Development of an ELISA for the detection of nifursol metabolite DNSH in meat and seafood and validation in accordance with Commission Implementing Regulation 2021/808

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Overview

- 3,5-dinitrosalicyclic acid hydrazide (DNSH) is a metabolite of the banned antibiotic nifursol.
- A polyclonal antibody for DNSH was produced in rabbits.
- An ELISA method for the specific detection of DNSH in meat and seafood with the detection capability of 0.25 µg/kg was developed and validated using both spiked and incurred samples.

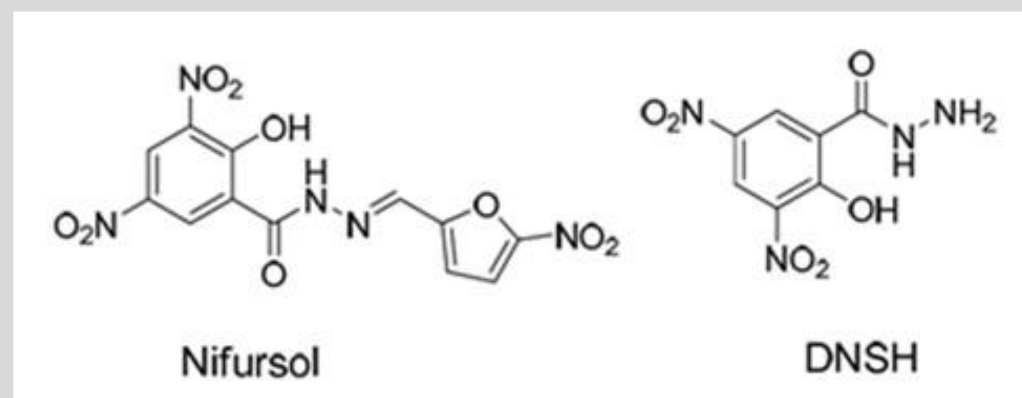


Fig. 1 Nifursol and DNSH structures.

Introduction

Nifursol is a nitrofuran antibiotic banned as a feed additive in the European Union and other countries. Nifursol (Fig. 1) is metabolised to 3,5-dinitrosalicyclic acid hydrazide (DNSH) in living organisms. DNSH is a marker for the detection of illegal use of nifursol in animal husbandry. The detection of DNSH by LC-MS/MS requires derivatisation of this metabolite with 2-nitrobenzaldehyde to NPDNSH, similar to other nitrofuran metabolites such as SEM, AHD, AMOZ and AOZ. In accordance with Commission Regulation (EU) 2019/1871 a reference point of action (RPA) of 0.5 µg/kg for DNSH and other nitrofuran metabolites applies from 28 November 2022.

Due to an increasing demand for monitoring for the presence of DNSH in meat and seafood, an ELISA method for the specific detection of DNSH was developed and validated in accordance with Commission Implementing Regulation 2021/808.

Method

ELISA procedure

A sequential antibody-coated competitive ELISA was developed and optimised based on a polyclonal antibody raised against DNSH hapten-conjugate. The ELISA procedure takes only 60 minutes. A typical standard curve is presented in Fig. 2.

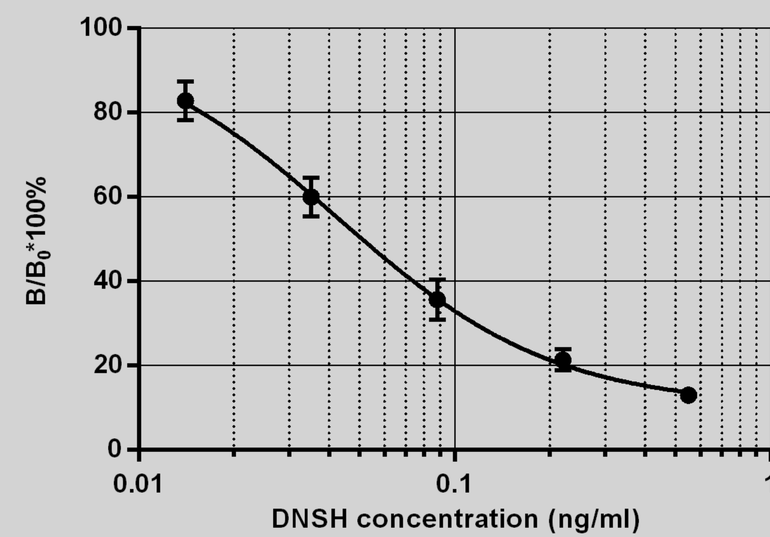
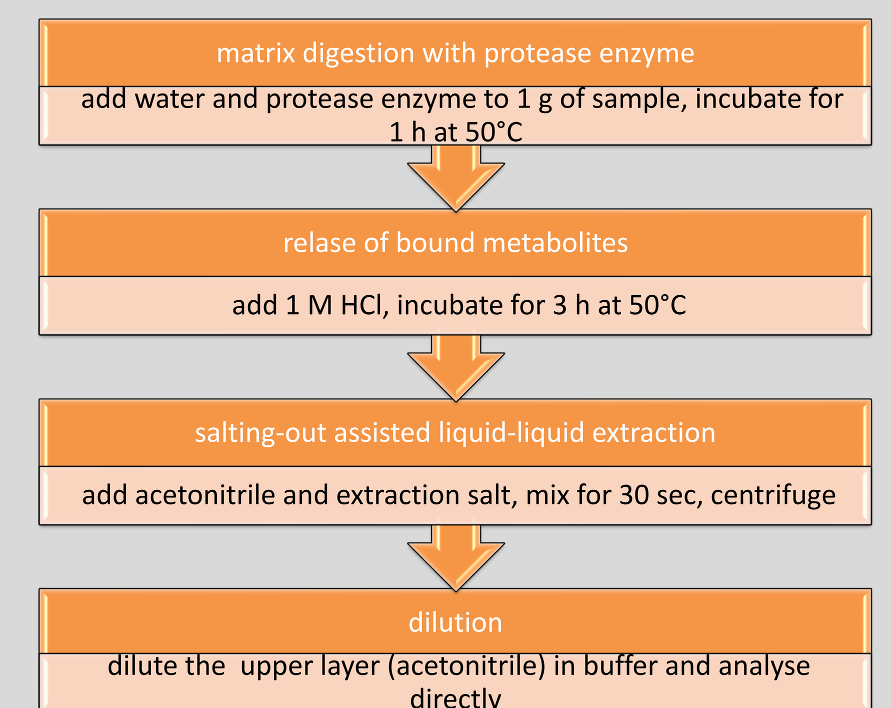


Fig. 2. Standard curve for DNSH (n=6).

Sample preparation method

A sample preparation method based on salting-out assisted liquid-liquid extraction was developed and optimised. The developed method is simple and does not require any evaporation step for analyte concentration. A sample is analysed directly after acetonitrile phase dilution in the assay buffer.



Results

Detection capability CCβ

For determination of the detection capability CCβ, a set of 20 different meat samples and 20 different seafood samples were analysed as blank and spiked at 0.25 µg/kg with DNSH (0.5×RPA) using three DNSH ELISA kit batches. There was no overlap between blank and spiked samples (Fig. 3).

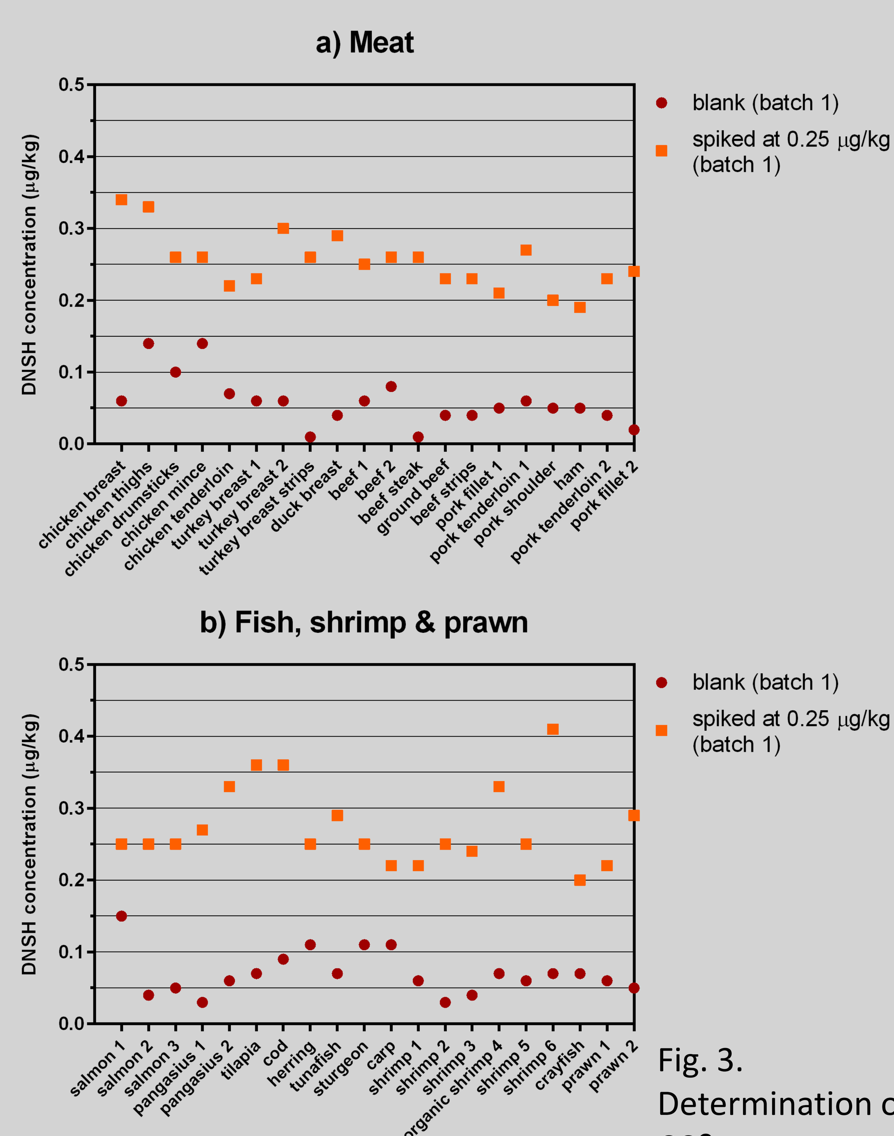


Fig. 3. Determination of CCβ.

Recovery and precision

Seven different samples for each product group spiked at 3 different levels were analysed on 3 different ELISA batches on 3 different days.
The repeatability (n=21 at each level) based on the data collected over 3 different days was calculated for each batch separately (Table 1) and then within laboratory-reproducibility (Table 2) for all 3 batches (n=63) was determined. CVs were from 14 to 26 %.

Table 1. Recovery and repeatability for 3 ELISA batches (n=21 at each level).

Matrix	Spiking level (µg/kg)	ELISA batch 1		ELISA batch 2		ELISA batch 3	
		Mean recovery (%)	RSD_r (%)	Mean recovery (%)	RSD_r (%)	Mean recovery (%)	RSD_r (%)
Meat	0.25	98	15.2	96	14.7	98	15.5
	0.5	88	16.7	97	14.0	96	17.0
	0.75	87	23.3	84	16.4	88	19.1
Fish, shrimp and prawn	0.25	98	26.4	103	18.8	105	18.2
	0.5	98	23.9	112	21.7	115	23.5
	0.75	94	21.9	110	20.0	106	21.7

Table 2. Recovery and within-laboratory reproducibility (n=63 at each level).

Matrix	Spiking level (µg/kg)	Mean recovery (%)	RSD_R (%)
Meat	0.25	97	14.9
	0.5	94	16.3
	0.75	86	19.6
Fish, shrimp & prawn	0.25	102	21.1
	0.5	108	23.7
	0.75	103	21.8

Comparison of DNSH ELISA and UPLC-MS/MS results

Incurred chicken samples were obtained in a feeding study with nifursol. A number of samples ware obtained by homogenising contaminated and non-contaminated chicken muscle to obtain DNSH concentration around 0.5×, 1× and 1.5× RPA values. Additional samples at higher concentrations were also prepared. The samples were analysed by UPLC-LC/MS method (SCIVP, Lviv, Ukraine) and DNSH ELISA. There was a good agreement between the results (Fig. 4).

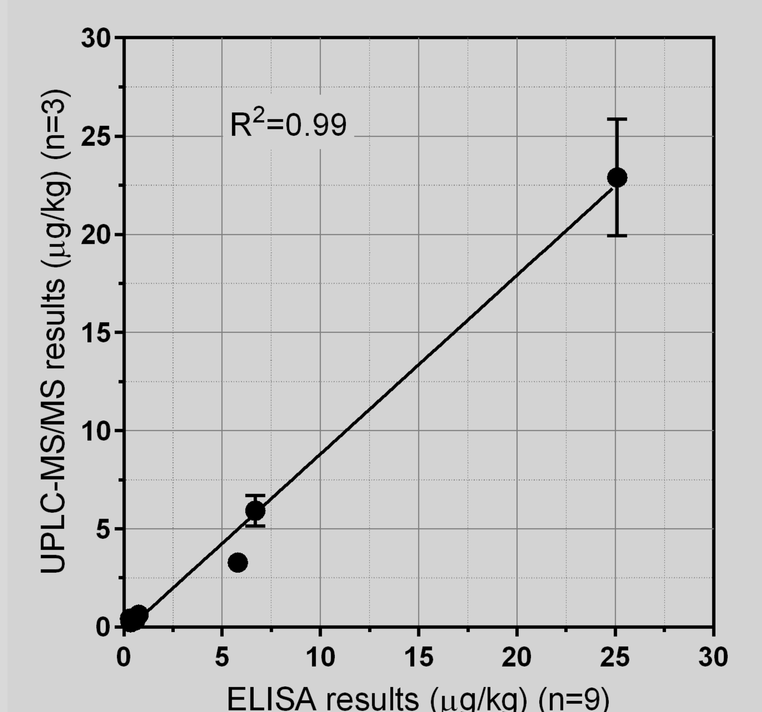


Fig. 4. Correlation between the concentrations of DNSH obtained by ELISA and UPLC-MS/Ms for incurred chicken samples.

Conclusions

- An ELISA screening method for the detection of nifursol metabolite DNSH in meat and seafood was developed and validated.
- The detection capability was found to be 0.25 µg/kg in meat and seafood, which is half of the refrence point of action (RPA) set by Commission Regulation (EU) 2019/1871 and applied from 28th of November 2022.
- The method will be transferred into the first commercial ELISA test kit for the specific and sensitive detection of DNSH.