI conditions. (a) and (c) Blank microsome incubate, (b) Microsome incubate with NFZ, (d) Microsome incubate with $^{13}C^{15}N_2$-NFZ. The compound with m/z 169 in Figure (b) and with m/z 172 in Figure (d) corresponds to the CN-metabolite. Indicated with an arrow is a minor dimeric component with mass 317 in the NFZ incubate, and as a component with mass 323 in the $^{13}C^{15}N_2$-NFZ incubate.

The metabolites identified by neutral loss scanning were verified by LC-MS/MS analysis of the corresponding transitions. In Figure 14 chromatograms of some selected MRM traces are presented as an example. The metabolites that were produced in significant amounts were investigated in more detail, by means of MS/MS scanning. For a number of compounds the observed fragments and

fragmentation pathways provided sufficient information to propose possible chemical structures for these metabolites.

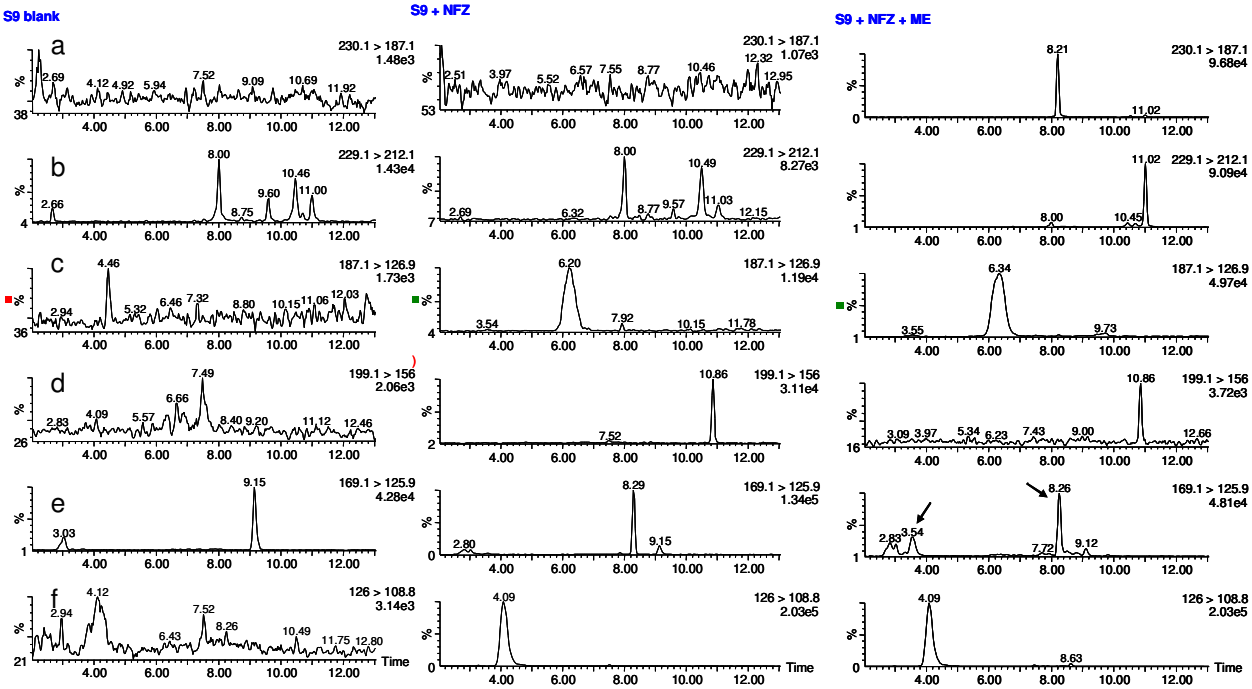

Figure 14. Chromatograms of blank, NFZ and NFZ + ME S9 incubates. Selected transitions to show the presence or absence of (a) a mercaptoethanol furan adduct ($M+H^+$: m/z 230, r.t. 8.2 min), (b) a mercaptoethanol pyrrole adduct ($M+H^+$: m/z 229, r.t. 11.0 min), (c) an amide metabolite ($M+H^+$: m/z 187, r.t. 6.2 min), (d) unreacted nitrofurazone ($M+H^+$: m/z 199, r.t. 10.9 min), (e) the cyano-metabolite ($M+H^+$: m/z 169, r.t. 8.3 min) together with an unknown metabolite at r.t. 3.5 min, and (f) a low molecular weight metabolite ($M+H^+$: m/z 126, r.t. 4.1 min) identified as aminofurfural hydrazone.

4.3.2 Identification of metabolites by LC-MS/MS

By comparing blank, NFZ and $^{13}C^{15}N_2$-NFZ composite spectra a list of potential metabolites of NFZ in microsome, S9 and *E. Coli* incubates has been compiled (Table 4). From this table it is evident that in all incubates a metabolite with mass 168 is produced. Combined with the information collected with the LC-ToF-MS and the standard synthesized by Eurofins (Dr. S. Weigel and Dr. R. Gatermann), this metabolite can be positively identified as the CN-metabolite. In the microsome incubates also a minor metabolite with mass 166 eluting at 8.9 min is observed. The obtained MS/MS spectrum is in accordance with the acrylonitrile-metabolite (see also section 4.3.3). This metabolite is not present in detectable amounts in the S9 and *E.Coli* incubates.

Table 4. Potential metabolites of nitrofurazone, detected by LC-MS/MS neutral loss scanning of microsome, S9 fraction and E. Coli incubates, retention times, observed neutral losses in positive and negative ESI and proposed molecular identities of metabolites.

Microsome S9 fraction *E. Coli*	Observed precursor nominal mass	Retention time (min)	Neutral loss fragments Positive/Negative		SEM side-chain	Suggested identity
			m/z 17	*m/z* 43/44		
M, S	125	4.1	P		Single[1]	Aminofurfural hydrazone
M, S, E	168	8.3		P, N	Single	Cyano-metabolite
M	166	8.9		N	Single	Acrylonitrile-metabolite
S, E	186	6.2	P	N	Single	Amide-metabolite
M	316	7.8 / 8.2	P	P, N	Double	Dimeric compound
E	332	9.3 / 9.7	P	N	Double	Dimeric compound
E	342	11.3	P	N	Double	Dimeric compound

[1] Mass increase of 2 units observed in the $^{13}C^{15}N_2$-metabolite

A metabolite with low molecular mass (125) was detected in microsome and S9 incubates at a relatively short retention time. A mass increase of only 2 units was observed for the $^{13}C^{15}N_2$-NFZ incubates indicating an incomplete SEM side-chain (loss of the terminal O=C-NH_2 / O=^{13}C-NH_2 group). MS/MS fragmentation (10 eV) of the protonated species (mass 126) yielded two fragments with mass 109 (loss of 17, NH_3) and mass 81 (additional loss of 28, CO) (Figure 15). MS/MS fragmentation of the isotope-labelled analog (mass 128) yielded m/z 111 and 83 fragments, respectively. Upon fragmentation the mass difference of 2 units is conserved, indicating that fragmentation occurs at the furan side of the molecule. Possible structures of the precursor molecule include a truncated CN-metabolite or an aminofurane metabolite (aminofurfural hydrazone). The latter is more probable because the precursor ion loses a fragment with mass *m/z* 17 (NH_3) instead of *m/z* 27 (HCN) under the conditions used. Furthermore, this NH_3 group does not originate from the SEM part of the molecule as this would have resulted in a loss of *m/z* 18 ($^{15}NH_3$) for the $^{13}C^{15}N_2$-NFZ incubate, but a regular loss of *m/z* 17 is observed for this compound instead. Probably the furan derivative with mass 109 rearranges to a more stable seven membered ring system. The fragment with mass 81 probably corresponds to a protonated pyridazine molecule.

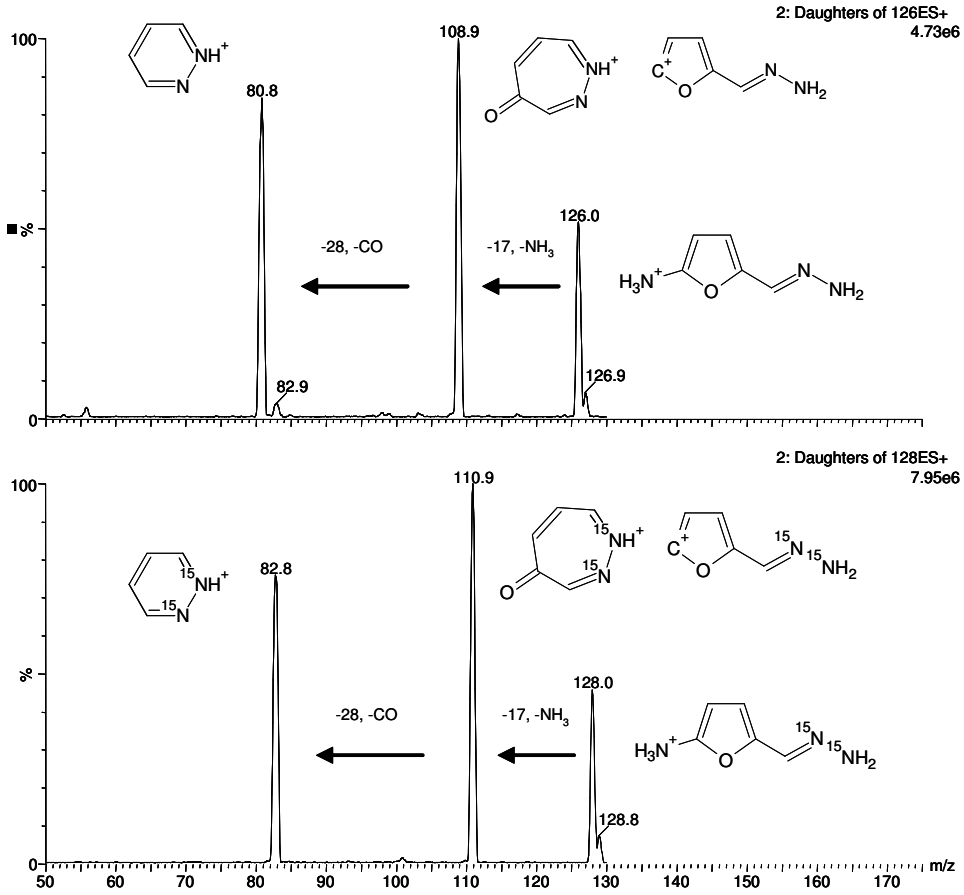

Figure 15: MS/MS spectra produced (collision energy: 10 eV) from the metabolite with MW 125 (aminofurfural hydrazone) and its isotope-labelled analog. Probable structures of the fragments are indicated.

Similarly, a metabolite eluting at 6.2 min and with mass 186 was observed in S9 and *E. Coli* incubates. A mass of 186 could correspond to a ring-opened metabolite with a terminal amide group instead of a cyano group. This metabolite was also detected by LC-ToF analysis. To further elucidate the structure, the protonated molecule was fragmented (10-20 eV) (Figure 16). At low collision energy the major fragments are *m/z* 170 (loss of 17, NH_3) and *m/z* 127 (additional loss of 43, HNCO). At higher collision energy the m/z 127 fragment loses a fragment with mass 28 (loss of CO or N_2) to 99 and further to 71 (loss of 28, CO or N_2). The corresponding fragments (data not shown) for the isotope-labelled analog are: *m/z* 190 produces 173 (loss of 17, NH_3) and *m/z* 129 (additional loss of 44, $HN^{13}CO$). Furthermore two products are formed with mass 99 (loss of 30, $^{15}N_2$) and 101 (loss of 28, CO) and a fragment with mass 71 (loss of 28, CO and loss of 30, $^{15}N_2$). In negative ESI mode the deprotonated molecule (*m/z* 185) produces fragments with mass 170 (loss of 18, H_2O), mass 142 (loss of 43, HNCO), mass 124 (loss of 18, H_2O from the *m/z* 142 fragment), and mass 96 (loss of 28, N_2, from the *m/z* 124 fragment). The fragments observed for the isotope-labelled analog are in accordance with the proposed structures. All data collected point to an open-chain amide metabolite.

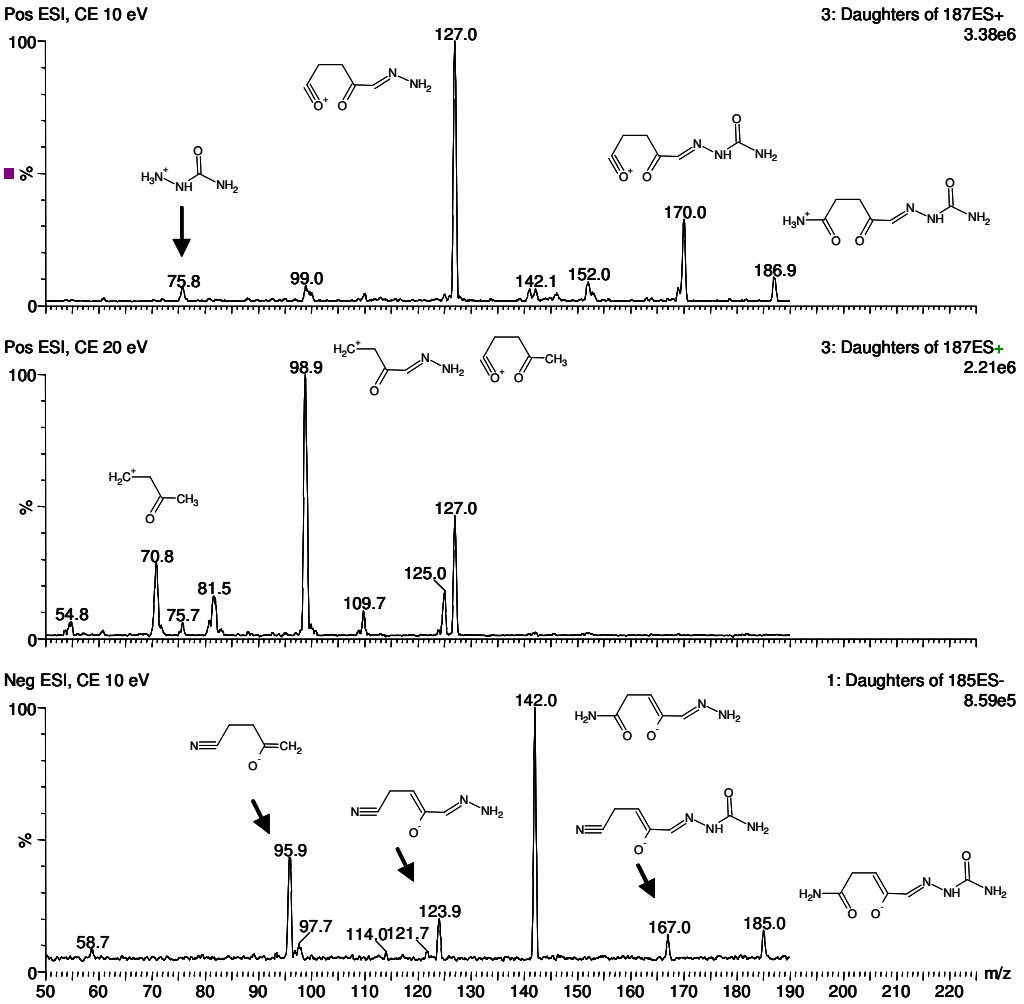

Figure 16: MS/MS spectra obtained for the metabolite with MW 186 and retention time of 6.3 min. Probable structures of the fragments are indicated.

Several dimeric compounds were detected in the microsome and *E. Coli* incubates. Peak intensities were quite low, however, especially when compared to the ToF-MS results (perhaps the loss of m/z 17 or 43 fragments is not very prominent for these compounds). The dimer with mass 312 formed in microsome incubates was also detected by LC-ToF-MS. The same is true for the MW 332 dimeric compound detected in the *E. Coli* incubate. The dimer with mass 366 observed in the ToF analysis of microsomal incubates was not detected by neutral loss scanning.

The same methodology was applied for incubates to which surplus cysteine or mercaptoethanol was added. In Table 5 and Annex IV the results are compiled. In most incubates the CN-metabolite could be detected. Its formation is reduced in the cysteine incubates. Similarly, the presence and identity of the low-molecular mass (125) metabolite could be confirmed. Dimeric compounds were hardly detected, which is in good agreement with the ToF-MS results. The formation of these compounds is strongly reduced by the presence of excess cysteine or mercaptoethanol.

Table 5. Potential metabolites of nitrofurazone, detected by LC-MS/MS neutral loss scanning of microsome, S9 fraction and E. Coli incubates in the presence of cysteine and mercaptoethanol. The observed retention time, neutral loss in positive and negative ESI and proposed molecular identity of metabolites is given.

Microsome S9 fraction *E. Coli*	Addition of Cysteine/ mercapto-ethanol	Observed precursor mass (m/z)	Retention time (min)	Neutral loss fragments *m/z* 17 Positive/Negative	Neutral loss fragments *m/z* 43/44 Positive/Negative	SEM side-chain	Suggested identity
M,S	C, M	125	4.1	P		Single[1]	Aminofurfural hydrazone
M, S, E	(C), M	168	8.3		P, N	Single	Cyano-metabolite
M, S	C	290	8.4	N	N	Single	Cysteine ester-metabolite
M, S	C	288	9.8 / 10.3	N	P	Single	Cysteine ester-dehydro-metabolite
M, S, E	M	168	3.5	P	P	Single	CN-metabolite analog
M, S, E	M	186	6.2	P	N	Single	Amide-metabolite
M, S	M	229	8.2	P	P	Single	Mercaptoethanol-furazone
M, S, E	M	228	11.0	P	P, N	Single	Mercaptoethanol adduct pyrrole ring
E	M	332	9.3 / 9.7	P	N	Double	Dimeric compound

[1] Mass increase of 2 units observed in the $^{13}C^{15}N_2$-metabolite

Several new metabolites were found in both cysteine and mercaptoethanol incubates. At least three different cysteine adducts were observed in both microsome and S9 incubates. However, *E. Coli* incubates failed to produce detectable amounts of cysteine adducts. The observed masses (288 and 290) and the prominent loss of *m/z* 17 (cysteine-NH_3) in negative mode are indicative for cysteine adducts. Interestingly, the synthesized standard of the cysteine-CN-metabolite (gift of Dr Weigel and Dr. Gatermann of Eurofins) does not match with any of these metabolites: the standard has a retention time of only 4 min and a mass of 287 (see section 4.3.3). A mass difference of 1 or 3 units indicates the loss of a NH group (or exchange to an O group). Tentative structures of the cysteine adducts are given in annex IV.

The mercaptoethanol incubates produced a variety of metabolites. The amide metabolite with mass 186 is abundantly formed. A metabolite with mass 168 was observed in all incubates at short retention time (3.5 min). As yet, the identity of this metabolite remains to be elucidated, but its short retention time suggests a polar nature. In conjunction with the LC-ToF results, all mercaptoethanol incubates produced a metabolite with mass 228, while in the microsome and S9 incubates also a metabolite with mass 229 was produced (but clearly absent in *E. coli*). The mass difference of 1 unit indicates the difference between an O and an NH group. For the metabolite with MW 229, the MS/MS fragmentation spectra collected point to a furazone compound in which the nitro-group has been substituted for a mercapoethanol substitutent (data not shown). The metabolite with MW 228 probably contains a similar structure, but with a pyrrole ring instead of a furan ring. These two metabolites were also identified by LC-ToF-MS (section 4.2.2 and Annex IV)

The list of tentatively assigned metabolites suggests that sulphur containing substances react with the intact furan ring (e.g. by attack on the nitro or amino group) rather than with the double bond of the acrylonitrile metabolite. The expected CN-metabolite containing a mercaptoethanol adduct with MW of 244 was not detected by LC-MS/MS (but it was detected in microsome and S9 incubates by LC-ToF-MS). It can not be excluded that acrylonitrile adducts are not detected because they do not produce the selected neutral loss fragments. Even though the structures proposed remain speculative, it can be concluded that there are several positions in the nitrofurazone molecule that may react with free thiol groups and may lead to a variety of tissue-bound and free metabolites.

4.3.3 Verification of synthesized metabolites by LC-MS/MS

The final step in the determination of potential metabolites in the microsome, S9 and *E. Coli* incubates comprised the analysis of selected compounds by LC-MS/MS. As has been discussed in section 4.3.2, the neutral loss scanning experiments have resulted in the identification of a number of potential metabolites. It should be noted that metabolites which do not yield a detectable neutral loss fragmentation of *m/z* 17 or 43 in either positive or negative mode are missed. The ToF-MS experiments indicated that some metabolites are formed that do not yield one of these neutral loss fragmentations (or in low abundance only). Unfortunately the ToF-MS experiments yield accurate mass and elementary composition data on the parent molecule only and not on potential product ions.

In addition to this, a number of nitrofurazone metabolites have been provided by the partner involved in lot 2, (Dr. Gatermann, Eurofins, Germany). Several of these metabolite standards have been incorporated in this study: the cyano-metabolite (CN-metabolite), acrylonitrile metabolite (AN-metabolite), glutathione-cyano-metabolite (GSH-CN-metabolite) and cysteine-cyano-metabolite (Cyst-CN-metabolite) were investigated by LC-MS/MS.

The partner involved in lot 2 had indicated that the sulphur-adducts of the CN-metabolite synthesized are unstable and react in the presence of a strongly basic alkyl amine like pyrrolidine or piperidine (poster presented by S. Weigel *et al* at 2nd Int Symp. on Recent Advances in Food Analysis, Prague, November 2-4, 2005). In order to study this reactivity in some detail (and to investigate the usefullness of piperidine as a trapping reagent of sulphur adducts *in vitro* and *in vivo*) a piperidine metabolite was synthesized in-house by reaction of a 0.2% solution of piperidine in water with a solution of the GSH-CN-metabolite or cyst-CN-metabolite. The available metabolites together with analytical standards of NFZ and GSH were analysed in positive and negative electrospray mode (Table 6 and 7):

Table 6: Analysis of nitrofurazone and synthesized metabolites by positive electrospray MS(/MS)

Metabolite	$[M+H]^+$ (m/z)	Cone (V)	Collision Energy (eV)	Fragments (m/z)
CN-metabolite[1]	169.1	15	15	81.7
			8	126.0
AN-metabolite[1]	167.1	15	10	67.6
			8	124.0
Cyst-CN-metabolite[1]	288.2	15	10	75.6
			10	213.0
GSH-CN-metabolite[1]	474.3	15	15	270.1
			15	345.1
NFZ[2]	199.1	15	13	156.0
			13	182.0
GSH[2]	308.2	25	15	162.0
			15	179.0
Piperidine-metabolite[3]	225.1	25	16	138.0
			16	208.1

[1] Provided by the partner involved in lot 2 (Dr Gatermann, Eurofins, Germany). [2] Included for completeness

[3] Standard obtained by reaction of GSH-CN-metabolite with piperidine

Table 7: Analysis of nitrofurazone and synthesized metabolites by negative electrospray MS(/MS)

Metabolite	$[M-H]^+$ (m/z)	Cone (V)	Collision Energy (eV)	Fragments (m/z)
CN-metabolite	167.1	10	8	124.0
			8	140.0
AN-metabolite	165.1	15	10	94.7
			10	122.0
Cyst-CN-metabolite[1]	286.2	10	10	122.0
			10	165.0
GSH-CN-metabolite[2]	472.2	15	15	306.2
NFZ	197.1	20	10	124.0
			10	150.0
GSH	306.2	20	20	128.0
			20	143.0
Piperidine-metabolite[2]	223.1	25	15	94.7

[1] The cyst-CN-metabolite proved very labile under the MS conditions used. Even at low cone voltage in-source fragmentation producing the m/z 165 fragment is prominent. [2] Only one fragment was observed in neg ESI.

The LC-MS/MS method described in section 4.3.1 for the neutral loss experiments was used for the analysis of the above mentioned metabolites. Typical retention times and ion ratios obtained for the metabolites are shown in Table 8. Two peaks were observed for the AN-metabolite, possibly representing the *cis* and *trans* isomers. It was noted that aqueous solutions of the standards were quite unstable. At pH 7 most of the GSH-CN-metabolite, the cyst-CN-metabolite as well as the AN-

metabolite(s) had decomposed to unidentified products within 24 h. At pH 3 (1% acetic acid solution) the standards were stable. The CN-metabolite proved to be stable in solution in a pH range of 2 to 12. A stability study indicated that solutions of the CN-metabolite in water ware stable for at least 4 weeks at 4°C.

Table 8: Retention times of the synthesized metabolites and ion ratios of the product ions described in Tables 6 and 7.

Component	Retention time (min)	Ion ratio (pos ESI)	Ion ratio (neg ESI)
CN-metabolite	8.2	60%	41%
AN-metabolite	9.6 (major)	23%	60%
	8.8 (minor)	24%	86%
GSH-CN-metabolite	8.5	56%	Only one fragment detected
Cyst-CN-metabolite[1]	5.3	23%	65%
NFZ	10.8	58%	83%
GSH	3.4	86%	51%
Piperidine-metabolite	10.2	83%	Only one fragment detected

[1] Low sentisitivity in neg ESI mode due to extensive in-source fragmentation.

The formation of the piperidine-metabolite was investigated in some detail at RIKILT. When the GSH-CN; cyst-CN or the AN-metabolite are added to a solution of 0.2% piperidine in water (pH = 12), the corresponding piperidine metabolite is formed instantaneously. At lower pH, the formation slows down, requiring more than 24 h at pH 7. At a pH lower than 7 no piperidine metabolite is formed at all.

In Figure 17 a tentative mechanism is depicted by which sulphur-containing metabolites can be transformed into a piperidine containing metabolite. Piperidine is likely acting both as base as well as the nucleophilic reagent. The driving force of this reaction is the formation of the relatively stable enamine derivative. It can be rationalized that 2-adducts are labile to basic conditions and prone to lose the sulphur substituent. In contrast 3-adducts are predicted to be more stable to basic conditions as the sulphur substituent cannot be cleaved very easily.

The stability of the piperidine metabolite at various pH was investigated as well. It was noted that at acidic pH the piperidine metabolite degrades quite rapidly. This is not surprising: enamines are hydrolyzed under acidic aqueous conditions (March, 1985).

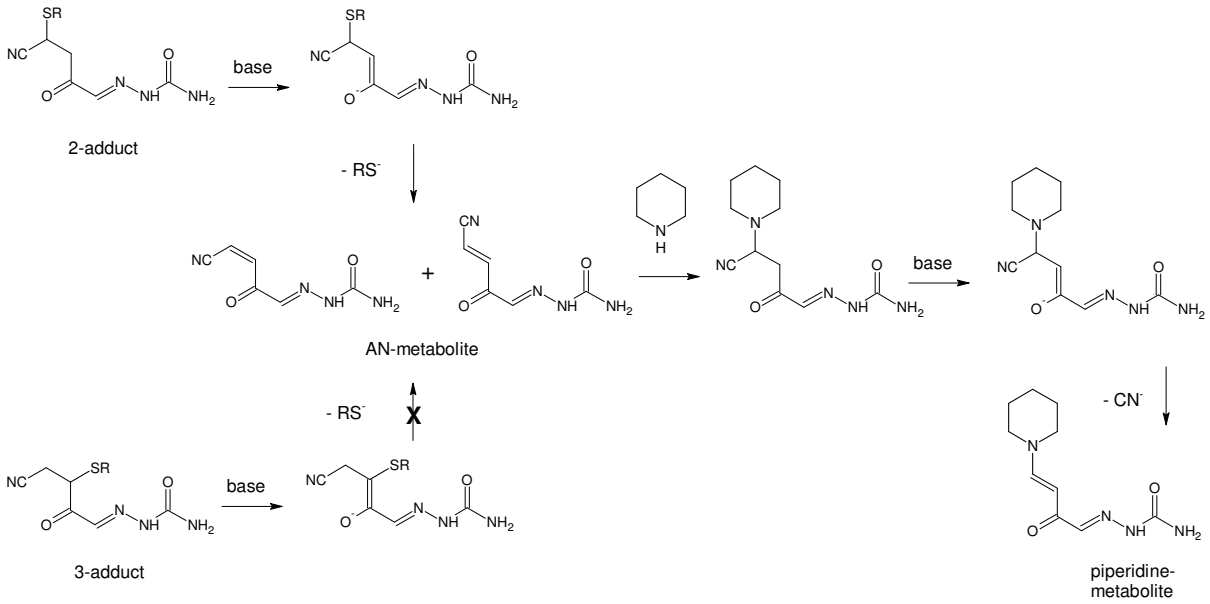

Figure 17: Tentative mechanism of the formation of a piperidine metabolite from sulphur-containing metabolites.

The microsomal, S9 and *E.coli* incubates were analysed for the presence of the selected metabolites by means of the MS conditions described in Table 5 and 6. NFZ and the CN-metabolite were positively identified in these incubates, thus confirming the results already obtained in sections 4.2.2 and 4.3.2. In the microsomal preparation a small amount of the AN-metabolite was identified (see also section 4.3.2). Interestingly, the synthesized standard exists of two compounds (cis and trans isomers?), while the microsome extract contains only one of them (likely the cis isomer). No evidence was found for the presence of the cyst-CN and GSH-CN metabolites in any of the incubates. This may indicate that they are formed in low concentrations only, or that their stability is rather limited.

A number of incubation extracts was treated with a 0.2% piperidine solution in order to produce the piperidine metabolite. Only trace amounts of this metabolite were formed, corroborating the finding that sulphur containing addition products to the AN metabolite are present in very low concentrations.

4.4 Overall discussion of results

The chemical reduction of nitrofurazone to its amino derivative has been described as early as 1957 by Austin. It was noted at the time that aminofurazone is a rather unstable compound, which slowly rearranges to form the corresponding CN-metabolite. Interestingly, the aminofurans produced upon reduction from other nitrofurans, like furazolidone and furaltadone were quite stable compared to nitrofurazone (Ebertino *et al*, 1962). The metabolism of nitrofurazone has been studied in limited detail only. Early reports mention the formation of the CN-metabolite as a major metabolite (Beckett and Robinson, 1957; Tasumi *et al*, 1975). The CN-metabolite was also found in the urine of rats administered with NFZ (Yeung *et al*, 1983). Based on this information combined with what is known for the metabolism of FZD, an overview of potential metabolites of NFZ was made. This overview is presented in Annex I.

Wrapping up the results obtained for the analysis of the microsome, S9 and *E. Coli* incubates, it can be concluded that several metabolites are produced. The chemical structures of these metabolites could be established in varying detail, depending on the availability of a synthesized standard or on the mass spectrometric data collected. The combined information of high-resolution ToF mass spectral data, MS/MS fragmentation patterns and the comparison of spectra of unlabelled and isotope-labelled analogs provided substantial evidence for the structure elucidation of several new metabolites. Three of them (CN-metabolite, amide metabolite and aminofurfural hydrazone metabolite) are of interest for further investigation. It should be noted that only the CN-metabolite is detected in all test systems and under all circumstances. The other two were detected in two out of three incubation systems. Another advantage of the CN-metabolite is that a synthesized standard is available. The microsome incubates produce several dimeric compounds, which are of limited interest for *in vivo* systems. A number of cysteine and mercaptoethanol adducts identified are only formed in detectable amounts when large amounts of the thiol component are added to the incubates.

The next step in the selection of an alternative marker for semicarbazide will be the study on the occurrence and depletion characteristics of the three selected metabolites. They will be investigated in incurred materials, in conjunction with the development of a LC-MS/MS confirmatory method for the metabolite that is finally chosen. The research conducted on this subject will be described in Chapter 5.

5 LC-MS ANALYSIS OF INCURRED MATERIALS AND DEVELOPMENT OF A LC-MS/MS CONFIRMATORY METHOD

5.1 LC-MS analysis of incurred materials

Having identified the major metabolites formed in *in vitro* incubates, the attention was directed to the analysis of incurred materials. Porcine muscle and liver samples were provided by the partner involved in lot 3, Dr Cooper of Chemical Surveillance Department, Veterinary Sciences Division, Belfast. These samples had originally been collected in the framework of the FoodBRAND FP5 project. Pigs had received 400 mg/kg nitrofurazone in feed for 10 days and were slaughtered at various withdrawal times. The muscle and liver samples of week 0 and week 6 were selected for investigation by LC-MS/MS and LC-ToF-MS. The muscle samples of week 0 at the time of collection contained approx. 2000 µg/kg tissue-bound SEM (plus an unknown amount of extractable metabolites!), the samples of week 6 contained 250 µg/kg. For liver the concentrations determined were 2500 and 40 µg/kg tissue-bound SEM at week 0 and 6, respectively.

Of each sample 500 mg of tissue was extracted with 2 ml water, centrifuged and filtered through a 0.45 µm acrodisc filter. The samples of week 6 were also spiked with a mix of metabolites at 500 µg/kg before extraction.

The samples were injected into the LC-ToF-MS and run with LC-gradient system 2, optimised for moderately polar metabolites. MetAlign™ software was used to analyse the obtained data from the LC-ToF-MS experiments. The full scan data of week 0 and week 6 were compared. MetAlign™ indicated a substantial number of differences between the two data sets. This could be explained, at least in part, by the fact that the tissues of two different animals were compared, which can be expected to differ more than two *in vitro* incubates. Except for the CN-metabolite, none of the obtained accurate mass data for these peaks could be correlated to previously identified metabolites. Only the CN-metabolite could be positively identified in the week 0 muscle tissue sample. If present, the concentration of other potential metabolites apparently is too low to allow detection under the conditions applied.

The same extracts were also subjected to LC-MS/MS analysis on a Micromass Micro system (Figure 18). Only two metabolites were detected In the muscle extracts of week 0: the CN-metabolite at a concentration of approx 500 ng/g, as well as the metabolite assigned as aminofurfural hydrazone with MW 125. The concentration of the latter cannot be determined as an analytical standard is not available. Both metabolites are not detectable in the week 6 extract. The amide metabolite as well as any of the other reference metabolites were not present in detectable concentrations in both week 0 and 6 extracts. Although the muscle tissue extracts contained large amouts of gluthatione (in excess of 10 µg/g, data not shown), the GSH-CN-metabolilte was not detected (detection limit: 25 ng/g). Additional analysis of the extracts on the more sensitive Micromass Ultima system revealed traces of NFZ (less than 5 ng/g) and of the GSH-CN-metabolite (5 ng/g) to be present in the week 0 sample (data not shown). No detectable amounts of the AN-metabolite and the cyst-CN-metabolite were found. Analysis of the spiked sample showed that the recovery of the latter two metabolites was very low. These metabolites either are not extracted by the extraction procedure used or are too unstable to be recovered. In the week 6 sample extracts as well as in the liver extracts concentrations of each of the metabolites were below or close to the limit of detection. Apparently the metabolites selected

accumulate to a lesser extent in liver tissue than in muscle tissue. It is possible that these metabolites are formed in the liver, but that they are quickly transformed to other products due to enzymatic and chemical action.

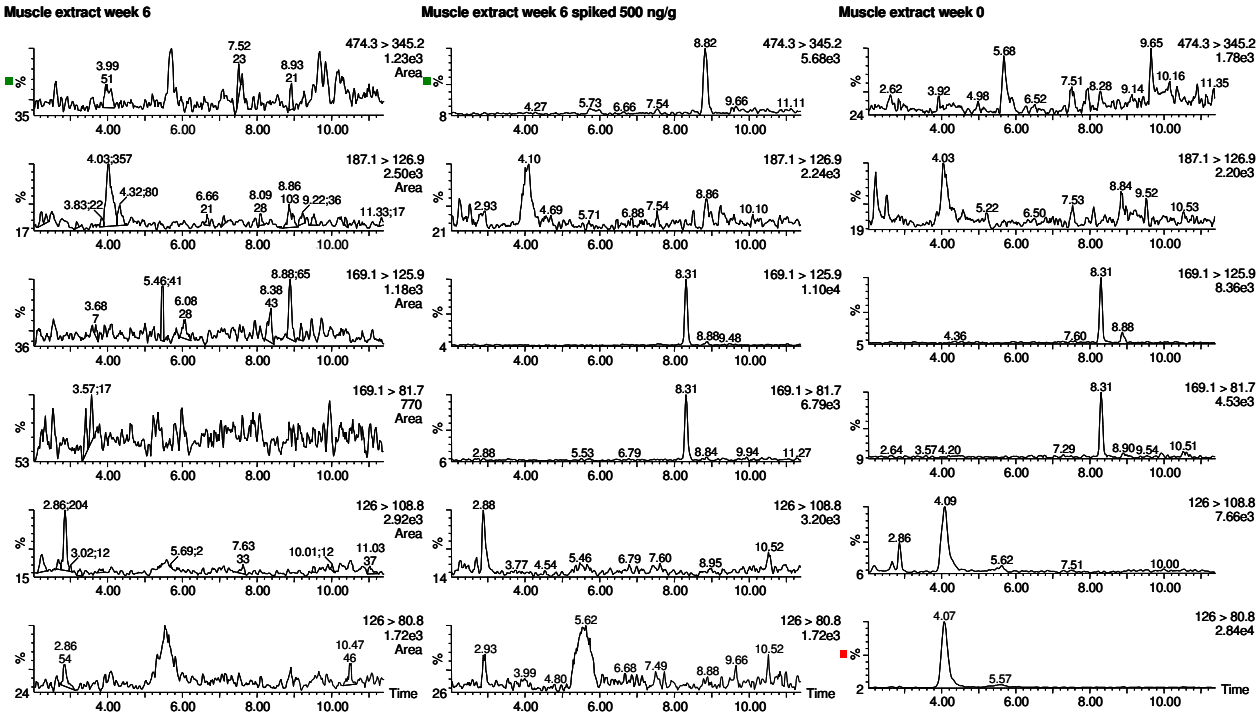

Figure 18: LC-MS/MS analysis of incurred porcine muscle tissue extracts of week 0 and 6. Some selected transitions are shown as an example: (a) GSH-CN-metabolite, (b): amide metabolite, (c) and (d) two MRM transitions of the CN-metabolite, (e) and (f) two MRM transitions of the 125-MW metabolite.

To test for the presence of sulphur-containing metabolites, the aqueous muscle extracts were treated with piperidine to a final concentration of 0.5%. The spiked sample of week 6 was used to check the complete conversion of the sulphur-containing metabolites into the piperidine derivative. As expected the CN-metabolite proved stable under the action of piperidine. A small amount of piperidine derivative (estimated to be not more than 25 ng/g) was detected in the week 0 extract. This concentration is somewhat higher that accounted for by the GSH-CN-metabolite (5 µg/kg), indicating the presence of other, unidentified, sulphur-containing adducts or metabolites.

It can be concluded that the CN-metabolite is detected in substantial amounts (approx. 500 µg/kg) in porcine muscle tissue during medication with nitrofurazone. In liver tissue the concentration is significantly lower. The low molecular weight metabolite (aminofurfural hydrazone) is present in a substantial amount in muscle tissue as well, but by lack of a standard it can not be quantified. All other targeted metabolites, if detected at all, are present in muscle as well as liver tissue only at extremely low concentrations, even during medication. It can not be excluded that adducts are formed by reaction of thiol-containing substances (amino acids, gluthatione, proteins), but these adducts may be of limited stability under the extraction conditions used.

5.2 Development of a confirmatory LC-MS/MS method for the cyano-metabolite of NFZ

All the experiments and work described in chapters 3 to 5 point to one single conclusion: the most likely candidate metabolite as an alternative to the current method of analysis is the CN-metabolite. The aminofurfural metabolite could be an interesting alternative only if method development fails for the CN-metabolite. All other metabolites (tentatively) identified are either metabolites formed in only one assay (e.g. dimeric compounds in the microsome incubates), or appear to be rather unstable and transient under the experimental conditions used (e.g. AN-metabolite) or are present in only very low concentrations even at zero-withdrawal time (e.g. GSH-CN-metabolite). The reaction with piperidine produces some derivatised product, but the amounts are relatively small compared to that of the CN-metabolite and therefore do not constitute a viable alternative. Hence, it was concluded that the CN-metabolite is the mostly likely alternative to SEM for the development of a confirmatory LC-MS/MS method suitable for official control.

To explore the possibility of a selective and sensitive analytical method for the determination of the CN-metabolite in muscle tissue, an improved extraction protocol was developed as described below.

Sample preparation procedure for the analysis of the CN-metabolite

One gram of sample is extracted with 10 ml of phosphate buffer pH 7 for 1 hour. The sample is centrifuged (10 min at 4000 rpm) and the supernatant is applied to a pre-conditioned Waters Oasis HLB 60 mg, 3 ml cartridge. The cartridge is washed with 3 ml water and eluted with 3 ml methanol. After evaporation to dryness the residue is reconstituted in 500 µl 0.2% formic acid. Analysis is performed by LC-MS/MS (Micromass Ultima) in negative ESI mode. Chromatographic conditions were applied as described in section 4.3.3.

To optimise extraction recovery several solvents and buffers of various pH were tested. Best results were obtained with a neutral or slightly acidic phosphate buffer. A single extraction with 10 ml of buffer followed by SPE over a Waters Oasis HLB column proved to give the most reliable results, with an overall recovery of 65%.

Several instruments (Micromass Ultima and Micromass Micro) and positive versus negative electrospray mode were compared to determine the optimal conditions for the analysis of the CN-metabolite. Best results were obtained on a Micromass Ultima instrument with negative ESI.

The method was applied to the incurred porcine materials obtained from VSD. Two muscle tissue samples of week 0 and one sample of week 1, 2, 3, 4 and 6 as well as a liver and a kidney sample of week 0 were analysed in duplicate. Quantification was performed against a matrix matched standard curve. The results are presented in table 8. At zero withdrawal time substantial amounts of the CN-metabolite are present in muscle tissue (around 500 µg/kg). The concentration in liver (20 µg/kg) and kidney (40 µg/kg) on the other hand is considerably lower. After one week of withdrawal the levels in muscle tissue have dropped to only 3 µg/kg, which is close to the limit of detection (2 µg/kg). There is no direct link between the amounts of tissue-bound SEM present and that of the CN-metabolite. The concentration of the CN-metabolite drops much more quickly than that of tissue-bound SEM. This indicates that when tissue-bound SEM is removed from the protein, it is not released in the form of the

CN-metabolite. Further improvement of the analytical method will be required to determine if the low concentrations detected of the CN-metabolite in the muscle samples of week 2 to 6 are real or just an artefact.

Table 8: Analysis of incurred porcine materials by LC-MS/MS: determination of the CN-metabolite. Results obtained by duplicate analysis.

Matrix	Sample ID	Week	Content (µg/kg)	Tissue-bound SEM (µg/kg)[a]	Remark
Porcine Muscle	0309845	0	403 ± 15	2000	Also NFZ detected at 7 µg/kg
	0309846	0	722 ± 52	1781	Also NFZ detected at 4 µg/kg
	0309859	1	3.0 ± 0.2	1006	
	0309869	2	0.8 ± 0.1	450	Only one product ion detected[b]
	0309881	3	1.1 ± 0.3	499	Only one product ion detected[b]
	0309893	4	0.9 ± 0.7	477	Only one product ion detected[b]
	0309905	6	1.2 ± 0.2	241	Only one product ion detected[b]
Porcine Liver	0309845	0	21.4 ± 0.4	2668	
Porcine Kidney	0309845	0	41.4 ± 3.6	3599	

[a] As determined by VSD. [b] The identity of the analyte could not be confirmed

In conclusion, the CN-metabolite, being the major metabolite detected in microsomal and *E. Coli* preparations, is also formed *in vivo*. Accumulation during medication largely occurs in muscle tissue. In liver and kidney tissues only low concentrations are observed, probably the metabolite is quickly metabolised to other yet unidentified metabolites. Although depletion in muscle tissue is relatively quick compared to the persistent tissue-bound metabolites, its presence can be detected up to one week after the end of medication. It should be realized that many authorized veterinary drugs have depletion half-lives ranging from only a few hours up to a few days at the most. This is also the case for other banned substances like nitroimidazoles and various growth promoting agents. It is therefore recommended that the LC-MS/MS method described for the CN-metabolite in this study should be further improved and that the substance should be made available as a reference material (including an isotopically-labelled analogue as IS).

6 CONCLUSIONS

At the start of the project a considerable uncertainty in completing the project was foreseen, originating from the fact that the analyte was unknown and hence no realistic estimate of the difficulty of this task could be made.

Due to the fact that the synthesis of ^{14}C-labelled standards and a $^{13}C^{15}N_2$-labelled standard was successful, the metabolic pathway of nitrofurazone could be elucidated in considerable detail from *in vitro* experiments using chicken liver microsomes and S9 fraction, and *Escherichia Coli* ATCC 8739.

The combination of high-resolution ToF mass spectrometry with MetAlign™ software for analysis of the full scan mass data proved very fruitfull in the detection and identification of a substantial number of metabolites. Based on the detected accurate mass elemental compositions could be derived for a number of potential metabolites. LC-MS/MS analysis of the incubates by means of neutral loss scanning yielded complementary information to LC-ToF-MS. Many of the metabolites detected by LC-ToF-MS were also identified by LC-MS/MS. MS/MS fragmentation yielded important information on the chemical structure of some of the newly identified metabolites.

The major metabolite identifed was the cyano-metabolite. It was detected in all test systems and under all circumstances. In addition, two other interesting metabolites were identified: a low molecular weight metabolite (aminofurfural hydrazone) in microsome and S9 incubates and an amide metabolite in S9 and *E. Coli* incubates. Several dimeric compounds were also detected in the microsome and *E. Coli* incubates, but these are of limited interest for *in vivo* systems. Furthermore, several cysteine and mercaptoethanol adducts were (tentatively) identified. However, these metabolites were only formed in detectable amounts when large amounts of the thiol component were added to the incubates. Based on the experiments conducted and the metabolites identified it can be concluded that several modes of metabolism occur simultaneously in the *in vitro* systems. The formation of the CN-metabolite is in good agreement with what is known from the metabolism of furazolidone and nitrofurazone. The CN-metabolite could therefore be considered as a potential alternative marker for NFZ.

Analysis of incurred material showed unequivocally that the CN-metabolite is present in substantial amounts (approx. 500 µg/kg) in porcine muscle tissue during medication with nitrofurazone. In liver tissue the concentration was significantly lower. Probably the metabolite is quickly metabolised to other yet unidentified metabolites. Of the other metabolites selected, the aminofurfural hydrazone metabolite was qualitatively detected in incurred muscle tissue at zero withdrawal time. All other potential metabolites, if detected at all, were present in muscle as well as liver tissue only at low concentrations, even at zero withdrawal time. It could not be excluded that adducts are formed by reaction of thiol-containing substances (amino acids, gluthatione, proteins), but these adducts may be of limited stability under the extraction conditions used, or extraction recoveries for these compounds may be low.

Accumulation of the CN-metabolite largely occurs in muscle tissue during medication. In muscle tissue it is detectable for at least one week after the end of medication. Although depletion in muscle tissue is relatively quick compared to the persistent tissue-bound adducts, its presence can be detected up to one week after the end of medication. It should be realized that many authorized veterinary drugs

have depletion half-lives ranging from only a few hours up to a few days at the most. This is also the case for other banned substances like nitroimidazoles and various growth promoting agents.

Overall it can be concluded that the main objective of the project, being "*The identification and structure elucidation of an alternative indicator (marker residue) for nitrofurazone use in animal derived food products"* has been completed successfully. The second objective, "D*evelopment of a routine method of analysis to identify this marker that fulfils the requirements of Decision 2002/657/EC"*, could not be completed fully, due to the time-consuming procedure to elucidate the metabolic pathway of nitrofurazone. Nonetheless, the preliminary results obtained indicate that such a method can be developed for the CN-metabolite of NFZ. It is recommended that a future prolongation of this research should include improvement of the LC-MS/MS method for the CN-metabolite and that the substance should be made available as a reference material (including an isotopically-labelled analogue as IS).

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