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# LC-MS-MS method for determining nifursol and other nitrofurans residues in meat

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## Introduction

Nifursol belongs to the antibacterial drug class of nitrofurans. It has been used to protect turkeys against histomoniasis (blackhead disease). Other nitrofurans like furazolidone, furaltadone, nitrofurazone and nitrofurantoin have been applied in aquaculture and poultry to combat against a variety of diseases affecting the live-stock. Nitrofurans were banned in a number of countries because of the data which indicates furazolidone as a possible mutagenic and genotoxic drug. Later, nifursol was also phased out in Switzerland and the EU because of its structural relationship with furazolidone and the incomplete toxicological datas.

The analysis of nitrofurans residues in meat became only possible after the discovery that residues of these fast metabolizing drugs are covalently bound to the tissue (1–3). Some of these bound metabolites still contain an intact side-chain of the original parent drug. In the case of furazolidone treatment, the substructure/side chain of 3-amino-2-oxazolidone (AOZ) can be acid-liberated from the tissue, derivatized and analytically detected. This metabolite can still be found in the meat, days or weeks after the application of the parent drug has been stopped (4, 5). Furaltadone, nitrofurazone and nitrofurantoin (6) were found to undergo the same degradation in the tissue and produce their corresponding metabolites which permits their analytical detection.

We are not aware of a published analytical method to detect nifursol residues in meat. However, methods are available for the quantification of unmetabolized nifursol in concentrates and premixes (7). There exists very limited data concerning the fate of this drug in the treated animals (8). Informations are available concerning the distribution of the whole radioactivity in the tissues after feeding C<sup>14</sup> labelled nifursol. Unmetabolized nifursol and 5-nitro-2-furanoic acid were reported to be below the limit of determination (0.02 mg/kg) in a number of organs, whatever the

withdrawal time. Some of these data were reported in undated studies or internal papers, probably not available to the wider public (8).

It is likely, that the degradation of the drug nifursol is related to the metabolism of the other nitrofurans. According to this assumption, 3,5-di-nitro-salicylic hydrazine (DNS) could be liberated, derivatized and detected by LC-MS-MS. However, this analytical approach is hampered by the same experimental problems as the analysis of some nitrofurans in the past. For analytical work, only semicarbazide (the metabolite of nitrofurazone) has been readily available from commercial sources. The three other metabolites were difficult to obtain. At present, this problem has been solved, although the cost for the reference material are still relatively high. According to our best knowledge, the postulated nifursol metabolite can not be purchased from a commercial source. Based on the fact that the covalently tissue bound nitrofuran side chains can be liberated, we found out that the corresponding metabolite can be produced by hydrolyzing the commercially available nifursol (parent drug). Since hydrolyzation takes place during the derivatisation step, there is no significant change of the established analysis protocol (derived from the work of Leitner) (6) needed. This alternative experimental approach was consequently exploited for the determination of nifursol residues and the other nitrofurans.

It is the aim of this paper to determine nifursol together with the four other nitrofurans at trace levels by using readily and inexpensively available reference substances.

## **Materials and methods**

### *Chemicals, reagents and solvents*

Nifursol was purchased from Ehrenstorfer (Augsburg, Germany), furazolidone, furaltadon, nitrofurazon and nitrofurantoin from Riedel de Haen (Seelze, Germany), 3-amino-2-oxazolidinone (AOZ) from Sigma (Milwaukee, USA). Methanol and acetone were from J.T. Baker (Deventer, Holland). Hydrochloric acid 37 % and ammonia solution 25 % from Scharlau (Barcelona, Spain), 2-nitrobenzaldehyde (NBA) and ammonia acetate from Fluka (Buchs, Switzerland), dimethylsulfoxide, di-potassium hydrogen phosphate anhydrous, sodium hydroxide pellets from Merck (Darmstadt, Germany).

Oasis HLB 6 ml (0.2 g) Waters (Massachusetts, USA)

### *Standard and reagent solutions*

Standard solutions were prepared and diluted in methanol/acetone (8:2 v:v). Standard solutions of 1; 10 and 100 µg/l were prepared. The spike solution contained 500 µg/l nifursol and 100 µg/l of the four other nitrofurans. Derivatization reagent: 22.8 g 2-nitrobenzaldehyd (NBA)/l dissolved in dimethylsulfoxide. Hydrochloric acid: 0.1 mol/l. Di-potassium hydrogen phosphate solution: 0.5 mol/l. Sodium hydroxide: 1 mol/l.



Mobile phase A: 8 mmol ammonium acetate and 5 mmol ammonia hydroxide in (200 ml methanol, 800 ml water). Mobile phase B: Methanol.

### *Sample preparation and derivatization conditions*

8 g of sample was homogenized in 80 ml of 0.1 mol/l hydrochloric acid and 0.8 ml of derivatization reagent solution (NBA) using a polytron PT 3000 (Littau, Switzerland). To test for recoveries, 0.4 ml of spike solution was added. The mixture was kept for 4 hours in a waterbath at 55 °C. After cooling, 15 ml of 0.5 mol/l dipotassium hydrogen phosphate was added and 6.4 ml of 1 mol/l sodium hydroxide was used to neutralize the mixture. A pH value between 6 and 8 should result. If beyond this range, the phosphate solution, respectively alkali was used to adjust. The mixture was centrifuged for 5 minutes at 14500 rotations per minute with a Sorvall RC 5C (Kenro Laboratory Zürich, Switzerland).

An SPE cartridge (Oasis HLB 6 ml (0.2 g) Waters (Massachusetts, USA)) was activated with 3 ml methanol and rinsed with 6 ml water. 40 ml of the reaction mixture was allowed to pass (applying gentle vacuum) through the SPE cartridge. The cartridge was rinsed with 6 ml water and afterwards air-dried. 10 ml of methanol was used to elute the trapped analytes. The eluate was evaporated under vacuum (130 mbar) at 50 °C on a rotavapor E120 Büchi (Flawil, Switzerland). The dry residue was dissolved with 0.4 ml of mobile phase and injected into the LC-MS-MS.

### *LC conditions*

The instrument used was an Agilent HPLC 1100 system (Waldbronn, Germany) consisting of a binary pump and an autosampler. Precolumn: xTerra MS, C18, 5 µm, 2.1\*20 mm. Column: xTerra MS, C18, 5 µm, 2.1\*100 mm Waters (Massachusetts, USA). The column flow was set at 0.3 ml/min, column temperature was maintained at 25 °C. 40 µl were injected. Linear gradient program: 0 min: 10 % B; 9 min: 90 % B; 10.5 min 90 % B, 10.6 min: 10 % B; 13 min: 10 % B.

### *MS parameters*

All measurements were done on a Quattro LCZ MS Micromass (Manchester, UK) equipped with an electrospray interface. Time programming was used to switch the instrument from positive (nitrofurazone etc.) to the negative mode (nifursol including derivatized DNS). Capillary voltage was 3.5 kV. Extractor and RF lense voltage were 3 resp. 0.3 V. The source and desolvation temperature were 150 °C respectively 350 °C. The desolvation gas flow was set to 570 l/h and the cone gas flow to 50 l/h nitrogen. The collision gas partial pressure (argon) was adjusted to obtain maximal MS-MS signals. The applied transitions are listed in table 1. The MS-MS tuning was performed by syringe flow injection of individually derivatized parent-dugs.

**Table 1**  
**Analyte specific MS-MS conditions**

| <i>Compound</i>            | <i>Polarity</i> | <i>Transition</i> | <i>Cone voltage</i><br>(V) | <i>Collision energy</i><br>(eV) |
|----------------------------|-----------------|-------------------|----------------------------|---------------------------------|
| Derivatized Furazolidone   | positive        | 236.3 > 134.1     | 25                         | 15                              |
|                            | positive        | 236.3 > 104       | 25                         | 20                              |
| Underivatized Furazolidone | positive        | 226.2 > 122       | 30                         | 20                              |
| Derivatized Furaltadone    | positive        | 335.3 > 262.3     | 20                         | 20                              |
|                            | positive        | 335.3 > 291.3     | 20                         | 15                              |
| Derivatized Nitrofurazone  | positive        | 209.3 > 192.2     | 20                         | 10                              |
|                            | positive        | 209.3 > 166.2     | 20                         | 10                              |
| Derivatized Nitrofurantoin | positive        | 249.3 > 134.0     | 25                         | 15                              |
|                            | positive        | 249.3 > 178       | 25                         | 15                              |
| Underivatized Nifursol     | negative        | 364.0 > 182.0     | 30                         | 20                              |
| Derivatized Nifursol       | negative        | 374.5 > 183.0     | 30                         | 25                              |
|                            | negative        | 374.5 > 226.0     | 30                         | 25                              |

### *Incurred samples*

Incurred samples (turkey breast and liver) were gratefully provided by frifag AG, 9562 Märwil Switzerland. The turkeys were fed with daily rations of 141 g feed containing 100 mg/kg of nifursol. One animal (age: 13 week) was sacrificed 25 days (withdrawal time) after feeding with medicated pellets was stopped. Another animal (age: 9 weeks) was sacrificed while still receiving medicated feed. The samples were immediately frozen and analyzed the following day.

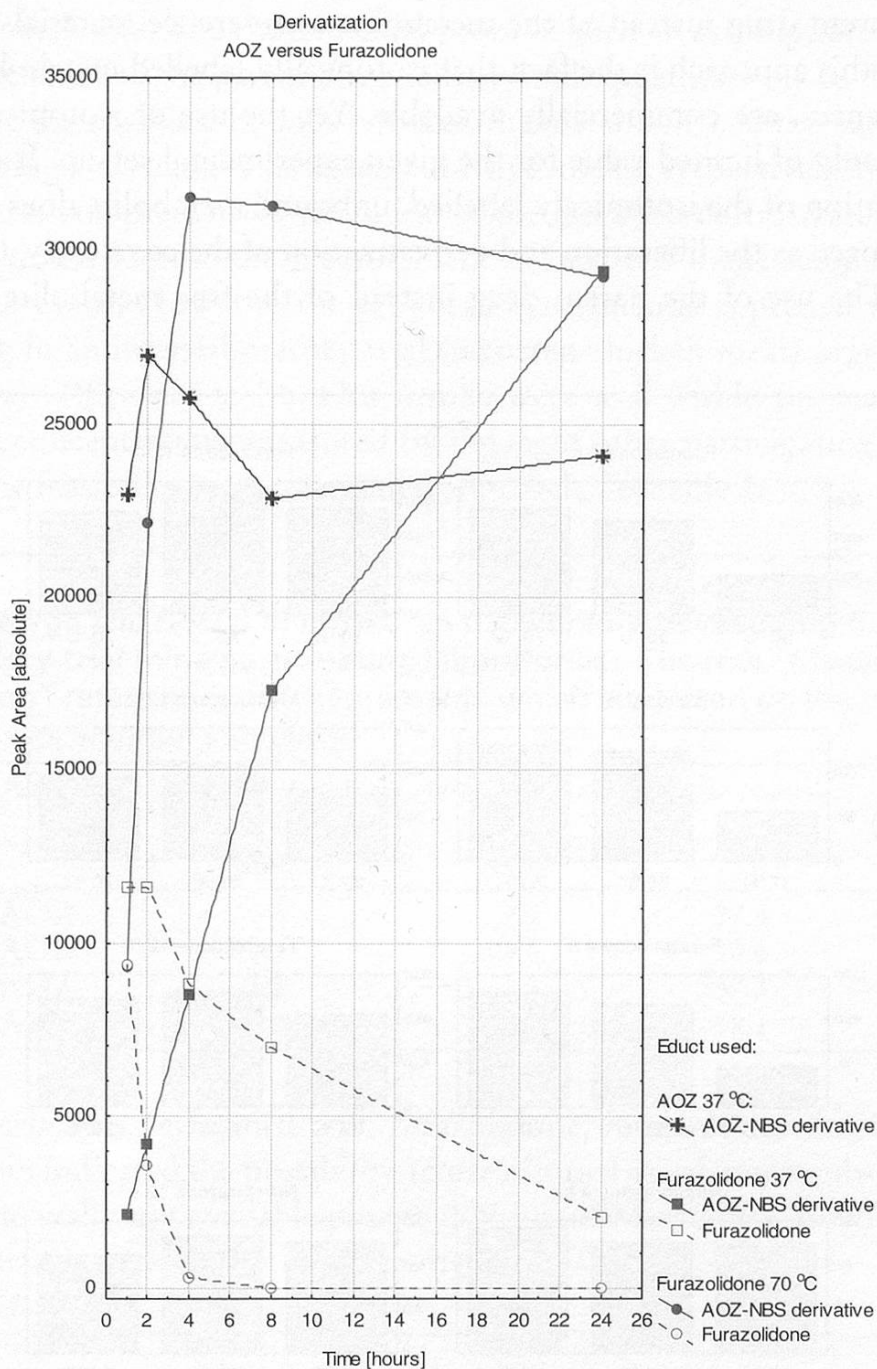
Furthermore, 20 turkey samples from Switzerland and a variety of EU countries were obtained from importers and local supermarkets.

## **Results and discussion**

### *Derivatization of nitrofurans*

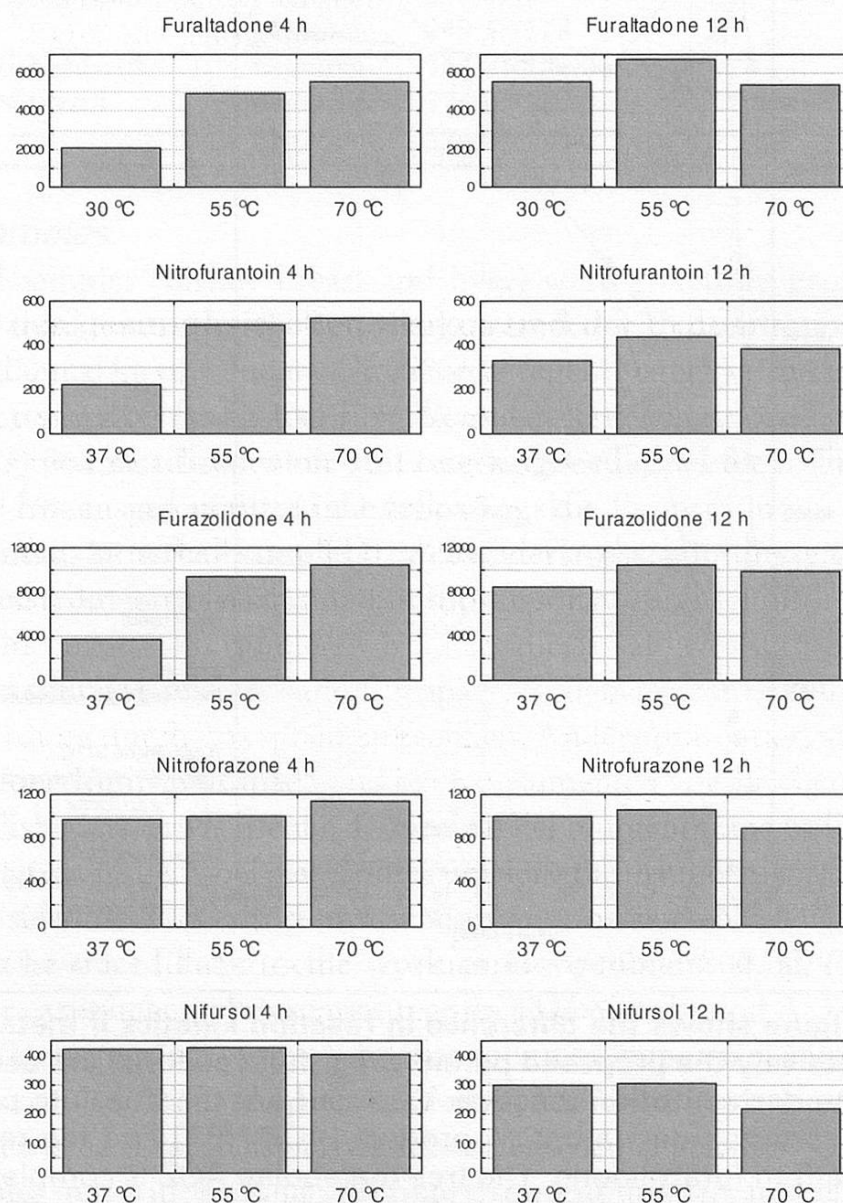
The published methods for the determination of nitrofurans utilize a derivatization temperature of 37°C. This reaction covers the hydrolysis of the covalently bound drug side chain and the derivatization reaction with NBA. The applied temperature can be traced back to the work of Hoogenboom et. al. (1) who used in some of their experiments the enzyme trypsin to liberate bound AOZ from the protein matrix. This approach was not further followed since there were no significant higher recoveries of AOZ-NBA derivative found. Yet, the physiological temperature of 37°C was maintained by Hoogenboom and other authors. Experiments with spiked samples performed in our laboratory showed that elevated temperatures can be used to reduce the rather long derivatization time of 16 to 4 hours. Since the derivatization permits the release of covalently tissue bound metabolites like AOZ, it should also be possible to liberate this side-chain by hydrolyzing and derivatizing the unmetabolized parent drug, furazolidone. This reaction was shown to proceed, albeit with a slower kinetic. See figure 1. The influence of temperature and the reac-





**Figure 1** The figure shows the difference in reaction kinetics if metabolite (AOZ) respectively the proposed parent drug (furazolidone) are used as educts for the derivatization reaction. Depicted are the absolute peak areas of their common derivatization product (AOZ-NBA) and the remaining underivatized furazolidone. The free metabolite AOZ is completely derivatized within 1 hour at 37°C. If the parent drug, furazolidone is used as educt, only 10% derivatization yield is observed within the same period. Furazolidone reacts significantly faster, if higher temperature is applied. After 24 hours, equal amounts of derivative is observed. Parallel to the increasing product peak areas, a corresponding drop of the furazolidone educt signal is observed.

tion time was studied for all five nitrofurans. See figure 2. Hence, it is possible to utilize the parent drug instead of the metabolite as reference materials. A possible drawback of this approach is the fact, that isotopically labelled metabolites, but not parent substances, are commercially available. Yet the use of isotopically labelled standards is only of limited value for the given experimental set-up. It is likely that the derivatization of the isotopically labelled, unbound metabolite does not proceed at the same speed as the liberation and derivatization of the covalently, tissue bound metabolite. The use of the parent drug instead of the free metabolite as standard



**Figure 2 Yield of derivatized nitrofurans, produced by varying derivatization time and temperature.** The height of the bars represent the absolute peak area (average of two independent experiments). Results refer to a turkey matrix spiked with equal amounts of parent drugs.



might be equally reasonable, because it resembles more closely to the covalently tissue bound metabolite. The experimental proof of such an assumption is not simple, since the side chain of the parent drug neither binds quantitatively to the tissue proteins, nor are the chemical bounds between the metabolite and the various possible protein binding sites equally accessible for hydrolysis, respectively the derivatization reagent. A comprehensive validation of the modified derivatization conditions (55 °C 4 h) would require the measurement of incurred tissue samples containing the five nitrofurans. However, the proposed experimental approach was tested by participating in an inter-laboratory trial (incurred chicken meat) organized by the Swiss Federal Office of Health. Our results were well within the measured AOZ and AMOZ concentrations measured by the eight other participating laboratories, indicating the feasibility of the suggested approach. See table 2.

Table 2

**Median, Average and RSD% of nitrofuran measurements resulting from an interlaboratory-trial (nine participating laboratories).** The row "Measured Concentration" refers to our measurements which are based on the use of the parent-drugs as external standards.

|          | Analyte | Median (n=9)<br>(ppb) | Average (n=9)<br>(ppb) | RSD (n=9)<br>% | Measured<br>Concentration<br>(n=1) (ppb) |
|----------|---------|-----------------------|------------------------|----------------|--|
| Sample 1 | AOZ     | 2.4                   | 2.2                    | 37.4           | 2.5                                      |
| Sample 2 | AOZ     | 99.3                  | 94.5                   | 30.6           | 66                                       |
| Sample 3 | AOZ     | 1.3                   | 1.3                    | 37.3           | 1.3                                      |
| Sample 4 | AMOZ    | 7.8                   | 8.9                    | 38.1           | 6.3                                      |

The experiments performed with furazolidone, furaltadone, nitrofurazone and nitrofurantoin indicated the possibility to use nifursol as reference substance instead of the commercially not available potential Nifursol-Metabolite (DNS). The postulated derivatization reaction is shown in figure 3.

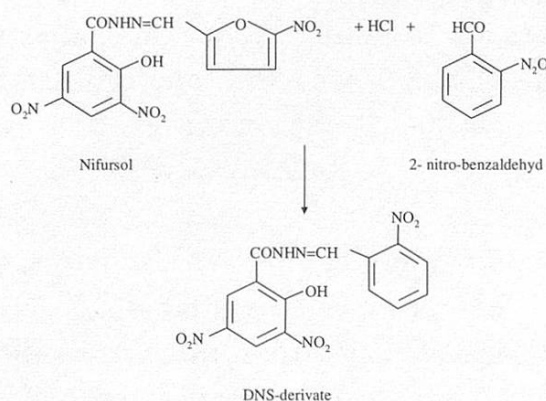


Figure 3 The proposed derivatization reaction



The progress of the derivatisation was monitored by measuring the remaining nifursol concentration and a signal corresponding to the expected mass of the derivatised nifursol (DNS-NBA). It was found out that the optimum derivatization conditions do not differ significantly between nifursol and the other nitrofurans. However, we observed a drop of the recovery values when spiked samples (turkey breast) were measured. Recovery varied between 30–40 % if compared to the derivatized aqueous nifursol solutions. Dilutions of the final sample solution did not induce an increase of the signal/concentration ratio of the studied analytes. We suspect therefore that rather the derivatization efficacy is reduced by the presence of matrix, than the analyte signals are suppressed in the MS interface by co-eluting components. A similar lowering of recoveries – although not to the same extent – was observed if furazolidone is derivatized in matrix. The reason for the somehow lower recoveries is unclear. However, the electron drawing effect of the two additional nitro groups in nifursol might affect the reactivity towards the derivatization reagent, while the low polarity of nifursol probably affects the extraction efficiency.

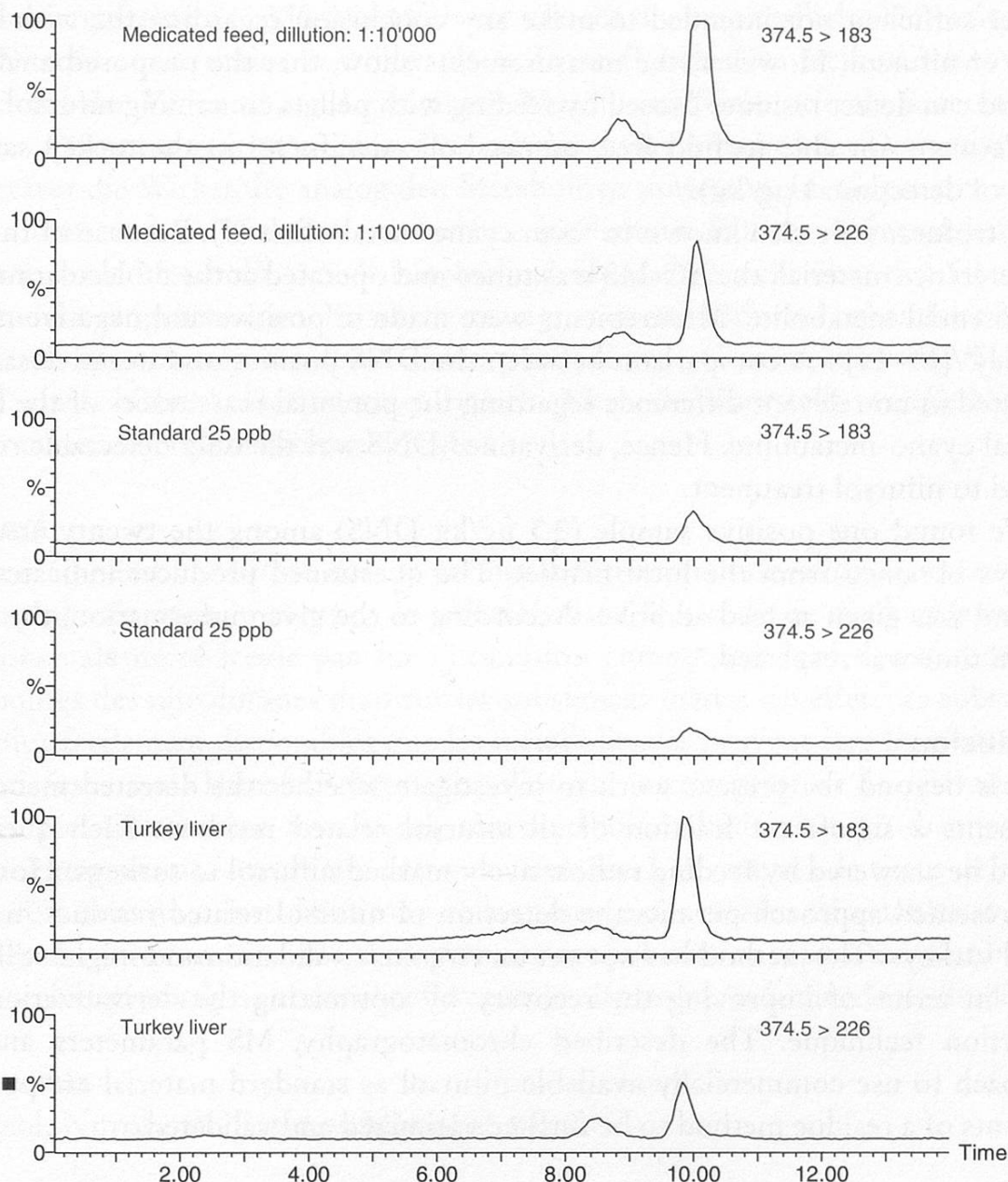
The calibration curves were found to be linear over two orders of magnitude, while the limit of determination was 0.2 µg/kg. Intra- and inter-day repeatability were 13 % RSD respectively 15 % RSD.

### *Chromatography*

Derivatized DNS elutes later from the analytical column than the other nitrofurans derivatives. Poor peak shape was observed, if mobile phase compositions described in published nitrofurans methods were used. This might be explained by the acidic hydrogen of nifursol and the low poor buffer capacity of the used MS compatible mobile phases. It was therefore necessary to increase the acetonitrile and the ammonium acetate concentration of the eluent. The mentioned mobile phase permits the separation and quantification of nifursol while maintaining the separation of the four other nitrofurans. Figure 4 shows typical chromatograms of highly diluted nifursol medicated feed, standard and the incurred turkey liver sample. There is a second peak which occurs on both mass traces, eluting ahead of the main peak. This peak is clearly related to the amount of injected nifursol. The ratio between the both peaks seems to be affected by the matrix in which the derivatization occurs. We speculate, that this behaviour might be explained by the formation of isomers.

### *Mass spectrometry*

We were not successful in applying positive electrospray (ESI) to detect derivatized Nifursol. Negative ESI produced acceptable signals and permitted the fragmentation of the  $[M-H]^-$  ion. The polarity switching of the MS allowed the detection of all five nitrofurans in one chromatographical run.



**Figure 4** The chromatograms show the two measured MS-MS traces of nifursol. From top to bottom: Medicated feed derivatized after a 1:10000 dilution, nifursol standard (25 µg/l), incurred turkey liver (68 µg/kg).

### *Measurement of samples.*

The viability of the proposed analytical approach is based on a postulated underlying metabolism. This assumption can not be proved by analyzing laboratory spiked matrix but only by analyzing incurred samples.

A turkey still being fed with medicated pellets showed residue levels of 6 µg/kg nifursol in turkey breast meat and 68 µg/kg in turkey liver. Another turkey, where nifursol feeding had stopped since 25 days (withdrawal time) showed neither in the



breast tissue nor in the liver measurable nifursol levels. ( $<0.2 \mu\text{g/kg}$ ). This data is neither sufficient nor intended to make any conclusion regarding the withdrawal times of nifursol. However, the measurements show, that the proposed analytical method can detect residues caused by feeding with pellets containing nifursol.

We were not able to find free, unmetabolized nifursol in the turkey samples (limit of detection:  $1 \mu\text{g/kg}$ ).

Nitrofurans are also known to form cyano metabolites (3). Because of the lack of a reference material, the LC-MS was tuned and operated at the molecular mass of the potential metabolite. Measurements were made in positive and negative modus  $[\text{M}+\text{H}]^+ / [\text{M}-\text{H}]^-$ . A comparison between the DNS positive and negative samples indicated in no relevant difference regarding the potential mass traces of the hypothetical cyano-metabolite. Hence, derivatized DNS was the only detectable residue related to nifursol treatment.

We found one positive sample ( $0.3 \mu\text{g/kg}$  DNS) among the twenty analyzed turkeys obtained from the local market. The questioned producer indicated that nifursol was given as feed additive. According to the given information, the withdrawal time was respected.

## Conclusion

It is beyond the present work to investigate whether the detected metabolite represents a significant fraction of all nifursol related residues. Such questions should be answered by feeding radioactively marked nifursol to turkeys. However, the presented approach permits the detection of nifursol related residues in medicated turkeys. The method has not yet undergone a validation and might still offer room in terms of improving the recovery by optimizing the derivatization and extraction technique. The described chromatography, MS parameters and the approach to use commercially available nifursol as standard material are possibly elements of a residue method to be further optimized and validated.

## Summary

The described LC-MS-MS method permits the detection of five nitrofurans including nifursol at residue levels in turkey meat. The experimental approach does not rely on the use of nitrofuran metabolites but on the parent drugs as reference material. It was shown that the parent drugs can be hydrolyzed and derivatized like the metabolites by applying a higher derivatization temperature. The parent drugs are more easily available than the metabolite. In the case of nifursol, the metabolite (3,5-di-nitro-salicylic hydrazine) can not be obtained from commercial sources. Turkeys feed with nifursol, produced tissues containing analytically detectable nifursol residues. According to our knowledge, there has been no other report of an analytical technique capable in detecting nifursol in incurred turkey meat.



## Zusammenfassung

Die vorgestellte LC-MS-MS Methode erlaubt die empfindliche Detektion von fünf Nitrofuranen (inklusive Nifursol) in Trutenfleisch. Der methodische Ansatz basiert auf der Verwendung der intakten Nitrofuran-Wirkstoffe als Referenzsubstanzen. Es konnte gezeigt werden, dass sich durch das Anheben der Derivatisations-temperatur die Wirkstoffe analog den Metaboliten umsetzen. Im Gegensatz zu den Metaboliten sind die Wirkstoffe einfacher erhältlich. Im Fall von Nifursol, kann der zu erwartende Metabolit (3,5-di-Nitrosalicyl-Hydrazin) nicht über den Chemikalienhandel bezogen werden. Es wurde gezeigt, dass mit Nifursol behandelte Truten analytisch quantifizierbare Rückstände produzieren. Gemäss unserem Wissensstand, wurde bisher keine andere analytische Methode vorgestellt, welche Nifursol in gewachsenen Proben nachweisen kann.

## Résumé

La méthode LC-MS/MS décrite permet la détection des résidus des métabolites de cinq nitrofuranes, y compris le nifursol, dans la viande de dinde. L'approche expérimentale ne se fonde pas sur l'utilisation comme standard de référence des métabolites des nitrofuranes mais sur les substances mères. En effet, ces substances sont plus facilement disponibles que les métabolites et peuvent être hydrolysées et dérivatisées comme les métabolites en appliquant une température de dérivatisation plus élevée. Concernant le nifursol, son métabolite (hydrazine 3,5-di-nitro-salicylique) ne peut pas être obtenu commercialement. Les analyses de tissus de dindes alimentées avec du nifursol ont montré la présence des résidus détectables et quantifiables. A notre connaissance, il n'existe aucune publication rapportant l'utilisation d'une technique analytique capable détecter le nifursol dans la viande de dinde.

## Key words

Nifursol, Nitrofuran, Residue, Meat, LC-MS-MS

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